Association of vitamin K with insulin resistance and body composition

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Abstract:
Increasing evidence supports an association between the skeleton and energy metabolism. These interactions are mediated by a variety of hormones, cytokines and nutrients. Osteocalcin (OC), a vitamin K-dependent protein, may have a role in the regulation of glucose metabolism and adiposity. To evaluate this role of OC in humans, one also needs to take into consideration the effect of vitamin K (VK). Because OC depends on VK for carboxylation, and thus function, the percentage of OC that is not carboxylated (%ucOC) has been used as a measure of VK status, with lower %ucOC representing higher circulating VK. In mice, the uncarboxylated form of OC (ucOC) is active in glucose metabolism and body composition. The data in humans are equivocal. Despite its dependence on VK, very few human studies examining the association between OC and insulin resistance (IR) or diabetes risk have taken VK status into account. To address this shortcoming, we examined the associations between VK, OC, IR and body weight in three well-characterized cohorts. Our specific aims were: 1: To determine if intentional weight loss is associated with a decrease in the carboxylation of OC in post-menopausal women when VK intake is held constant, 2: To determine if increased carboxylation of OC through VK supplementation is associated with lower markers of IR in younger and older men and women, and 3: To determine the cross-sectional associations between circulating VK concentrations and markers of IR in older men and women. For specific aim 1, data were obtained from healthy post-menopausal women (n=71) undergoing a twenty-week weight loss program. Measures of body weight, body fat percentage, and serum OC forms were assessed before and after the

intervention. All participants lost weight (-10.9±3.9 kg) and body fat (-3.9±2.0 %), but their weight-loss was not associated with changes in any form of serum OC (all p>0.31). For specific aim 2, data were obtained from 42 healthy younger (age= 18-40y) and older (age= ±65y) men and women who received a VK supplement for 21 days. Circulating measures of OC, VK, and markers of IR were assessed before and after supplementation. With VK supplementation, circulating uncarboxylated OC (ucOC) decreased (pre= 4.4±3.0, post= 1.1±2.1 ng/mL, p<0.001) with no concomitant change in the homeostatic model of insulin resistance (HOMA-IR, p=0.78). No significant correlation between any form of OC (total OC (tOC), ucOC or %ucOC) and HOMA-IR was observed (p>0.12). For specific aim 3, data were obtained from a large multi-center cohort of community dwelling older men and women (n= 932). Circulating measures of VK and IR were measured. Serum lipids were also measured since VK is transported on triglyceride-rich lipoproteins. A trend for higher plasma VK to be associated with lower measures of HOMA-IR was observed in men with normal triglycerides only (β= -0.077, p=0.08). No significant associations were noted for women, consistent with prior literature. The approach was limited to secondary analysis. However, the data from three independent cohorts were consistent in that ucOC was not associated with measures of IR or weight loss in community-dwelling men and women. In contrast, there was a trend for higher circulating VK to be associated with lower HOMA-IR cross-sectionally, particularly in older men with normal triglycerides. Carefully designed studies are required to define the role of VK in the regulation of glucose metabolism in humans.

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1. Introduction

1.1 Significance of proposed research

The world-wide prevalence of diabetes and obesity have steadily risen.[1] In addition to increasing a person's mortality risk independently, obesity contributes to a person's risk of developing diabetes.[2,3] Weight loss, in contrast, can reduce this risk. Dietary patterns and nutritional interventions may mitigate risk of developing diabetes and unintentional weight gain. One such nutrient, vitamin K, and the associated vitamin-K dependent protein (VKDP), osteocalcin (OC) have been implicated in the regulation of both insulin sensitivity and thus progression to diabetes, as well as weight regulation.

Vitamin K is a fat soluble vitamin whose main function is in the post-translational modification of VKDPs.[4] The biological functions of some of these VKDPs in the promotion of health and reduction of disease have been well established.[5] For example, there are multiple VKDPs that function in blood coagulation, including but not limited to prothrombin (Factor II) and Factor X. However, VKDPs are found in multiple tissue throughout the body with functions beyond that of coagulation.[6] Osteocalcin is a VKDP that has been well researched for its role in bone formation and regulation of the mineral phase. However, recent experiments using a mouse model suggest that OC and specifically, the uncarboxylated form of OC (ucOC), may also impact weight loss and glucose metabolism.[7,8] The direct administration of ucOC to humans is not feasible,[9] hence the role OC may have in these processes in humans is still under investigation. The work described in this thesis details our extensive analysis of vitamin K, osteocalcin carboxylation and the effect that these two factors may have on insulin resistance and weight in humans.

1.2 Hypotheses &Specific Aims

We utilized three well-characterized independent studies to examine the associations between vitamin K (VK) and markers of insulin resistance (IR) and weight in community-dwelling men and women. For circulating concentrations of VK, we limited our analysis to phylloquinone (PK), which is the primary form in both circulation and in Western diets.

Hypothesis 1: Intentional weight loss will be associated with increased carboxylation of the vitamin K-dependent protein osteocalcin.

Specific Aim #1: Determine if intentional weight loss through caloric restriction and/or increased exercise is associated with a decrease in the carboxylation of osteocalcin (OC) in post-menopausal women when VK intake is held constant.

Hypothesis 2: Higher circulating concentrations of the undercarboxylated form of the vitamin K-dependent protein osteocalcin (ucOC) are associated with reduced insulin resistance and increased bone turnover.

Specific Aim #2a: To determine if higher carboxylation status of osteocalcin is associated with lower concentrations of markers of IR (as defined by fasting glucose, insulin and the homeostatic model for insulin resistance) in younger and older adults participating in a metabolic study designed to examine the dietary and non-dietary influences on vitamin K metabolism.

Specific Aim #2b: To determine if the relationship between ucOC and measures of IR is the same as that of other bone turnover makers and IR.

Hypothesis 3: Higher circulating concentrations of vitamin K will be associated with decreased insulin resistance.

Specific Aim #3a: To determine the cross sectional associations between circulating PK concentrations and measures of IR in a cohort of community-dwelling older adults.

2. Review of the Literature:

Background and function of vitamin K

Vitamin K (VK) is a fat soluble vitamin (FSV) found in two forms. Phylloquinone (PK) is found in vegetables and plant-based oils and is the most abundant form of VK found in the diet.[10,11] The second form of VK, menaquinones (MK), is predominantly found in animal sources and fermented products, as well as being produced by the gut bacteria.[11] Both forms of VK share the chemical structure 2-methyl-1, 4-napthoquinone but differ in the length and saturation of their phytl side chain.[12] Currently, little is known regarding the contribution of MK to overall VK nutrition, so the focus of this thesis will be on PK.

Since VK is a FSV, its absorption from the diet is enhanced when consumed with lipids.[13] Once absorbed, it is incorporated into chylomicrons and transported through circulation by triglyceride-rich lipoproteins.[14] The current adequate intake for VK is 90 and 120 µg/d for women and men respectively; however, it is estimated that close to one third of Americans do not meet this recommended intake.[10,15] Populations at greatest risk of not attaining adequate VK in the diet include children and the elderly.[10,16]

The classical function of VK is as an enzyme cofactor in the gamma-carboxylation of VK-dependent proteins (VKDP). Vitamin K-dependent proteins are synthesized with one or more glutamic acid (Glu) residues that can be carboxylated. These proteins are considered inactive until the Glu residues are carboxylated by a VK-dependent process.[17] The most well-known VKDPs are those involved in blood coagulation, including coagulation factors VII, IX, X and prothrombin. However, other VKDPs are known to be involved in bone and vascular homeostasis (Table 1).[18] In the

case of the VKDP osteocalcin (OC), for example, carboxylation allows the protein to bind to hydroxyapatite, the mineral component of bone.[19]

Currently there is growing evidence that other biological functions of VK may exist outside of its role in the post-translational carboxylation of Glu residues.[20,21] These include expression and production of inflammatory cytokines and the role of VK in mitigating insulin resistance (IR).[22,23]

Table 1. Known vitamin K-dependent proteins and their proposed function

Vitamin K-dependent Protein	***Proposed Function***
Prothrombin, Factor VII, IX, X, Proteins C, S, Z	Blood Coagulation
Growth Arrest Specific protein-6 (GAS-6)	Mediate cell survival in vascular endothelium
Osteocalcin (OC)	Bone formation
Periostin	Regulating biomechanical properties of connective tissues
Matrix gla protein (MGP)	Regulate bone, arterial calcification
Gla-rich protein (GRP)	Unknown; may modulate calcium availability

Insulin and insulin resistance

Insulin is an anabolic hormone synthesized by the beta cells of the pancreas and is released in response to increased blood glucose.[24] After its release, insulin travels through the circulation and binds to the insulin receptor on peripheral tissues such as adipose and muscle. The binding of insulin to its receptor causes a cascade of reactions which ultimately leads to the translocation of glucose transporters to the cell surface where they can take glucose from circulation into the cell, among other cellular processes.[24]

When the peripheral cells become less responsive to the actions of insulin, blood glucose remains elevated. This is referred to as insulin resistance (IR).[25] The pancreas attempts to compensate for the sustained high blood glucose concentrations by secreting more insulin. Eventually, this increased production can lead to beta cell failure and diabetes.

There are several tests used to measure glucose and insulin sensitivity with the gold standard being the hyperinsulinemiceuglycemic glucose clamp.[26] The methodologies for properly carrying out this technique can be technically challenging and difficult to undertake in larger research studies. Therefore surrogate methods have been developed and validated against it. The simplest are fasting serum measures of glucose and insulin, but the use of these measures individually pose many limitations.[26] Therefore, modifications have been proposed to these measures to make them more sensitive to determining a person's insulin sensitivity. One of the most widely used calculations is the homeostatic model of insulin resistance (HOMA-IR) which uses a formula incorporating both fasting insulin and glucose measures.[27] High-correlations have been reported between HOMA-IR calculations and the euglyemic clamp demonstrating its utility in acting as a valid measure of insulin sensitivity.[26] A final method utilized in many studies is the oral glucose tolerance test (OGTT) which requires persons to take a bolus amount of glucose followed by measurement of blood glucose for two hours. The result is typically indicative of peripheral glucose sensitivity, but can be costly for large population-based studies.[26]

The prevalence of IR has steadily increased in the US[28] and globally.[29] With that, research into the factors relevant to the etiology and prevention of IR has become more

critical. These include genetic and environmental factors as well as how specific lifestyle choices like nutrient intakes can impact IR. One specific nutrient of investigation is VK and its related VKDP OC.

Vitamin K and insulin resistance

Although VK has been implicated in the alteration of IR,[30–33] the exact mechanism is unknown. One theory suggests a protective role of VK in reducing IR. Available published data from human studies are summarized in **Table 2**. In studies where participants were given a VK supplement, a decrease in IR was observed in men, as indicated by HOMA-IR.[31,34,35] Only one study reported no change in IR with VK supplementation, which may be due to small sample size (n=21 per group) and/or the fact that the study was only conducted in postmenopausal women.[36] Additionally, higher VK intakes, as assessed by food frequency questionnaires, were associated with lower markers of IR in two independent cohort studies conducted in different countries.[32,37] Although the exact mechanism by which VK may reduce IR is unknown, it is biologically plausible that it may be via a reduction in pro-inflammatory cytokines,[38] VK measures tracking healthy lifestyles[39] or a currently unrealized mechanism.

Table 2: Human studies of the association between VK and measure of glucose homeostasis

VK Measure	Duration	Populaton	Outcome	Reference
PK intake assessed by FFQ	n/a	Men and women enrolled in the Framingham Offspring Study (n=2719)	Higher phylloquinone associated with greater insulin sensitivity and glycemic status	[37]
Supplementation with 500ug/d PK	36 Months	Older men and women, 60-80 y	HOMA-IR significantly lower	[31]

		(n=355)	in supplemented men only	
PK & MK intake assessed by FFQ	n/a	Men and women enrolled in the Dutch Epic cohort (n=38,094)	Higher intakes of PK & MK associated with lower IR incidence	[32]
Supplementation with 90mg/d of MK-4	1 Week	Young men (n=12)	After one week supplementation, relative glucose intolerance was improved	[35]
Supplementation with 30mg/d of MK-4	4 Weeks	Young men (n=33)	Increase in insulin sensitivity index, no change in glucose	[34]
Supplementation with 1mg/d PK	1 Year	Post-menopausal women (n=42)	No change in HOMA-IR	[36]

Body fat and its role in insulin resistance, inflammation and VK status

Independent of VK, obesity influences a person's susceptibility for IR in part through an increase in circulating pro-inflammatory cytokines. As adipocytes increase in size and number, the macrophages associated with them, known as adipose tissue macrophages (ATMs), also increase.[38] These ATMs secrete inflammatory cytokines which causes an obese individual to be in a systemic low grade pro-inflammatory state.[38,40–43] Weight loss can lead to improvements in circulating pro-inflammatory markers and insulin sensitivity. Although these improvements in IR may be through a decrease in the amount of pro-inflammatory cytokines produced and secreted by ATMs, decreases in these cytokines may also be observed if the fat loss associated with weight loss leads to the liberation of fat-soluble compounds that can modulate inflammation. Arguably, greater circulating VK associated with loss of fat could reduce pro-inflammatory cytokines. This, however, has yet to be determined.

Osteocalcin and Insulin Resistance Risk

A different theory that implicates VK in IR, albeit in the opposite direction, proposes that IR is reduced by increasing circulating concentrations of the undercarboxylated form of OC (ucOC).[7,44] This theory is based on experiments that used genetically-modified mice that lacked the OC gene. Over time, these mice became fat and developed IR.[7] In a follow up study using male C57Bl/6J mice, injections of 3ng ucOC/hr increased circulating insulin and decreased circulating glucose concentrations.[44] In vitro and in vivo evidence suggested that fully carboxylated osteocalcin was not functional in this regard. Although this theory may hold true in mice, it has limited application to humans due to species-specific differences in OC which includes differences in VK intake.[9]

Additionally, alteration in ucOC concentrations in mice via osteoblast specific targeted mutations have produced results that do not fully support the original model.[45,46] For example, when floxed mice had a loss of tuberous sclerosis 2 (Tsc2) in the osteoblast which ordinarily regulates the mammalian target of rapamycin (mTOR), signaling by mTOR is increased. This results in a mouse with a phenotype of impaired glucose metabolism. Paradoxically, circulating ucOC was increased in these mice. The authors speculated that exposure to high levels of ucOC may desensitize its target tissue or that Tsc2 may regulate the gamma-glutamyl carboxylase.[45] However, when a separate group of researchers knocked out the gamma glutamyl carboxylase in the osteoblasts of mice the expected increase in ucOC was observed. With this increased ucOC, no differences were observed in body weight, blood glucose or insulin concentrations, as well as pancreas weight, compared to control mice at 32 weeks.[46]

These two contrasting results call into question the actions of ucOC on glucose metabolism in mice.

The carboxylation of OC is dependent on VK. Osteocalcin is secreted by osteoblasts and in the presence of VK, undergoes a posttranslational modification of three glutamic acid residues to become carboxylated; and with the addition of calcium, osteocalcin undergoes a conformational change that facilitates its binding to hydroxyapatite.[47] Both carboxylated OC (cOC) and ucOC can be found in human circulation and measured either alone or as a composite known as total OC (tOC). Both reflect osteoblastic synthesis of the protein. Measuring the different forms of OC in circulation is thought to reflect different physiological processes, as described in Table 3.

Table 3: OC measures and their proposed utility as a physiological marker

OC Form	Indicator of	Species	Reference
Total OC (tOC)	Bone formation	Multiple, including human and mouse	[48]
Carboxylated OC (cOC)	Bone formation	Unknown	
Undercarboxylated OC (ucOC)	Stimulate insulin secretion	Mouse	[7,44,49]
Percent ucOC (%ucOC)	Vitamin K status	Human	[50]

The study proposing a role for ucOC in IR was conducted in a transgenic mouse model. Since the publication of these results, multiple secondary analyses have been conducted to determine if the association can also be observed in humans (Table 4).[9] These secondary analyses, however, possess many limitations. In the mouse model, it was the uncarboxylated form of OC that was associated with a reduction in IR.[44,51,52] In contrast, in most human studies, tOC was the form of OC associated with measures of IR, presumably as a measure of convenience given that tOC is a standard measure of

bone formation and ucOC is both difficult to measure and encompassed in the measurement of tOC. However, authors have attributed the mechanism underlying any positive associations between tOC and IR to that of ucOC, as proposed in the mouse model.[9] In addition, almost all circulating OC is fully carboxylated in wild type mice whereas approximately fifty percent of OC is fully carboxylated in humans.[9] Finally, it is important to express ucOC as a percentage of the total OC to account for the difference between tOC and ucOC – which depends on vitamin K status/intake. This percentage of undercarboxyled OC (%ucOC) has been used as an indicator of VK status.[53] Vitamin K's role as an enzyme co-factor is post-translational, which means that VK status has no impact on tOC concentrations, but is critical for the relative amounts that are carboxylated or undercarboxylated. Higher %ucOC concentrations may be attributed to inadequate consumption of VK in humans.[10,54] In contrast, mice rarely have VK deficiency, in part because the VK in standard chow is high compared to human VK intake and due to the practice of coprophagy, which provides an unmeasured consumption of MK and contribute to carboxylation of OC if absorbed.[55] Unfortunately, the majority of research to date have not interpreted their data in context of this important nuance regarding the biochemical role of VK in OC carboxylation.

Manipulation of VK is the only known mechanism to change OC carboxylation in humans.[56] Therefore in studies elucidating the association between OC and IR, it is important to account or control for a person's VK intake to minimize this confounding feature. To our knowledge few of the studies of ucOC and IR in humans have accounted for this. Those that did have reported null findings between measures of ucOC and IR.[50]

Bone and insulin resistance

The association between bone mineral density, glucose metabolism and diabetes is well established. Alterations in bone turnover have been observed with IR, as well as insulin- and non-insulin dependent diabetes (T1DM and T2DM respectively).[57–62] This is through the anabolic actions of insulin.[57,58] Osteoblasts possess an insulin receptor, that when stimulated, leads to increases in bone turnover as indicated by higher concentrations of bone turnover markers, including OC.[58,63] Although individuals with T2DM have higher bone mineral density (BMD) compared to control participants, they are also at a higher risk for fracture. The underlying mechanism(s) by which these two phenomena occur is not clearly understood. Human studies conducted to determine if bone turnover is up- or down-regulated in T2DM have reported mixed results,[62,64] which may be explained by not controlling for variance in treatment modalities (such as diet vs insulin injection).[65] Changes in bone turnover markers in T2DM, however seem to be universal and not unique to one particular protein or bone marker.[64] This would indicate an overall effect of glucose metabolism on bone turnover. Therefore, to rule out an overall effect of changes in glucose metabolism on bone turnover, compared to an effect specific of OC on IR, it would be important to measure an additional bone marker. This would clarify if OC is acting independently or simply acting as a marker of changes in bone turnover. Few studies have reported using multiple bone markers, making it difficult to isolate the role of OC per se. In a recent study, the bone formation marker, procollagen type 1 amino-terminal propeptide (P1NP), but not OC, was associated with HOMA-IR in overweight subjects.[60] The study population was young (34.9 ± 8.3) and

VK was not assessed. It is unknown if similar results would be observed in an older population and whether controlling for VK would influence the observed association. Nonetheless, these data challenge the assumption that exclusive measurement of OC is sufficient to conclude causality. In addition, it would be beneficial to look at %ucOC since higher absolute amounts of circulating ucOC are not informative if changes in tOC are not accounted for. I propose to address this limitation of prior studies by including tOC, cOC ucOC and %ucOC as well as VK in a well-characterized cohort with corresponding measures of IR, body composition and bone turnover. In addition, I will include additional measures of bone formation, such as P1NP, to differentiate between overall bone effects versus a unique effect of OC on IR and body composition.

Table 4. Cross-sectional associations between bone turnover markers and glucose metabolism and adiposity in non-diabetic children and adults (Adapted from Booth et al, 2012[9])

Measure of Glucose Metabolism and Adiposity	n	Measures of Bone Formation			Measures of Bone Resorption	Reference
		tOC[a]	ucOC	Other		
Glucose Metabolism						
HOMA-IR	2493 M+F	inverse [b]	-[c]	-	-	66
	1597 M	inverse	inverse	-	no effect (TRACP)	67
	580 M+F	inverse	-	-	-	68
	380 M+F	inverse	-	-	no effect (NTX)	69
	348 M+F	inverse	no effect	-	no effect (NTX)	50
	199 M	-	inverse	-	-	70
	106 M+F (children)	no effect	inverse (Caucasian only)	-	-	71
	36 F (children)	no effect	-	positive (PICP)	positive (NTX)	72
Insulin (Fasting)	2493 M+F	inverse	-	-	-	66

	380 M+F	inverse	-	-	no effect (NTX)	69
	348 M+F	no effect	no effect	-	no effect (NTX)	50
	199 M	-	no effect	-	-	70
	140 M+F (children)	no effect	no effect	-	-	61
	106 M+F (children)	no effect	no effect	-	-	71
	83 M	no effect	no effect	-	-	73
	36 F (children)	no effect	-	positive (PICP)	positive (NTX)	72
Glucose (Fasting)	2493 M+F	inverse	-	-	-	66
	1597 M	inverse	inverse	-	no effect (TRACP)	67
	380 M+F	inverse	-	-	no effect (NTX)	69
	348 M+F	inverse	no effect	-	no effect (NTX)	50
	199 M	-	inverse	-	-	70
	140 M+F (children)	no effect	no effect	-	-	61
	106 M+F (children)	positive	no effect	-	-	71

	83 M	no effect	inverse (obese only)	-	-	[73]
	64 M+F (obese)	inverse	-	no effect (P1NP)	-	[74]
Adiposity						
% Body Fat	443 M+F	inverse (just in women)	-	-	-	[75]
	380 M+F	inverse	-	-	no effect (NTX)	[69]
	307 M+F	inverse	-	-	no effect (NTX)	[76]
	106 M+F (children)	inverse	no effect	-	-	[71]
BMI	2493 M+F	inverse	-		-	[66]
	380 M+F	inverse	-	-	no effect (NTX)	[69]
	106 M+F (children)	inverse	inverse (males only)	-	-	[71]
	83 M	no effect	no effect			[73]

[a]F, females; M, males; tOC, total osteocalcin; ucOC, uncarboxylated osteocalcin, [b]Only significantly statistically ($P<0.05$) associations were indicated by direction, [c]No measures were reported

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3. Chapters

3.1 Osteocalcin carboxylation is not associated with body weight or percent fat changes during weight loss in post-menopausal women

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Abstract: Osteocalcin (OC) is a vitamin K-dependent bone protein used as a marker of bone formation. Mouse models have demonstrated a role for the uncarboxylated form of OC (ucOC) in energy metabolism, including energy expenditure and adiposity, but human data are equivocal. Purpose: To determine the associations between changes in measures of OC and changes in body weight and percent body fat in obese, but otherwise healthy post-menopausal women undergoing a twenty-week weight loss program. Methods: All participants received supplemental vitamins K and D and calcium. Body weight and body fat percentage (%BF) were assessed before and after the intervention. Serum OC [(total (tOC), ucOC, percent uncarboxylated (%ucOC)], and procollagen type 1 N-terminal propeptide (P1NP; a measure of bone formation) were measured. Results: Women lost an average of 10.9±3.9 kilograms and 4%BF. Serum concentrations of tOC, ucOC, %ucOC, and P1NP did not significantly change over the twenty-week intervention, nor were these measures associated with changes in weight (all $p>0.27$) or %BF (all $p>0.54$). Conclusion: Our data do not support an association between any serum measure of OC and weight or %BF loss in post-menopausal women supplemented with nutrients implicated in bone health.

Key words: Osteocalcin, vitamin K, weight loss, body fat

Introduction:

Osteocalcin (OC) is a vitamin K- (VK) dependent protein found primarily in bone. It is synthesized in osteoblasts[1] and undergoes a VK-dependent post-translational modification that results in carboxylation of specific glutamate residues. In its carboxylated (cOC) form, OC binds to hydroxyapatite in bone.[2] Serum concentrations of OC have been used to assess bone formation,[3] whereas the percent of OC that is not carboxylated (%ucOC) is used as a measure of VK status.[4] Although the Adequate Intake for VK in the form of phylloquinone (vitamin K_1) has been established at 120 and 90 μg phylloquinone per day for men and women respectively, intakes of 100μg phylloquinone per day do not result in complete carboxylation of osteocalcin.[5]

Based on studies in mice, OC, specifically the uncarboxylated (ucOC) form, regulates energy metabolism, including energy expenditure and measures of adiposity.[6] Subsequent studies corroborated the findings in mice and extended the hypothesis regarding the influence of ucOC to glucose metabolism, male fertility, and brain development.[7–14] Attempts to replicate these findings in humans have been inconclusive. Most analyses of the association between OC and metabolic outcomes in humans are limited by the reliance on the exclusive measurement of total OC (tOC) and not ucOC.[2] Furthermore, observed associations may be simply tracking a larger influence of changes in energy balance on bone turnover.

The purpose of this study was to determine the associations between measures of OC and weight loss or body fat changes in healthy overweight/obese post-menopausal women undergoing a weight loss program. All women received supplemental vitamins D and K and calcium, which would minimize the potential

influence of inadequate or varied micronutrient intake on bone loss and OC carboxylation.[15–18] Multiple forms of OC were measured, along with an additional marker of bone formation to control for changes in bone turnover that might accompany weight loss or reduction in body fat.

Materials and Methods:

Data were obtained from the Diet, Exercise and Metabolism for Older Women (DEMO) study, a twenty-week randomized trial comparing the effects of caloric restriction (CR) with and without aerobic exercise on body composition changes and weight loss described elsewhere.[19] This study and protocol were approved by the Wake Forest University School of Medicine Institutional Review Board and the Tufts University School of Medicine Institutional Review Board, and all participants provided written informed consent (clinicaltrials.gov #NCT00664729).

Study Participants

Women were recruited from the Forsyth County, NC area. Inclusion criteria were sedentary post-menopausal women between 50-70 years of age, self-reported not receiving hormone replacement therapy of any type, self-reported weight stable for six months prior to enrollment, with a BMI between 25-40 kg/m^2 and waist circumference >88cm. Exclusion criteria included those with systemic disorders, uncontrolled diabetes and/or taking diabetic medications. Of the 1078 women initially screened, a total of 112 were randomly assigned to one of three interventions.[19] For this study, only women who

had sufficient archived sera to conduct complete measurements from both time points were included (n=71).

Anthropometrics

Absolute total, lean and fat body mass were measured pre- and post-intervention using dual-energy x-ray absorptiometry (DXA, Hologic Delphi QDR, Bedford, MA) and used to calculate percent body fat (%BF). Height was measured using a stadiometer and weight with a body weight scale. Height and weight were measured with the participant shoeless and wearing minimal clothing.[19]

Intervention Groups

The three intervention groups were controlled for an average 400kcal/d (2800 kcal/wk) deficit with similar diet composition between groups (25-30% fat, 15-20% protein and 50-60% carbohydrate). Participants received counselling on breakfast choices while food for lunch, dinner and snacks were provided for the course of the study. The caloric restriction-only group (CR) had all caloric deficits through diet restriction alone. In addition to diet restriction, womenin the Moderate Intensity Exercise and Vigorous Intensity Exercise groups were prescribed an exercise regimen equal in intensity to 45-50% (moderate) or 70-75% (vigorous) heart rate reserve (HRR) three times per week as described in detail in the parent paper.[19] All participants regardless of intervention were also given a daily dietary supplement (Viactiv®, Viactiv Lifestyle®) that provided 1000 mg/d calcium carbonate, 200 IU/d Vitamin D3 and 80 µg/d VK (phylloquinone).

