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ORIGINAL ARTICLE

Effects of calcium supplementation on circulating osteocalcin and glycated haemoglobin in older women

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Abstract

Summary One year of calcium supplementation in older women led to modest reductions in total osteocalcin and undercarboxylated osteocalcin (ucOC), with no changes in muscle or fat mass, or glycated haemoglobin. Future studies should explore whether treatments with more profound effects of suppressing ucOC may lead to impaired glycaemic control.

Introduction Total osteocalcin (TOC) is a marker of bone turnover, while its undercarboxylated form has beneficial effects on glucose metabolism in mice. This post hoc analysis of a randomised double-blind, placebo-controlled trial examined whether 1 year of calcium supplementation affected circulating TOC, undercarboxylated osteocalcin (ucOC) or glycated haemoglobin (HbA1c) in 1368 older community-dwelling women (mean age 75.2 ± 2.7 years).

Methods Women enrolled in the Calcium Intake Fracture Outcome Study trial (1998–2003) were supplemented with 1.2 g/d of elemental calcium (in the form of calcium carbonate) or placebo. Circulating TOC, ucOC and HbA1c was measured at 1 year (1999).

Results After 1 year of calcium supplementation, TOC and ucOC levels were 17% and 22% lower compared with placebo (mean 22.7 ± 9.1 vs. 27.3 ± 10.9 μg/L and 11.1 ± 4.9 vs. 13.0 ± 5.7 μg/L, both $P < 0.001$). Carboxylated osteocalcin/ucOC was 6% lower after calcium supplementation ($P < 0.05$). Despite this, no differences in HbA1c were observed (calcium, 5.2 ± 0.6 vs. placebo, 5.3 ± 0.8%; $P = 0.08$). Calcium supplementation did not affect BMI, whole body lean or fat mass. In exploratory analyses, total calcium (dietary and supplemental) was inversely related to TOC and ucOC, indicating calcium intake is an important dietary determinant of osteocalcin levels.

Conclusion One year of calcium supplementation in older women led to modest reductions in TOC and ucOC, with no changes in muscle or fat mass, or HbA1c. Future studies should explore whether treatments with more profound effects of suppressing ucOC may lead to impaired glycaemic control.

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Keywords Bone · Diabetes · Lean mass · Osteocalcin · Vitamin K

Introduction

The polypeptide protein, osteocalcin, is synthesised and secreted by mature osteoblasts during bone formation [1]. It is also released from the bone matrix during bone resorption and, as such, is used clinically as a serum marker of bone remodelling. Osteocalcin undergoes vitamin K-dependent post-translational modification whereby three available glutamic acid residues (Glu) are γ-carboxylated (Gla) [2, 3] with the negatively charged γ-carboxyglutamic acid groups having a high binding affinity for bone; in particular, the exposed calcium ions at the surface of bone mineral [4]. Both carboxylated (cOC) and undercarboxylated osteocalcin (ucOC) are abundant in the circulation [5].

High-quality mouse genetic-based studies indicate that osteocalcin stimulates pancreatic insulin production and secretion, as well as insulin sensitivity to reduce average glucose concentrations [6–8]. These effects on glucose metabolism are thought to be primarily due to the uncarboxylated form of osteocalcin (ucOC) in which fewer than three glutamic acid residues are carboxylated. Lower ucOC concentrations are also linked to reduced muscle mass and function in animal models [6–11].

Despite this evidence from in vivo and ex vivo studies in mice, the findings cannot be directly applied to humans. There are substantial differences in the regulation of the osteocalcin gene between mice and human [12]. In the few observational studies that have been conducted in humans, the data are inconsistent [13–18]. Furthermore, given that the carboxylation of osteocalcin in humans is vitamin K dependent, the vitamin K status of the participant must be evaluated [12]. Thus, the role of osteocalcin in the regulation of glucose metabolism and lean and fat mass in humans remains unclear; indeed, it remains to be determined whether in human's osteocalcin reflects or determines the connection between bone and glucose metabolism [12, 19] .

