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*Association between circulating osteocalcin and cardiometabolic risk factors following a 4-week leafy green vitamin K-rich diet*

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Running Title: Osteocalcin and cardiovascular risk factors following vitamin K-rich diet

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## **Abstract**

### **Background**

Evidence suggests that lower serum undercarboxylated osteocalcin (ucOC) may be negatively associated with cardiometabolic health. We investigated whether individuals with the largest suppression of ucOC following an increase in dietary vitamin K1, exhibit a relative worsening of cardiometabolic risk factors.

### **Materials and Methods**

Men (n = 20) and women (n = 10) aged  $62 \pm 10$  years participated in a randomised, controlled, cross-over study. The primary analysis involved using data obtained from participants following a high vitamin K1 diet (HK; 4-week intervention of increased leafy green vegetable intake). High and low responders were defined based on the median percent reduction (30%) in ucOC following the HK diet. Blood pressure (resting and 24-hour), arterial stiffness, plasma glucose and lipid concentrations, and serum OC forms were assessed.

### **Results**

Following the HK diet, ucOC and ucOC/tOC were suppressed more ( $p < 0.01$ ) in high responders (41% and 29%) versus low responders (12% and 10%). The reduction in ucOC and ucOC/tOC was not associated with changes in blood pressure, arterial stiffness, plasma glucose or lipid concentrations in the high responders ( $p > 0.05$ ).

### **Discussion/Conclusion**

Suppression of ucOC via consumption of leafy green vegetables has no negative effects on cardiometabolic health, perhaps, in part, because of cross-talk mechanisms.

## 1. Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide [1]. A diet rich in fruit and vegetables is an important, non-therapeutic approach to reduce CVD development and progression [2, 3]. Evidence suggests that diets rich in green leafy vegetables increase nitric oxide bioavailability and can improve vascular health [4, 5]. However, we have previously shown that a 4-week dietary intervention involving an increased intake of leafy green vegetables, did not reduce blood pressure (BP) or arterial stiffness [6]. One potential explanation for the absence of a beneficial effect on BP and arterial stiffness may be related to other bioactive components found in leafy green vegetables that concomitantly influence vascular health. For example, vitamin K1 is abundant in leafy green vegetables and regulates several coagulation factors including vitamin K-dependent proteins (VKDP) [7].

One such protein is osteocalcin (OC), a VKDP derived from osteoblasts that exists in two forms: carboxylated OC (cOC) and undercarboxylated OC (ucOC) [8-10]. cOC has a high affinity to hydroxyapatite within the bone matrix and is therefore thought to reflect bone mineralisation [11, 12], whereas ucOC is proposed as the bioactive form of OC in several target tissues [13]. Growing evidence suggests an association between OC, in particular total OC (tOC) and ucOC with hypertension, vascular calcification, atherosclerosis and CVD mortality [14-17]. However, the literature is conflicting and it is unclear whether tOC or its isoforms are associated with positive or negative effects on cardiometabolic health [18, 19]. We have previously shown that a diet rich in leafy green vegetables, and thus vitamin K1, reduces circulating ucOC levels [20].

The current study was a sub-analysis examining the cardiometabolic implications of ucOC suppression following an increased intake of predominantly leafy green vegetables. It was of interest to investigate whether a reduction in ucOC levels was correlated with changes in cardiometabolic risk factors, and whether this could explain, at least in part, the lack of a beneficial effect on blood pressure following an increase in dietary nitrate. Participants from the high vitamin K1 intervention were divided into high/low responders based on the suppression of ucOC following the intervention. The aim was to determine if a large reduction in ucOC (high responders) would be associated with alterations in cardiometabolic risk factors including blood pressure, arterial stiffness, blood glucose and lipid concentrations.