Biochemical Measures

Blood samples were collected from each participant pre- and post- intervention (0 and 20 weeks respectively) in commercial EDTA-containing evacuated tubes by venipuncture in the early morning after a 12-h fast. All samples were stored for less than five years at -70^{o}C without multiple freeze-thaw cycles. Samples were then shipped frozen to the Human Nutrition Research Center on Aging at Tufts University (Boston, MA) for biochemical analyses. The stability of tOC under these conditions has been tested out to one year with no significant difference between fresh and stored frozen sample.[20]

Total and uncarboxylated plasma OC concentrations were determined by radioimmunoassay before (to determine tOC) and after (to determine ucOC) separation by hydroxyapatite binding, respectively, at the Human Nutrition Research Center on Aging at Tufts University (Boston, MA) as described in detail elsewhere.[21] The inter-assay coefficients of variation (CV) for total OC was 24.7%. Serum phylloquinone (vitamin K1) was determined using high-performance liquid chromatography (HPLC).[22] Plasma vitamin D (25-(OH)D) was measured by radioimmunoassay (DiaSorin, Stillwater, MN).[23] Plasma triglycerides were measured using standard methods as previously described.[19] Intact N-terminal propeptide of type I procollagen (P1NP) was assessed by radioimmunoassay (Orion Diagnostica, Finland).[24] According to the Orion website, the sensitivity of the P1NP assay is 2 µg/l with specificity of 2.7% (http://www.oriondiagnostica.com/).

Statistical Analysis

In the parent study, there were no significant differences in baseline weight or weight loss when compared among intervention groups ($p>0.29$).[19] Similarly, there were no statistical differences in measures of OC at either time point among the three groups (all $p>0.13$, **Supplementary Table 1**). Therefore, the participants from all three groups were pooled for the current analysis. Baseline and follow up characteristics were summarized using mean ± standard deviation (normally distributed data), medians± interquartile range (skewed data) or proportions as appropriate. General linear models were used to determine if changes in serum concentrations of OC (ucOC, tOC and %ucOC) predicted change in body weight or %BF. Covariates included age, race (non-Hispanic white or African-American), and treatment group. Similar methods were used to determine if changes in serum phylloquinone, 25-(OH)D and P1NP predicted change in either body weight or %BF. Since VK transport is dependent on triglyceride-rich lipoproteins,[25] plasma triglycerides were included as a covariate in the VK the model while cholesterol was included in the 25-(OH)D model. Spearman correlations between tOC and ucOC (ng/mL) and tOC and %ucOC (%) were calculated. The statistical software SAS version 9.3 (SAS Institute Inc, Cary, NC) was used to analyze all data. Significance was set at $p<0.05$.

Results

At baseline, women were 58.7±5.4 years of age and had a mean BMI of 33.0±3.8 kg/m^2 (**Table 1)**. Women lost an average of 10.9±3.9 kg (12.5%) and 3.9±2.0 %BF during the intervention. The median ± interquartile range for ucOC was 1.8±3.0 ng/mL

at baseline, and did not significantly change over the course of the intervention (p=0.09). At baseline, fasting plasma phylloquinone concentrations were higher than observed in the general population.[22,26,27] while 25-(OH)D concentrations were similar to those reported in the general population (47-65nmol/L).[28,29] Plasma phylloquinone did not significantly change (p=0.17) whereas 25-(OH)D concentrations increased (p<0.05) over the course of the intervention.

Percent change in OC, independent of form, did not predict change in body weight or %BF (**Table 2**). Additionally, changes in phylloquinone, 25-(OH)D and P1NP did not predict changes in body weight or %BF (**Table 2)**. Total OC and ucOC at baseline were significantly correlated (r=0.64, p<0.001). A similar correlation was observed post-intervention (r=0.65, p<0.001). In contrast, there was no significant correlation between tOC and %ucOC at baseline(r=-0.13, p=0.29) or post intervention (r=-0.06, p=0.60), indicating that the absolute amount of ucOC may simply be tracking overall tOC.

Discussion

In this study of post-menopausal women enrolled in weight loss interventions, changes in tOC, ucOC and %ucOC were not significantly associated with weight loss or changes in %BF.

Prior human studies on the role of OC in energy metabolism have been equivocal. Some cross-sectional studies have reported higher serum concentrations of ucOC and tOC associated with lower body weight and measures of body fat.[30–39] Others have reported no associations.[40,41] In the one longitudinal study, a three-month increase

in ucOC was associated with a twelve-month decrease in body fat.[42] However, we demonstrated in our study that absolute ucOC in humans is correlated with tOC. Total OC is a robust marker of bone formation so significant findings using absolute ucOC concentrations may simply be tracking a larger influence of changes in energy metabolism on bone turnover, independent of any unique effect of ucOC.

To account for the high correlation between absolute ucOC and tOC concentrations, we calculated %ucOC to differentiate between carboxylation and total amount of circulating OC. In our study, measures of %ucOC were also not associated with weight loss or changes in %BF. Since both ucOC and %ucOC did not change but women still lost weight, it is unlikely that ucOC has a unique role in weight or %BF loss. This is supported by our previous work which found no form of OC associated with weight loss in older men and women.[40] Additionally, weight loss in a VK supplementation trial was not associated with ucOC.[41] However neither of these studies were designed to specifically promote weight loss in a controlled manner whereas the strength of the current study design is the intentional weight loss through clearly defined interventions combining diet and exercise. Women achieved weight loss through caloric restriction and participation in an exercise regimen of moderate or vigorous intensity three times per week.[19] Similar to earlier reports finding no link between intensity of aerobic exercise and OC concentrations, we also found that no form of OC was associated with any intensity of aerobic exercise performed by post-menopausal women.[43–47]

Another strength of our study is the inclusion of an additional independent bone formation marker to separate the putative OC effect from an overall bone effect. In our

study neither ucOC nor P1NP was associated with body weight or %BF changes. This indicates no effect of bone turnover on weight loss, or conversely, no effect of the degree of weight loss on bone turnover. Additionally, intake of nutrients known to influence bone turnover was supplemented, which is likely to mitigate their influence.[48,49] Since the majority of previously published studies did not take into account the status of nutrients involved in bone turnover, it is unclear how this difference in nutrient status may affect the reported results.[31,38,42]

In the mouse model, only the ucOC form was responsible for weight loss.[6] In humans, the only known mechanism by which carboxylation of OC is modified is through manipulation ofVK.[50] In the current study, the carboxylation of OC was kept constant in part because of low dose VK supplementation. However, participants still lost weight which suggests that the carboxylation of OC is unrelated to weight loss in humans. In other studies using much higher doses of VK supplementation (500 to 1,000 µg/day compared to the current 80 µg/day), there was also no observed association between ucOC and measures of body weight or body fat.[40,41]

Limitations of this study merit consideration. This was a secondary analysis and not the primary outcome of the overall study. Because this study was an observational analysis, we cannot establish causality. Our sample limits the generalizability of the results to post-menopausal women. Additionally, there is a high amount of bone turnover in the immediate time point following onset of menopause which subsides after about 5 years. Since there were some women who were less than five years post-menopausal, we conducted an analysis to determine if OC was different between these two groups and found no difference. Therefore, we included all women in the analysis.

The stability of OC has not been published for greater than one year timeframe, therefore, it is unknown if some degradation occurred from serum collection to analysis. Finally, since this study was conducted, the Institute of Medicine modified the Recommended Dietary Allowance for vitamin D from 400 to 600 IU/d.[51] Although at the time these women were considered vitamin D-replete, their intakes would fall below current recommendations. In contrast, our study was strengthened by the controlled feeding design because calorie and nutrient intake as well as weight change were closely monitored.

Conclusions

Our data do not support a role for the carboxylation of OC to affect body weight or fat loss in post-menopausal women undergoing a weight loss intervention with and without aerobic exercise of differing intensities in which nutrients known to affect bone turnover are supplemented. That P1NP was also not associated with weight or %BF loss additionally suggests that bone formation is not associated with changes in body composition in nutrient-supplemented conditions.

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Table 1. Participant characteristics at baseline and 20-week follow-up (n=71) (mean ± SD or median ± IQR)

	Baseline	20 –Week Follow-up	p-value
Age at Baseline (yrs)	58.7±5.4		
Years Postmenopausal (yrs)	14.2±10.3		
White, non-Hispanic (%)	72%		
Weight (kg)	88.5±11.3	77.6±10.4	<0.001
BMI (kg/m^2)	33.0±3.8	29.0±3.5	<0.001
Body Fat (%)	42.8±3.3	38.8±4.1	<0.001
Abdominal Visceral Fat (cm^3)	2210±698	1639±579	<0.001
Plasma Phylloquinone (nmol/L)	1.3±0.9*	1.4±1.3*	0.17
Plasma 25-(OH)D (nmol/L)	52.4±18.5	62.2±18.2	<0.001
Triglycerides (mmol/L)	1.35±0.85*	1.12±0.56*	<0.001
Total Osteocalcin (ng/mL)	5.9±3.3	5.8±3.4	0.85
Uncarboxylated Osteocalcin (ng/mL)	1.8±3.0*	1.8±2.3*	0.09
Uncarboxylated Osteocalcin (%)	39.5±23.8	33.9±20.1	0.06
P1NP (ug/L)	53.3±23.0*	54.0±25.6*	0.54

*presented as median ± interquartile range

Table 2: Predicted Percent change in body fat percentage, body weight or BMI associated with 1 unit change in biomarker

	Outcome					
	% Change in %Body Fat		% Change in Body Weight (kg)		% Change in BMI (kg/m^2)	
	Beta Coefficient	p-value	Beta Coefficient	p-value	Beta Coefficient	p-value
% Change in OC						
ucOC (ng/mL)*	0.005	0.42	-0.005	0.31	-0.005	0.29
tOC (ng/mL)*	0.004	0.49	0.005	0.27	0.006	0.25
%ucOC (%)*	0.005	0.42	-0.005	0.31	-0.005	0.29
% Change in vitamin K (nmol/L)**	-0.006	0.26	-0.006	0.14	-0.006	0.14
% Change in P1NP (ug/L)*	0.003	0.86	-0.003	0.84	-0.003	0.84
% Change in 25-(OH)D (nmol/L) [x]	0.003	0.77	0.006	0.48	0.006	0.49

*adjusted for age, race, and treatment group; **adjusted for age, race, treatment group, and percent change in triglycerides

[x]adjusted for age, race, treatment group, and percent change in cholesterol

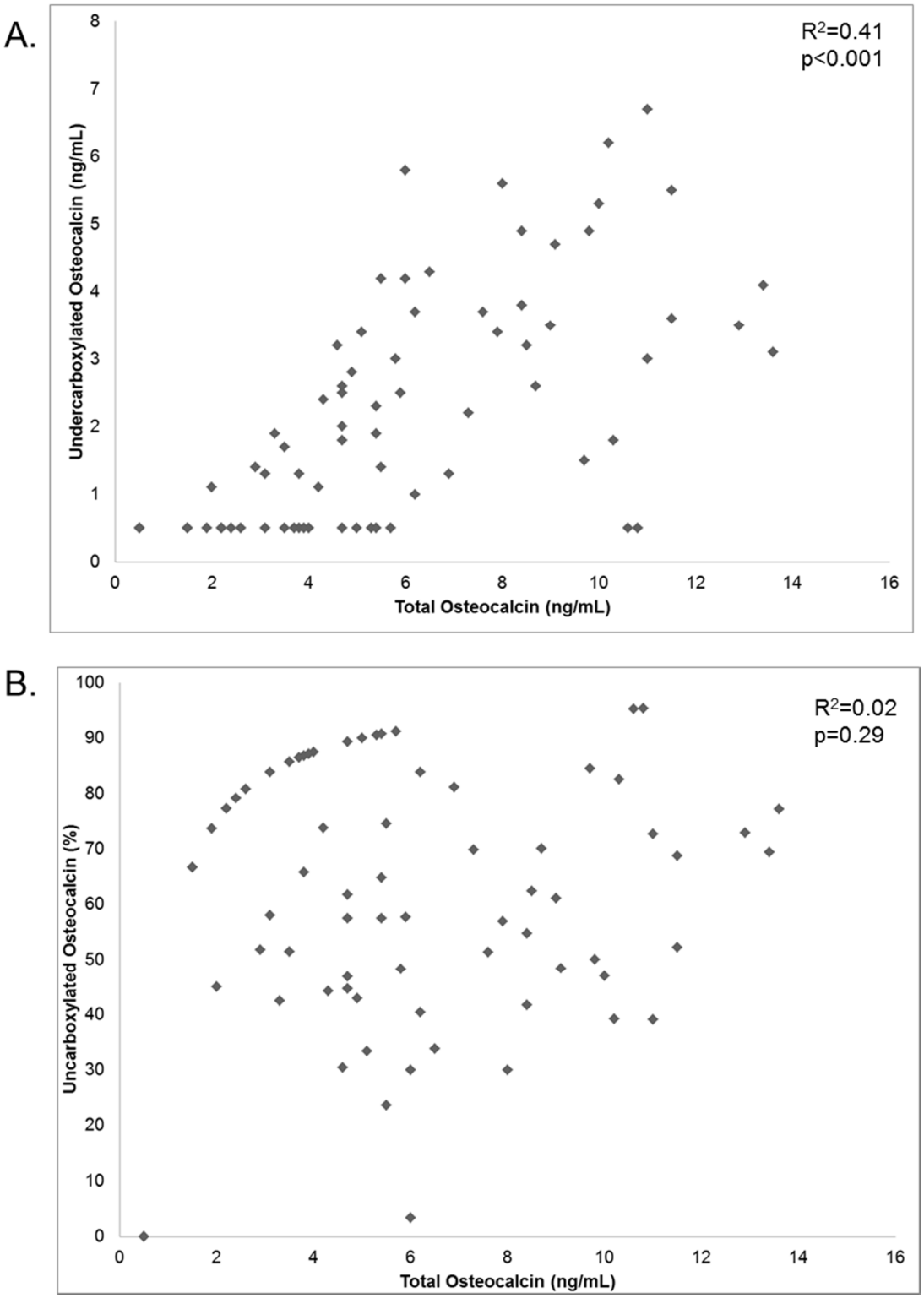

Figure 1: Correlations of total osteocalcin to ucOC (A) and %ucOC (B).

Supplementary Table 1. Baseline and absolute change in measures of OC by group*.

	Caloric Restriction (n=20)		**Caloric Restriction + Moderate Exercise (n=28)**		**Caloric Restriction + Vigorous Exercise (n=23)**	
	Baseline	Absolute Change	Baseline	Absolute Change	Baseline	Absolute Change
Total OC (ng/mL)	4.86±2.96	-1.22±17.15	6.79±3.27	-5.85±27.22	5.74±3.48	-9.17±28.38
ucOC (ng/mL)	1.68±1.47	0.45±2.58	2.66±1.92	-0.10±3.32	2.12±1.60	-0.48±3.03
%ucOC (%)	34.49±21.34	0.02±0.91	40.31±22.60	-0.35±1.73	42.95±27.40	-0.42±1.11

*No significant difference between groups (p>0.05).

3.2 Circulating Undercarboxylated Osteocalcin is Not Associated with HOMA-IR

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Abstract:

Background: The under carboxylated form of osteocalcin (ucOC) has been linked to improved glucose metabolism. Increasing the intake of vitamin K (VK) is the only known factor to reduce the proportion of circulating ucOC in humans. The purpose of this study was to determine if decreasing ucOC via VK supplementation would increase insulin resistance measures in humans. *Materials & Methods:* Healthy younger and older men and women (n=42, age=20-40y or 55-80y, 50% female) were given a VK supplement (500 µg phylloquinone/d) for twenty one days. Circulating measures of ucOC, VK, fasting glucose and insulin were measured before and after supplementation. Glucose and insulin measures were used to calculate the homeostatic model (HOMA-IR). General linear modeling was utilized to determine if changes in ucOC or VK would influence insulin resistance. Measured covariates included body mass index, sex, age-group and triglycerides. Pearson correlations were also calculated between ucOC, VK and HOMA-IR. *Results:* With supplementation, circulating ucOC decreased (pre= 4.79 ± 2.61ng/mL post= 1.59 ± 2.11ng/mL, $p<0.001$) whereas there was no change in HOMA-IR (pre= 2.20 ± 0.98 post= 2.25 ± 1.12, $p=0.78$). No statistically significant correlation between ucOC and HOMA-IR was observed (all $p>0.09$). *Conclusion:* The lack of association between changes in ucOC and HOMA-IR suggests that in humans, ucOC does not have a role in insulin resistance.

Clinical Relevancy Statement

Vitamin K determines the carboxylation of the bone formation protein, osteocalcin (OC), in humans. The undercarboxylated form of OC (ucOC) has been reported to increase insulin sensitivity, which by inference would suggest that vitamin K supplementation would increase insulin resistance in patients. When vitamin K status was controlled for in the present study, ucOC has no association with insulin resistance. These findings are clinically relevant to clinicians who are looking to give their patients information on specific nutrient dietary components that may influence insulin resistance.

Introduction

The rates of insulin resistance (IR) and diabetes have steadily increased.[1–3] Alterations to biochemical pathways and protein action that can lead to increased risk of developing IR have been the focus of much research, including the protein osteocalcin (OC). Higher concentrations of circulating undercarboxylated osteocalcin (ucOC) are associated with improved glucose metabolism in mice.[4,5] However, similar studies in humans are equivocal.[6]

Osteocalcin is a well-characterized protein known to be involved in bone formation.[7] It is produced in the uncarboxylated form, and undergoes a vitamin K (VK) - dependent post-translational gamma-carboxylation to convert its three Glu residues into Gla residues. It is only when these three residues are fully carboxylated that OC is able to bind to hydroxyapatite and calcium in bone.[8] Because of its dependence on VK for carboxylation to occur, the percentage of OC that is not fully carboxylated (%ucOC) has been used as a measure of VK status, with lower %ucOC representing higher circulating VK. Despite the relationship between OC carboxylation and VK, few human studies have taken VK status into account when associating OC with IR. Observational studies of VK intake and IR have found higher intakes of VK associated with lower IR.[9] Additionally, VK intake has been used as a marker of a healthy diet.[10] Both of these are in contrast to the theory that high ucOC (and thus low VK) would be protective against IR. In this study, we sought to elucidate whether a decrease in in circulating ucOC

through VK supplementation would increase insulin resistance (IR) measures in humans.

Research Design and Methods

Data were obtained from a metabolic study determining the dietary and non-dietary influences of VK status.[11] The study and protocol were approved by the Tufts University Institutional Review Board, and all participants provided written informed consent (clinicaltrials.gov # NCT00336232).

Subjects

This study enrolled forty-two healthy younger (n=21, age=18-40y) and older (n=21, age=55-80y) men and women. For the purpose of this analysis, we utilized two time points from the larger study: 1) Pre-supplementation, after a 21-d VK deplete diet (10 μg/d phylloquinone) and 2) Post-supplementation, after a 21-day VK supplemented diet (500 μg/d phylloquinone), to determine if manipulation of OC carboxylation would affect HOMA-IR. All meals and beverages during the course of this study were provided to control for kilocalories and other nutrients. Height in meters and weight in kilograms were measured and used to calculate body mass index (BMI).

Biochemical Assessment

Serum was collected pre- and post-supplementation following a 12-h fast. All samples were stored at -70ºC without multiple freeze-thaw cycles. Radioimmunoassays were used to measure total OC (tOC, a measure of bone formation), ucOC and insulin.[7] Glucose was measured by an enzymatic kinetic method. Plasma triglycerides (TG) were measured with a Hitachi 911 automated analyzer (Hitachi Ltd, Japan).

Statistical Methods

The homeostatic model assessment of IR (HOMA-IR) was calculated[12] from fasting measures of insulin and glucose. Percentage of ucOC (%ucOC, a measure of VK status[13]) was also calculated. Repeated measures ANOVA via mixed linear models with random intercepts (proc mixed, SAS v 9.3, SAS Institute Inc, Cary, NC) was used

to determine if ucOC, %ucOC, tOC and HOMA-IR changed with VK supplementation. Since previous research has reported a significant difference between sexes,[9] we tested for effect modification by sex, but none was detected (all interactions p>0.13). All models were adjusted for sex, BMI, age-group and TG because TGs are required for VK absorption and transport.[14] We also determined the Pearson correlations between all measures of OC (ucOC, tOC, %ucOC) with HOMA-IR. Baseline and follow up characteristics were summarized using mean ± standard deviation (normally distributed data), medians ± interquartile range (skewed data) or proportions as appropriate. Significance was set at $p<0.05$.

Results

Participant characteristics pre- and post-supplementation are provided in Table 1. At baseline, participants mean BMI was 25.4 kg/m^2. Baseline fasting glucose and insulin measures were 94.5 mg/dL and 9.4 uU/dL, respectively. As expected with VK depletion, ucOC and %ucOC were higher at pre-supplementation vs. post-supplementation.

The percent change in %ucOC was not correlated with the percent change in HOMA-IR (p=0.87). In addition, no measure of OC was correlated with HOMA-IR pre- or post-supplementation (ucOC both r<-0.24, p>0.12, %ucOC both r<-0.26, p>0.09, tOC both r<-0.22, p>0.16).

No sex differences were observed in ucOC, %ucOC, tOC or HOMA-IR at either pre- or post-supplementation (ucOC all p>0.56, %ucOC all p>0.35, tOC all p>0.69, HOMA-IR all p>0.21, adjusted for BMI, age, and triglycerides). Undercarboxylated OC and %ucOC decreased by nearly 67% and 70% respectively from pre- to post-supplementation (both p<0.001) and tOC decreased by nearly 8% (p=0.015). No change in HOMA-IR (p=0.78) was observed with supplementation (Figure 1).

Discussion and Conclusions

Through the use of a controlled dietary study which manipulated VK intake, we demonstrated that a 67% reduction in circulating ucOC had no impact on HOMA-IR in younger and older healthy adults. Our findings do not support prior observational data

that report an association between high circulating concentrations of ucOC and decreased risk of IR.[15–17]

The proposed association between ucOC and IR in humans originated from a mouse model where it was determined that injecting mice with ucOC led to improved insulin sensitivity.[5] While ucOC may have an endocrine function in mice, there have been challenges in translating this theory to humans.[18] A number of secondary analyses in humans have used tOC in place of ucOC as the primary OC exposure since tOC is a common biomarker for bone turnover.[6] Generalizing the use of ucOC in rodent studies[6] to the use of tOC in human studies is inappropriate since tOC and ucOC concentrations do not reflect identical influences and because human and rodent OC metabolism differs.[18] In the current study, we were able to analyze tOC, in addition to ucOC and %ucOC. The lack of change in HOMA-IR associated with changes in any OC form would suggest no role for any form of OC in human glucose metabolism.

A strength of our study was the direct manipulation of VK intake, which allowed us to directly influence the carboxylation of OC and compare the HOMA-IR within individuals during VK repletion and VK depletion. Another was control for factors that can independently influence circulating ucOC, including VK status, sex, triglycerides and age. The following limitations of this study merit consideration. This was a secondary analysis and not the primary outcome of the overall study.[11] The study participants were overall metabolically healthy and had average body weights on the lower limit of overweight, which may have attenuated our ability to detect measurable differences in HOMA-IR. However, we felt it was important to elucidate any association in healthy individuals first since it is in this group of individuals where interventions and preventative measures would be most beneficial.

In conclusion, although circulating concentrations of ucOC significantly decreased by 67%, no associated increase in HOMA-IR was observed in younger and older men and women. This would imply that ucOC does not play a direct role in glucose metabolism in metabolically healthy adults.

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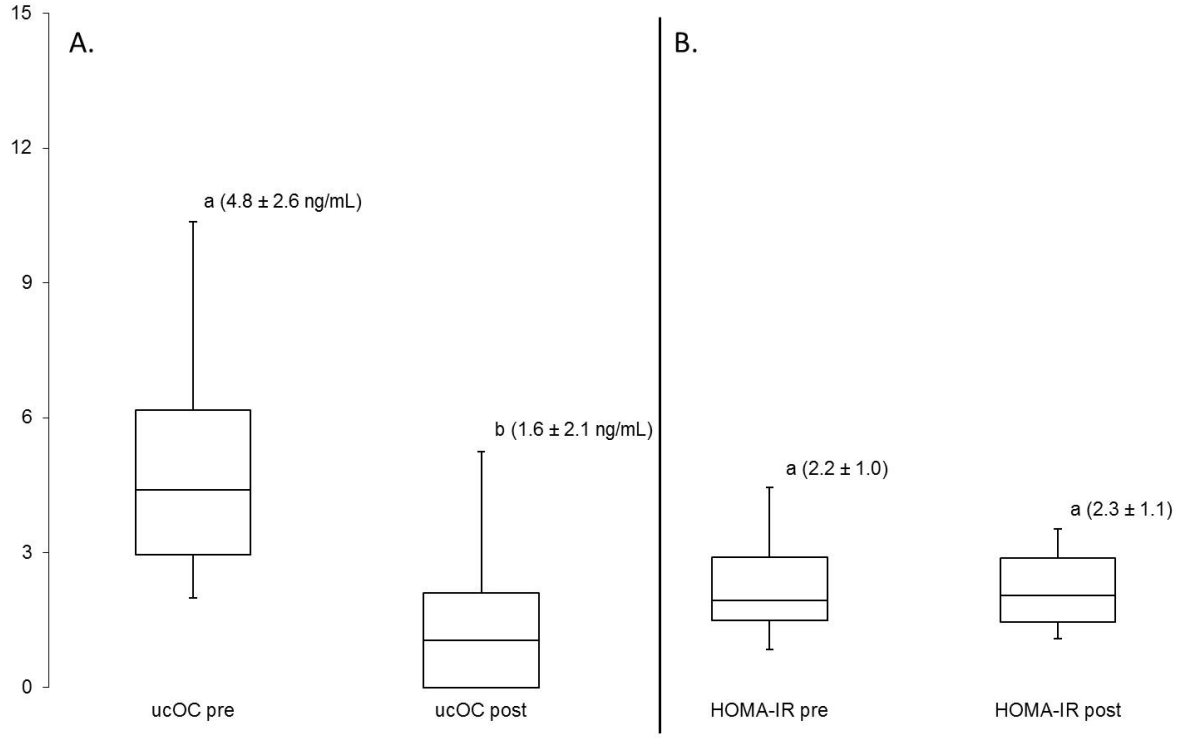

Figure 1. Box and whisker plot for A) ucOC and B) HOMA-IR in all participants (n=42). Different letter superscripts denote pre-post differences.

Table 1: Pre- Post- Characteristics (mean ± SD) of 42 healthy younger (n=21, age=18-40y) and older (n=21, age=55-80y) men (n=21) and women (n=21).

	Pre (n=42)	Post (n=42)	p-value
BMI (kg/m^2)	25.4±4.0	25.0±3.9	<0.001
Weight (kg)	74.8±13.9	73.0±13.6	<0.001
tOC (ng/mL)	9.2±4.1	8.5±3.9	0.015
ucOC (ng/mL)[a]	4.4±3.3	1.1±2.1	<0.001
%ucOC (%)	50.6±12.6	15.0±14.9	<0.001
Plasma vitamin K (nmol/L)[a]	0.2±0.2	1.5±1.2	<0.001
Triglycerides (mg/dL)[a]	96±52	98±59	0.48
Insulin (uU/dL)	9.4±4.0	9.6±4.7	0.60
Glucose (mg/dL)	94.5±8.1	95.4±9.3	0.48
HOMA-IR	2.2±1.0	2.3±1.1	0.66

[a]denotes data presented as median ± IQR

3.3 The association between vitamin K status and markers of insulin resistance in older adults: The Health, Aging and Body Composition Study

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Abstract

Background: Emerging evidence suggests a role for the vitamin K in the form of phylloquinone (PK) in insulin resistance (IR).

Purpose: To determine the cross-sectional associations between plasma PK and measures of IR in a large well-characterized cohort of older community-dwelling men and women

Methods: Participants enrolled in the Healthy Aging and Body Composition Study (HABC) with measures of plasma phylloquinone (PK) were utilized in this study. Measures of IR included fasting glucose and insulin, the homeostatic model of insulin resistance (HOMA-IR), glycated hemoglobin, 2-hour post oral glucose tolerance test glucose, and the homeostatic model of beta cell function. Linear modeling was used to determine the cross sectional associations between these measures of IR and plasma PK measures. Analyses were stratified by sex and triglycerides (<150 vs ≥150 mg/dl). Covariates included body mass index, study site location, education, presence of knee pain, race, average walking time per week and caloric and vegetable intake.