Calcium is an essential mineral for both the accrual of peak bone mass and preventing bone loss in later life, particularly in postmenopausal women [20]. The recommended dietary intake calcium for postmenopausal women is 1200–1300 mg per day [21, 22]. Calcium supplementation is known to reduce bone turnover [23]; thus, we hypothesised that women with 1 year of calcium supplementation would have lower total osteocalcin (TOC) and ucOC levels compared with placebo and that these lower levels may identify effects on glycated haemoglobin (HbA1c) levels as changes in lean and fat mass.

Methods

Ethics statement

The Human Ethics Committee of the University of Western Australia approved the study and written informed consents were obtained from all participants. Human ethics approval for the use of linked data for the project was provided by the Human Research Ethics Committee of the Western Australian Department of Health (DOHWA HREC), project number **no.** 2009/24.

Study design

We took advantage of the CAIFOS RCT study design in which the participants involved were recruited in 1998 to a 5-year, randomised, controlled trial of oral calcium supplements compared with placebo to prevent osteoporotic fractures as described previously [24].

Study participants

Briefly, women were recruited from the Western Australian general population of women aged over 70 years by mail using the electoral roll as a requirement of citizenship. Over 99% of Australians of this age are registered on the roll. Of the 5586 women who responded to a letter inviting participation, 1510 women were willing and eligible. Of these women, 1460 were recruited for the study. Participants were ambulant and did not have any medical conditions likely to influence 5-year survival. They were excluded if they were receiving any bone-active agents, including hormone replacement therapy. Women were similar in terms of disease burden and pharmaceutical consumption to whole populations of this age, but they were more likely to be from higher socio-economic groups [25]. In the 5 years of the trial, participants received 1.2 g of elemental calcium, as calcium carbonate, daily or a matched placebo. As this trial commenced and was completed prior to the advent of the clinical trials registry, the trial was retrospectively registered in the Australian New Zealand Clinical Trials Registry ACTRN12615000750583. We assessed osteocalcin in 2014/2015 from blood samples collected in 1999 in the CAIFOS RCT that had not been defrosted previously. The year-1 clinical visit was attended by 1368 women, of which 1265 (92%) had samples collected in 1999 for osteocalcin measures and 1255 (91%) for HbA1c.

CAIFOS randomised controlled trial

Patients received calcium carbonate tablets, 0.6 g twice per day (with morning and evening meals), or identical placebo tablets (Wyeth Consumer Healthcare, Baulkham Hills, Australia). The randomisation list was produced by generating 146 blocks of 10 numbers. In each block, five positions representing placebo and five positions representing calcium treatment were ordered using a letter code according to a random number generator. The numbered blocks were ordered according to randomly generated numbers, and an identification number was assigned, in order, to each letter code in the randomised list. The Pharmacy Department of the Sir Charles Gairdner Hospital, Nedlands, Australia, assigned a treatment to the letter code and assigned the appropriate medications to the patient according to this list. The randomisation was stratified by allocating patients to blocks according to whether a prevalent non-traumatic fracture had occurred after age 50 years, ensuring that an equal number of patients with and without a prevalent fracture received placebo or calcium. Medication compliance was checked at the completion of the study by counting returned tablets at each 12-month review and was calculated as a percentage of the optimum. For the current study, only the year-1 compliance data was used.

Participant characteristics

Participants provided their previous medical history and current medications which was verified by their general practitioner where available. Data were coded using the International Classification of Primary Care – Plus (ICPC-Plus) method [26]. The coding methodology allows aggregation of different terms for similar pathologic entities as defined by the ICD-10 coding system. Information about pre-existing diabetes (T89001-90009) was obtained from the patient's previous medical history and current medications. Participants were asked to verify this information with their general practitioner, where available.

Biochemistry

Fasting blood samples were collected at year 1 (1999) for assessment. HbA1c was determined immunoturbidimetrically using standard colorimetric and enzymatic methods on a Cobas Integra 800 analyser with reagents supplied by Roche Diagnostics (Castle Hill, NSW, Australia). Serum TOC was measured by sandwich electrochemiluminescence immunoassay using the Roche Cobas N-Mid Osteocalcin assay (Roche Diagnostics, Mannheim). The inter-assay coefficients of variation (CV) were 2.3% and 4.8% at levels of 18 and 90 ng/mL respectively. Serum ucOC was determined using the same reagent assay with pre-treatment of the serum samples using 5 mg/mL of hydroxyapatite (Calbiochem) following the method of Gundberg et al. [27]. The inter-assay imprecision for percentage binding of cOC was 8% and 12% at osteocalcin concentrations of 100 and 15 ng/mL respectively.