## 2. Methods

The data for this paper was collected for the Vegetable Intake and Blood Pressure (VIABP) study (ACTRN12615000194561). The study was approved by The University of Western Australia Human Research Ethics Committee and was completed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants. The study was a randomised, controlled crossover trial and methodology has been described in full elsewhere [6]. In brief, middle and older aged (40 to 74 years of age) community dwelling men and women with pre-hypertension or untreated grade one hypertension were recruited to participate. Each participant received three 4-week dietary interventions, each interspersed with a 4-week washout period. The VIABP study was originally designed with the following dietary interventions: (1) increased intake of nitrate-rich leafy green vegetables (high nitrate); (2) increased intake of nitrate-poor vegetables (low nitrate); and (3) no increase in vegetables (control). As vitamin K1 is also found predominately in leafy green vegetables, these three dietary interventions have been equated to: (1) high vitamin K1 intake (HK); (2) low vitamin K1 intake (LK); and (3) control diet (CON) [20]. Considering the primary aim of this study is to examine the association between the suppression of ucOC and cardiometabolic risk factors (and given the LK diet did not suppress ucOC), we predominantly considered data from the HK intervention.

Resting BP and pulse wave velocity (PWV) (SphygmoCor XCEL 2012, AtCor Medical Pty. Ltd.) were measured pre and post the 4-week dietary intervention, as previously described [6]. Ambulatory BP was recorded over a 24-hour period, every 20 minutes during the day and every 30 minutes during the night, mean BP was determined for the 24-hour period [6]. Plasma concentrations of glucose, triglycerides, total cholesterol, HDL cholesterol and calculated LDL cholesterol were analysed by PathWest laboratories (Fiona Stanley Hospital, Perth, Australia). Serum tOC was measured by sandwich electrochemiluminescence immunoassay using the Roche Cobas N-Mid OC assay (Roche Diagnostics, Mannheim). The inter-assay coefficients of variation were 2.3% and 4.8% at levels of 18 and 90 ng/mL, respectively. Serum ucOC was determined using the hydroxyapatite binding method (Calbiochem) [21]. The inter-assay imprecision for percentage binding of cOC was 8% and 12% at OC of 100 and 15 ng/mL, respectively. Plasma creatinine was measured at baseline and glomerular filtration rate (GFR) was estimated using plasma creatinine levels based on the known equation [22]. Vitamin K intake was estimated as previously described [20].

### *Statistical analysis*

All statistical analysis was performed using Statistical Package for the Social Sciences (SPSS Inc. Chicago, IL, USA, version 22). Independent samples t-tests were conducted to examine OC concentrations between males and females and if characteristics known to influence ucOC (BMI, age, vitamin K intake and GFR) were different between the high responders and low responders at baseline. Spearman rho correlations were used to assess the relationship between pre-intervention OC concentrations and pre-intervention outcome measures. Spearman partial correlations were used for the additional adjustments of age and body mass index (BMI) as they are strong influencers of ucOC levels [23, 24].

When considering post intervention data from the HK diet intervention, participants were divided into high responders (suppression of ucOC  $\geq$  median [ $\geq 30\%$ ]) and low responders (suppression of ucOC  $<$  median [ $< 30\%$ ]), based on the percent change in ucOC. The between groups (high versus low responders) effect of the HK diet on changes in OC, vascular and metabolic outcomes were assessed using one-way ANOVA. Within groups effects for pre- and post-intervention were assessed using paired samples t-tests, as previously reported [20]. All data reported as mean  $\pm$  SEM and statistical analysis was conducted at the 95% confidence level of significance ( $p < 0.05$ ).

### **3. Results**

Baseline characteristics are presented in **Table 1**. Serum tOC, cOC and ucOC levels at pre-intervention data points were not different between women ( $n = 10$ ) or men ( $n = 20$ ) ( $p > 0.05$  for all, **Table 1**). With pre-intervention data points combined together, a higher ucOC/tOC ratio was associated with lower PWV when adjusted for BMI and age ( $r = -0.493$ ,  $p < 0.05$ ). A higher concentration of cOC was associated with a higher PWV when adjusted for BMI and age ( $r = .638$ ,  $p < 0.01$ ). All other pre-intervention correlations were not significant ( $p > 0.05$  for all, **Supplementary Table 1**).