Results: A trend for HOMA-IR to be inversely associated with PK was observed in men with normal triglycerides. No significant associations were observed in women or those with high triglycerides.

Conclusion: Similar to previous studies, we found an inverse association between HOMA-IR and PK status in men; however, this association only existed in men with normal triglyceride concentrations and did not reach statistical significance.

Introduction

In the United States, the prevalence of obesity has nearly tripled since the 1960s. The rates of insulin resistance (IR) and diabetes have also steadily increased with the CDC estimating 9.3% of American living with diabetes in 2014.[1–5] The benefits of modifying a person's diet to decrease IR and diabetic symptoms have proven beneficial,[6] which has led some researchers to focus on specific nutrient components that can impact IR. While some of the data has determined strong associations between specific nutrients or dietary components and IR (such as saturated fat[7]), others remain to be fully elucidated. One such nutrient is vitamin K (VK).

Vitamin K is a fat soluble vitamin found primarily in green leafy vegetables and plant oils. The classical function of VK is as an enzyme cofactor in the post-translational gamma-carboxylation of glutamic acid (Glu) residues of VK-dependent proteins (VKDP).[8] However, there is growing evidence that other biological functions of VK may exist outside of this classical function.[9,10] These include expression and production of inflammatory cytokines and the role of VK in mitigating insulin resistance (IR).[11,12]

Although VK has been implicated in the alteration of IR,[13–16] the exact mechanism is unknown. Observational studies of VK and IR have found higher intakes associated with lower IR.[14] However, none of these studies have measured plasma concentrations of VK to determine if there is an inverse association with markers of IR. Additionally, in studies where participants were given a VK supplement, a decrease in IR was observed, as indicated by the homeostatic model assessment for IR (HOMA-IR).[14,17,18] However, these studies have found sex-specific associations, with decreases only being observed in men.[14,17,18] Therefore, we determined the cross-sectional

associations between the plasma VK form phylloquinone (PK) and measures of IR in a large well-characterized cohort of older community-dwelling men and women.

Methods

Subjects

The Healthy Aging and Body Composition study is a multi-site study seeking to understand the influencers of age-related body-composition changes and how these changes may impact disease risk. Approximately 3075 black and white community dwelling men and women were enrolled in this study between 1997-1998 from Pittsburgh, Pennsylvania and Memphis, Tennessee. Inclusion criteria included ability to walk for one-quarter of a mile, able to perform activities of daily living and ability to climb ten steps. All participants provided written informed consent and all protocols were approved by the Institutional Review Boards at both study sites.

Biochemical Analysis

For the current analysis, subjects who were selected as part of the knee osteoarthritis and VK sub-study were included.[19] Plasma PK (the primary circulating form of vitamin K) was measured from samples obtained at the year 2 clinic visit (1998-99) using reversed-phase HPLC at the Vitamin K Laboratory at the USDA Human Nutrition Research Center on Aging at Tufts University.[20] The lower limit of detection using samples volumes available was <0.2 nmol/L. Because there were no available measures of triglycerides at year 2, we used those available from the year 1 clinic visit (1997-98). Fasting measures of triglycerides were determined using a commercially available analyzer (Vitros 950; Johnson & Johnson, Rochester, NY). Interassay

coefficient of variation for triglycerides was 2.3%. Fasting measures of insulin and glucose were measured before participants underwent an oral glucose tolerance test that consisted of consuming a 75 g glucose solution.[21] Glucose was also measured two hours post glucose solution consumption. The percentage of hemoglobin with glycated proteins, an indicator of longer blood glucose regulation, was measured using Tosoh 2.2 Plus (Tosoh Bioscience Inc., Tokyo, Japan).[22]

Other Covariate Measures

Body weight and height were measured using a standard balance-beam scale and Harpenden stadiometer (Holtain Ltd., Crosswell, UK. Body Mass Index (BMI) was calculated as weight (kg)/ (height $(m)^2$). Dietary intake was estimated using a 108-item Food Frequency Questionnaire (FFQ). This FFQ was designed for the Health ABC cohort and based off of information collected from the National Health and Nutrition Examination Survey (NHANES) III.[23] Physical activity was based on self-reported time spent walking (in minutes) over the previous week. Demographic information such as age, sex and education were collected via questionnaire.

Statistical Methods

Our primary outcome was the homeostatic model of insulin resistance (HOMA-IR), a validated estimate of steady state insulin sensitivity which is calculated from fasting measures of plasma glucose (FPG) and insulin (FPI).[24] In addition to HOMA-IR, FPG, and FPI, other measures of IR included, glycated hemoglobin (HG-A1C), 2-hour post oral glucose tolerance test glucose measure (OGTT-120), and the homeostatic model of beta cell function, a measure of pancreatic beta cell activity (HOMA-B%).[24] All of these measures, excluding HG-A1C, were skewed; therefore logged values were

used in the analysis. Participants were excluded from the analysis if they were taking warfarin (since it is a VK antagonist) or diabetic drugs. An ANOVA (proc mixed, SAS v 9.3, SAS Institute Inc, Cary, NC) was used to determine if measures of IR (HOMA-IR, FPI, FPG, HG-A1C, OGTT-120, HOMA-B%) were associated with plasma PK. Since previous research from our laboratory has reported a significant difference between sexes,[14] we stratified our analyses by sex. Plasma PK is transported on triglyceride (TG)-rich lipoproteins,[25] which can confound its association with IR,[26] so we also stratified by normal (<150 mg/dL) and high TG (≥150 mg/dL).[27] All models were adjusted for BMI, study site location, education, presence of knee pain, race, average walking time per week and caloric and vegetable intake as assessed by the FFQ. Baseline characteristics are summarized using mean ± standard deviation (normally distributed data), medians ± interquartile range (skewed data) or proportions as appropriate. Significance was set at $p<0.05$.

Results:

A total of 932 participants were included in this cross sectional analysis. Baseline characteristics are included in Table 1. Since there was a significant interaction ($p<0.001$) between PK and TG for our main outcome HOMA-IR, analyses were stratified by normal (>150 mg/dL) and high TG (<150 mg/dL). In general, participants were approximately 74.6 years old and had a mean BMI of 27.7 kg/m^2. Mean plasma PK concentrations were 1.0±1.1 nmol/L while the mean HOMA-IR was 2.06±1.59. Overall, men and women with TG ≥150 mg/dL had higher markers of IR compared to those with

TG <150 mg/dL. Vitamin K was also higher in those in the high triglyceride groups compared to normal triglycerides in both sexes (both $p<0.003$).

We detected a trend for higher PK status to be associated with lower HOMA-IR in men with normal triglycerides only ($p=0.08$, Table 2), such that for 1nM increase in PK, there was an approximate 1% decrease in HOMA-IR. Otherwise plasma phylloquinone was not associated with any other measures of IR in men or with any measure of IR in women.

Discussion:

In this study, we determined the association between measures of IR and plasma PK concentrations in older, healthy community-dwelling men and women. We found a trend for PK to be inversely associated in men with normal triglycerides only. Otherwise, plasma PK was not associated with any measure of IR in older men or women. These results could suggest a limited protective role for PK in reducing IR in the absence of hyperlipidemia.

Since VK is a fat soluble vitamin, its absorption from the diet is enhanced when consumed with lipids.[28] Once absorbed, it is incorporated into triglyceride rich lipoproteins and transported through circulation.[29] Therefore, it is important to adjust for TGs in studies like the current where PK is the main exposure. While this is typically done by including TGs as a covariate in statistical models, to our knowledge this is the first study to stratify by TG status. Our findings suggest PK may be beneficial with respect to IR when TG status is normal, but not when TGs are elevated. High TGs are a known risk factor for IR,[26] and would most likely override any beneficial effect of PK in

reducing IR risk. Therefore stratification by TG status may be important to minimize the confounding that high TG can have on observed high values of circulating PK.

Consistent with others,[14,17,18,30] our findings suggest the protective effect of VK in IR may be present in males but not females, although our results only approach significance. In a previous study conducted by this laboratory, three years of PK supplementation (500ug/d) reduced HOMA-IR in men.[14] Additionally, others have reported a beneficial effect of VK supplementation with measures of glucose sensitivity in two separate groups of men.[17,18] These studies did not report any results in women. Observational studies have also reported higher VK intake was associated with lower IR.[13] However, these studies of VK intake may have been influenced by recall bias and confounded by the consumption of a healthy diet since high VK intake is a marker of a healthy diet.[31] Further research is warranted to clarify the association between PK status and IR using objectively measured biomarkers and to determine the underlying causes that may be driving different observations between sexes. The finding of PK only being associated with HOMA-IR in men with normal triglycerides is novel. We theorize that although PK may have an impact on IR, its contribution is inconsequential when larger influences like high triglycerides are present. This theory however requires further investigation.

We observed a significant trend for PK to be associated with HOMA-IR, but not HOMA-B%. Since HOMA-B% is an indicator of pancreatic beta cell activity, this may indicate that any effect VK may have on IR is outside of the pancreas, perhaps in either glucose uptake or sensitivity in the periphery or in glycogen breakdown and production in the liver. Since measures of glucose including OGTT and HGA1C were not

associated with VK, this may additionally indicate that VK influences peripheral action specifically. The mechanism by which VK may influence IR is also uncertain. One theory is that it may be through a modulation of inflammation, independent of any effect on carboxylation of VKDP. Both in vivo and in vitro studies have proposed that VK can reduce inflammatory cytokine expression and production.[25,32] Additionally, observational studies have also associated higher VK with lower inflammation markers.[33,34]

A strength of our study is the large racially diverse sample, multiple measures of IR, and stratification by TG status. Additionally, we directly associated plasma PK measures with markers of IR which to our knowledge has not been reported before. Unlike other studies determining the relationship of VK and IR,[13–18,30] we were able to adjust for both vegetable intake and physical activity, markers of a healthy lifestyle, which can reflect VK status.[31] To be included in Health ABC cohort, men and women had to be between 70-79 years old, and physically well-functioning; therefore, results may not be generalizable to dissimilar groups. The study participants were overall metabolically healthy and had average body weights on the lower limit of overweight, which may have attenuated our ability to detect measurable differences in HOMA-IR. Additionally, we did not include participants taking diabetic drugs so it is unknown if this relationship can be expanded to treated diabetics. It is our notion however that the influence of VK would be less relevant once a person reaches frank diabetes. We felt it was first important to elucidate any association in individuals who had not been diagnosed with diabetes since it is in this group where interventions and preventative measures would be most beneficial. Measures of IR were conducted at year 1 while PK measures were conducted at year 2. This may have led to misclassification of IR in

some individuals. Finally, although our trend for an association between HOMA-IR and VK is consistent with previous reports, it may also be accounted for by the multiple testing we conducted as part of this analysis which increases the likelihood of Type I error.

In conclusion, we observed a trend for PK status to be inversely association with HOMA-IR in men with normal triglycerides. The observed confounding by TG status in the association between plasma PK and HOMA-IR is novel and suggests TG status be considered in future studies utilizing circulating PK as a biomarker of VK nutritional status. Further research is also needed to substantiate a role for PK in IR.

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Table 1: Baseline Characteristics of men and women participating in Health ABC included in this sub-study (not taking diabetic drugs or warfarin) (n=932)

Variable	**Men (n=363)**			**Women (n=569)**		
	Trigs <150mg/dL (n=268)	Trigs ≥150mg/dL (n=95)	p-value	Trigs <150mg/dL (n=394)	Trigs ≥150mg/dL (n=175)	p-value
Age (yrs)	74.7±2.9	74.6±3.0	0.76	74.7±3.0	74.4±2.8	0.39
Weight (kg)	81.2±13.9	84.9±11.6	0.02	71.1±16.0	73.0±13.5	0.17
BMI (kg/m^2)	26.8±4.2	28.2±3.6	0.003	27.9±5.7	28.6±4.8	0.17
Race (% white)	58%	81%	<0.001	46%	69%	<0.001
Education (College Graduate)	41%	55%	0.005	38%	44%	0.171
HOMA_IR*	1.4±1.2	2.4±2.7	<0.001	1.5±1.2	2.2±1.6	<0.001
Glucose (mg/dL)*	94±12.5	99±19	0.001	91±12	95±15	0.001
Insulin (IU/mL)*	6.1±4.3	9.1±9.0	<0.001	6.8±4.9	8.6±6.0	<0.001
HGA1C (%)	6.1±0.9	6.1±0.9	0.23	6.0±0.7	6.1±0.7	0.07
HOMA-B%*	77.3±51.9	86.6±65.0	0.01	85.9±58.3	104.4±69.4	0.004
2 Hour Post OGTT Glucose	125.0±52.8	157.0±73.0	<0.001	125.6±43.6	150.6±53.6	<0.001
PK (nmol/L)*	0.7±0.8	0.9±0.9	0.02	0.7±0.7	1.1±1.2	<0.001
Triglycerides (mg/dL)*	104±42	192±82	<0.001	101±45	192±77	<0.001
Vegetable Intake (servings/day)	3±2	3±2	0.21	3±2	3±2	0.81
Caloric Intake (calories/day)	2103±808	2142±796	0.70	1691±672	1709±634	0.77
Walk Time (minutes/week)	161±336	148±236	0.71	105±211	118±260	0.57

*Median(IQR)

Table 2: Cross Sectional Results for insulin resistance outcome measures stratified by sex and normal vs high triglycerides.

	Triglycerides <150 mg/dL			Triglycerides ≥150 mg/dL		
	n	PK B-Coefficient (SE)	p-value	n	PK B-Coefficient (SE)	p-value
Log HOMA-IR						
Males	258	-0.077 (0.044)	0.08	93	-0.009 (0.068)	0.89
Females	377	0.011 (0.030)	0.71	167	0.020 (0.020)	0.32
Log Glucose						
Males	262	-0.018 (0.012)	0.14	95	-0.037 (0.026)	0.17
Females	383	-0.007 (0.008)	0.37	171	0.010 (0.007)	0.15
Log Insulin						
Males	258	-0.059 (0.0414)	0.15	93	0.028 (0.060)	0.64
Females	378	0.018 (0.0268)	0.51	167	0.009 (0.017)	0.60
HG-A1C						
Males	259	0.001 (0.071)	0.995	95	-0.073 (0.130)	0.58
Females	377	0.008 (0.042)	0.85	170	-0.018 (0.028)	0.52
Log HOMA-B%						
Males	258	-0.006 (0.045)	0.90	93	0.119 (0.077)	0.12
Females	377	0.041 (0.029)	0.16	167	-0.021 (0.020)	0.28
Log 2-Hour Post OGTT Glucose						
Males	248	-0.025 (0.029)	0.39	89	0.014 (0.055)	0.80
Females	366	-0.003 (0.020)	0.87	158	-0.008 (0.014)	0.58

Adjusted for race, study site, BMI, triglycerides, education, daily vegetable intake, daily caloric intake, walk time per week, knee pain.

4. Summary and Discussion

4.1 Research summary

A hormonal role for OC has been proposed that includes regulation of glucose and insulin.[1] Despite its dependence on VK for gamma-carboxylation, few human studies examining the association between OC and IR have taken VK status into account. The overall goal of my thesis was to better understand the role that VK status has on this purported association between OC and measures of IR. Specifically, I examined associations between ucOC and IR when VK intake was controlled for. In addition, I examined associations between biochemical measures of VK status, including serum phylloquinone, and IR in a community-based cohort of older men and women.

The proposed theory that OC has function outside of its known role in bone metabolism was based on a series of studies in a genetically-modified mouse model. Specifically, mice that lacked the OC gene were observed to develop adiposity as well as decreased insulin sensitivity.[2] When these genetically-modified mice were injected with ucOC within a narrow range of doses, their metabolic profiles improved.[2,3] Direct administration of ucOC to humans with IR is currently not feasible so to understand the translational potential of these findings in mice, investigators have conducted secondary analysis of cross-sectional observational studies. While some cross-sectional studies have reported that higher serum concentrations of ucOC were associated with lower body weight and body fat, others have reported no associations.[1] To our knowledge, studies that have reported higher ucOC was associated with more weight and body fat loss in humans have not taken VK status into account and very few have taken into account the influence of other factors influencing bone turnover.

To determine if ucOC was associated with weight or percent fat loss, I conducted a secondary analysis of data and samples collected as part of a 20-week exercise and calorie-restriction intervention study in post-menopausal women. All participants received a daily supplemental intake of VK which controlled for any influence of VK on the carboxylation of OC. Participants also received supplemental calcium and vitamin D to minimize fluctuations in bone metabolism during weight loss. Serum measures of ucOC did not change over the course of the study, nor did any measure of OC (total, ucOC or %ucOC) predict weight or body fat loss. These findings are consistent with previous studies [4,5] and reinforce the need to account for an individual's VK status when assessing the function(s) of ucOC in observational studies since VK is the only known mechanism to modify OC carboxylation in humans.

For the second aim, I examined the effect of changes in carboxylation of ucOC through manipulation of VK on measures of IR. This was accomplished through secondary analysis of data collected as part of a VK depletion-supplementation study conducted in older and younger men and women. Specifically, I compared OC (tOC, ucOC and %ucOC) and IR measures in participants following intake of low VK compared to intake of supplemental VK. As predicted, ucOC was substantially reduced in response to VK supplementation. However there were no concomitant changes in IR measures. These findings, which are the first to directly manipulate ucOC in humans through VK depletion-supplementation, challenge the hypothesis that ucOC has a direct hormonal effect on IR measures in humans.

The final aim of this thesis was to conduct a cross-sectional analysis of a large, well-characterized older cohort for which objective measures of IR and VK status were

available. Guided by the previously-reported sex-specific differences in the associations between VK and IR,[6,7] the analysis was stratified by sex. In addition, I noted a statistically-significant interaction between plasma PK and triglyceride concentrations so I further stratified our analysis by normal vs high triglycerides. Although there were no statistically-significant associations, as defined by a $p<0.05$, there was a non-significant trend towards an inverse associations between plasma VK concentrations and HOMA-IR in men with normal triglycerides. That these trends were observed in men but not women, is consistent with previous findings from our laboratory, [7] as well as others.[6,8,9,10] There is currently no explanation for these sex-specific differences.

To the best of my knowledge, this is the first report that high triglycerides may strongly confound associations between VK status and health outcome measures. While there was a non-significant trend for an inverse association between VK serum concentrations and IR in men with normal triglycerides, there was no association in men with high triglycerides. The interplay between triglycerides and VK in IR risk is not surprising because circulating VK is transported by the triglyceride-rich lipoproteins, and VK serum concentrations are highly correlated with serum triglycerides.[11] Previous studies have addressed this mechanism through exclusion of non-fasting samples and/or controlling for triglycerides in the statistical model. However, these approaches do not differentiate between the effect of diet and the effect of high triglycerides on a high serum VK concentration. The results of this study suggest that VK only has a potential role in reducing IR within a normal range of circulating triglycerides. In

contrast, the detrimental effect of elevated triglycerides and their associated influence on IR most likely overrides any potential benefit of VK on IR.

It was beyond the scope of this thesis to elucidate the mechanism(s) by which VK may be protective in IR risk. Previous research has proposed a role for VK in reducing inflammatory cytokine production.[12–15] It is plausible that VK indirectly reduces IR through reduction in low-grade systemic inflammatory production. Large-well designed studies are required to determine the application of this theory in humans.

This thesis had several limitations that merit consideration. All three hypotheses were conducted as secondary analyses of previously-collected data. Due to the nature of the statistical analyses and the original study designs, the mechanisms by which VK status may affect measures of IR cannot be determined in this thesis. In addition, although I used three different cohorts from three different areas of the United States, participants were generally healthy and mostly older. Therefore, these findings may not be entirely generalizable. Finally, all of these cohorts used validated, surrogate markers of IR and not the direct method of a euglycemic hyperinsulinemic clamp technique. While considered the gold standard of measuring IR, the clamp technique is not only invasive and difficult to perform, but also not feasible in large sample size studies, such as those utilized in this thesis.

The limitations of this dissertation were offset by its strengths. In all three cohorts, circulating concentrations of PK were measured using the same method in the same laboratory, which provides consistency across all three cohorts. In all three studies, vitamin K status was accounted for through VK supplementation, manipulation of dietary VK and/or VK status. In the two cohorts that utilized OC as an exposure, all three

measures of OC (total, ucOC and %ucOC) were measured using the same method in the same laboratory to directly address the question of ucOC and IR in humans.

In conclusion, when VK status is accounted for, there is no association between ucOC and IR or measures of body composition in older adults. Although VK may have a protective effect in IR, this role may be limited to specific subgroups of the population. Future studies are necessary to elucidate the exact mechanism by which VK may influence IR.

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5. APENDICES

5.1 PUBLICATIONS

5.1.1Changes in the content and forms of vitamin K in processed foods

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Abstract:

High intake of *trans* fatty acids has been linked to deleterious health effects including increased risk of cardiovascular disease. Since 2006, the Food and Drug Administration requirescompanies to label the *trans* fatty acid content of foods. This has resulted in an overall decrease of commercially-hydrogenated oils in the food supply. Hydrogenation of vitamin K (VK)- rich plant oils changes form and content of VK. It is not known if changes in use of hydrogenated oil in the food supply resulted in a change in the forms and amount of VK of processedfoods. To test this, we compared 253 foods for total and individual forms of VK in foods analyzed pre- and post-2006 as part of the U.S. Department of Agriculture (USDA) Nutrient and Food Analysis Program. Overall, foods identified as rich sources of the hydrogenated form of VK pre-2006 (dihydrophylloquinone; dK), had lower amounts of dK with a concomitant increase in the parent form of VK, phylloquinone. However, the range of dK was large within foods, suggestive of a wide range of current practices regarding use of hydrogenated oils in the U.S. food supply.

Keywords: Trans-fatty acids, vitamin K, dihydrophylloquinone, phylloquinone, food composition Food Analysis, Food Composition

Introduction:

In response to the evidence regarding the deleterious health effects associated with the consumption of *trans* fatty acids,(Booyens, Louwrens, & Katzeff, 1988; Katan, Zock, & Mensink, 1995; Korver & Katan, 2006) the Food and Drug Administration (FDA) requireslabelling of the amount of *trans* fatty acids contained per serving of food. *Trans* fatty acids are primarily produced when vegetable oils are commercially hydrogenated.(Davidson, Booth, Dolnikowski, & Sadowski, 1996)Since this FDA policy went into place in 2006, the presence of *trans* fatty acids in the food supply has been diminishingwith a decrease in the use of partially hydrogenated oils and the increased use of *trans* fat-free spreads.(Korver & Katan, 2006; Vesper, Kuiper, Mirrel, Johnson, & Pirkle, 2012)

Whereas the focus of the health consequences of hydrogenated oils has been on the formation and subsequent consumption of *trans* fatty acids, other nutrients, including vitamin K (VK), are similarly transformed during the process of hydrogenation of plant oils. The most common dietary form of VK is phylloquinone (PK), which is found primarily in green leafy vegetables and plant oils. When PK-rich plant oils are hydrogenated, overall VK content is reduced, with the majority of remaining PK being chemically transformed into dihydrophylloquinone (dK).(Davidson et al., 1996) Unlike *trans* fatty acids, which can be naturally occurring, dK is the exclusive product of commercial hydrogenation of plant oils, with no known natural sources. As such, dKis limited tothose foods containing hydrogenated oils, such as commercially fried and

baked foods.(Dumont, Peterson, Haytowitz, & Booth, 2003; Elder, Haytowitz, Howe, Peterson, & Booth, 2006; Ferreira, Haytowitz, Tassinari, Peterson, & Booth, 2006; Peterson et al., 2002)The recommended adequate intake (AI) for VK is 120 and 90 µg/d for men and women respectively. It has been demonstrated that dK is absorbed less than equimolar amounts of PK,(Booth, O'Brien-Morse, Dallal, Davidson, & Gundberg, 1999) and its intake is associated with lower bone mass.[11]Since the presence of dK reflects the amount of hydrogenated oils in these food sources, it has also been used as a marker of an unhealthy diet.(Troy et al., 2007)Therefore, reduction of*trans* fatty acids in the food supply should also reduce the presence of dK.

The purpose of this study was to analyze the PK and dK contents of commonly consumed foods that had previously been identified as rich in hydrogenated oils and compare their concentrations pre- and post-2006, since this was the implementation date of the FDA policy on *trans* fatty acid labelling.

Methods:

The analysis of PK and dKin U.S. food samples is an ongoing collaborative agreement of the Vitamin K Laboratory at Tufts University with the U.S. Department of Agriculture (USDA). Food samples were obtained from the USDA Nutrient Data Laboratory as part of the National Food and Nutrient Analysis Program.(Haytowitz, Pehrsson, & Holden, 2008) Samples were collected in 12 cities in the U.S. and combined to form either brand-specific or subnational composites as appropriate to the food item. This food sampling plan

provides aliquots of homogenized foods that are representative of key foods consumed in the U.S.(Pehrsson, Haytowitz, Holden, Perry, & Beckler, 2000)Food samples were shipped to the Food Analysis Laboratory Control Center at Virginia Polytechnic Institute and State University in Blacksburg, Virginia, for the preparation of aliquots and quality-control materials. Aliquots ofthe foods were then shipped on dry ice to the Vitamin K Laboratory atTufts University, Boston, Massachusetts, and stored at –80°C until analyzed. VitaminK is stable under these conditions for a minimum of fiveyears (unpublisheddata).

The PK and dK contents ofthe food samples were determined using a high-performance liquid chromatography (HPLC) proceduredescribed elsewhere.(Ferreira et al., 2006)The assay used to generate data pre-2006 is the same as used for current analysis and has remained stable, as confirmed through use of in-house control samples.

All samples were analyzed in duplicate. If the CV of duplicates was greater than 15% (for sampleswith PK concentrations >5 μg/100 g) the assay was repeated.For samples containing <5 μg/100 gof PK, the assay was repeatedif the duplicate differed by greater than 0.75 μg/100 g. A control sample(consisting of an aliquot of baby food chicken vegetable dinner, Beechnut®, Amsterdam, NY)was run with each batch of foods. If the determined concentrations of the control sample varied by more than 2.5 standard deviations, the entire sample batch was rejected and rerun. Quantification wasachieved by direct comparison of peak area ratios (PK or dK to the assay standardK1(25) generated from thecalibration standard to those generated by the

sample. Peak integrationand sample concentration calculations were performedusing Waters Millennium32 software, version 3.20.

Food composition data for PK and dK obtained prior to 2006 have been previously reported(Dumont et al., 2003; Elder et al., 2006; Ferreira et al., 2006; Peterson et al., 2002)and incorporated into USDA National Nutrient Database for Standard Reference (http://www.ars.usda.gov/nutrientdata/sr). For the purpose of this study, data were selected for those foods analyzed prior to 2006 in which dK was present and for which we had repeated VK analysis in samples collected post-2006. We did not conduct further laboratory analysis on these samples for which data were available. Food samples were not matched for brand or sampling location in the pre- and post-2006 comparisons, and therefore can only be used to monitor overall trends.

Data are presented as the mean ± standard deviation (SD) for each food product. Ranges are also given if there were greater than 2 samples per food product.

Results and Discussion:

Overall, foods that contained high amounts of dK prior to the 2006 *trans* fatty acid labelling requirement hadlower amounts after the labelling requirement in 2006 (Table 1). In foods where dK was reduced post-2006, a concomitant increase in PK concentrations was often observed. This was most obviousin commercially-baked goods, such as chocolate chip cookies, which had almost a 100% decrease in average dK concentration and a concomitant increase in

average PK concentration post-2006. This was an expected change because the hydrogenation process reduces the overall VK content of the plant oils during the conversion of PK to dK, such that hydrogenated oils contain up to 50-80% less VK (PK + dK) than the parent form of the plant oil.(Davidson et al., 1996)Use of non-hydrogenated plant oils would predictably contain PK in higher amounts.However in some foodsin which the dK was reduced post-2006, the PK content either did not change or was overall reduced.This may reflect a change in the use, or lack thereof, of hydrogenated oilsin addition to an overall reduction in oils used or a shift to the use of different plant-based oils that do not contain high amounts of PK, such as peanut or cottonseed oils.(Peterson et al., 2002) The bioavailability of PK obtained from plant-based oils is greater thanPK obtained from vegetables,(Booth, Lichtenstein, & Dallal, 2002) so these trends in changes in PK content of processed foodsmayinfluence thestability of oral anticoagulant therapy if these food items are consumed in large quantities.