Body composition

Body composition was measured in a randomly selected subgroup of participants at 12 months by whole body dual-energy X-ray absorptiometry (DXA), using a Hologic 4500A bone densitometer (Hologic Corp., Waltham, MA) with CVs under 2% in our laboratory. All data and analyses presented exclude the head. Lean body mass refers to bone-free lean mass.

Dietary calcium intake

A validated semi-quantitative food-frequency questionnaire (FFQ) developed by the Cancer Council of Victoria was used to assess baseline dietary intake, including calcium [28] in 1050/1103 (95.2%) of participants. The process of collection was identical, whereby a research assistant supervised the completion of the questionnaire in small groups. Food models, cups, spoons and charts for frequency were provided. Energy and nutrient intakes were estimated based on frequency of consumption and an overall estimate of usual portion size [29]. Total calcium intake (mg/d) was calculated by multiplying the 12-month percentage tablet compliance by 12 to convert to mg/d and adding dietary calcium intake in mg/d.

Dietary vitamin K intake

As vitamin K is related to the ratio of cOC to ucOC, we assessed dietary vitamin K intake from the aforementioned FFQ. Dietary vitamin K intake was calculated at baseline from all listed food items ($n = 101$) included on the FFQ, by multiplying the food item consumed (g/d) by the mean vitamin K value (μg/g). Vitamin K_1 (phylloquinone) values for FFQ food items ($n = 96$) were obtained from the US Department of Agriculture National Nutrient Database for Standard Reference (Release 28) [30]. Vitamin K_2 (menaquinone; MK-4 to MK-9) values ($n = 43$) for FFQ food items were obtained from Schurgers and Vermeer [31] and vitamin K_2 (menaquinone; MK-10) values ($n = 6$) for FFQ food items were obtained from Manoury et al [32]. Where foods containing vitamin K were not available for FFQ food items, a value of 0 μg/g was applied where no values were available for FFQ food items (phylloquinone $n = 5$; MK-4 to MK-9 $n = 58$; and MK-10 $n = 95$).

Statistical analysis

Continuous variables were presented as mean ± standard deviation (SD), or number and percentage. To investigate a 'dose' relationship between dietary calcium and calcium supplements, total calcium intake was calculated and tested in unadjusted and age-adjusted analyses. Differences between normally distributed characteristics of the treatment groups were determined by univariate analysis of covariance with adjustments for age. The Mann-Whitney test was used to determine the differences between the groups for non-normally distributed variables. All P values less than 5% (< 0.05) were considered statistically significant. All analyses were undertaken using IBM SPSS Statistics Version 22 (2012, Armonk, NY: IBM Corp) or Stata (version 13 StataCorp LP, College Station, TX).

Results

Details of the women included in the RCT are shown in Fig. 1. Participant characteristics at baseline are displayed in Table 1. The characteristics of the participants who did not attend the 12-month clinic visit and those with missing osteocalcin or HbA1c data not included in the study were similar (data not shown).

Effect of calcium supplements on circulating osteocalcin

After 12 months, the mean ± SD TOC and ucOC in women randomised to the calcium supplemented group (22.7 ± 9.1 and 11.1 ± 4.9 μg/L, respectively) was significantly lower than placebo (27.3 ± 10.9 and 13.0 ± 5.7 μg/L, respectively). These results were similar in age-adjusted models (Fig. 2). The ratio of cOC/ucOC was ~ 6% lower in the calcium supplemented group in the unadjusted and age-adjusted models ($P < 0.05$). To determine whether threshold effects were evident, we examined the relationship between tablet compliance (0–100%) and osteocalcin measures. A linear inverse relationship between tablet compliance and tOC and ucOC levels was observed in the calcium supplemented group but not in the placebo group; suggesting a linear relationship between calcium and osteocalcin forms (Supplementary Fig. 1). Considering that vitamin K and age are known to influence both ucOC and the ratio of cOC/ucOC, additional analyses were performed where dietary vitamin K and age were included as covariates when examining the effects of calcium supplements on these outcomes. The inclusion of the aforementioned covariates did not change the interpretation of the results for ucOC (estimated mean (EM) ± standard error (SE), placebo 13.0 ± 0.2 μg/L and calcium 11.0 ± 0.2 μg/L) or the ratio of cOC/ucOC (EM ± SE, placebo 1.22 ± 0.02 and calcium 1.15 ± 0.02) (all $P < 0.05$). Further analysis of individual participant dietary and supplemental (total) calcium intake identified a linear relationship between calcium intake and TOC and ucOC (Fig. 3).