We have previously shown that the HK intervention, but not the LK or CON intervention suppressed tOC, ucOC and the ucOC/tOC ratio [20]. In the high responders tOC, ucOC and ucOC/tOC were reduced post-intervention compared to pre-intervention, following the 4-week HK diet ( $p < 0.001$  for all, **Table 2**). Whilst in the low responders, ucOC ( $p < 0.001$ ) and ucOC/tOC ( $p < 0.01$ ), as well as resting systolic BP (2%,  $p < 0.05$ ) were reduced post intervention. As expected, the change in ucOC and ucOC/tOC ratio was significantly greater in the high responders versus low responders ( $p < 0.05$  for both, **Table 2**). The change in tOC,



cOC, markers of vascular (ambulatory systolic BP, ambulatory diastolic BP, resting systolic BP, resting diastolic BP or PWV) and metabolic (glucose, total cholesterol, LDL, HDL or triglycerides) health were not significantly different between the low and high responders (**Table 2**). There was no difference in BMI, vitamin K intake, age or estimated GFR (eGFR) between the high or low responders at baseline ( $p > 0.05$  for all, **Supplementary Table 2**).

Using unadjusted Spearman rho correlation and Spearman partial correlation there was no association between the change in ucOC or the ucOC/tOC ratio with the change in any cardiometabolic risk factor in the high responders ( $p > 0.05$  for all, **Table 3**). Using unadjusted spearman rho correlation, a positive association was present between the change in ucOC and the change in LDL when all participants were combined (i.e. high and low responders combined) ( $p < 0.05$ , **Table 3**). When adjusted for age and BMI using Spearman partial correlations, a positive correlation was present between the change in ucOC/tOC ratio and change in ambulatory diastolic BP when all participants were combined ( $r = .435$ ,  $p < 0.05$ ). In low responders only, there was a strong positive correlation between the change in ucOC/tOC ratio and change in glucose levels ( $r = .793$ ,  $p < 0.05$ ). All other correlations were not significant ( $p > 0.05$  for all, **Table 3**).

#### **4. Discussion**

The major finding of this study is that the suppression of ucOC was not associated with increased cardiometabolic risk factors, even in individuals who responded the most to the intervention (high responders). As such, it appears that the suppression of ucOC following a leafy green-rich diet does not impact, either negatively or positively, on cardiometabolic risk factors.

Currently, there are conflicting reports regarding the relationship between OC and blood pressure. Some have reported that lower tOC levels are associated with a higher prevalence of hypertension in adult men and women [25, 26]. Others however, have described no association between tOC and systolic or diastolic BP in adult men and women [27, 28]. As cOC and ucOC may have diverse biological functions, the examination of tOC alone, as often reported in these studies, limits our understanding of the exact function of each form of OC [23, 29]. In the current study, we have examined each form of OC and report that a reduction in ucOC and ucOC/tOC ratio via dietary modification is not correlated with changes in BP. This is interesting and suggests several possibilities. Firstly, ucOC may simply not have a

regulatory role in the maintenance of blood vessel function and BP. Secondly, the HK (leafy green rich) diet may regulate other bioactive factors that influence vascular health. For example, we have previously shown that the 4-week leafy green-rich diet increased plasma nitrate levels [6]. An increase in plasma nitrate enhances the bioavailability of nitric oxide, an anti-atherogenic molecule that regulates blood vessel function and BP [4, 30]. ucOC has also been implicated as a regulatory factor responsible for the maintenance of blood vessel function and BP [19]. Therefore, it is possible that the reduction in ucOC was offset by an increase in NO bioavailability. Consequently, cross-talk mechanisms may exist, which may explain the lack of changes in BP. This hypothesis should be explored in further mechanistic studies.