An unexpected finding was the large variation in the dK content of foods analyzed post-2006. Whereas for some foods, dK was present in all samples analyzed pre-2006, the range of dK in samples analyzed post-2006 indicated a wide range of practices in terms of use of hydrogenated plant oils. For example, butter-flavored popcorn contained large amounts of dK in all samples analyzed pre-2006 but post-2006, dk content ranged from non-detectable to 80 µg/100g of popcorn. This largerange emphasizes the need for clear labelling for consumers to identify ingredients such as hydrogenated plant oils in their foods.

There were some food items analyzed in this study for which we do not have comparative data prior to 2006. However we chose to include these foodsthat contained dK, and hence hydrogenated plant oils. For example, fried tortillas contained high concentrations of both PK and dK, yet are not food items usually identified as a good dietary source of VK.

This analysis contains some limitations. This study addressed trends in VK content in a limited number of processed foods available in the U.S. food supply, and no attempt was made to identify changes at the regional or specific brand level. In addition, the list of foods reported here are not exhaustive nor did we have concomitant *trans* fatty acid contents for the same food samples as a direct comparison. However, given that dK is exclusively formed from commercial hydrogenation of plant oils, it is likely that the dK concentrations reflect similar trends in *trans* fatty acids in these same foods.

Since dK tracks hydrogenation of foods containing *trans* fatty acids,(Troy et al., 2007) its reduction in the foods analyzed is consistent with reports by others that *trans* fatty acids in the U.S. food supply have decreased following the *trans* fatty acid labelling requirement.(Angell, Cobb, Curtis, Konty, & Silver, 2012; Downs, Thow, & Leeder, 2013; Vesper et al., 2012) Indeed, multiple studies have observed a decrease in the concentration of *trans* fatty acids in both food and human consumption and circulation.(Angell et al., 2012; Downs et al., 2013; Vesper et al., 2012) Recently, the FDA has proposed removing partially hydrogenated oils from the generally recognized as safe (GRAS) list. If this were

to occur, we would expect a further decrease in the dK content of processed foods.(FDA, 2013)

Conclusion:

Intakes of VK may be inadvertently variable in terms of form and absolute amounts due to the wide range in amounts and types of oils used in food manufacturing. These changes may in part be due to decreased amounts of hydrogenated oils used in processed foods in response to labelling requirements for *trans* fatty acids that have been in effect since 2006.

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Table 1
Changes in the PK and dK contents of selected foods pre and post *trans* fatty acid labeling.

Food	PK (μg/100 g)						dK (μg/100 g)*			
	Pre-2006			Post-2006			Pre-2006		Post-2006	
	N	Mean (SD)	Range	*N*	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range
Baked goods										
Cake										
Yellow cake, with chocolate icing	NA[a]	NA (NA)	NA	3	25.1 (3.6)	21.1–28.3	NA (NA)	NA	8.5 (8.1)	ND[b]–16.2
Chocolate cake, with chocolate icing	NA	NA (NA)	NA	3	28.9 (3.3)	25.3–31.7	NA (NA)	NA	5.8 (8.5)	ND–15.6
Cinnamon buns, packaged	4	14.0 (0.9)	12.8–14.9	6	10.1 (7.6)	1.7–20.4	22.8 (2.4)	19.4–24.5	2.5 (2.1)	ND–5.0
Cookies										
Oatmeal	NA	NA (NA)	NA	3	25.1 (16.3)	7.7–40.0	NA (NA)	NA	7.6 (13.2)	ND–22.9
Sugar Wafer	NA	NA (NA)	NA	4	2.1 (0.1)	2.0–2.2	NA (NA)	NA	2.0 (1.2)	2.0–2.1
Chocolate Chip	6	11.9 (9.0)	4.3–29.1	8	22.5 (17.1)	1.8–41.3	48.2 (38.5)	ND–79.7	0.3 (0.6)	ND–1.5
Chocolate Sandwich	2	22.8 (9.0)	16.4–29.2	7	27.4 (11.1)	5.6–40.9	0.0 (–[c])	NA	0.1 (0.2)	ND–0.5
Muffins, blueberry	2	39.3 (–)	32.6–45.9	6	30.7 (11.6)	16.3–44.8	1.0 (–)	ND–1.9	1.2 (1.3)	ND–2.6
Bread, rice and pasta										
Bread, specialty										
Sweet Bread	NA	NA (NA)	NA	2	1.7 (–)	1.6–1.8	NA (NA)	NA	10.5 (–)	3.1–17.8
Corn Bread	NA	NA (NA)	NA	2	7.8 (–)	6.2–9.3	NA (NA)	NA	9.7 (–)	7.9–11.5
Crackers										
Crackers, cheese	NA	NA (NA)	NA	2	9.4 (–)	8.1–10.6	NA (NA)	NA	ND (–)	–
Crackers, no cheese	20	24.3 (14.8)	7.1–52.0	13	19.5 (17.3)	2.2–69.55	17.3 (33.2)	ND–141.4	7.9 (9.7)	ND–24.7
Tortillas, fried	NA	NA (NA)	NA	3	25.8 (20.7)	11.9–49.7	NA (NA)	NA	21.9 (25.3)	ND–49.7
Lasagna with meat sauce	4	4.4 (3.1)	2.1–8.8	12	7.1 (1.7)	5.3–10.8	1.1 (0.5)	0.4–1.5	0.1 (0.2)	ND–0.4
Rice										
Spanish Rice Mix, prepared	NA	NA (NA)	NA	2	2.7 (–)	2.3–3.1	NA (NA)	NA	ND (–)	–
Spanish Rice Mix, unprepared	NA	NA (NA)	NA	2	3.1 (–)	2.5–3.7	NA (NA)	NA	0.8 (–)	ND–1.6
Fats and oils										
Margarine, 80% fat	8	93 (46)	50.8–163.0	3	53.2 (5.0)	47.4–56.5	111 (48)	68.9–182.0	67.4 (1.2)	66.6–68.7
Meat										
Chicken, breaded, fried	8	7.9 (6.4)	3.8–23.5	18	29.8 (8.9)	17.3–53.3	20.1 (16.7)	ND–35.3	0.1 (0.2)	ND–0.9
Chicken Pot Pie	7	3.9 (6.4)	0.5–18.2	2	14.0 (–)	1.5–26.5	1.8 (3.4)	ND–9.0	0.4 (–)	ND–0.8
Hush Puppies	NA	NA (NA)	NA	2	3.8 (–)	2.4–5.3	NA (NA)	NA	16.0 (–)	12.6–19.5
Corn dogs	NA	NA (NA)	NA	4	4.0 (4.7)	1.55–11.0	NA (NA)	NA	0.8 (0.7)	ND–1.5
Shrimp, breaded, fried	NA	NA (NA)	NA	2	6.4 (–)	1.9–11.0	NA (NA)	NA	32.3 (–)	39.9–24.8
Fast food										
French Fries	12	11.2 (4.5)	5.3–17.0	13	32.9 (7.7)	20.6–48.1	42.8 (18.1)	14.5–64.0	ND (0.1)	ND–0.2
Onion Rings, fried	NA	NA (NA)	NA	8	43.3 (9.6)	26.1–53.8	NA (NA)	NA	0.1 (0.1)	ND–0.4
Hamburger	14	11.2 (7.7)	1.1–23.4	3	6.2 (1.5)	4.7–7.7	0.3 (0.5)	ND–1.5	ND (0.0)	–
Miscellaneous										
Toaster Pastry	10	6.1 (1.4)	4.7–8.3	2	19.75 (–)	19.0–20.5	23.0 (5.0)	15.7–29.1	ND (–)	–
Popcorn, microwave, butter flavor, prepared	4	4.2 (2.0)	2.6–7.0	8	4.3 (1.7)	1.3–6.8	68.9 (16.3)	49.0–88.3	30.2 (34.9)	ND–80.1
Ramen noodle soup mix, unprepared	4	2.6 (0.3)	2.2–2.9	2	2.7 (–)	1.9–3.5	0.9 (0.4)	0.5–1.4	ND (–)	–
Tamale, corn	NA	NA (NA)	NA	3	5.4 (5.8)	1.1–12.0	NA (NA)	NA	1.1 (1.8)	ND–3.2

* Data entries for dK are the same as the corresponding PK values and can be found in the PK columns.
[a] NA = not available.
[b] ND = below the lower limit of detection (0.2 μg/100 g).
[c] For food items with 2 or less samples, no standard deviation was reported.

5.1.2 Influence of Kidney Function of Risk of Supratherapeutic International Normalized Ratio- Related Hemorrhage in Warfarin Users: A Prospective Cohort Study. American Journal of Kidney Diseases, *In Press*

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Abstract

Background: Anticoagulation management is difficult in chronic kidney disease, with frequent supratherapeutic international normalized ratios (INRs ≥ 4) increasing hemorrhagic risk. We evaluated whether the interaction of INR and lower estimated glomerular filtration rate (eGFR) increases hemorrhage risk and whether patients with lower eGFRs experience slower anticoagulation reversal.

Study Design: Prospective cohort study.

Setting & Participants: Warfarin pharmacogenetics cohort (1,273 long-term warfarin users); warfarin reversal cohort (74 warfarin users admitted with INRs ≥ 4).

Predictor: eGFR, INR as time-dependent covariate, and their interaction in the pharmacogenetics cohort; eGFR in the reversal cohort.

Outcomes & Measurements: In the pharmacogenetics cohort, hemorrhagic (serious, life-threatening, and fatal bleeding) risk was assessed using proportional hazards regression. In the reversal cohort, anticoagulation reversal was assessed from changes in INR, warfarin and metabolite concentrations, clotting factors (II, VII, IX, and X), and PIVKA-II (protein induced by vitamin K absence or antagonist II) levels at presentation and after reversal, using linear regression and path analysis.

Results: In the pharmacogenetics cohort, 454 (35.7%) had eGFRs < 60 mL/min/1.73 m^2 . There were 137 hemorrhages in 119 patients over 1,802 person-years of follow-up (incidence rate, 7.6 [95% CI, 6.4-8.9]/100 person-years). Patients with lower eGFRs had a higher frequency of INR ≥ 4

(*P* <0.001). Risk of hemorrhage was affected significantly by eGFR-INR interaction. At INR < 4, there was no difference in hemorrhage risk by eGFR (all *P* ≥ 0.4). At INR ≥ 4, patients with eGFRs of 30 to 44 and <30 mL/min/1.73 m^2 had 2.2-fold (95% CI, 0.8-6.1; *P* = 0.1) and 5.8-fold (95% CI, 2.9-11.4; *P* < 0.001) higher hemorrhage risks, respectively, versus those with eGFRs ≥ 60mL/min/1.73 m^2 . In the reversal cohort, 35 (47%) had eGFRs < 45 mL/min/1.73 m^2 . Patients with eGFRs < 45 mL/min/1.73 m^2 experienced slower anticoagulation reversal as assessed by INR (*P* = 0.04) and PIVKA-II level (*P* = 0.008) than those with eGFRs ≥ 45 mL/min/1.73 m^2 .

Limitations: Limited sample size in the reversal cohort, unavailability of antibiotic use and urine albumin data.

Conclusions: Patients with lower eGFRs have differentially higher hemorrhage risk at INR ≥ 4. Moreover, because the INR reversal rate is slower, hemorrhage risk is prolonged.

Introduction

Therapy with warfarin, the most commonly prescribed oral anticoagulant, is challenging because of the many factors that influence its pharmacokinetics and pharmacodynamics. [1] Despite concerted efforts, anticoagulation management remains suboptimal, with frequent supratherapeutic international normalized ratios (INRs) often associated with hemorrhagic complications. [2] [3] This reality has earned warfarin a consistent ranking among the top 10 drugs associated with serious adverse events. [4]

There has been growing appreciation that decreased kidney function affects the clearance of (and response to) drugs that are metabolized mainly by the liver, such as warfarin. [5] [6] [7] Although anticoagulation management among patients with chronic kidney disease (CKD) is particularly challenging, initiation and management of warfarin therapy in patients with CKD are similar to those in the general medical population. [8] [9] We previously have reported that patients with CKD require lower warfarin doses to maintain a therapeutic INR, have worse anticoagulation control, and have a higher risk of hemorrhage compared with patients with normal kidney function. [10] [11] [12]

The goal of the present study was to evaluate whether patients with CKD have a differentially higher risk of hemorrhage during episodes of supratherapeutic INR (INR ≥ 4) in the warfarin pharmacogenetics cohort, and whether decreased kidney function influences the rate of INR reversal among patients with episodes of supratherapeutic INR in the warfarin reversal cohort. Finally, we provide preliminary data on a potential mechanism by which decreased kidney function

influences supratherapeutic INR, facilitated by assessment of PIVKA-II (protein induced by vitamin K absence or antagonist II) in the warfarin reversal cohort.

Methods

Patient Characteristics and Study Design

The warfarin pharmacogenetics cohort (institutional review board protocol numbers X030102003 [Pharmacogenetic Optimization of Anticoagulation Therapy] and X080114012 [Genetic and Environmental Determinants of Warfarin]) recruited patients 20 years or older initiating warfarin therapy with a target INR range of 2 to 3. The aims of the study were to identify the influence of clinical and genetic factors on warfarin dose and hemorrhage. These data supported evaluating the interaction of kidney function and supratherapeutic INR (INR ≥ 4) on risk of hemorrhage.

A detailed history documented information including race, demographics, height and weight, indication for warfarin therapy, comorbid conditions, medications, and socioeconomic factors, in addition to laboratory values (serum urea nitrogen, serum creatinine, hemoglobin, and hematocrit) as detailed in recent publications. Genotyping methodology for the cytochrome P450 (CYP) genes *CYP2C9* and *CYP4F2* and the gene encoding vitamin K oxidoreductase complex subunit 1 (*VKORC1*) has been reported previously. [10] [13] [14] All patients were followed up at least monthly [8] for up to 2 years from initiation of therapy (or for the duration of therapy if <2 years). Variables influencing warfarin response, such as warfarin dose, INR, concurrent medications (such as statins, antiplatelet

agents, and amiodarone), dietary vitamin K and alcohol intake, and medication adherence were recorded at each visit.

Patients using warfarin with supratherapeutic INRs reported on admission were identified. The treating physicians were contacted and patients were enrolled in the warfarin reversal cohort (institutional review board protocol number X090911007) if they were to receive vitamin K to reverse the INR. Warfarin users (n = 102; age ≥ 20 years) hospitalized with supratherapeutic INRs (INR ≥ 4; visit 1) were recruited prior to administration of vitamin K per guidelines. [8] A structured interview form was used at the time of enrollment to obtain a detailed medical lifestyle, social, and concomitant medication history as in the other cohort. Patients were followed up until INR had decreased by > 50% from the initial INR (visit 2). Patients who received plasma or clotting factors (due to medical necessity; n = 28) were excluded from the analysis. The other 74 patients were followed up until INR had decreased by > 50% from the initial INR (visit 2). Blood samples (DNA, plasma, and serum) were collected at both times. Single-nucleotide polymorphisms (SNPs) in *CYP2C9* , *VKORC1* , and γ-glutamyl carboxylase (*GGCX* ; reference SNP identification number rs11676382) were assessed. This supported the assessment of influence of kidney function on anticoagulation reversal among warfarin users hospitalized with supratherapeutic INRs.

All plasma and serum samples were processed within 30 minutes of blood collection and archived at −70°C. For both visits 1 and 2, plasma samples were analyzed for vitamin K–dependent clotting factors (factors II, VII, IX, and X;

University of Alabama at Birmingham Hospital laboratories) using the coagulation analyzer STAR (Stago). PIVKA-II was used to assess functional vitamin K status. The PIVKA-II assay was performed on plasma using a murine monoclonal antibody available in an enzyme immunoassay kit (Asserachrom PIVKA-II; Stago) at the Tufts University vitamin K laboratory as previously reported. Serum samples were analyzed to determine total warfarin and metabolite concentrations (see Item S1 , available as online supplementary material) at the University of Pittsburgh.

Assessment of Kidney Function

Kidney function was assessed using estimated glomerular filtration rate (eGFR) calculated using the CKD-EPI (CKD Epidemiology Collaboration) creatinine equation. [15] [16] Serum creatinine was determined by the Jaffé rate method standardized to isotope-dilution mass spectrometry. Patients were categorized into 4 groups based on eGFR: ≥60 (reference group), 45 to 59 (CKD stage 3a), 30 to 44 (CKD stage 3b), and <30 mL/min/1.73 m ^{2} (CKD stages 4 and 5). Patients receiving maintenance dialysis were categorized in the latter group. Both studies were conducted under the approval of the Institutional Review Board of the University of Alabama at Birmingham.

Outcome Definitions and Statistical Analysis

Supratherapeutic INR was defined as an episode of INR ≥ 4 among patients on warfarin therapy.[3] [17] Major hemorrhages included serious, life-threatening, and fatal bleeding episodes. [18] For all hemorrhagic events, complication site (eg, gastrointestinal), gravity of the event (eg, requiring medical/surgical intervention),

and laboratory findings at the time of the event were objectively documented. Isolated sub- or supratherapeutic INRs in the absence of evidence of bleeding were not classified as events. Minor hemorrhages (nosebleeds, microscopic hematuria, bruising, and mild hemorrhoidal bleeding) were not included. During follow-up, all hemorrhagic complications were captured and verified through review of admissions and emergency department visits. Only medically documented events were included in the analyses. The Alabama Center for Health Statistics was queried to verify cause of death for all deceased patients to ensure inclusion of deaths due to hemorrhagic complications. All complications were reviewed and adjudicated by a blinded reviewer.

Statistical Methods

To assess unadjusted between-group differences across eGFR categories in both cohorts, we performed analysis of variance models for continuous variables and χ^2 tests for categorical variables. To determine whether proportions of INRs ≥ 4 across the eGFR categories in the warfarin pharmacogenetics cohort were significantly different, we used generalized estimating equations with the autoregressive lag-1 covariance structure to account for multiple INR measurements from the same patient because the density of the INRs differs across patients during clinical care.

Incidence rate of hemorrhage and confidence intervals (CIs) were calculated using SAS, version 9.3 (SAS Institute Inc). After adjusting for age, race, sex, genotype, concomitant medications, clinical comorbid conditions, and INR at the time of the event, the interaction between kidney function and INR (eGFR-INR)

was evaluated using multivariable Cox proportional hazards regression with the counting process format. [19] This allowed us to account for multiple events and account for the INRs as a time-dependent covariate. Departures from the proportional hazards assumption were assessed by evaluating interactions of the predictors and a function of survival time.

We calculated rates of changes per hour for INR, PIVKA-II, warfarin concentrations, and clotting factor levels (II, VII, IX, and X) by dividing the difference in measured levels by the time in hours between visit 1 and visit 2 for each participant. To assess differences between time of enrollment (visit 1) and follow-up (visit 2), we performed paired *t* tests.

The influence of kidney function on anticoagulation reversal, including rate of change in INR per hour and rate of change in PIVKA-II per hour, were assessed using multivariable linear regression analyses with adjustment for age, race, sex, body mass index (BMI), vitamin K dose, and genotype (*CYP2C9* , *VKORC1* , *CYP4F2* , and *GGCX*). To understand the indirect effect of eGFR on rate of change in INR through rate of change in PIVKA-II after adjusting for sex, race, vitamin K dose, genotype (*CYP2C9* , *VKORC1* , and *CYP4F2*), and change in clotting factor levels (factors VII, IX, and X), we conducted path analysis with nonparametric bootstrap estimates of the adjusted indirect effects and CIs (see Item S1). All analyses were performed using SAS, version 9.3, at a nondirectional significance level of $\alpha = 0.05$.

Results

Study Participants

Clinical and genetic characteristics of participants in the warfarin pharmacogenetics cohort are presented in Table 1 . Among the study participants, 35.7% had eGFRs < 60 mL/min/1.73 m^2 , including 17.5%, 9.0%, and 9.1% with eGFRs of 45 to 59, 30 to 44, and <30 mL/min/1.73 m^2 or on dialysis therapy, respectively. These eGFR levels were associated significantly with race, indications for therapy, number of comorbid conditions, antiplatelet and amiodarone use, and*VKORC1* and *CYP4F2* genotypes. These variables were included as covariates in subsequent multivariable analyses.

Decreased kidney function (eGFR < 45 mL/min/1.73 m ^{2}) was associated with an increased frequency of supratherapeutic INR (*P* < 0.001) and hemorrhage (Table 2). During the 1,802 person-years of follow-up, 137 major hemorrhages were encountered in 119 patients (incidence rate, 7.6 [95% CI, 6.4-8.9]/100 person-years). Gastrointestinal hemorrhage was most common (n= 82), followed by hematoma (n = 25), genitourinary (n = 12), intracranial hemorrhage (n = 11), and other (n = 7). The incidence of hemorrhage in patients with eGFRs of 45 to 59 mL/min/1.73m ^{2} was similar to that of patients with eGFRs ≥ 60 mL/min/1.73 m ^{2} (*P* = 0.6). Compared with patients with eGFRs ≥ 60 mL/min/1.73 m ^{2} , those with eGFRs of 30 to 44 (incidence rate ratio, 1.8; 95% CI, 1.1-3.0; *P* = 0.03) and <30 mL/min/1.73 m ^{2} (incidence rate ratio, 3.5; 95% CI, 2.3-5.4; *P* < 0.001) experienced hemorrhage more frequently. Of the 137 major hemorrhages, INR at the time of event was <4 at 91 events and ≥4 in 44 (Table 2). After adjusting for age, race, sex, genotype, concomitant medications, clinical comorbid conditions, time in target range, and INR at the time of the event, the eGFR-INR interaction was statistically significant (*P* < 0.001; Fig 1).

Among patients with eGFRs ≥ 60 and those with eGFRs of 45 to 59 mL/min/1.73 m ^{2} , INR did not influence risk of hemorrhage (*P* = 0.8). Among patients with eGFRs of 30 to 44 mL/min/1.73m ^{2} , INR ≥ 4 was associated with a 2.2-fold (hazard ratio [HR], 2.2; 95% CI, 0.8-6.1; *P* = 0.1) higher risk of hemorrhage, although this was not statistically significant. Among patients with

eGFRs < 30 mL/min/1.73 m ^{2} , INR ≥ 4 was associated with a 5.8-fold (HR, 5.8; 95% CI, 2.9-11.4;*P* < 0.001) higher risk. This differentially higher risk of hemorrhage among patients with eGFRs of 30 to 44 and <30 mL/min/1.73 m ^{2} when INR is ≥4 after adjustment for clinical and genetic factors is illustrated in Fig 1 .

Given the significant increase in risk of hemorrhage among patients with eGFRs < 45mL/min/1.73 m ^{2} when INR is ≥4, we evaluated the influence of kidney function on anticoagulation reversal among 74 patients (mean age, 61 years; 54% women; and 45% African American) who made up the warfarin reversal cohort. In 47% of patients, eGFR < 60mL/min/1.73 m ^{2} was present. Venous thromboembolism (46%) was the major indication for warfarin therapy, followed by atrial fibrillation (31%). Temporary discontinuation of warfarin was the sole treatment implemented in 31 patients, while 43 were treated with vitamin K in addition to temporary discontinuation of warfarin therapy. The institution of vitamin K treatment did not vary by kidney function. Patients with severe CKD (eGFR < 30 mL/min/1.73 m ^{2}) received higher vitamin K doses, although this finding was not significant (*P* = 0.1; Table 3).

Anticoagulation, clotting factor, and warfarin (and metabolite) levels at time of enrollment (visit 1) and follow-up (visit 2) are shown in Table 4 . As expected, INR and PIVKA-II levels declined with a parallel increase in clotting factor activity. Similarly, warfarin and metabolite levels decreased, although changes in 7-hydroxy and 10-hydroxy warfarin levels were not statistically significant.

Influence of Kidney Function on INR Reversal and PIVKA-II Levels

After adjustment for age, race, sex, BMI, vitamin K dose, and genotype (*CYP2C9* , *VKORC1* ,*CYP4F2* , and *GGCX*), kidney function had a significant influence on rate of INR reversal (*P* =0.04). The rate of INR decline was faster (0.11 U/h) among patients with eGFRs ≥ 45mL/min/1.73 m^2 compared to the rate (0.05 U/h) among those with eGFRs < 45 mL/min/1.73 m^2 . Additionally, sex (*P* = 0.02), vitamin K dose (*P* = 0.001), and *CYP2C9* (*P* = 0.08) influenced rate of INR reversal. After adjustment for age, race, sex, BMI, vitamin K dose, and genotype (*CYP2C9* , *VKORC1* , *CYP4F2* , and *GGCX*), kidney function had a significant influence on the rate of decrease in PIVKA-II levels (*P* = 0.008). Because kidney function has a significant influence on the rate of decrease in INR and PIVKA-II levels and the rate of decrease in PIVKA-II levels was related significantly to the rate of change in INR (*P* = 0.004), we investigated the indirect effects of kidney function on rate of change in PIVKA-II and rate of change in INR using path analyses (Fig 2). Race; sex; vitamin K dose; variant *CYP2C9* , *VKORC1* , *CYP4F2* , and *GGCX* genotypes; and rate of change in factors VII, IX, and X accounted for 21.9% of the variance in rate of change in PIVKA-II, although this finding did not reach statistical significance (*P* = 0.07). However, the addition of kidney function uniquely explained an additional 8.2% of the variance in rate of change in PIVKA-II (*P* = 0.01). Overall, these factors explained 30.1% of the variance in rate of change in PIVKA-II (*P* = 0.01). Similarly race; sex; vitamin K dose; variant *CYP2C9* , *VKORC1* , *CYP4F2* , and *GGCX* genotypes; and rate of

change in factors VII, IX, and X accounted for 59.5% of the variance in rate of change in INR (*P* <0.001). Although kidney function did not explain additional variance in rate of change in INR (*P*= 0.3), the addition of rate of change in PIVKA-II uniquely explained 6.8% (*P* = 0.004) of the variance in rate of change in INR. Overall, the model explained 66.3% of the variation in rate of change in INR.

Discussion

This prospective study demonstrates that supratherapeutic INR (INR ≥ 4) increases the risk of major hemorrhage among warfarin users with decreased kidney function (eGFR < 45mL/min/1.73 m^2 ; CKD stages 3b, 4, and 5; and patients on dialysis therapy), but not in those with eGFRs ≥ 45 mL/min/1.73 m^2 . Inclusion of patients across the spectrum of kidney function improves the generalizability of these results.

There is extensive literature on the increased risk of hemorrhage among warfarin users during episodes of supratherapeutic INR. [1] [18] [20] [21] [22] [23] [24] [25] We previously have shown that patients with eGFRs < 30 [10] and those with eGFRs < 45 [12] mL/min/1.73 m^2 are at increased risk of hemorrhage. To our knowledge, our work is the first to demonstrate that kidney function modifies the association between supratherapeutic INR and risk of hemorrhage. At INRs < 4, the risk of hemorrhage is similar among warfarin users independent of kidney function. Episodes of supratherapeutic INR are more frequent in patients with eGFRs < 45 mL/min/1.73 m^2 compared with patients with

eGFRs ≥ 45 mL/min/1.73 m ^{2} . Moreover, compared with patients with eGFRs≥ 60 mL/min/1.73 m ^{2} , those with eGFRs < 45 mL/min/1.73 m ^{2} are at a 2.2-fold higher risk of hemorrhage and those with eGFRs < 30 mL/min/1.73 m ^{2} are at a 5.8-fold higher risk. In the warfarin pharmacogenetics cohort, 18% had eGFRs < 45 mL/min/1.73 m ^{2} and accounted for 47 (34.3%) major hemorrhages encountered, highlighting the importance of this finding.