Effect of calcium supplements on HbA1c

After 12 months, mean ± SD HbA1c in the women randomised to placebo was 5.3 ± 0.8% and was not significantly different ($P = 0.080$) to the calcium supplemented group (5.2 ± 0.6%). Results remained similar in age-adjusted models (Fig. 2) with no indication of any relationship with tablet compliance with HbA1c levels, strengthening the confidence in the null findings ($P > 0.05$). Additionally, we investigated total calcium intake and HbA1c levels and there was no relationship ($r^2 = 0$, $P > 0.05$).

Effect of calcium supplements on body mass index and body composition

Despite the reduction in TOC and ucOC, after 12 months, the change in body mass index (BMI, mean ± SD) in the women randomised to placebo (− 0.03 ± 1.1 kg/m^2) was not

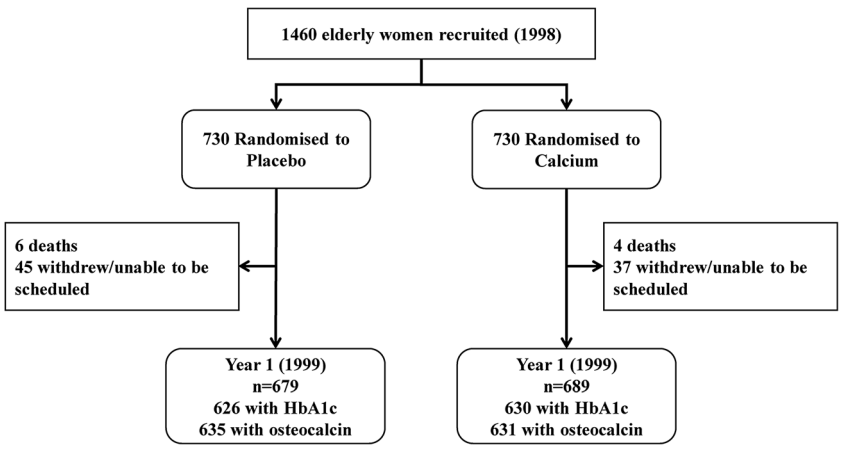

Fig. 1 Flowchart of randomised controlled trial participants

Table 1 Characteristics of the women included in the post hoc analysis

Characteristics	All participants	Placebo	Calcium
Number, *n* (%)	1368 (100)	679 (49.6)	689 (50.4)
Age, years, mean ± SD	75.2 ± 2.7	75.1 ± 2.7	75.2 ± 2.7
Body mass index, kg/m^2, mean ± SD	27.2 ± 4.7	27.4 ± 4.7	27.1 ± 4.7
Smoking history, previous or current (%)	501 (36.8)	238 (35.1)	263 (38.5)
Dietary vitamin K intake (μg/d)	120 ± 47	119 ± 45	120 ± 48
Dietary calcium intake (mg/d)	960 ± 355	962 ± 357	957 ± 48
Diabetes, yes (%)	85 (6.2)	43 (6.3)	42 (6.1)

Data are presented as either mean ± standard deviation or number (percentage) for all participants at 1 year of follow-up

significantly different to the calcium supplemented group (−0.11 ± 1.34 kg/m^2, $P = 0.229$). Results were similar in age-adjusted models ($P > 0.05$), with no indication of any differences by tablet compliance (data not shown). Similarly, in the women with DXA-derived measures of body composition at baseline and 12 months ($n = 201$, placebo $n = 104$ and calcium $n = 97$), no difference in the change of lean mass (placebo, −0.10 ± 1.09 vs. calcium, −0.07 ± 0.98 kg; $P = 0.792$) or fat mass was recorded over this time (placebo, −0.43 ± 2.05 vs. calcium, −0.43 ± 2.17 kg; $P = 0.995$). Results remained similar after age adjustments (data not shown).