ucOC has been established as a regulator of energy homeostasis, at least in animal models [31, 32]. A large number of cross-sectional studies in humans show that ucOC is associated with metabolic responses and diseases. For example, a reduction in circulating ucOC is associated with an increased risk or presence of metabolic disorders, such as metabolic syndrome and type 2 diabetes [17]. Lower circulating tOC and ucOC has been associated with increased concentrations of blood glucose and triglycerides and decreased levels of HDL [33, 34]. However, few interventional studies have modified ucOC and examined the effect on metabolic outcomes. One study administered a single dose of prednisolone, a glucocorticoid, which suppressed circulating tOC and ucOC and also caused a reduction in insulin sensitivity and fasting blood glucose [35, 36]. In the current study, despite a 41% reduction in ucOC and 29% reduction in ucOC/tOC after the HK diet, there were no changes in fasting glucose or lipid levels in the high responders. Potential mechanisms for the lack of change are not clear, but it may be related to other bioactive components present in green leafy vegetables that can cause a compensatory effect and prevented any change in metabolic variables.

The development of vascular calcification is a process comparable to the development of bone within the skeleton. As OC is involved in bone mineralisation within the skeleton, it has also been implicated in the development of mineralisation within the vasculature [37, 23]. cOC, is the form of OC most involved with bone development in the skeleton, as such, it is possible that cOC is the form of OC involved in the development of calcification within the vasculature. However, research in this area is lacking. We have shown that baseline cOC is associated with baseline PWV, a measure of arterial stiffness which suggests the presence of vascular calcification [38]. However, we saw no correlation of cOC with PWV following the

HK diet in the high or low responders. Whilst, it is possible that OC is involved in vascular calcification, future large scale studies are needed to assess the effect of each form of OC, in particular cOC, on arterial stiffness and the development of vascular calcification.

A limitation of the current study is that the 4-week intervention period may not have been long enough or the dose of vitamin K1 large enough to observe a change in measures of cardiometabolic risk. Previous studies administering vitamin K1 supplementation (500 - 1000 $\mu$ g p/day) for 3 years found improvements in vascular compliance and reductions in coronary artery calcification [39, 40]. In the current study, it was estimated that participants increased their vitamin K1 intake by  $\sim$ 150  $\mu$ g p/day over the 4-weeks [20]. As such, a prolonged intervention may be needed to demonstrate changes in cardiometabolic risk factors. Another potential limitation was the inclusion of people who are relatively healthy. It is possible that those with diabetes or cardiovascular disease will respond differently to the intervention and that the correlation between ucOC and cardiovascular risk factors may be apparent in these populations. Finally, the generalisation of the results are somewhat limited due to the relatively small sample size. As such, further large scale studies, in particular RCTs, are needed to confirm our findings.

In conclusion, this study demonstrated that the suppression of ucOC following increased daily intake of leafy green vitamin K1-rich vegetables over 4-weeks was not associated with unfavourable changes in cardiometabolic risk factors. This may be due to the presence of compensatory mechanisms, or the fact that ucOC has a limited regulatory role over cardiometabolic risk factors in apparently healthy individuals. Such hypothesis should be explored by future mechanistic studies.

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## **Statement of Ethics**

The Vegetable Intake and Blood Pressure (VIABP) Study (registered at [www.anzctr.org.au](http://www.anzctr.org.au) as ACTRN12615000194561) was approved by the University of Western Australia Human Research Ethics Committee and was carried out in accordance with the Declaration of Helsinki.

## **Disclosure Statement**

The authors have no conflicts of interest to declare.

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## **Author Contributions**

The Author contributions were as follows: MS, JRL, JMH, LCB designed the research; EB, LCB conducted the research; AT, CS, MW, IL analysed the data; AT wrote the first draft manuscript; all authors revised the manuscript and approve the final version.

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