Recognition of the hemorrhagic risk associated with supratherapeutic INR has led to the development of guidelines to mitigate the risk. [8 26] Reversal strategies are based on the patient's INR and the presence (or absence) of bleeding. [8 26] In nonbleeding patients with elevated INRs, administration of vitamin K, which is the essential cofactor for synthesis of vitamin K–dependent proteins, is the first-line treatment. In more urgent situations (actively bleeding patient or patient at imminent risk of bleeding), fresh frozen plasma and factor replacement (prothrombin complex concentrate or recombinant factor VIIa) are administered. Although factor replacement provides reliable warfarin reversal, the more commonly used strategy of temporarily withholding warfarin with or without administering vitamin K leads to unpredictable anticoagulation reversal, with significant variation in the rate and extent of INR reversal. [2 27 28 29]

To our knowledge, our work is the first to demonstrate that kidney function influences the rate of INR reversal among warfarin users with supratherapeutic INRs. Among patients with supratherapeutic INRs, those with poor kidney function (eGFR < 30 mL/min/1.73 m ^{2}) experienced a slower rate of INR reversal.

This indicates that these patients are at an increased risk of hemorrhage during episodes of supratherapeutic INR and experience slower reversal of anticoagulation (withholding doses with or without vitamin K administration), thereby prolonging the period of heightened risk.

Assessment of the indirect effects of decreased kidney function on reversal of anticoagulation (measured by INR decrease) and PIVKA-II (measured by decrease in PIVKA-II levels) enabled us to explore a potential mechanism through which decreased kidney function influences coagulation. In the presence of vitamin K, clotting factors II, VII, IX, and X are carboxylated by GGCX. PIVKA-II, the uncarboxylated clotting factor II, represents a functional measure of vitamin K antagonism in patients on warfarin therapy. [30] [31] [32] [33] [34] [35] [36] Therefore, PIVKA-II is a functional measure of vitamin K status and the rate of decrease in PIVKA-II represents the rate of carboxylation of clotting factors. [37] [38] Our analysis shows that kidney function explains 9.4% of the variance in change in PIVKA-II. The slower decrease in PIVKA-II levels among patients with decreased kidney function implies that it is associated with a decreased rate of carboxylation of clotting factors.

Recognition of the influence of CKD on response to medications that are metabolized predominantly by the liver is growing. [7] [39] [40] Animal studies in CKD have demonstrated significant downregulation (40%-85%) of hepatic CYP metabolism. [41] [42] Our findings led us to hypothesize that the mechanism by which decreased kidney function influences anticoagulation is through a slower rate of carboxylation of clotting factors, as indicated by slower rates of INR and PIVKA-II

reversal in patients presenting with overanticoagulation. This is supported by the influence of decreased kidney function on the carboxylation of matrix Gla-protein, another vitamin K–dependent protein. [43] [44] [45] The slower rate of INR decline, together with other factors (eg, uremia and platelet dysfunction) known to be associated with kidney disease, could explain the higher risk of hemorrhagic complications.

Our study had several strengths, including the large sample size in the warfarin pharmacogenetics cohort with prospective data collection that enabled assessment of the influence of the eGFR-INR interaction on risk of hemorrhage. [10] [12] [14] Our focus was on major hemorrhage because these events are associated with morbidity, mortality, and health care costs. Furthermore, clinical (comorbid conditions and medications) and genetic factors, overall anticoagulation control, and anticoagulation intensity (INR) at the time of hemorrhage were taken into account in our analysis. However, we recognize its limitations. First, urine albumin was not ascertained uniformly and therefore could not be included in classifying CKD stages. Second, the small (n = 74) warfarin reversal cohort allowed for assessment of INR reversal in only 2 broad categories (eGFR of 45 vs ≥45 mL/min/1.73 m^2). Third, among patients in the warfarin reversal cohort, data for recent antibiotic use were not complete and therefore were not included in the analysis.

Although the warfarin reversal cohort allowed us to evaluate the influence of kidney function on INR reversal and enabled us to propose a potential mechanism, our findings should be considered exploratory and hypothesis

generating. Moreover, development in assay methodologies that can facilitate the assessment of changes in noncarboxylated forms of other vitamin K–dependent clotting factors (factors VII, IX, and X) would allow us to further vet this hypothesis. Finally, further research in larger cohorts is needed to confirm these findings and better understand the influence of kidney function on coagulation processes.

The institution of oral anticoagulation therapy in patients with decreased kidney function is particularly challenging because these patients are under-represented in clinical trials. The decision to initiate therapy should weigh the risk of thromboembolism and risk of hemorrhage judiciously. Recently, investigators demonstrated that for hemodialysis patients with incident atrial fibrillation, warfarin use was associated with a decreased risk of all-cause mortality and a composite outcome of gastrointestinal bleeding, any stroke, and death, indicating net benefit of warfarin use in this indication. [46] [47] As the population ages and the prevalence of CKD increases, research that addresses the use and management of oral anticoagulation in this high-risk/high-benefit population is greatly needed.

In summary, patients with poor kidney function have more frequent episodes of supratherapeutic INR, are at a differentially higher risk of hemorrhage during episodes of supratherapeutic INR, and experience slower reversal of anticoagulation (with vitamin K treatment), prolonging the period of heightened risk. Given the increased hemorrhagic risk, guidelines for reversing the effects of warfarin should provide specific guidance for patients with decreased kidney function.

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Table 1

Clinical and Demographic Characteristics of 1,273 Patients Receiving Long-term Warfarin Therapy by Baseline eGFR Category

Characteristic	eGFR ≥60 (n = 819)	eGFR 45-59 (n = 223)	eGFR 30-44 (n = 115)	eGFR <30 (n = 116)	*P* Trend[a]
Average follow-up (y)	1.4 ± 0.9	1.5 ± 1.0	1.5 ± 0.9	1.2 ± 0.8	0.03
Age (y)	57.9 ± 15.8	68.4 ± 12.5	72.5 ± 12.7	57.7 ± 15.9	0.5
BMI (kg/m ^{2})	30.4 ± 7.7	29.4 ± 7.2	29.3 ± 7.1	30.1 ± 6.8	0.6
Race					0.1
African American	364 (44.4)	73 (32.7)	37 (32.2)	77 (66.4)	
European American	448 (54.7)	149 (66.8)	78 (67.8)	39 (33.6)	
Other	7 (0.9)	1 (0.5)	0 (0.0)	0 (0.0)	
Female sex	381 (46.5)	118 (52.9)	60 (52.2)	58 (50.0)	0.2
Indication for warfarin					0.2
Atrial fibrillation	311 (38.0)	128 (57.4)	63 (54.8)	38 (32.8)	
Stroke	50 (6.1)	16 (7.2)	9 (7.8)	1 (0.9)	
Venous thromboembolism	382 (46.6)	65 (29.1)	33 (28.7)	60 (51.7)	
Other	76 (9.3)	14 (6.3)	10 (8.7)	17 (14.7)	
No. of comorbid conditions					<0.001
Low: 0 or 1	280 (34.2)	43 (19.3)	14 (12.2)	14 (12.1)	
Moderate: 2-3	284 (34.7)	77 (34.5)	33 (28.7)	45 (38.8)	

Characteristic	eGFR undefined≥ undefined60 (n undefined= undefined819)	eGFR undefined= undefined45-59 (n undefined= undefined223)	eGFR undefined= undefined30-44 (n undefined= undefined115)	eGFR undefined< undefined30 (n undefined= undefined116)	*P*Trend[a]
High: ≥4	255 (31.1)	103 (46.2)	68 (59.1)	57 (49.1)	
Concurrent medications					
Antiplatelet agents	431 (53.1)	147 (65.9)	78 (67.8)	61 (52.6)	0.06
Statins	176 (21.7)	57 (25.6)	27 (23.5)	29 (25.0)	0.3
Amiodarone	57 (7.0)	30 (13.5)	17 (14.8)	10 (8.6)	0.03
Genotype					
CYP2C9 variant	189 (24.8)	59 (27.7)	28 (27.5)	22 (21.4)	0.9
VKORC1 variant	320 (40.4)	99 (45.4)	61 (56.5)	37 (33.6)	0.5
CYP4F2 variant	259 (37.3)	88 (44.2)	36 (37.9)	23 (26.1)	0.3

a *P* is significant at α = 0.05 and denote differences across kidney function categories.

b Includes 3 Asians and 5 Hispanics.

c Includes cardiac thrombus, myocardial infarction, peripheral vascular disease, low ejection fraction, etc.

d Defined as concomitant diseases (eg, hypertension, high cholesterol, diabetes, and congestive heart failure).

e Include aspirin, clopidogrel, and dipyridamole as mono or dual therapy; 7 individuals were missing information on concurrent medication for antiplatelet, statins, and amiodarone therapy in the eGFR ≥ 60 category.

f Includes *2 and *3 alleles among European Americans and *2, *3, *5, *6, and *11 alleles among African Americans. Samples for 92 patients had not been typed at the time of this analysis: there were 56, 10, 13, and 13 missing

genotypes for eGFRs ≥ 60, 45 to 59, 30 to 44, and <30 mL/min/1.73 m ^{2}, respectively.

g Variant VKORC1-1173C/T (rs9934438) corresponds to TT or CT. Samples for 44 patients had not been typed at the time of this analysis: there were 26, 5, 7, and 6 missing genotypes for eGFRs ≥ 60, 45 to 59, 30 to 44, and <30 mL/min/1.73 m ^{2}, respectively.

h Variant *CYP4F2* (rs2108622; V433M) corresponds to GA or AA. Samples for 196 patients had not been typed at the time of this analysis: there were 124, 24, 20, and 28 missing genotypes for eGFRs ≥ 60, 45 to 59, 30 to 44, and <30 mL/min/1.73 m ^{2}, respectively.

Table 2
Frequency of Supratherapeutic INR and Hemorrhage Among Warfarin Users by eGFR Category

	eGFR undefined≥ undefined60 (n undefined= undefined819)	**eGFR undefined= undefined45-59 (n undefined= undefined223)**	**eGFR undefined= undefined30-44 (n undefined= undefined115)**	**eGFR undefined< undefined30 (n undefined= undefined116)**	***P*Trend [a]**
INR ≥ 4					
No. of INRs	20,953	6,027	3,286	3,359	
INRs ≥ 4	1,029 (4.9)	292 (4.8)	199 (6.1)	249 (7.4)	<0.001
Major hemorrhage					
No. of events	68	22	18	29	
Person-y	1,160.5	331.5	169.7	140.1	
Incidence rate	5.6 (4.6-7.4)	6.6 (4.3-9.9)	10.6 (6.5-16.4)	20.7 (14.3-29.3)	
INR at event < 4	51 (75.0)	19 (86.4)	11 (61.1)	10 (37.0)	
INR at	17 (25.0)	3 (13.6)	7 (38.9)	17 (63.0)	<0.001

	eGFR undefined≥ undefined60 (n undefined= undefined819)	eGFR undefined= undefined45-59 (n undefined= undefined223)	eGFR undefined= undefined30-44 (n undefined= undefined115)	eGFR undefined< undefined30 (n undefined= undefined116)	*P*Trend [a]
event ≥ 4					

a *P* is significant at α = 0.05 and denotes differences across kidney function categories.

b *P* values were obtained using the generalized estimating equation with the autoregressive lag-1 covariance structure to account for multiple INR measurements from the same patient because the density of INRs differs across patients during clinical care.

c There were 137 major hemorrhages (2 INRs not available); 91 occurred at INR < 4 and 44 occurred at INR ≥ 4. INR not available for 2 events. Breslow-Day test for interaction of rate ratio over kidney function strata *P*= 0.002. Breslow-Day test for interaction of risk difference over kidney function strata *P*= 0.003.

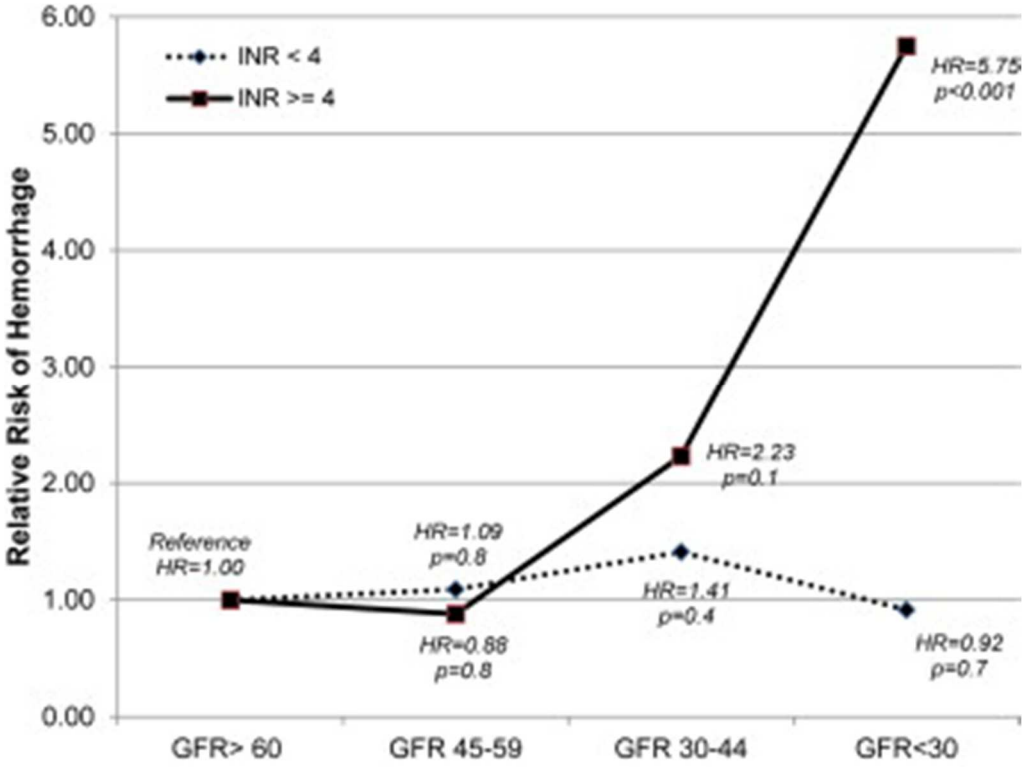

Figure 1
Relative risk of hemorrhage among patients with varying kidney function by international normalized ratio (INR) at the time of the event (estimated glomerular filtration rate [GFR] undefined≥ undefined60 undefinedmL/min/1.73 undefinedm ^{2} is the reference group). Abbreviation: HR, hazard ratio.

Table 3
Characteristics of Participants in Warfarin Reversal Cohort by eGFR Category

Characteristic	eGFR undefined≥ undefined60 (n undefined= undefined39)	eGFR undefined= undefined45-59 (n undefined= undefined15)	eGFR undefined= undefined30-44 (n undefined= undefined71)	eGFR undefined< undefined30 (n undefined= undefined13)	*P*Trend [a]
Age (y)	57.9 ± 18.9	64.3 ± 16.5	58.0 ± 15.4	69.9 ± 14.5	0.1
BMI (kg/m ^{2})	27.4 ± 5.7	26.0 ± 9.5	29.4 ± 2.9	27.8 ± 7.7	0.5
INR at visit 1	7.4 ± 2.5	6.5 ± 2.3	6.1 ± 2.0	6.6 ± 2.3	0.3
INR at visit 2	2.6 ± 1.2	3.0 ± 1.0	1.8 ± 0.8	2.9 ± 1.7	0.8
Difference in	4.8 ± 2.6	3.5 ± 1.6	4.2 ± 2.2	3.7 ± 1.9	0.3

Characteristic	eGFR undefined≥ undefined60 (n undefined= undefined39)	eGFR undefined= undefined45-59 (n undefined= undefined15)	eGFR undefined= undefined30-44 (n undefined= undefined7)	eGFR undefined< undefined30 (n undefined= undefined13)	*P*Trend [a]
INR					
Warfarin dose (mg/wk)	38.4 ± 20.7	35.6 ± 15.4	30.4 ± 11.7	31.5 ± 11.6	0.2
Time between visits 1 and 2 (h)	33.3 [23.3-65.0]	42.5 [23.7-66.3]	43.0 [23.3-73.9]	39.4 [22.0-74.8]	0.9
Vitamin K dose (mg)	4.8 ± 3.5	6.3 ± 3.4	2.8 ± 1.5	6.6 ± 3.8	0.7
Female sex	21 (54)	6 (40)	3 (43)	10 (77)	0.3
African American	15 (39)	8 (53)	2 (29)	8 (62)	0.3
Receiving vitamin K	22 (56)	7 (47)	4 (57)	10 (77)	0.3
Indication for warfarin therapy					0.6
Venous thromboembolism	20 (51)	3 (20)	4 (57)	7 (54)	
Stroke/TIA	1 (3)	1 (7)	0 (0)	0 (0)	
Atrial fibrillation	12 (31)	5 (33)	3 (43)	3 (23)	
Other	6 (15)	6 (40)	0 (0)	3 (23)	
Genotype					
CYP2C9 variant	7 (18)	1 (7)	2 (29)	1 (8)	0.5
VKORC1 variant	21 (55)	7 (47)	5 (71)	6 (46)	0.8
CYP4F2 variant	10 (26)	3 (21)	2 (29)	3 (23)	0.9

Characteristic	eGFR undefined≥ undefined60 (n undefined= undefined39)	eGFR undefined= undefined45-59 (n undefined= undefined15)	eGFR undefined= undefined30-44 (n undefined= undefined7)	eGFR undefined< undefined30 (n undefined= undefined13)	*P*Trend a
GGCX variant	4 (11)	1 (7)	1 (14)	1 (8)	0.9

a *P* is significant at α = 0.05 and denotes differences across kidney function categories; *P* values for continuous variables derived from Kruskal-Wallis test; for categorical variables, from χ^2 test.

b Value at visit 2 less the value at visit 1.

c Include cardiac thrombus, myocardial infarction, peripheral vascular disease, etc.

d See notes to .

Table 4
Anticoagulation, Clotting Factor, and Warfarin Levels at Both Visits

	Visit 1	Visit 2	*P* a
INR	6.9 ± 2.4	2.7 ± 1.3	<0.001
PIVKA-II	2,861.7 ± 1,717.1	1,685.1 ± 1,501.7	<0.001
Vitamin K–dependent clotting			
Factor II	14.9% ± 7.9%	30.7% ± 17.4%	<0.001
Factor VII	12.4% ± 9.8%	52.4% ± 29.7%	<0.001
Factor IX	25.7% ± 18.3%	81.4% ± 52.9%	<0.001
Factor X	9.5% ± 5.4%	22.0% ± 16.1%	<0.001
Total warfarin (mg/dL)	1,409.9 ± 1,216.8	1,221.5 ± 1,002.7	0.07
Warfarin metabolites			
4-OH (mg/dL)	2.9 ± 3.2	1.5 ± 2.1	<0.001
6-OH (mg/dL)	6.0 ± 9.6	4.4 ± 6.3	0.02
7-OH (mg/dL)	93.0 ± 102.0	82.4 ± 93.4	0.2
8-OH (mg/dL)	19.3 ± 20.8	15.2 ± 15.7	0.02

	Visit 1	Visit 2	*P* a
10-OH (mg/dL)	87.1 ± 162.8	82.2 ± 131.7	0.4

a *P* is significant at α = 0.05 and denotes statistical difference in measurements across visits 1 and 2.

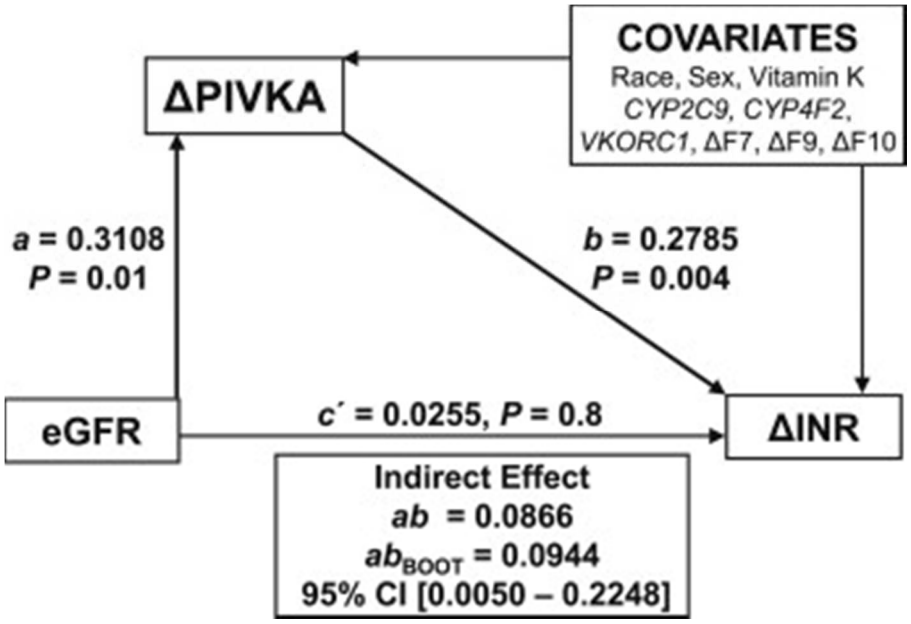

Figure 2
Indirect effect of estimated glomerular filtration rate (eGFR) on change in international normalized ratio (INR) by change in PIVKA (protein induced by vitamin K absence or antagonist).The *a* coefficient represents the effect of GFR on rate of change in PIVKA-II, and the *b* coefficient represents the association of rate of change in PIVKA-II. Abbreviations: CI, confidence interval; CYP, cytochrome P450; F7 (9, 10), factor VII (IX, X).

5.1.3 Bone as an Endocrine Organ Relevant to Diabetes, Current Diabetes Reports. 2014 Dec;14(12):556.

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Abstract

There are well-established associations between diabetes and fracture risk and yet the mechanism underlying these associations are controversial. Guided by a series of mouse studies, a specific form of the bone protein, osteocalcin, was proposed to be the mechanistic link between these two chronic diseases. Translation to humans initially appeared elusive in part because serum concentrations of osteocalcin are a biomarker of bone turnover and not necessarily specific to the biology of this protein. The suitability of the mouse model for the study of osteocalcin as a therapeutic target also appears ambiguous. With greater discrimination of the different forms of osteocalcin present in circulation and inclusion of multiple measures of bone turnover, evidence currently does not support osteocalcin as a protein critical to the diabetes and fracture association in humans.

Introduction

For almost a century, there has been evidence in the medical literature documenting increased risk of fracture among diabetic patients [1]. The public health implications of this observation are of importance as the prevalence of confirmed diabetic cases continues to rise [2]. As extensively reviewed elsewhere [3, 4], the mechanisms are still being elucidated, which underscores the complexity of this established association. In patients with type 1 diabetes mellitus (T1DM), low bone mineral density (BMD) has been observed, which is consistent with an increased fracture risk [5]. Given that insulin, through an osteoblastic insulin receptor, is anabolic for bone [6] low insulin levels in T1DM result in reduced bone formation. This decrease is especially pronounced before the introduction of insulin as a treatment [5]. Individuals with type 2 diabetes mellitus (T2DM) are also at increased fracture risk [7]; however, BMD tends to be normal or higher. Many factors can contribute to this dichotomy including changes in bone architecture, cortical porosity, and bone turnover [8, 9, 10•]. This has led to an intensive search for factors that explain the diabetes-bone link. Beginning in 2007, Karsenty and colleagues proposed that bone is an endocrine organ based on a series of studies conducted in mice [11]. As reviewed elsewhere [12•, 13], this team proposed that the osteoblast-specific protein, osteocalcin, was a hormone that affected pancreatic β cells [14]. The osteocalcin-null mice were obese with elevated glucose and lipid concentrations and reduced numbers of β cells [14, 15]. Both insulin secretion and sensitivity were decreased, which was attributed to a decrease in adiponectin expression in

adipose tissue [14, 15]. These studies established that uncarboxylated osteocalcin was the active form, but fully carboxylated osteocalcin was inactive. In a subsequent series of experiments in male C57Bl/6 J mice, osmotic minipumps delivering 3 ng of uncarboxylated osteocalcin per hour reduced circulating glucose and increased circulating insulin levels [16]. Relative to the impressive favorable changes in glucose and insulin in response to these doses, the uncarboxylated osteocalcin had only a very modest increase ranging from 10 to 14 % of the total circulating osteocalcin in adult mice fed standard rodent chows.

That the uncarboxylated form of osteocalcin was identified by Karsenty and colleagues as the protein involved in glucose metabolism represented a paradigm shift in our current understanding of the biochemistry of osteocalcin. Its ability to bind calcium is dependent on the vitamin K-dependent γ-carboxylation of three glutamic acid residues (Glus) [17, 18]. In most species, all three Glus are fully carboxylated, thereby resulting in little if any uncarboxylated osteocalcin in circulation [12•]. In the original model, Lee et al. found that osteoblast-conditioned media from a tyrosine phosphatase-null mouse contained a factor that regulated insulin sensitivity (OST-PTP) [14]. They proposed that OST-PTP, which is expressed only in osteoblasts and Leydig cells, regulated the carboxylation of osteocalcin. However, this initial hypothesis was rejected when no orthologue for OST-PTP could be located in humans. An alternative model was proposed for the origin of the uncarboxylated form of osteocalcin that no longer challenged the unique role of vitamin K in the carboxylation of osteocalcin.

Insulin signaling in osteoblasts decreases the expression of osteoprotegerin, which activates bone resorption [19, 20]. In this proposed model, bone resorption produces a highly acidic environment which decarboxylates osteocalcin at the first of three γ-carboxyglutamic acid residues. This decarboxylation of osteocalcin is sufficient to promote its action on glucose and insulin [13]. In an accompanying paper, the Clemens laboratory confirmed that insulin receptor signaling promotes osteoblast proliferation and differentiation [20, 21]. Mice lacking the insulin receptor (IR) in osteoblasts (Ob-ΔIR) had increased adiposity and insulin resistance along with low bone formation and reduced numbers of osteoblasts. Infusion of uncarboxylated osteocalcin via minipump to Ob-ΔIR mice resulted in increased but not significant reductions in glucose during insulin-tolerance tests [20, 22]. Consistent with the low osteoblast activity, osteocalcin was reduced in serum and in conditioned media from osteoblasts isolated from these mice. However, uncarboxylated osteocalcin was also reduced in both serum and media, in conflict with the requirement for osteoclast decarboxylation of osteocalcin. A more recent paper, however, suggested that other osteoblast-derived factors may also contribute to the regulation of energy metabolism in mice [23].

In order to determine if moderate changes in insulin signaling in the osteoblast would have an effect on glucose tolerance, Karsenty and his colleagues generated mice over- ($Insr^{tr}$) or under- ($Insr^{+/-}$) expressing the insulin receptor [24]. When animals were fed a normal diet, no changes were observed in glucose metabolism as compared to wild type. However, when the animals were

fed a high-fat diet, insulin resistance was weakened or exacerbated in the animals over- and under-expressing the insulin receptor, respectively, compared to wild-type mice on a high-fat diet. In these models, bone turnover parameters were lower in animals on the high-fat diet with relatively greater reduction in those animals under-expressing the insulin receptor. Circulating levels of both total and the uncarboxylated form of osteocalcin decreased in parallel to the decrease in bone turnover as collectively measured by C-terminal telopeptide (CTx) and procollagen type 1 amino-terminal propeptide (P1NP). When uncarboxylated osteocalcin was given by daily injections to $Insr^{+/-}$ mice, glucose tolerance and insulin sensitivity were improved. Paradoxically, total circulating osteocalcin increased three times that of uncarboxylated osteocalcin, suggesting non-equivalency of the assays.

Several recent studies further the complexity of any impact that osteocalcin may have on glucose homeostasis. Brennan-Speranza and colleagues sought to test the hypothesis that osteocalcin could moderate the glucose intolerance associated with glucocorticoid therapy [*25*]. However, when either carboxylated or uncarboxylated osteocalcin was administered via injection or slow-release osmotic pumps at concentrations in accordance with published values, no changes were observed in circulating levels of either form of osteocalcin nor were there any effects on metabolic parameters in untreated or corticosterone-treated animals. Even very high concentrations of osteocalcin failed to provide an effect by these routes of administration. To overcome this problem, the authors targeted osteocalcin to hepatocytes via hydrodynamic tail vein injection in vivo

resulting in high expression levels in the liver and moderately increased levels in the circulation. Corticosterone-treated mice transfected with the osteocalcin vector regained a partial sensitivity to insulin but there was no effect on pancreatic insulin secretion [*25*].