Discussion

In this post hoc analysis of a large RCT of 1.2 g/day of elemental calcium in older women, we found that compared with control, 12 months of calcium supplementation lowered circulating TOC and ucOC but had no significant effect glycaemic control as measured by HbA1c or on lean body or fat mass before or after adjusting for vitamin K consumption. Given the high-quality studies in mice demonstrating reducing ucOC impairs glycaemic control [6–8], it would be expected that a 22% reduction in ucOC may be accompanied by an increase in HbA1c. However, we did not observe differences in glycated haemoglobin in this study which was well powered to detect even modest differences between groups suggesting these findings may not translate to humans. Interestingly, it has been suggested the rat osteocalcin gene shares greater similarities and synteny to the human osteocalcin gene than the mouse osteocalcin gene [33]. A study using the CRISPR/Cas9 technology to knockout osteocalcin in rats found that 5-month old knockout rats had higher trabecular thickness, density and volume but did not display the profound metabolic disturbances observed in mice [33], supporting the concept of different species-specific roles of osteocalcin on metabolism. To our knowledge, this is the first RCT investigating calcium supplementation in older women to have assessed glycaemic control and body composition in the same patients.

Two previous short-term interventions in men reported that circulating levels of ucOC was related to glycaemic control at rest and after exercise [34, 35]. The association between ucOC and metabolic parameters may be stronger in men than women [36]. Other human observational studies of men and women report that increases in serum TOC levels were associated with an increase in insulin secretion, while increases in ucOC were associated with an improvement in insulin secretion and sensitivity [13]. In other studies, individuals with higher serum osteocalcin were reported to experience lesser increments in fasting plasma glucose at their 3-year clinical follow-up [14]. A 10-year longitudinal study also reported that lower baseline osteocalcin, but not ucOC, in middle-aged men, was associated with reduced risk of developing type-2 diabetes [16].

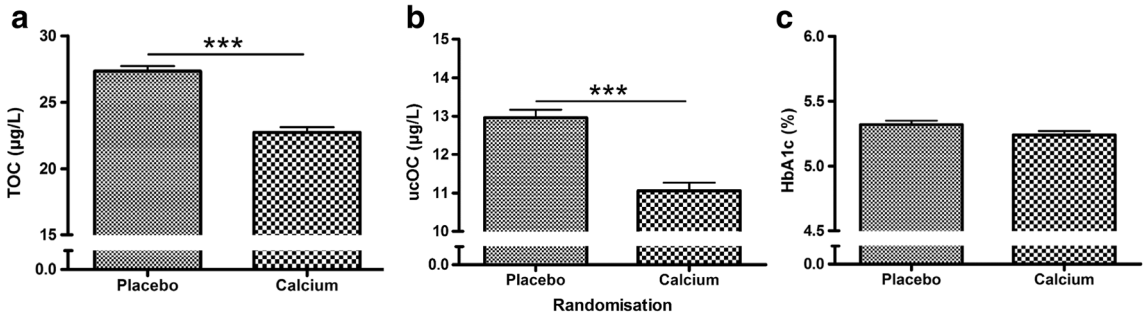

Fig. 2 Age-adjusted mean and SEM of total osteocalcin (TOC) (**a**), undercarboxylated osteocalcin (ucOC) (**b**), and glycated Hb (**c**) in the patients randomised to placebo or calcium

