11 Granado-Lorencio/c/p_04 LORD_c 27/04/12 16:36 Page33

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SERUM ß-CRYPTOXANTHIN AS A PREDICTOR OF BONE FORMATION MARKERS IN POST-MENOPAUSAL WOMEN

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Abstract: *Objective:* To assess the associations between fat-soluble vitamin and antioxidant status and levels of bone remodelling markers in post-menopausal women. Design: A cross-sectional study. *Setting;* A nutrition laboratory from a terciary hospital. *Participants:* Sixty-one post-menopausal women were contacted to participate in a dietary intervention trial and 36 women were finally included (age 45 to 65years, BMI <35 kg/m^2, amenorrhea over 12 months, no hormone therapy, no anabolic or antiresorptive therapy). *Mearurements:* Baseline blood samples were processed for the assessment of retinol, α-tocopherol, 25-OH-D3, lutein/zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene (by UHPLC), parathyroid hormone and bone metabolism markers (osteocalcin, N-terminal peptide of procollagen I (P1NP) and β-crosslaps), sex hormones (17-β-estradiol, FSH, LH) and lipid profile. *Results:* Mean values of all the parameters except total cholesterol were within reference ranges even when mean concentrations of 25-OH-vitamin D could be considered as insufficient (mean, [95% confidence interval]: 47.7 nmol/l [41.1 to 54.2 nmol/l]). Bone remodelling markers in serum were not significantly correlated to age, age of menopause, weight, BMI, sex hormones, lipids, retinol, α-tocopherol (or α-tocopherol /cholesterol ratio) or 25-OH-vitamin D. Only crude concentrations and cholesterol-adjusted values of β-cryptoxanthin showed a positive and significant correlation with bone formation markers (r= 0.53, p=0.001 for osteocalcin; r=0.41, p=0.013 for P1NP) while lycopene showed an inverse trend with P1NP (r= -0.32, p=0.06). In the regression analysis, β-cryptoxanthin alone could explain 28% of the variation of serum osteocalcin (r2= 0.28; B= 0.60; p=0.001). *Conclusion:* Serum β-cryptoxanthin is a predictor of bone formation markers in post-menopausal women and dietary advice regarding β-cryptoxanthin-rich foods may be a safe and sustainable approach to improve bone health.

Key words: β-cryptoxanthin, bone markers, osteoporosis, carotenoids, human study.

Introduction

Nutrition is an important modifiable factor in the development and maintenance of bone health (1-4). Osteoporosis is a highly prevalent condition in the elderly and constitutes a major health problem that involves bone loss and a high risk of bone fracture (5). Pharmacological and nutritional factors may prevent bone loss with increasing age, although the chemical compounds in food that act on bone metabolism are poorly understood (1, 6).

Several dietary factors and antioxidants (i.e., vitamin A, vitamin E, carotenoids) have attracted much attention due to their potential relevance in bone health. High oxidative stress is associated with reduced bone mineral density (BMD) and bone metabolism markers (7-9) and serum α-tocopherol levels seem to modify the association between urinary 8-iso-PGF2α and BMD in the elderly (9). Also, a negative association between osteocalcin (a bone formation marker) and retinol has been reported (10) although epidemiological studies suggest a potential beneficial role of carotenoids in general, and β-cryptoxanthin and lycopene in particular, on bone health. In this context, associations between carotenoid intake (i.e. β-cryptoxanthin, lycopene), bone resorption markers and loss of bone mineral density has been reported (3, 11, 12). Moreover, both dietary intake and serum levels of β-cryptoxanthin have been inversely related to different bone and joint disorders (e.g., knee osteoarthritis, rheumatoid arthritis) (7, 13- 17), a consistent finding with in vitro, animal, and human evidence suggesting a unique anabolic effect on bone calcification and osteoporosis prevention of β-cryptoxanthin (18, 19).

Thus, because of the potential role of several dietary factors in bone metabolism, our aim was to assess the associations between markers of fat-soluble vitamin and antioxidant status and circulating levels of bone

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11 Granado-Lorencio/c/p_04 LORD_c 27/04/12 16:36 Page34

remodelling markers in post-menopausal women.

Material and Methods

Baseline samples from a dietary study conducted by a nutrition laboratory from a terciary hospital were used to perform a cross-sectional study. Sixty-one post-menopausal women were contacted and invited to participate in a dietary intervention trial (NCT 01074623). Enrolment criteria included age (45-65 years), BMI <35 kg/m^2, amenorrhea (> 12 months), no hormone therapy, no anabolic or antiresorptive therapy, no use of cholesterol-lowering drugs (i.e. fibrates, statins), w-3 fatty acids or phytosterol-containing foods or supplements, no dieting, and no consumption of supplements, herbs extracts or foods enriched with vitamins, carotenoids or other dietary bioactive components (i.e. phytoestrogens). Additionally, inclusion criteria include serum levels of vitamin A, E and D, and biochemical and hemotological profile within reference ranges (except for total and LDL cholesterol which was considered as an inclusion criteria as it was a "target" endpoint of the intervention study). A total of 38 women were finally accepted to take part in the study but 2 of them refused to participate just after beginning the trial. The study protocol was approved by the research ethics committee of the Hospital Universitario Puerta de Hierro-Majadahonda (Madrid, Spain), and all subjects were informed and gave their written consent.

Baseline blood samples (before intervention) were obtained after overnight fast, between 8.00-10.00AM and within 3 consecutive days in winter (January) to minimize circadian and seasonal variations of some analytes (i.e. bone remodelling markers, 25-OH-vitamin D3). Samples were processed for the assessment of fat-soluble vitamins (retinol, α-tocopherol, and 25-OH-D3), major carotenoids in serum (lutein/zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene), sex hormones, lipid profile (total-cholesterol, LDL and HDL-cholesterol and triglycerides), parathyroid hormone (iPTH) and bone remodelling markers including osteocalcin, β-crosslaps (β-CTx), and N-terminal peptide of procollagen I (P1NP).