More recently, Clemens, Karsenty, and colleagues studied mice with a loss of tuberous sclerosis 2 (Tsc2) in the osteoblast which functions to regulate mTOR, a protein kinase important for nutrient and growth factor signaling [*26*]. The mice develop metabolic features similar to mice lacking the IR in the osteoblast. In contrast to the Ob-ΔIR mice, bone formation was markedly increased but resorption decreased, resulting in sclerotic bone [*27*•]. Uncarboxylated osteocalcin was dramatically increased, despite the fact that resorption was reduced. The authors speculated that the vitamin K-dependent carboxylase may be regulated by the Tsc2-mTOR pathway, but this remains untested at this time. All of these studies utilize animal models with disruptions in insulin signaling in the osteoblast and support the notion insulin has an effect on both whole body glucose homeostasis and bone turnover. The current hypothesis is that osteocalcin links the two. However, transgenic mice overexpressing ΔFosB, an activator protein transcription factor present in bone and other tissues, exhibit an increase in bone density, a decrease in adipose mass with increased insulin sensitivity and glucose tolerance, all of which are independent of osteocalcin [*28*]. We could find no reports of studies that target only the osteoblast or osteoclast and look for effects on glucose homeostasis. Similarly, we could not find any studies in animals that manipulate osteoclast or osteoblast activity with

agents such as parathyroid hormone (PTH) or bisphosphonate and evaluate glucose homeostasis. Likewise, studies in animals that directly manipulate the degree of uncarboxylated osteocalcin by a low vitamin K diet or warfarin administration did not find changes in body weight but did not evaluate fasting glucose levels or insulin resistance [29]. Clearly, further refinement is required in understanding the molecular physiology of the osteocalcin protein as it related to its putative hormonal function. Additionally, explorations into the origin of uncarboxylated osteocalcin remain critical to the translation of this model to other species.

Despite inconsistencies between the osteocalcin knockout model and data generated from other rodent models of diabetes and cross-species comparisons, there emerged a proliferation of publications that claimed to substantiate the findings of the osteocalcin knockout model in humans. This immediate proliferation in studies on the topic of osteocalcin and diabetes risk merits some discussion on scientific bias, interpretation of individual results relative to the biology of osteocalcin, and robustness of study designs used. In the case of osteocalcin being the putative mediator of glucose metabolism, there appears to be an assumption that the hypothesis is indeed true in humans in the absence of robust evidence.

With some of the aforementioned refinements to the model as summarized in Fig. 1, the hypothesis that uncarboxylated osteocalcin is the link between diabetes and fracture risk has gained tremendous popularity. As summarized in Table 1, translation of the osteocalcin hypothesis has primarily been in the form

of post hoc, secondary analyses of observational and interventional studies that were not initially designed to test this hypothesis. These studies have generally not controlled for well-established factors that influence total and uncarboxylated osteocalcin concentrations, such as age, sex, and vitamin K status [*12*•]. Based on the current model described in Fig. 1, the proportion of osteocalcin that is not carboxylated would need to be manipulated in order to confirm that the uncarboxylated form of osteocalcin mediates glucose metabolism. In humans, it is well established that manipulation of dietary vitamin K alters the degree of carboxylation of osteocalcin [*50*]. Thus, diet can lead to changes in circulating concentrations of uncarboxylated osteocalcin independent of any signaling mechanism from bone. Therefore, one would predict that increasing vitamin K, which would decrease the uncarboxylated from of osteocalcin, would result in abnormal glucose metabolism and reduced insulin sensitivity. This, however, has not been observed. In contrast, the majority of studies of vitamin K intake and supplementation have been associated with a lower risk of insulin resistance and T2DM [*30*, *51*–*54*]. In secondary analysis of individuals participating in vitamin K supplementation trials, there has been no association between uncarboxylated osteocalcin and insulin resistance, as measured by the homeostatic model of insulin resistance (HOMA-IR) [*30*, *52*]. As previously stated, Karsenty and colleagues proposed the decarboxylation of osteocalcin through bone resorption [*19*]. In humans, this can be tested directly through the secondary analysis of studies conducted in patients prescribed anti-resorptive agents for treatment of osteoporosis [*55*]. By inference, if bone resorption was critical for generating

circulating forms of uncarboxylated osteocalcin, anti-resorptive agents would be associated with increased risk of insulin resistance and fracture risk. However, when examined in three independent cohorts that were characterized for measures of bone and glucose metabolism as well as anti-resorptive use, there were no differences in rates of diabetes or glucose metabolism among users and non-users of anti-resorptives [*10*•]. In addition, data from the national Danish database reported a reduced incidence of developing diabetes in persons taking anti-resorptive drugs [*56*]. Although there were no measures of osteocalcin in these larger analyses, a smaller analysis of postmenopausal women receiving bisphosphonates for 16 weeks indicated reductions in both total and uncarboxylated osteocalcin with no concomitant increase in measures of glucose homeostasis [*57*]. Moreover, changes in measures of osteocalcin were not correlated with measures of glucose homeostasis [*57*]. Similarly, other drugs known to alter bone turnover such as hormone replacement therapies as well as parathyroid hormone have not been associated with risk of diabetes in women [*58*–*62*]. This would indicate that at least in humans, altering the rate of bone resorption either does not lead to an increase in circulating forms of uncarboxylated osteocalcin and/or that increased uncarboxylated osteocalcin does not alter glucose metabolism.

This apparent contradiction in findings between humans and mice merits some discussion regarding the potential species differences in osteocalcin. Perhaps the most obvious disparity between humans and mice is the proportion of circulating osteocalcin that is in the uncarboxylated form. Whereas in mice,

osteocalcin is fully carboxylated in circulation when fed on standard chow, there is only about 50 % carboxylation of osteocalcin in humans [63]. Furthermore, the total circulating concentration of osteocalcin in humans is about 20 % that of other species [12•]. In the mouse osteocalcin knockout model, Ferron et al. [16] only increased uncarboxylated osteocalcin from 10 to 14 % of the total circulating osteocalcin in order to demonstrate improvement in glucose and insulin. This change is well within the intra-individual variability of uncarboxylated osteocalcin in humans in response to dietary fluctuations of vitamin K, and controlled metabolic studies demonstrate that providing adults with a dietary supplement of 500 μg vitamin K per day decreases the proportion of osteocalcin that is uncarboxylated from 55 to 15 % within a 5-day period [50]. This suggests that the narrow therapeutic range of uncarboxylated osteocalcin in mice is not applicable to humans given differences in absolute circulating concentrations between species. There are also other differences in osteocalcin between mice and humans including the homology between the genes encoding osteocalcin in mice and humans, which is only about 60 % [64]. In addition, whereas humans have a single copy of the osteocalcin gene that can be upregulated by 1,25$(OH)_2$ vitamin D, mice have three copies and downregulated by 1,25$(OH)_2$ vitamin D [12•, 65]. Collectively, these data seem to indicate that the species-specific differences in this protein require caution in the translation of the findings to humans.

Given that there is an overwhelming body of evidence indicating that manipulation of the uncarboxylated form of osteocalcin in humans does not result in effects proposed in the mouse models, it is somewhat surprising that the

majority of the initial human studies published concluded that the data indeed do [31–39,45, 47, 48]. The mouse model has attributed the endocrine signal from bone to the pancreas and insulin-sensitive tissue as the uncarboxylated form of osteocalcin, regardless of its origin. In contrast, many of the human studies have examined associations between the total osteocalcin as the exposure and measures of fasting glucose, insulin and/or HOMA-IR model as the outcome [31–39, 45, 47, 48]. Total osteocalcin is a standard measure of bone formation and is routinely used in epidemiological and intervention studies examining bone health as an outcome [66]. Therefore, it is not unexpected that secondary analysis of total osteocalcin and glucose measures can present an opportunity of convenience in existing cohorts to verify the century-old observation that diabetes is associated with alterations in bone health and specifically bone turnover. Unfortunately, very few authors have measured the uncarboxylated form of osteocalcin, which is not widely available, so conclusions which erroneously assume that total and uncarboxylated forms have the same molecular physiological role present an unintentional bias in the literature used to support this model. Additionally, serum uncarboxylated osteocalcin is highly correlated with total osteocalcin while the percentage of uncarboxylated osteocalcin (%ucOC) is not [12•]. This would indicate that the percent of osteocalcin that is uncarboxylated is indicative of vitamin K status, while the absolute uncarboxylated osteocalcin concentration is suggestive of osteoblast activity and cannot be used to isolate the hormonal role of uncarboxylated osteocalcin.

The use of total osteocalcin can also present other challenges in its interpretation because it is a biomarker of bone turnover. Bone is in a constant state of turnover, and it is estimated that the entire skeleton is regenerated every 10 years. Therefore, it has been difficult, if not impossible, to differentiate the role of osteocalcin as a marker of bone turnover from its role as a mediator of glucose metabolism through use of secondary analysis of completed osteoporosis studies.

With the recognition that total osteocalcin was not a robust marker for isolating its effects within bone on glucose metabolism, there was an emergence of studies that included other measures of bone turnover in their analyses. Gower and colleagues measured osteocalcin in the total and uncarboxylated forms, in addition to another measure of bone formation, P1NP. In this study, the proportion of osteocalcin that was not carboxylated was not different between participants with normal and impaired fasting glucose (NFG and IFG, respectively) [*40*•]. More importantly was the finding that both total osteocalcin and P1NP were higher in IFG participants compared to NFG participants [*40*•]. This study not only showed the discrepancy that can occur when studies interchange the concepts of total osteocalcin with uncarboxylated osteocalcin but also indicated the larger association that may exist between bone and glucose homeostasis beyond that of osteocalcin. Indeed, we were unable to identify any studies that included both multiple measures of bone turnover and identified a unique inverse association between total osteocalcin and insulin resistance among non-diabetics (Table 1).

In male, but not female, mice, the uncarboxylated form of osteocalcin has also been theorized to play a role in fertility via an increase in testosterone production [*67*, *68*•]. It was observed that testosterone production in Leydig cells was significantly diminished when co-cultured with primary osteoblasts lacking the osteocalcin gene. Furthermore, osteocalcin was reported to be a GPRC6A agonist, a receptor on Leydig cells [*24*, *68*•, *69*]. However, extensive investigation by the laboratory of Bräuner-Osborne could not confirm that osteocalcin is a ligand for GPRC6A [*70*•]. Although total osteocalcin decreased in men with anti-resorptive use, no change was observed in circulating testosterone concentrations [*71*]. The relationship between osteocalcin and testosterone may also be reflective of an overall relationship between bone and testosterone since additional measures of bone formation and resorption have also been associated with testosterone [*72*]. The logical next step in humans would be to determine if the uncarboxylated form of osteocalcin has any impact on male spermatogenesis.

Conclusions

Collectively, the evidence to support the hypothesis that osteocalcin is the factor responsible for the diabetes-fracture risk association has weakened as studies in humans accumulate. Whether this relates to species-specific differences that preclude translation of findings from the mouse model to humans or if the original model needs to be refined is not known. There has been improper use of causal language in citing results such that the different forms of osteocalcin have been used interchangeably in the literature even though only the uncarboxylated form

of osteocalcin was found to have an effect in the mouse model. Given that the total form of osteocalcin is a biomarker of bone turnover, the observed consistent inverse association between bone turnover and insulin resistance in non-diabetic patients has been interpreted by some as direct evidence that uncarboxylated osteocalcin is a hormone involved in human glucose metabolism. Randomized clinical trials designed specifically to test this hypothesis are required. Based on the evidence at this point in time, it is uncertain that uncarboxylated osteocalcin has potential as a therapeutic target for regulation of glucose metabolism in humans.

Compliance with Ethics Guidelines

Conflict of Interest

Sarah L. Booth declares that she has no conflict of interest.

Amanda J. Centi declares that she has no conflict of interest.

Caren Gundberg declares that she has no conflict of interest.

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Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

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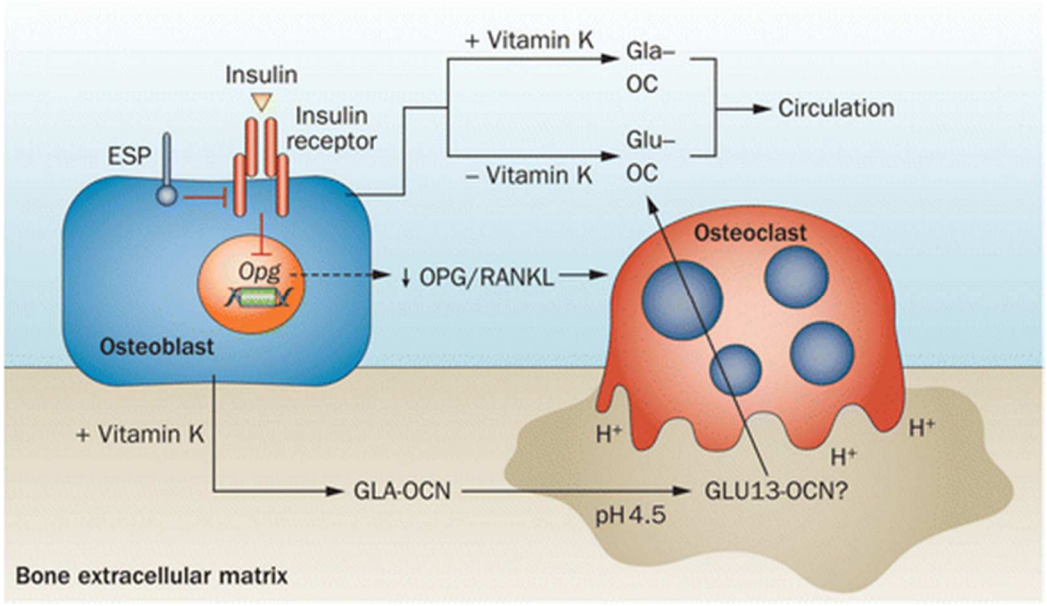

Fig. 1
Speculative model of the role of osteocalcin in glucose metabolism. From Booth et al. [12•], courtesy of Nature Publishing Group

Table 1
Publications reporting associations between osteocalcin and markers of glucose metabolism in non-diabetic adults. Updated and modified from [12•]

	Osteocalcin measure			**Additional bone formation marker**	**Additional bone resorption marker**
	tOC	**ucOC**	**%ucOC**		
HOMA-IR					
Inverse effect	[30–37]	[31, 38]	–	–	–[a]
Positive effect	–	–	–	P1NP [37]	–
No effect	[39, 40•,41–43]	[30, 39, 40•,42–44]	[30, 40•]	BAP [39] P1NP [40•]	CTX [39] NTX [30, 32, 39] TRACP [31]
Fasting plasma insulin					
Inverse effect	[32, 33,35–37, 45]	[31]	–	–	TRACP [31]
	–	–	–	–	–

	Osteocalcin measure			Additional bone formation marker	Additional bone resorption marker
	tOC	**ucOC**	**%ucOC**		
Positive effect					
No effect	[30, 39,41–43, 46]	[30, 35, 38,39, 42, 43,46]	[30]	BAP [39] P1NP [37]	CTX [39] NTX [30, 32, 39]
Fasting plasma glucose					
Inverse effect	[30–33, 35,37, 39, 47,48]	[31, 38, 46]	–	P1NP [37]	CTX [48]
Positive effect	–	–	–	–	–
No effect	[36, 41–43,46, 49]	[30, 39,42–44]	[30]	BAP [39] P1NP [47]	CTX [39] NTX [30, 32, 39] TRACP [31]

HOMA-IR homeostatic model assessment of insulin resistance, *tOC* total osteocalcin, *ucOC*uncarboxylated osteocalcin, *%ucOC* percentage of

uncarboxylated osteocalcin, *P1NP* procollagen type 1 amino-terminal propeptide, *BAP* bone-specific alkaline phosphatase, *CTX* C-terminal telopeptide, *NTX* N-terminal telopeptide, *TRACP* tartrate-resistant acid phosphatase
[a]No data reported

5.1.4 The role of osteocalcin in human glucose metabolism: marker or mediator? Nature Reviews: Endocrinology, 2013: 9(1), 43–55

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Competing interests

The authors declare no competing interests.

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Abstract

Abstract: | Increasing evidence supports an association between the skeleton and energy metabolism. These interactions are mediated by a variety of hormones, cytokines and nutrients. Here, the evidence for a role of osteocalcin in the regulation of glucose metabolism in humans is reviewed. Osteocalcin is a bone matrix protein that regulates hydroxyapatite size and shape through its vitamin-K-dependent, [gamma]-carboxylated form. The concentration of osteocalcin in the circulation is a measure of bone formation. The undercarboxylated form of osteocalcin is active in glucose metabolism in mice. Total serum osteocalcin concentrations in humans are inversely associated with measures of glucose metabolism; however, human data are inconclusive with regard to the role of uncarboxylated osteocalcin in glucose metabolism because most studies do not account for the influence of vitamin K on the proportion of undercarboxylated osteocalcin or differentiate between the total and uncarboxylated forms of osteocalcin. Furthermore, most human studies do not concomitantly measure other bone turnover markers to isolate the role of osteocalcin as a measure of bone formation from its effect on glucose metabolism. Carefully designed studies are required to define the role of osteocalcin and its carboxylated or undercarboxylated forms in the regulation of glucose metabolism in humans.

Introduction

In 2007, Lee *et al*.5 suggested that in mice osteocalcin acts as a hormone to affect insulin sensitivity and energy expenditure. Osteocalcin was reported to improve glucose tolerance by increasing [beta]-cell proliferation and insulin expression and secretion. Osteocalcin also increased insulin sensitivity in peripheral tissues, increased adiponectin expression and protected the mice from adiposity. The most surprising finding was that, although osteocalcin nominally contains three [gamma]-carboxyglutamic acid residues, only the uncarboxylated or undercarboxylated (that is, containing only one or two [gamma]-carboxyglutamic acid residues) forms of osteocalcin were biologically active.5–12 Since those reports, a growing number of studies have linked serum osteocalcin concentrations to glucose homeostasis in humans. This research has led to speculation that serum osteocalcin concentrations have clinical utility as a risk marker for diabetes mellitus.

The bone-fat axis hypothesis has been extensively reviewed elsewhere.9,11,13–16Here, the unique species-specific features of vitamin-K-dependent osteocalcin are reviewed and evidence for a role of osteocalcin in energy metabolism in humans is discussed.

A vitamin-K-dependent protein

Proteins that include [gamma]-carboxyglutamic acid contain a propeptide recognition site that is essential for their binding to the vitamin-K-dependent carboxylase. After carboxylation, the propeptide is removed and the mature

protein is secreted.19 Aside from the vitamin-K-dependent clotting factors, the requirement for vitamin-K-dependent [gamma]-carboxylation to activate proteins has been demonstrated in many tissues with diverse biological functions. In the skeleton, matrix Gla protein (MGP) and Gla-rich protein (also known as UCMA) require the presence of [gamma]-carboxyglutamic acid to function as potent inhibitors of calcification in cartilage and other soft tissues,20,21 whereas the [gamma]-carboxyglutamic acid residues in osteocalcin are involved in regulation of the size and shape of bone mineral.

Regulation of crystal size and shape

Early studies to investigate the function of osteocalcin and other proteins containing [gamma]-carboxyglutamic acid were designed based on the knowledge that the [gamma]-carboxyglutamic acid residues in the vitamin-K-dependent clotting factors were essential in order to bind Ca^{2+} and interact with phospholipid membranes to facilitate the clotting cascade. Similarly, the [gamma]-carboxyglutamic acid residues in osteocalcin bind free Ca^{2+} and hydroxyapatite.

Analysis by circular dichroism, NMR imaging and X-ray crystallography 22–24 has revealed that osteocalcin is a globular protein comprised of three [alpha]-helices, a hydrophobic core, an unstructured N-terminus and an exposed C-terminus (Figure 2). All three [gamma]-carboxyglutamic acid residues are found in the first helical region (at amino acid positions 17,21 and 24 in human osteocalcin), and when bound to free Ca^{2+}, facilitate a conformational change that aligns them in a complementary fashion to the Ca^{2+} ions on the C-axis of the

hydroxyapatite crystal lattice.

The importance of the osteocalcin-hydroxyapatite interaction is illustrated by the fact that osteocalcin amino acid sequences from all animal species investigated share extensive amino acid sequence homology at the central [gamma]-carboxyglutamic acid region. However, considerable sequence variation exists at other regions. Compared with the human sequence, the overall protein sequence is highly conserved in most species (80-95%), but to a lesser extent in the frog and chicken (70%), mouse (60%) and bony fish (40%).24 Human osteocalcin is encoded by a single-copy gene located at the distal long arm of chromosome 1. By contrast, mice have a cluster of three osteocalcin genes in a 23 kb span oriented in the same transcriptional direction. Two of the genes (*Bglap* and *Bglap2,* also known as *OG1* and *OG2*) are expressed only in bone, whilst the third, the osteocalcin-related gene (*Bglap-rsl,* also known as *ORG*), is expressed at low levels in brain, lung and kidney, but not in bone.25 Examination of the promoters of the human and rat genes reveal that they are very similar with respect to the organization of regulatory elements and their response to hormones and growth factors, whereas the mouse gene exhibits differences, particularly in response to 1,25-dihydroxyvitamin D_3.26 The human and rat osteocalcin genes are dose-dependently upregulated by 1,25-dihydroxyvitamin D_3, whereas the mouse osteocalcin gene is downregulated.26,27

The function of osteocalcin in bone is thought to be dictated by its structure. Immunolocalization studies show that the protein is distributed throughout the

mineralized regions of bone matrix, dentine and calcified cartilage in rats.28 *In vitro*growth of hydroxyapatite is inhibited by low concentrations of osteocalcin, which suggests that this protein's function is related to the control of crystal morphology. In the bone of all species studied, osteocalcin first appears coincident with the onset of mineralization *in utero,* and its levels increase in tandem with hydroxyapatite deposition during skeletal growth.29,30 In rat and mouse osteoblast cultures, osteocalcin appears only after the extracellular matrix accumulates and begins to mineralize.31

In 1996, Ducy *et al.*32 generated an osteocalcin-deficient mouse model. Given the abundance of osteocalcin, its restriction to bone and specificity to hydroxyapatite, it was surprising that the mice had no overt phenotypic abnormalities.32 The mice were reported to be morphologically normal at birth, viable and fertile with no skeletal defects. By the age of 6 months, however, they exhibited a small increase in cortical bone thickness, a consequence of an increased bone formation rate, but had normal osteoclast activity. Evaluation of the structural properties by whole bone biomechanical testing showed significant differences in bone strength. Bone mineral content was unaffected, but subsequent analysis of the crystal properties of hydroxyapatite showed altered mineral composition in the cortical bone of osteocalcin-knockout mice.33

Hydroxyapatite varies in crystallinity as a result of impurities, primarily carbonate ions, which accumulate with time as the crystal matures. In osteocalcin-deficient mice, the hydroxyapatite crystals showed decreased

carbonate substitution, which suggested they consisted of less-mature and/or remodelled mineral compared to that of the wild-type mice.33 Small-angle X-ray scattering confirmed that the hydroxyapatite crystals were immature (and also thinner and smaller) in the osteocalcin-deficient mice than in the wild-type mice. Furthermore, crystals in the osteocalcin-deficient mice were less well aligned along the collagen fibrils than those of the wild-type mice.34 Bone strength is a function of both bone quantity and quality, and changes in optimal crystal size and orientation have been associated with increased brittleness.35

A marker of bone remodelling

The skeleton undergoes continuous remodelling (turnover) of bone, with removal of old bone by osteoclasts and coordinated replacement with new bone by osteoblasts. In the steady state, this coupling of bone formation and resorption maintains bone mass. The discovery of osteocalcin presented an opportunity for clinicians searching for noninvasive markers to aid in the management of patients with osteoporosis. Histomorphometric analysis and calcium kinetics demonstrated that serum osteocalcin levels were correlated with bone formation and osteoblast number.36–38 As a product synthesized by osteoblasts, osteocalcin has been used as a marker of bone formation. Furthermore, in normal rats, circulating osteocalcin originates primarily from new bone synthesis rather than from the breakdown of bone.39

The majority of commercially available osteocalcin assays measure the intact molecule and a large osteocalcin$_{1\text{-}43}$ fragment that is generated by tryptic activity

in the circulation.40 Fragments of osteocalcin encompassing the mid-molecular region 41also circulate. These fragments are derived from the action of osteoclastic matrix metalloproteinases and cathepsin K during bone resorption,42,43 and are rapidly cleared in individuals with normal renal function. Although some single antibody assays have limited capacity to detect these fragments in serum, only urine-based, mid-molecular assays have sufficient sensitivity and specificity to measure them accurately.44

Current markers of bone formation include the N-terminal propeptide of type I collagen, bone-specific alkaline phosphatase, and intact or N-mid-molecule osteocalcin in serum. Resorption markers include collagen N-telopeptide and C-telopeptide, tartrate-resistant acid phosphatase, and urinary mid-molecule osteocalcin. Most have a circadian variation and respond to changes in bone formation and resorption accompanying growth, age, menopause, metabolic bone diseases and medications that affect bone turnover.45,46

[gamma]-Carboxylation of osteocalcin

Undercarboxylation in humans

In most species, all three vitamin-K-dependent [gamma]-carboxyglutamic acid sites in the osteocalcin molecule are fully carboxylated. However, in humans, osteocalcin in bone and serum is incompletely carboxylated (undercarboxylated osteocalcin). High-dose warfarin co-administered with high-dose vitamin K maintains adequate blood clotting but the osteocalcin is not fully carboxylated,47 which suggests that the liver sequesters vitamin K at the expense

of bone. *In vitro*, carboxylation is an ordered process with Glu22 and Glu24 in osteocalcin being carboxylated first, followed by Glu17 in humans (corresponding to Glu13 in mice).48 Analysis of osteocalcin isolated from 20 human bone samples found carboxylation to be (mean ± SD) 67% ± 14%, 88% ± 9%, and 93% ± 4% at residues Glu17, Glu21 and Glu24, respectively,49 reflecting long-term variability in vitamin K availability to the bone. Circulating osteocalcin is similarly undercarboxylated. Estimates of the percentage of undercarboxylated osteocalcin by direct ELISA or by differential binding to hydroxyapatite coupled to an immunoassay suggest that up to 50% of osteocalcin in serum is undercarboxylated in normal individuals, and that the percentage of undercarboxylated osteocalcin reflects current vitamin K intake.50 Human osteocalcin concentrations in bone and in the circulation are only 20% of those found in other species.

Response to vitamin K manipulation

In humans, the current recommendations for dietary intake of vitamin K are 90-120 µg per day based on median phylloquinone intakes.51 Some menaquinones are produced by gut flora in the large intestine, but the extent to which endogenous menaquinone production contributes to the daily requirement for vitamin K is not known in humans.52 However, a subclinical deficiency of vitamin K can be created within days by limiting dietary intakes of phylloquinone without a concomitant change in gut flora or menaquinone status.53

The degree to which a vitamin-K-dependent protein such as osteocalcin is

carboxylated is used as a functional indicator of vitamin K status. However, biomarkers of vitamin K status are consistently fluctuating in humans, as human diets contain varying vitamin K content from day to day. Dietary restriction of phylloquinone to <35 μg per day causes rapid and marked increases in undercarboxylated osteocalcin (expressed as the proportion of total osteocalcin that is not fully [gamma]-carboxylated, or percentage of undercarboxylated osteocalcin).54 Conversely, supplementation with phylloquinone in doses ranging from 250 μg per day for 14 days to 5,000 μg per day for 24 months markedly decreases the percentage of undercarboxylated osteocalcin to less than 10% (Figure 3).55–59 This response is consistent for the percentage of undercarboxylated osteocalcin regardless of the forms of vitamin K studied.56,60 The rapidity of the observed changes noted in short-term studies suggests that carboxylation of the three Glu residues in osteocalcin may change in response to fluctuations in intakes of vitamin K on a daily basis in humans. Therefore, any discussion regarding the role of carboxylation of osteocalcin in energy metabolism in humans requires consideration of the vitamin K status at the time of measurement.