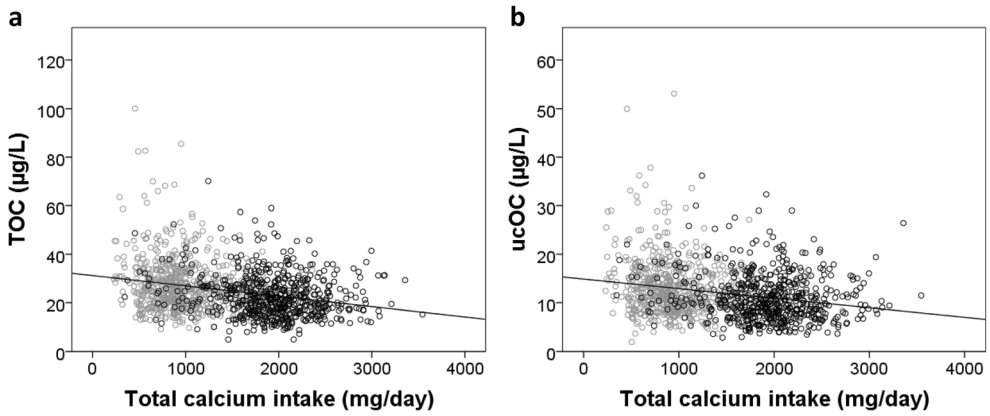

Fig. 3 Total (diet + supplemental) calcium intake (placebo grey circles and calcium black circles) and total osteocalcin (TOC; $r^2 = 0.07$) (**a**) and undercarboxylated osteocalcin (ucOC; $r^2 = 0.06$) (**b**)

In regard to changes in muscle and fat mass, a previous study of daily doses of 500 mg/d of vitamin K (phylloquinone) over 3 years in older women ($n = 237$, aged 60–80 years), ucOC was reduced by 58% without effect on lean mass loss or fat gain over 3 years. This led the authors to conclude that ucOC may not be implicated in the age-related changes in skeletal muscle or adipose tissue mass in older community-dwelling adults [17]. Our data in 201 women with more modest reductions to ucOC due to calcium supplementation add further support to the concept that lowering ucOC may not affect skeletal muscle or adipose tissue mass.

In our investigation, we also report that dietary calcium can influence circulating TOC and ucOC. Physiologically, it would be plausible as calcium is a weak anti-resorptive agent and should lead to less osteocalcin being released into the circulation. However, to date, few studies reporting the association between circulating forms of osteocalcin and metabolic outcomes have considered calcium intake. Our work provides evidence for the importance of adjusting for dietary and supplemental calcium intake for studies planning to investigate the association of either TOC or ucOC with disease outcomes.

It is notable here that we assessed both TOC (carboxylated and undercarboxylated) and ucOC in a large RCT of older women taking calcium or placebo supplements. Secondly, the assessment of HbA1c, which is an established measure of longer term (3 months) glucose control compared with blood glucose alone, provides high-quality evidence that despite 12 months of calcium supplementation lowering both ucOC and TOC, there were no detrimental effects on glucose control.

There are several limitations within our study that should be considered. Firstly, baseline osteocalcin measures were not performed. Therefore, we cannot exclude the possibility of residual confounding by unmeasured differences in osteocalcin measures at baseline; however, this is unlikely given the RCT design. Secondly, as the average age of these women was 75 years, these findings may not extrapolate to other populations. Despite the observed reductions in both TOC and ucOC with calcium intake over 12 months, the final values were higher compared with values previously observed for healthy young females [37]. In addition, as this was a post hoc analysis, bias may have been inadvertently been introduced into the study. Thirdly, as this study was based on the 1-year follow-up, with no assessment of osteocalcin and HbA1c levels beyond 12 months, we cannot rule out different effects beyond this time frame. Finally, participants in this study had other comorbidities and prescription medications that may have affected the circulating levels of osteocalcin and ucOC; however, these would likely have been randomly distributed between groups.

In conclusion, this study demonstrates that increased calcium intake inversely affects circulating TOC and ucOC levels. However, the changes in TOC and/or ucOC did not appear to have any effects on glucose control or body composition. These findings further suggest that bone cell activity as measured by osteocalcin or under cOC may not regulate glucose metabolism or body composition.

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Compliance with ethical standards

The Human Ethics Committee of the University of Western Australia approved the study and written informed consents were obtained from all participants. Human ethics approval for the use of linked data for the project was provided by the Human Research Ethics Committee of the Western Australian Department of Health (DOHWA HREC), project number no. 2009/24.

Conflicts of interest None.

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