Correlation among osteocalcin measures

The percentage of undercarboxylated osteocalcin is influenced by vitamin K status, whereas serum total concentrations of osteocalcin are influenced by osteoblastic synthesis independent of vitamin K (Figure 4). This interdependence of the different available measures of circulating osteocalcin in humans has given

rise to some confusion in the published literature regarding the predictive value of serum osteocalcin for the risk of type 2 diabetes mellitus (T2DM).

By use of either the current ELISA or hydroxyapatite binding assays, absolute concentrations of undercarboxylated osteocalcin are highly correlated with total osteocalcin, whereas the percentage of undercarboxylated osteocalcin is not (Figure 4). As reviewed elsewhere,[61,62] the accurate interpretation of hydroxyapatite-binding assays requires equivalent detection of both carboxylated and undercarboxylated forms of osteocalcin. Unfortunately, few studies in humans that used ELISA have assessed the carboxylated forms and, hence, the percentage of undercarboxylated osteocalcin could not be calculated.

Osteocalcin and glucose metabolism

Overview

The existence of altered bone metabolism among patients with diabetes mellitus is a well-characterized phenomenon, although the mechanisms are not well understood.[63] Bone mass is low in patients with type 1 diabetes mellitus (T1DM) but higher than normal in those with T2DM. Yet, in both patients with T1DM and those with T2DM, increased fracture risk is observed at a given BMD as compared to that in individuals without diabetes mellitus.[64–67] The effects of glucose toxicity on osteoblasts, low levels of insulin and insulin-like growth factor 1, altered vitamin D metabolism, low bone formation, raised levels of advanced glycation end products, increased cortical bone porosity, and biomechanical forces have all been implicated in the pathology associated with changes in bone

mass and fracture risk in patients with diabetes mellitus, but these processes do not completely explain the clinical findings.68

Osteoblast precursors and adipocytes reside within the bone marrow and are thought to differentiate from a common precursor.69,70 Increased commitment of bone-marrow-derived mesenchymal stem cells to adipocytogenesis occurs at the expense of osteoblastogenesis, and high levels of marrow fat are associated with an increased risk of both osteoporosis and diabetes mellitus.71 A growing body of evidence shows that the pancreas and adipocytes secrete bone-active hormones.13–16 Adipose tissue produces leptin, which regulates bone remodelling via the central nervous system.72–74 These relationships pose an important question: namely, are these relationships bidirectional, such that bone has a regulatory role in energy metabolism?

Genetic studies in mice

In a search for potential mediators of metabolism in mice, Lee *et al*.5 found two candidate genes for which protein biosynthesis is restricted to bone. The first of these,*Esp* (also known as *Ptprv*), is a gene expressed only in osteoblasts and Sertoli cells and encodes a transmembrane protein, tyrosine phosphatase (OST-PTP, also known as R-PTP-V). Both global and osteoblast-specific deletion of *Esp* produced animals that were lean, hypoglycaemic, and had increased [beta]-cell proliferation, insulin secretion and insulin sensitivity. When adipocytes from normal mice were grown in the presence of osteoblast-conditioned media from either wild-type mice or mice with global deletion of*Esp*, expression of

adiponectin was increased by 40% and 100%, respectively. Likewise, insulin expression was increased in wild-type islets grown in the presence of osteoblast-conditioned media from wild-type mice (40%) or from mice with global deletion of *Esp*(100%). These results suggest that osteoblasts secrete a factor or factors that affect [beta] cells and adipocytes and that OST-PTP regulates the activity of this factor. Because of its osteoblast-specific expression, a logical candidate for this factor was osteocalcin (Figure 5).

Lee *et al.* re-examined the phenotype of their osteocalcin-deficient mice,5 which had been described earlier.32 Osteocalcin-knockout mice were obese, with elevated glucose and lipid concentrations, reduced insulin levels, reduced numbers of [beta] cells, and were both glucose-intolerant and insulin-insensitive. However, in the original description of the osteocalcin-knockout mouse,32 bone formation was elevated compared with wild-type mice, a finding that conflicts with other animal models of diabetes mellitus, in which bone formation is reduced.13 In contrast to observations in humans with T2DM, both insulin secretion and sensitivity were decreased in these mice, effects that were attributed to a decrease in adiponectin expression in adipose tissue.6Overall, the phenotype was the exact opposite of that observed in the $Esp^{-/-}$ mice. Islets and adipocytes from wild-type mice, cultured with osteoblast-conditioned media derived from osteocalcin-knockout mice, showed decreases in insulin and adiponectin. Furthermore, the metabolic phenotype was normalized in $Esp^{-/-}$ mice lacking one allele of osteocalcin, further supporting the notion that OST-PTP and osteocalcin are in the same pathway.

The circulating levels and expression of osteocalcin were normal in *Esp*$^{-/-}$ mice, which suggests that OST-PTP does not regulate the biosynthesis of osteocalcin, but rather regulates its metabolic function. Given that the only known modifiable aspect of osteocalcin is its [gamma]-carboxyglutamic acid residues, Lee *et al.*5 showed that uncarboxylated osteocalcin, but not carboxylated osteocalcin, induces expression of both adiponectin in adipocytes and insulin in islets. This finding presents a major paradigm shift, given that all known vitamin-K-dependent proteins require the presence of [gamma]-carboxyglutamic acid for function, including the carboxylating enzyme itself.18

To establish the role of osteocalcin in glucose metabolism, Ferron *et al*.6 implanted mice with osmotic minipumps containing uncarboxylated osteocalcin. Doses that delivered up to 3 ng/ml to the circulation were given for 4 weeks, and resulted in low blood glucose levels and an increase in serum insulin levels. In these mice, the circulatinglevel of undercarboxylated osteocalcin was 7ng/ml,6 approximately 10% of total circulating osteocalcin levels measured in adult mice fed standard rodent chows. At the doses given to the mice, the proportion of undercarboxylated osteocalcin increased only to 14%, which is within the intraindividual variation that is normal in humans consuming a varied diet. This observation highlights the need to compare uncarboxylated or undercarboxylated osteocalcin to total osteocalcin in both human and animal studies to understand any relevant changes that are related to metabolism or vitamin K intake.

Insulin signalling in osteoblasts

Ferron *et al*.7 considered OST-PTP to be a regulatory factor for enzymes of the vitamin K cycle but no tyrosine phosphorylation was found on either the enzymes or osteocalcin itself. However, substrate trapping showed that the insulin receptor in osteoblasts was a substrate for OST-PTP and that increased phosphorylation of the osteoblast insulin receptor is found in *Esp*$^{-/-}$ mice.7 Orthologues for all rodent protein tyrosine phosphatases have been found in humans, with the exception of OST-PTP. Ferron *et al*.7 searched for other protein tyrosine phosphatases that might compensate for this absence in humans. They showed that tyrosine-protein phosphatase nonreceptor type 1 (PTN1, also known as PTP-1B) was expressed in human osteoblasts. PTN1 is a ubiquitously expressed tyrosine phosphatase that has been implicated in multiple signalling pathways.75 Subsequently, Zee *et al*.76 demonstrated that tyrosine-protein phosphatase nonreceptor type 2 (PTN2, also known as TC-PTP) also regulates insulin receptor phosphorylation in human osteoblasts and is, in fact, more highly expressed in bone than either OST-PTP or PTN1 (Figure 5).

Insulin signalling in osteoblasts decreases the expression of osteoprotegerin, an inhibitor of osteoclast maturation, and hence activates bone resorption.7,8 Osteoclast bone resorption produces an acid environment which, as proposed by Ferron *et al*.,7may decarboxylate osteocalcin.15 Furthermore, in this model, decarboxylation occurs at only the first [gamma]-carboxyglutamic acid residue at position 17 (or position 13 in mice) and is sufficient for osteocalcin

hormonal action. The final regulation of this process is via leptin which, through sympathetic tone, regulates the expression of*Esp*.74

This scenario raises several questions. First, it has been established that hydrolytic side reactions can occur during acidification of osteocalcin in solution, producing multiple osteocalcin fragments rather than intact decarboxylated osteocalcin.77Furthermore, when human osteoclasts are cultured on bovine bone, only a small amount of intact osteocalcin is released during acidification. When the matrix osteocalcin is subsequently degraded enzymatically, fragments of osteocalcin are produced.44 These observations are consistent with clinical studies showing that increased levels of urinary osteocalcin fragments are associated with bone resorption.78Secondly, the requirement for bone resorption to activate matrix-bound osteocalcin is inconsistent with results of Lee *et al.*,5 in which osteoblast-conditioned media from wild-type and *Esp*-knockout mice affected the expression of adiponectin and insulin. Finally, undercarboxylated osteocalcin in human circulation could be the consequence of two separate processes: incomplete carboxylation of osteocalcin due to suboptimal vitamin K intake or decarboxylation during osteoclast resorption (Figure 5).

G-coupled protein receptor

The final element necessary to complete a putative endocrine loop mediated by osteocalcin was the identification of a tissue-specific receptor. This identification was achieved in an indirect way. On the basis of the fact that OST-PTP was expressed in osteoblasts and Leydig cells of the testes, Oury *et*

*al.*79 showed that uncarboxylated osteocalcin also regulated male fertility. Furthermore, the researchers showed that binding of osteocalcin *in vitro* to a G-protein-coupled receptor (Gprc6a) in isolated mouse Leydig cells was associated with an increase in the biosynthesis of testosterone.79

Ample evidence exists for the involvement of GPRC6A in the regulation of biological processes in humans. GPRC6A is a seven-transmembrane receptor that mediates signalling of a wide range of L-[alpha]-amino acids, predominantly the basic amino acids, arginine, lysine and orthinine.80 The calcium-sensing receptor is its closest homologue.80 GPRC6A is widely expressed in brain and peripheral tissues of humans, including kidney, skeletal muscle, testis and leucocytes.81 GPRC6A is directly activated by high concentrations of Ca^{2+}, a response that is augmented by carboxylated osteocalcin.82 Mice lacking Gprc6a have been produced by two separate laboratories. Wellendorph *et al.*83 found that knockout mice were viable and fertile, developed normally and exhibited no significant differences in body weight or skeletal manifestations compared with their wild-type littermates. By contrast, Pi *et al.*84reported a complex metabolic phenotype, decreased BMD and impaired mineralization. Given the wide expression of this receptor, the question remains how osteocalcin functions as a cell-specific ligand. Perhaps a co-receptor is required for tissue specificity, as seen for FGF-23 (another bone-derived factor) and its co-receptor, Klotho.85

Bone turnover and glucose metabolism

Bone biopsy data in humans, and in mice, show that the cortical bone

formation rate is low in the setting of diabetes mellitus.86 These studies are corroborated in humans by measures of bone turnover, including serum total osteocalcin and urinary N-telopeptide, which are lower in patients with T1DM 87,88 and T2DM 88 than in individuals without diabetes mellitus. Conversely, interventions that improve glycaemic control are associated with a concomitant increase in total osteocalcin serum concentrations in patients with T1DM 89 and T2DM.90,91 These changes occur in parallel to changes in other measures of bone formation and resorption, which implies that a single measurement of total osteocalcin does not confirm its independent hormonal role. Furthermore, in studies that measure only undercarboxylated forms of osteocalcin, interpretation of results are complicated by the high correlation between undercarboxylated and total osteocalcin concentrations (Figure 4). Few studies, however, have measured multiple forms of osteocalcin in response to improvement in glycaemic control. In one study of improved glycaemic control, primarily achieved through dietary modification, total osteocalcin levels increased but levels of the undercarboxylated form of osteocalcin did not change.91

Epidemiological studies

A plethora of studies have examined cross-sectional associations between serum concentrations of different forms of osteocalcin and various measures of glucose metabolism and adiposity among nondiabetic individuals. In cross-sectional studies of nondiabetic adults and children, some of which are summarized in SupplementaryTable 1 online,92–104 total osteocalcin serum

concentrations are, in general, inversely associated with measures of glycaemia, consistent with the hypothesis that either osteocalcin influences [beta]-cell function and insulin sensitivity or that increased glucose levels affect bone turnover.5,6 Similarly, total osteocalcin serum concentrations seem to be inversely associated with measures of adiposity, such as percentage body fat and BMI (Supplementary Table 1 online). However, some researchers have reported a positive association between serum total osteocalcin concentrations and insulin sensitivity index among lean, but not obese, adult men.105

Whereas an abundance of studies have assessed serum total osteocalcin, few studies have measured serum undercarboxylated osteocalcin. Of those that have, an overall lack of association was found between the undercarboxylated form of osteocalcin and measures of glucose metabolism or adiposity. A few studies have reported an inverse association in some subgroups, such as obese individuals 96 and male adolescents.101 In one longitudinal study in older men (55-80 years) at risk of cardiovascular disease and not taking antidiabetic medication, baseline concentrations of total osteocalcin, but not undercarboxylated osteocalcin, were positively associated with a 2-year change in fasting insulin levels and insulin resistance, as captured by the homeostasis model assessment of insulin resistance (HOMA-IR).106 In another 3-year study of older men and women (60-80 years), concentrations of carboxylated osteocalcin were associated with a decrease in fasting insulin and HOMA-IR but neither total nor undercarboxylated osteocalcin was associated with changes in HOMA-IR.94 Similarly, baseline concentrations of total osteocalcin, undercarboxylated

osteocalcin and percentage of undercarboxylated osteocalcin did not predict development of diabetes mellitus in a community-based adult population.107

The regulation of insulin sensitivity by osteocalcin has been proposed to act through an effect on an adipocytederived hormone, adiponectin.5 This hormone, levels of which decrease as fat mass increases, is recognized to be an important regulator of insulin sensitivity.108 In the past 5 years, some researchers have proposed that leptin also has an indirect role in osteocalcin's hormonal action.5,74,109 In the few cross-sectional studies that measured adiponectin and total osteocalcin serum concentrations, adiponectin was positively associated with total osteocalcin in adults,94but was inversely associated in some subgroups, including Asian-American children.101Neither the uncarboxylated form of osteocalcin nor N-telopeptide levels were associated with adiponectin levels in adults.94 Similarly, levels of total osteocalcin were not associated with leptin concentrations in adults with or without diabetes mellitus.110 The data are currently too sparse and equivocal to draw conclusions.

The overall lack of association between undercarboxylated osteocalcin and glucose metabolism in humans contrasts with findings in mouse models, in which the uncarboxylated form of osteocalcin is the active hormonal form. If total osteocalcin, but not undercarboxylated osteocalcin, is inversely correlated with glucose metabolism and adiposity in humans, it becomes important to discern if the osteocalcin protein is mediating this effect or if it is an independent indication of an impaired osteoblast, as suggested by studies in an insulin-resistant T2DM

rat model.111 Unfortunately, most studies do not include independent measures of bone formation and resorption, which limits our ability to address the question of whether osteocalcin acts as a mediator or marker. In the few studies that included multiple bone turnover markers there did not seem to be any consistent trends; indeed, the preponderance of studies that included bone resorption markers reported no association between bone resorption and glucose metabolism measures (Supplementary Table 1).

The lack of human evidence to support an association of bone resorption with glucose metabolism is inconsistent with the hypothesis that uncarboxylated osteocalcin is the consequence of decarboxylation during osteoclastic resorption in mice,15 but consistent with the lack of effect observed in rats.111 Not enough studies have included bone formation measures other than osteocalcin to differentiate between an osteocalcin effect *per se* or specific changes in bone formation that relate to glucose metabolism. Certainly, confirmation through changes in multiple bone formation markers would be consistent with the known effects of insulin on the osteoblast.12 In fact, in mice, insulin signalling stimulates the synthesis not only of osteocalcin but also of other major matrix proteins, as well as MGP, in osteoblasts.8

Comparisons of observational studies are problematic because most *are post hoc,*secondary analyses of studies that were not designed to examine the role of osteocalcin on glucose metabolism. Furthermore, neither longitudinal nor cross-sectional studies take into account the many factors that independently influence

osteocalcin serum concentrations in humans, such as age, diet, ethnicity and sex. The majority of studies have measured total osteocalcin as the exclusive measure of bone formation. Similarly, many researchers have interpreted the findings of their studies (using total osteocalcin serum concentrations in humans) as providing support for the mouse models that identified uncarboxylated osteocalcin as the hormonal form. Few studies directly measured both the total and the undercarboxylated forms of osteocalcin, which precludes forming conclusions regarding the importance of osteocalcin carboxylation in glucose metabolism. Similarly, very few studies used assays that measure the percentage of undercarboxylated osteocalcin, which would address any concern related to the strong correlation between total and undercarboxylated osteocalcin concentrations (Figure 4).

The influence of bone-active agents

The notion that osteocalcin is activated by osteoclastic resorption of the matrix led to the question of whether agents that target the osteoclast would also increase the risk of diabetes mellitus or affect insulin requirements. A study of elderly patients with T1DM found that those who were taking the bisphosphonate alendronate had a reduced insulin requirement compared with those receiving only calcium and vitamin D.112 In a large population-based study in Denmark, a reduced risk of T2DM was found in individuals receiving alendronate, etidronate and raloxifene.113 However, markers of bone resorption and bone formation, including osteocalcin, are equally decreased in women with and without diabetes

mellitus during antiresorptive therapy.114 Although these observations conflict with the hypothesis that a reduction in uncarboxylated osteocalcin is detrimental to glucose metabolism, positive effects of the bisphosphonates and raloxifene on circulating lipids have been noted, potentially confounding these observations.115,116

A study published in 2011 evaluated the relationship between 3-month changes in bone turnover and 12-month measures of body weight, fat mass, and levels of adiponectin, leptin and glucose in participants treated with either parathyroid hormone 1-84 (PTH_{1-84}) or alendronate.117 As expected, both total and undercarboxylated osteocalcin increased with PTH_{1-84} and decreased with alendronate treatment. 3-month changes in undercarboxylated osteocalcin were negatively associated only with 12-month changes in body weight and fat mass, and positively with 12-month changes in adiponectin in those individuals receiving PTH_{1-84}. No significant relationships were found between changes in undercarboxylated osteocalcin and changes in any measured parameters in individuals receiving alendronate. No other information was provided regarding potential relationships with total osteocalcin or other markers of bone formation and resorption.

Vitamin K and glucose metabolism

The capacity to experimentally manipulate the percentage of osteocalcin that is carboxylated without affecting bone turnover through the use of vitamin K supplementation has been well established in humans.54 Therefore, manipulation

of vitamin K levels provides an ideal model for testing the hypothesized link between uncarboxylated osteocalcin and glucose metabolism in humans.

High vitamin K intakes are associated with a low percentage of undercarboxylated osteocalcin,118 and are also associated with reduced insulin resistance.119–121 The results of these observational studies infer that a low percentage of uncarboxylated osteocalcin actually improves glucose metabolism in humans. As major sources of phylloquinone in the diet are green leafy vegetables, high phylloquinone intakes are generally associated with healthier lifestyle and dietary habits,122 which may independently contribute to reduced insulin resistance.123 Therefore, it is critical to isolate the effect of vitamin K manipulation on carboxylation of osteocalcin and its subsequent effect on glucose metabolism in clinical trials. Short-term supplementation of supraphysiological doses of menaquinone-4 (vitamin K_2) improved acute insulin response after an oral glucose load in individuals with a high percentage of undercarboxylated osteocalcin at baseline.124 In studies using high doses of menaquinone-4, no effect on body weight was observed despite the reduction in the percentage of undercarboxylated osteocalcin.125,126 In the studies that measured glucose metabolism, administration of both menaquinone-4 and phylloquinone reduced the percentage of undercarboxylated osteocalcin and improved HOMA-IR in men (Table 1).120,125 Similar findings, however, were not noted in women.120,127 The role of vitamin K in regulating insulin sensitivity still requires more systematic investigation in humans.

Warfarin

On the basis of the presence of osteocalcin and other vitamin-K-dependent proteins in bone, patients receiving warfarin have been compared with age-matched individuals not receiving warfarin to explore the physiological effects of vitamin K antagonism and deficiency on bone mass, with varying levels of success.128 Warfarin is among the 15 most widely prescribed drugs in the USA,129 and is used for the prevention and treatment of thrombosis.130 Of importance to the role of osteocalcin in energy metabolism is the interruption of the carboxylation reaction for all vitamin-K-dependent proteins, including osteocalcin, in response to warfarin treatment (Figure 1). Carboxylation of osteocalcin is dramatically decreased by warfarin in both mice and humans.62,131 In mice, the first [gamma]-carboxyglutamic acid residue (Glu13) is the most sensitive to warfarin 131 because, as in humans, it is least likely to be carboxylated when vitamin K availability in the osteoblast is reduced.47

With regard to glucose metabolism, one case study reported hypoglycaemia in a premature infant exposed to warfarin *in utero.*132 However, the infant had neonatal hepatopathy, which may have impaired glucose metabolism independently of undercarboxylated osteocalcin. No other reports of hypoglycaemia exist in an otherwise large body of literature of case studies of warfarin embryopathy. One of the major challenges in studying the role of undercarboxylated osteocalcin in glucose metabolism among patients using warfarin is the potential interaction with glucose-lowering drugs that result in

increased absorption of warfarin independent of the carboxylation of osteocalcin.133,134 There are no reports of changes in insulin resistance in patients treated with warfarin alone. Therefore, it is unlikely that warfarin studies will provide insight into the role of osteocalcin in energy metabolism.

Exercise and weight loss

Physical inactivity decreases BMD and increases insulin resistance.135 Levinger *et al.* proposed the hypothesis that the increase in glucose disposal rate associated with exercise is driven by the forces exerted on bone by muscles, which increases bone formation and insulin action through uncertain mechanisms.136 Resistance training increases measures of bone formation whilst transiently suppressing measures of bone resorption.137 However, the current data are inconsistent, as resistance training is associated with either no effect 138–140 or an increase 105,139,141 in total serum osteocalcin concentrations. In one study of resistance training in combination with dietary counselling for weight loss, an increase in serum osteocalcin concentrations was observed, but these changes were not statistically associated with changes in insulin resistance or circulating adiponectin levels.105

Only a few exercise studies have actually directly measured the undercarboxylated form of osteocalcin. Among obese men, acute aerobic activity resulted in an increase in the levels of total and uncarboxylated osteocalcin and adiponectin.136 In a subgroup analysis of participants with diabetes mellitus, acute exercise increased the proportion of undercarboxylated osteocalcin by 4%,

and this change was correlated with a decrease in glucose levels. By comparison, an increase in vitamin K intake of an amount that is equivalent to two servings of green, leafy vegetables, can decrease the percentage of undercarboxylated osteocalcin by ~15% through reduction in the undercarboxylated form of osteocalcin but not the total osteocalcin form.54 This finding would suggest that the well-documented daily variation in vitamin K intake,142 which causes larger changes in uncarboxylated osteocalcin levels than are achieved though exercise, would result in rapid and large fluctuations in glucose concentrations. Therefore, it is unclear if this is an independent osteocalcin effect on glucose metabolism or if osteocalcin is a marker for the short-term changes in bone turnover created by acute exercise.

In another study, obese children who engaged in a 6-month exercise programme had an increase in insulin levels with a concomitant increase in serum concentrations of both total and undercarboxylated osteocalcin.143 By contrast, no associations between insulin levels and serum concentrations of total or undercarboxylated osteocalcin were found among obese children not participating in the exercise program. This result led the authors to conclude that there was a regulatory loop, in which an increase in osteocalcin synthesis in response to exercise stimulates insulin secretion up to a currently undefined level, at which point the high insulin level exerts a negative effect on osteocalcin secretion.

Currently the human data are inconclusive regarding the role of undercarboxylated osteocalcin in improving glucose metabolism in response to

exercise. Furthermore, the study designs used do not differentiate the changes in total osteocalcin concentrations that occur in response to changes in bone formation (regardless of causality) from the changes in total osteocalcin concentrations that may be responsible for direct effects on insulin action through feed-forward loops.

Conclusions

The well-established observation that diabetes mellitus is associated with altered bone metabolism has stimulated tremendous interest in identifying potential mechanisms that link adipose tissue and the skeleton. The hypothesis that osteocalcin, a vitamin-K-dependent protein produced in the osteoblast, acts as a hormone to affect insulin sensitivity and energy expenditure was first proposed based on a series of studies conducted *in vitro* and *in vivo* knockout mice models. In these models, the uncarboxylated form of osteocalcin was identified as having the hormonal function. Since that time, a surge of reports in humans have used *post hoc* correlative analysis to examine associations between osteocalcin and glucose metabolism in studies with varied designs.

The existence of species-specific differences in osteocalcin challenges the extrapolation of findings from the mouse models to humans. In most species, osteocalcin is fully carboxylated, whereas in humans osteocalcin in bone and serum is incompletely carboxylated, and the degree of carboxylation is determined by vitamin K availability in the diet. The concentration of osteocalcin in human bone and blood is only 20% of that observed in other species. Whereas

mouse models have tremendous value in studies of diabetes mellitus, several examples of genetically modified mouse models exist for which one cannot extrapolate directly from mouse to humans.144 Osteocalcin may be a protein that would fall in this category and caution needs to be used in the interpretation of the current literature.

Overall, a lack of consensus exists among current literature to support a unique effect of undercarboxylated osteocalcin on the regulation of glucose metabolism or on measures of adiposity in humans. However, few studies have measured the undercarboxylated form of osteocalcin. Furthermore, interpretation of the effect of uncarboxylated osteocalcin is confounded by its close correlation with total osteocalcin levels when measured using hydroxyapatite-binding assays or specific immunoassays, which reduces its utility as an independent biomarker. Manipulation of vitamin K intake can alter the proportion the osteocalcin that is undercarboxylated without altering bone turnover. Interestingly, a high intake of vitamin K, which results in a low proportion of undercarboxylated osteocalcin, has been reported to reduce insulin resistance, which is the opposite to what would be expected based on the mouse model.

Another limitation of human studies to date is the reliance on a single biomarker of bone turnover. Serum concentrations of total ostecalcin are established measures of bone formation. However, in the absence of other bone turnover markers, one cannot isolate the putative role of osteocalcin in glucose metabolism from its role as a measure of bone formation. In fact, several studies

in the past few years show that the relationship between adipose tissue and the skeleton is complex, involving not only pancreatic and adipose tissue hormones but also nutritional factors and enteric hormones.145–147 It seems likely that additional new factors will be added to the repertoire of agents that connect bone and energy metabolism. In the interim, further studies are required to precisely define the effect of insulin on osteoclast activity and the nature of osteocalcin receptor binding in humans.

Review criteria

Articles cited in this Review were obtained using a PubMed database search using the following terms: “osteocalcin”, “glucose”, “diabetes”, “vitamin K”, “bone”, “bone formation”, “GPRC6A”. No restrictions were placed on the year the papers were published. Searches were conducted up to June 2012. However, only English-language articles were considered. Similarly, only full-text, peer-reviewed publications were used in preparing this manuscript.

Key points

- Osteocalcin is a calcium-binding bone matrix protein that contains the vitamin-K-dependent amino acid, [gamma]-carboxyglutamic acid; circulating osteocalcin concentrations are a measure of bone formation

- Studies in mice show that osteocalcin acts as a hormone to affect insulin sensitivity and energy expenditure; only the undercarboxylated form of

osteocalcin is active

- Human dietary intake of vitamin K is suboptimal, in contrast to that in mice—and, as a consequence, both bone and serum osteocalcin are undercarboxylated in humans
- Most human studies examining the association between serum osteocalcin and measures of glucose metabolism do not differentiate between the total and undercarboxylated forms or take into account vitamin K intake
- Most human studies also do not measure other bone turnover markers to distinguish circulating osteocalcin as a measure of bone turnover from its effect on glucose metabolism
- In mice, the uncarboxylated form of osteocalcin is linked to glucose homeostasis, whereas in humans the data are inconclusive

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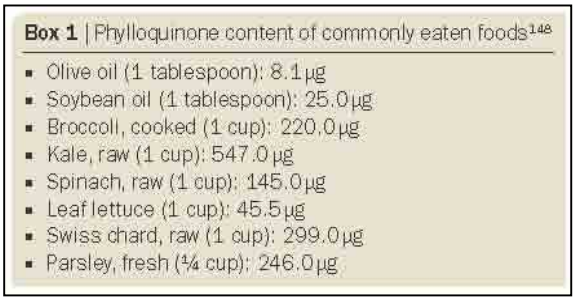

Box 1 | Phylloquinone content of commonly eaten foods 148

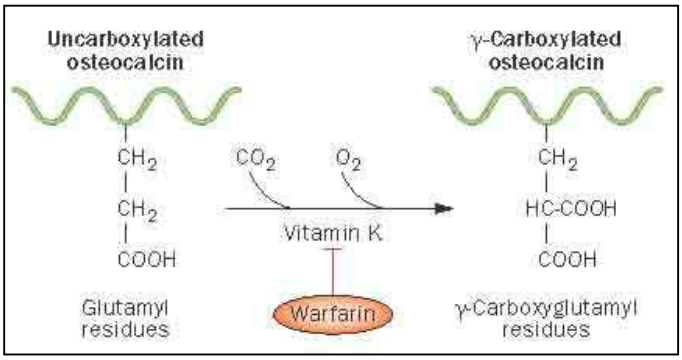

Figure 1 | Vitamin K is required for the formation of y-carboxyglutamic acid. [gamma]-Carboxyglutamic acid (Gla) is a unique amino acid that is created by vitamin-K-dependent post-translational modification of specific glutamic acid residues in all Gla-containing proteins, including osteocalcin. This process is inhibited by warfarin.

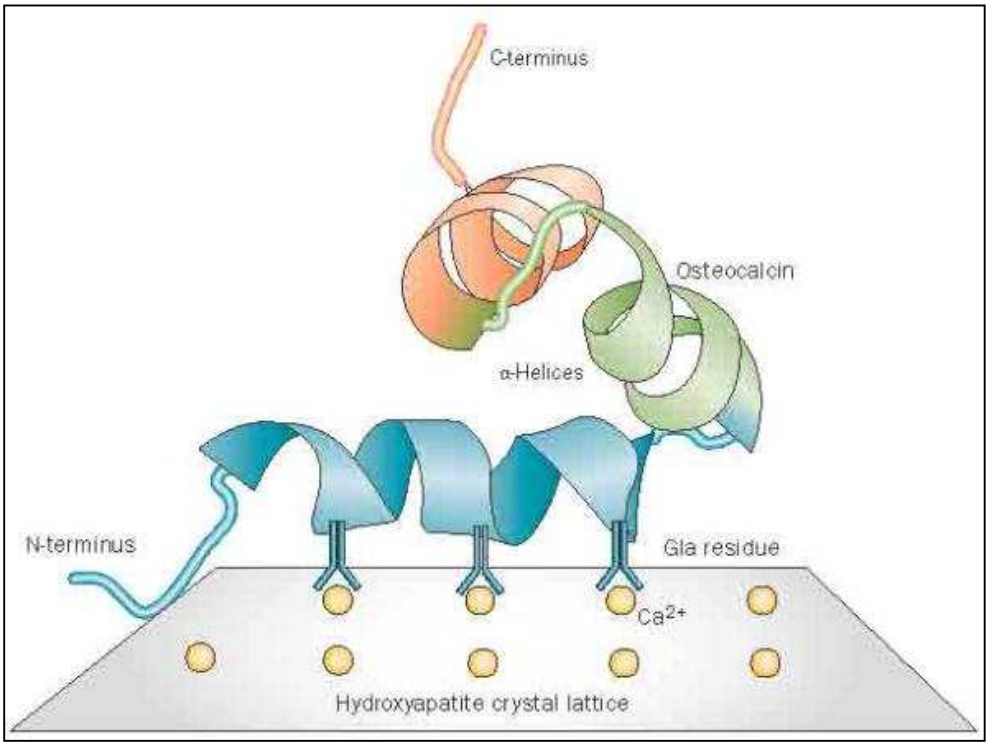

Figure 2 | Direct structural analysis of osteocalcin by NMR imaging and X-ray crystallography predicts a tight globular structure comprised of three [alpha]-helices, a C-terminal hydrophobic core and an unstructured N-terminus. All three [gamma]-carboxyglutamic acid (Gla) residues are found in the first helical region. Osteocalcin amino acid sequences from all species share extensive homology at the central region containing the [gamma]-carboxyglutamic acids, but there is considerable sequence variation in other regions. The [gamma]-carboxyglutamic acid residues are complementary to the calcium ions on the c-axis of the hydroxyapatite crystal lattice, and are positioned to control crystal size and shape within the constraints of the collagen fibril. Permission obtained from Nature Publishing Group (C) Hoang, Q. Q. et al. Nature 425, 977-980 (2003).

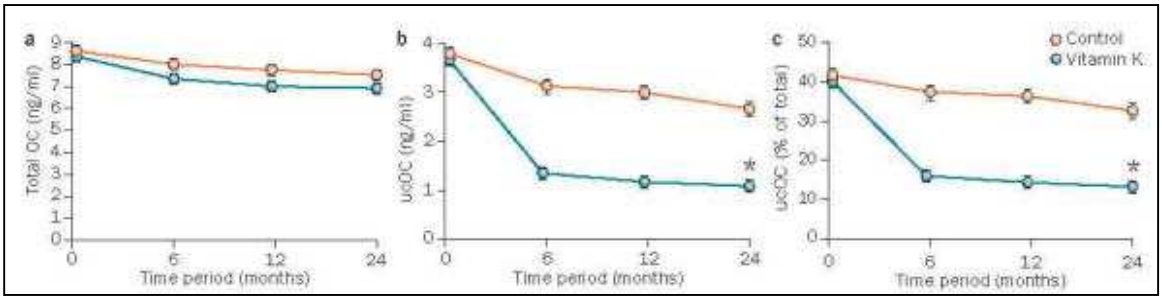

Figure 3 | Response of all forms of osteocalcin to vitamin K supplementation in humans. 2-year changes (mean +/- SEM) in a | serum total osteocalcin, b | serum undercarboxylated osteocalcin and c | percentage undercarboxylated osteocalcin in 396 men and women (age range 60-80 years) in response to 500 [mu]g per day of vitamin K in the form of phylloquinone or no vitamin K

supplementation (control) in a randomized, double-blind clinical trial.55 2-year changes in total osteocalcin concentrations did not differ between groups. By contrast, vitamin K supplementation significantly decreased concentrations of undercarboxylated osteocalcin and the percentage of undercarboxylated osteocalcin in the phylloquinone group (*P<0.05) but not in the control group, which demonstrates the need for vitamin K status to be considered when discussing the role of undercarboxylated osteocalcin in energy metabolism in humans. Abbreviations: OC, osteocalcin; ucOC, undercarboxylated osteocalcin.

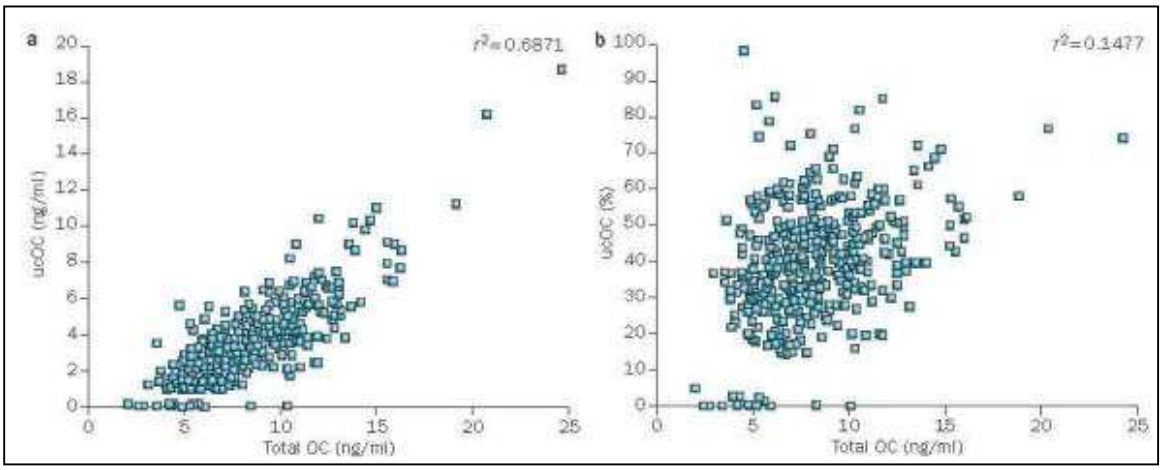

Figure 4 | Correlations among osteocalcin measures. a | Serum undercarboxylated osteocalcin is highly correlated with total serum concentrations of osteocalcin, whereas b | the serum percentage of undercarboxylated osteocalcin does not correlate with total serum concentrations of osteocalcin. The 426 men and women who participated were community-based older adults (age range 60-80 years) free of osteoporosis and cardiovascular disease at the time of enrolment in a randomized double-blind clinical trial examining the effect of vitamin K supplementation on bone health.55 Osteocalcin measures were made prior to vitamin K supplementation.55 Abbreviations: OC, osteocalcin; ucOC, undercarboxylated osteocalcin.

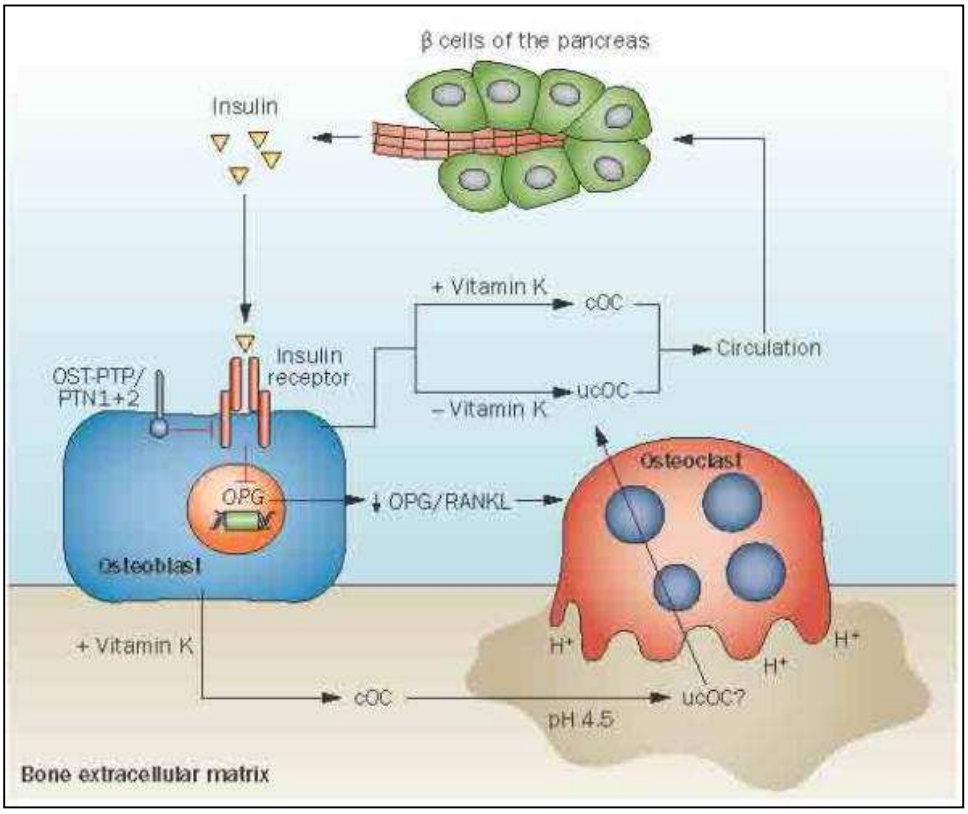

Figure 5 | Speculative model of the role of osteocalcin in glucose metabolism. The presence of undercarboxylated osteocalcin in human circulation could be the consequence of two separate processes: incomplete carboxylation of osteocalcin due to suboptimal vitamin K intake or decarboxylation during osteoclast resorption. Insulin signaling in osteoblasts limits the production of osteoprotegerin, an inhibitor of osteoclast maturation. This mechanism facilitates osteoclast bone resorption, producing an acid environment that decarboxylates (and hence activates) intact osteocalcin. OST-PTP/PTN 1+2 dephosphorylates the insulin receptor in osteoblasts leading to inhibition of insulin signalling. Abbreviations: cOC, carboxylated osteocalcin; OPG, osteoprotegerin; ucOC, undercarboxylated osteocalcin. Permission to adapt obtained from Nature Publishing Group (C) Karsenty, G & Ferron, M. Nature 481, 314-320 (2012).

Table 1 | Response of osteocalcin forms and measures of glucose metabolism to vitamin K supplementation

Form of vitamin K	Daily dose	Duration	Change in treatment group compared with control group				Body weight	Reference
			Total osteocalcin	Undercarboxylated osteocalcin	% Undercarboxylated osteocalcin	Glucose metabolism		
Menaquinone-4	30 mg	4 weeks	Not indicated	Decrease	Decrease	No change in glucose; increase in insulin sensitivity index	No change	125
Menaquinone-4	45 mg	36 months	Not indicated	Decrease	Decrease	Not indicated	No change	126
Phylloquinone	1 mg	12 months	No change	Decrease	Decrease	No change in glucose, HOMA-IR or insulin	NR	127
Phylloquinone	500 µg	36 months	No change	Decrease	Decrease	No change in glucose; reduction in HOMA-IR and insulin in men only	NR	120

Abbreviations: HOMA-IR, homeostasis model assessment of insulin resistance; NR, not reported.

Supplementary Table 1: Cross-sectional Associations between Bone Turnover Markers and Glucose Metabolism and Adiposity in Non-Diabetic Children and Adults

Measure of Glucose Metabolism and Adiposity	n	Measures of Bone Formation			Measures of Bone Resorption	Reference
		TOC[a]	undOC	Other		
Glucose Metabolism						
HOMA-IR	2493 M+F	inverse [b]	-[c]	-	-	92
	1597 M	inverse	inverse	-	no effect (TRACP)	99
	580 M+F	inverse	-	-	-	100
	380 M+F	inverse	-	-	no effect (NTX)	93
	348 M+F	inverse	no effect	-	no effect (NTX)	94
	199 M	-	inverse	-	-	95
	106 M+F (children)	no effect	inverse (Caucasian only)	-	-	101
	36 F (children)	no effect	-	positive (PICP)	positive (NTX)	102

Insulin (Fasting)	2493 M+F	inverse	-	-	-	[92]
	380 M+F	inverse	-	-	no effect (NTX)	[93]
	348 M+F	no effect	no effect	-	no effect (NTX)	[94]
	199 M	-	no effect	-	-	[95]
	140 M+F (children)	no effect	no effect	-	-	[103]
	106 M+F (children)	no effect	no effect	-	-	[101]
	83 M	no effect	no effect	-	-	[96]
	36 F (children)	no effect	-	positive (PICP)	positive (NTX)	[102]
Glucose (Fasting)	2493 M+F	inverse	-	-	-	[92]
	1597 M	inverse	inverse	-	no effect (TRACP)	[99]
	380 M+F	inverse	-	-	no effect (NTX)	[93]
	348 M+F	inverse	no effect	-	no effect (NTX)	[94]

	199 M	-	inverse	-	-	[95]
	140 M+F (children)	no effect	no effect	-	-	[103]
	106 M+F (children)	positive	no effect	-	-	[101]
	83 M	no effect	inverse (obese only)	-	-	[96]
	64 M+F (obese)	inverse	-	no effect (P1NP)	-	[104]
Adiposity						
% Body Fat	443 M+F	inverse (just in women)	-	-	-	[97]
	380 M+F	inverse	-	-	no effect (NTX)	[93]
	307 M+F	inverse	-	-	no effect (NTX)	[98]
	106 M+F (children)	inverse	no effect	-	-	[101]
BMI	2493 M+F	inverse	-		-	[92]
	380 M+F	inverse	-	-	no effect (NTX)	[93]

	106 M+F (children)	inverse	inverse (males only)	-	-	101
	83 M	no effect	no effect			96

[a] F, females; M, males; TOC, total osteocalcin; undOC, undercarboxylated osteocalcin

[b] Only significantly statistically ($P<0.05$) associations were indicated by direction.

[c] No measures were reported

5.2 Abstracts

5.2.1 Phylloquinone (Vitamin K) and dihydrophylloquinone content of selected baked products.

David B. Haytowitz, BAShowell, Amanda J Centi, Monica Ramos-Brown, Sarah L Booth

Presented at the 2015 Institute of Food Technologists Annual Meeting & Food Expo

Justification: Phylloquinone (Vitamin K) is involved in blood coagulation. It may also play a role in reduction of risk in coronary artery calcification. In recent years, as these products have been reformulated to reduce trans fatty acids, a concomitant reduction in dihydrophylloquinone has been observed, requiring new, up-to-date data.

Objective: The objective of this research is to generate nationally representative vitamin K values for baked products. These data will update and expand the data available on vitamin K in USDA's National Nutrient Database for Standard Reference (SR).

Methods: Sample units for a variety of baked products were collected at 12 locations in the US from retail outlets. The composites were prepared and shipped to the Vitamin K Laboratory at the Jean Mayer Human Nutrition Research Center on Aging for analysis. Vitamin K forms (Phylloquinone, dihydrophylloquinone, and menaquinone-4) were determined by reversed-phase HPLC with fluorescence detection.

Results: The phylloquinone content of selected baked products ranged from 0.2 mcg/100g in bagels to 30.6 mcg/100g in shortbread. Values for shortbread ranged from 25.4 to 37.7 mcg/100g. Values for dihydrophyloquinone in most baked products were quite low, while only a few were higher. Sugar cookies contained 8.9 mcg/100g, with a range of 8-9.9 mcg/100g and shortbread contained 1.8 mcg/100g, with a range of 0-2.7 mcg/100g. Menaquinone-4 values for all baked products were very low.

Significance: Phylloquinone values for baked products, along with those for other foods, will be added to SR, which in turn will be used to assess vitamin K intake in the NHANES-What We Eat in America survey. These values will enable investigators to monitor dietary intakes of phylloquinone, and to assess the impact of vitamin K intake on specific health conditions.

5.2.2 Decreases in Circulating Uncarboxylated Osteocalcin are not associated with HOMA-IR

Amanda J. Centi, M Kyla Shea, Caren Gundberg, Edward Saltzman, Sarah L. Booth

Presented at: Experimental Biology 2015

Osteocalcin (OC) in its uncarboxylated form (ucOC) may improve glucose metabolism in mice. However, human data are equivocal. Vitamin K (VK) is the only known factor to reduce the proportion of circulating OC that is uncarboxylated. We hypothesized that a decrease in circulating ucOC through VK supplementation would increase insulin resistance (IR) measures in humans.
Serum was collected from weight-stable older and younger men and women (n=42) before and after 28d of VK supplementation (500 µg phylloquinone/d). All meals and beverages were provided to control for other nutrients. The primary outcome was defined as IR change as assessed by the homeostatic model (HOMA-IR). Total OC (tOC), ucOC and insulin were measured by radioimmuno assay. Glucose was measured by an enzymatic kinetic method. Measured covariates included triglycerides, BMI, age group and sex. A repeated measures ANOVA was used to determine if decreases in ucOC increased HOMA-IR.
In response to VK supplementation, significant decreases in circulating ucOC (pre=4.79± 2.61 ng/mL post = 1.59±2.11ng/mL, $p<0.001$) and tOC (pre=9.19±4.05ng/mL post = 8.48±3.86ng/mL, $p=0.015$) were observed. However, there were no significant changes in HOMA-IR (pre=2.20± 0.98 post = 2.25±1.12, $p=0.78$). Further, no differences in HOMA-IR were observed between sexes ($p=0.39$) or age groups ($p=0.20$) in relation to decreases in circulating ucOC or tOC.

The lack of association between changes in circulating ucOC and HOMA-IR suggests that in humans, ucOC does not have a role in IR.

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

Stephanie G. Harshman, Donald Smith, **Amanda Centi,** J Philip Karl, Xiaohua Shen, M Kyla Shea, Xueyan Fu, Sarah L Booth

Presented at: Experimental Biology, 2015

Phylloquinone (PK) and menaquinone (MK) are naturally-occurring forms of vitamin K (VK).There is selective tissue distribution and conversion of dietary PK to MK4, providing indirect evidence of unique MK4 functions beyond those established for PK. We determined the effect of dietary PK manipulation on the ratio of PK:MK4 in tissues of male lean Zucker rats (n=24, 4mo). Rats were pair-fed a PK-supplemented (S) (10±0.5 mg PK/kg) or PK-deficient (D) (0.03±0.003 mg PK/kg) diet for 12 weeks. PK was the sole dietary VK form provided. Liver, kidney, brain, pancreas, mesenteric adipose tissue, serum and diet PK and MK4 concentrations were measured by HPLC. Data were analyzed by ANOVA, with $p<0.01$ considered significant based on a Bonferroni adjustment. Data presented as mean±SD.

PK:MK4 was significantly higher in kidney, liver and mesenteric adipose tissue on the S diet (1.41±0.14 ; 78.8±15.5 and 21.8±4.8 pmol/g, respectively) compared to D diet (0.54±0.3, 7.4±4.9 and 12.2±5.4 pmol/g, respectively) ($p<0.01$). In contrast, PK:MK4 in brain (0.34±0.03, S; 0.55±0.3, D) and pancreas (0.86±0.3, S; 0.87±0.26, D) did not significantly differ between diet groups (both $p>0.147$).

PK supplementation resulted in an increase in PK:MK4 in liver, kidney and adipose tissue. In brain and pancreas, tissues that have a preferential conversion of PK to MK4, there was concomitant increase in MK4 such that the PK:MK4 ratio is maintained. The implications of this ratio merits further investigation in order to elucidate the roles of MK4.

5.2.4 Trend in the Content and Forms of Vitamin K in Processed Foods

Monica Brown-Ramos, **Amanda J. Centi,** David B. Haytowitz, Sarah L. Booth

Presented at: Experimental Biology 2015

High intake of *trans* fatty acids has been linked to deleterious health effects including increased risk of cardiovascular disease. Since 2006, the Food and Drug Administration requires companies to label the *trans* fatty acid content of foods, which has resulted in an overall decrease of commercially-hydrogenated oils in the food supply. It is not known if an unanticipated consequence due to changes in use of hydrogenated oils in the food supply resulted in a change in the forms and amount of vitamin K (VK) of processed foods. To test this, we compared 253 foods for individual forms of VK in foods analyzed pre- and post-2006 as part of the U.S. Department of Agriculture (USDA) Nutrient and Food Analysis Program. Forms of VK were analyzed via HPLC. Overall, foods identified as rich sources of the hydrogenated form of VK pre-2006 (dihydrophylloquinone; dK), had lower amounts of dK with a concomitant increase in the parent form of VK, phylloquinone (PK). For example, dK in chocolate chip cookies decreased (48.2 ± 38.5 µg/100g pre-2006 vs 0.3 ± 0.6 µg/100g post-2006) but PK increased (11.9 ± 9.0 µg/100g pre-2006 vs 22.5 ± 17.1 µg/100g post-2006). However, the range of dK was large within foods, suggestive of a wide range of current practices regarding use of hydrogenated oils in the U.S. food supply. Updated VK food composition data that captures trends in processed foods is beneficial to health care providers who counsel patients on oral anticoagulants where monitoring of dietary VK is necessary to sustain stability of oral anticoagulants.

5.2.5 Response of serum osteocalcin to caloric restriction with and without exercise in post-menopausal women

Amanda J. Centi, Sarah L. Booth, Caren M. Gundber, Barbara Nicklas, M Kyla Shea

Presented at: Experimental Biology 2013

Osteocalcin (OC) is a vitamin K-dependent bone protein that has been linked to regulation of glucose metabolism and body weight in mice. We determined the association between OC, weight loss and insulin resistance in 74 post-menopausal women replete in vitamins K, D and calcium, who were randomized to caloric restriction alone (CR) or caloric restriction + exercise (CR+Ex) (age 59±6yrs; BMI 33.0±3.7kg/m^2). Serum total (tOC), carboxylated (cOC) and uncarboxylated OC (ucOC), fasting blood glucose and insulin were measured pre- and post-intervention. HOMA-IR was calculated. Multivariable linear regression determined if absolute changes in OC forms and HOMA-IR were associated with weight loss. After 16 weeks, participants lost a similar amount of body fat (9.27±4.98%, $p \geq 0.60$). No changes in serum OC forms were observed post weight-loss (all $p \geq 0.21$). There were no significant associations between change in OC forms and change in weight (all $p \geq 0.12$) or between change in OC forms and change in HOMA-IR in either group (all $p \geq 0.17$). Our findings do not support a regulatory role of OC in energy metabolism or insulin resistance in post-menopausal women who are replete in nutrients implicated in bone health.

5.2.6 The Impact of Vitamin K1 Supplementation on Biomarkers of Vitamin K in Hemodialysis Patients: A Randomized Trial

Rachel M. Holden, Wilma H. Hopman, **AJ Antczak**

Presented at: American Society of Nephrology Annual Meeting 2012

Background: Sub-clinical vitamin K deficiency is common in hemodialysis (HD) patients and may be a modifiable risk factor for cardiovascular disease. We aimed to investigate whether short-term vitamin K_1 supplementation improved biomarkers of vitamin K status in HD patients, assessed by circulating vitamin K and uncarboxylated prothrombin (PIVKA-II [protein induced by vitamin K absence]).

Study Design: Interventional randomized placebo-controlled crossover trial. Investigators and patients were blinded to the participant's order of treatment allocation

Setting and Participants: 20 (12 male, 8 female) prevalent stable HD patients

Intervention: 1 mg of vitamin K_1 daily versus matching placebo for 2 weeks with a 4 week wash-out period between treatments.

Outcomes: Absolute and percent change in plasma levels of vitamin K_1 and PIVKA-II.

Measurements: Fasting vitamin K_1 concentrations were measured using HPLC. PIVKA-II concentrations were determined by ELISA.

Results: Two subjects were withdrawn from the study due to acute hospitalization. The baseline mean vitamin K_1 level was 0.9±1.0 and 0.7±0.6 for vitamin K_1 and placebo, respectively (P=0.3). The mean increase in vitamin K_1 level in response to vitamin K_1 supplementation was 6.7 nmol/L (4.0-10.0, P<0.0001) versus 0.6 nmol/L (0.3-1.0, p=.001) in the placebo (P<0.001). There was a 36.5% reduction in PIVKA-II levels in the vitamin K_1 (1.9 ± 1.3 to1.0 ± 0.5, p=0.002) versus a 10% increase in the -placebo (1.5±1.2 to 1.7±1.4, p=0.07). Post-vitamin K_1 supplementation, 94.1% of subjects were in the normal range for PIVKA-II. There was no impact of treatment allocation order on the absolute or percent change of vitamin K_1 or PIVKA-II.

Conclusions: This study shows that biomarkers of vitamin K status respond to short-term supplementation with vitamin K_1. Studies evaluating the impact of vitamin K_1 supplementation on cardiovascular outcomes are warranted.

5.2.7 Response of osteocalcin to weight loss and exercise in post menopausal women

Amanda J. Centi, Sarah L. Booth, Caren Gundberg, Barbara Nicklas, M Kyla Shea

Presented at: FASEB Summer Research Conference on molecular, Structural & Clinical Aspects of Vitamin K& Vitamin K-Dependent Proteins

Osteocalcin (OC) is a vitamin K-dependent bone protein that has been linked to regulation of glucose metabolism and body weight in mice. We determined the association between OC, weight loss and insulin resistance in 74 post-menopausal women replete in vitamins K, D and calcium, who were randomized to caloric restriction alone (CR) or caloric restriction + exercise (CR+Ex) (age 59±6yrs; BMI 33.0±3.7kg/m^2). Serum total (tOC), carboxylated (cOC) and uncarboxylated OC (ucOC), fasting blood glucose and insulin were measured pre- and post-intervention. HOMA-IR was calculated. Multivariable linear regression determined if absolute changes in OC forms and HOMA-IR were associated with weight loss. After 16 weeks, participants lost a similar amount of body fat (9.27±4.98%, $p \geq 0.60$). No changes in serum OC forms were observed post weight-loss (all $p \geq 0.21$). There were no significant associations between change in OC forms and change in weight (all $p \geq 0.12$) or between change in OC forms and change in HOMA-IR in either group (all $p \geq 0.17$). Our findings do not support a regulatory role of OC in energy metabolism or insulin resistance in post-menopausal women who are replete in nutrients implicated in bone health.