For the analysis of fast-soluble vitamin and carotenoids in serum, 0.5 ml serum was mixed with 0.5 ml ethanol, vortexed, and extracted twice with 2 ml methylene chloride/hexane (1:5). Organic phases were pooled, evaporated to dryness, and reconstituted to be injected (THF/EtOH) onto the ultra performance liquid chromatograph (UHPLC Aquity System, Waters) (20). Detection was carried out by a photodiode array (Waters Associates, Milford, MA) set at 268nm for 25-OH-vitamin D3, 325nm for retinol, 295 for α-tocopherol and 450 nm for carotenoids. Identification of the analytes was performed by comparing retention times with those of authentic standards and on-line UV–visible spectra.

Osteocalcin, iPTH, CTx, and P1NP were determined using commercially available electrochemiluminescence immunoassays (Elecsys 2010, Roche Diagnostics), with variation coefficients <6.5% for all the analytes (Manufacturer information sheets and in-house tests). Other serum biochemical determinations (i.e., sex hormones, lipid profile) were carried out at the General Biochemistry Laboratory of the hospital according to routine quality-controlled standard methods. The validity of the UHPLC method is contrasted periodically through our participation in the Fat-Soluble Quality Assurance Programme (National Institute of Standards and Technology, Gaithersburg, MD, USA) and the Vitamin D External Quality Assurance Survey (Charing Cross Hospital, London, UK).

Statistical analysis

All data are expressed as mean and standard deviations. Relationships between fat-soluble vitamins, carotenoids, and bone markers were assessed using Pearson correlation coefficients. Linear regression analysis was computed using PROC GENMOD from SAS version 9.2 (SAS Institute Inc., Cary, NC) Likelihood ratio tests were used to compute statistical significances using P values less than 0.05 as the statistical threshold.

Results

Characteristics of the women and serum markers analyzed are showed in Table 1. Mean values of most parameters evaluated were within reference ranges, even when mean concentrations of 25-OH-vitamin D could be considered as insufficient. Also, as expected due to the inclusion criteria, mean total cholesterol was above the reference range in our laboratory. Thus, fat-soluble antioxidants mostly transported by lipoproteins (i.e. α-tocopherol and carotenoids) were also expressed as cholesterol-adjusted concentrations and used for calculating the correlations among the parameters assessed.

Serum levels of bone remodelling markers were unrelated to age, weight, BMI, lipid markers, serum retinol, α-tocopherol (or α-tocopherol /cholesterol ratio) or 25-OH-vitamin D3, although a trend was observed between bone formation markers and sex hormones (r= 0.30, p=0.087 and r=0.31, p=0.077 for osteocalcin and FSH and LH, respectively; r=0.30, p=0.079 for P1NP and FSH) and between age of menopause and the resorption marker CTx (r=0.32, p=0.064). Only serum β-cryptoxanthin showed a significant positive correlation with the bone formation marker osteocalcin (r= 0.45, p=0.005) and a positive trend with P1NP (r= 0.31, p=0.068) (Figure 1), while lycopene showed an inverse relationship with P1NP (r= -0.34; p=0.043) and CTx (r= -

11 Granado-Lorencio/c/p_04 LORD_c 27/04/12 16:36 Page35

0.28, p=0.099). When cholesterol-adjusted values were used, β-cryptoxanthin was the unique carotenoid showing a positive and significant correlation with bone formation markers (r= 0.53, p=0.001 for osteocalcin; r=0.41, p=0.013 for P1NP) while lycopene maintained an inverse trend with P1NP (r= -0.32, p=0.06).

Table 1
Characteristics of the post-menopausal women enrolled in the study (n=36)

	Post-menopausal group (mean, SD)
Age (y)	55 ± 4.1
Age of menopause (y)	47 ± 5.1
Weight (kg)	67.1 ± 10.5
BMI (kg/m²)	25.6 ± 4.0
17-B-estradiol (pg/ml)	19.0 ± 12.5
FSH (mUI/ml)	54.1 ± 24.5
LH (mUI/ml)	31.1 ± 15.3
Cholesterol (mg/dl)	225 ± 35.7
HDL (mg/dl)	62 ± 13.2
LDL (mg/dl)	146 ± 31.8
Triglycerides (mg/dl)	84 ± 32.6
P1NP (ng/ml)	54.5 ± 20.3
Osteocalcin (ng/ml)	24.3 ± 8.8
Beta-crosslaps (pg/ml)	496 ± 224
25-OH-vitamina D (nmol/l)	47.7 ± 19.2
Retinol (μg/dl)	56.6 ± 12.1
α-tocopherol (μg/dl)	1275 ± 247
α-tocopherol/cholesterol (μg/mg)	5.7 ± 0.8
Lutein + zeaxanthin (μg/dl)	17.9 ± 6.4
β-cryptoxanthin (μg/dl)	19.7 ± 11.2
Total Lycopene (μg/dl)	27.7 ± 13.7
α-carotene (μg/dl)	6.4 ± 4.5
Total β-carotene (μg/dl)	23.1 ± 15.1

SD stands for standard devation.

To assess the contribution of each analyte to the variability of bone markers in serum, two models were constructed; the model 1 included only those analytes correlated with bone markers and the model 2, in addition to the variables included in model 1 all the vitamins (A, E and D) and carotenoids as potential biological contributors were added. In model 1, only cholesterol-adjusted β-cryptoxanthin was a significant predictor of serum osteocalcin (change of serum osteocalcin for every 1 μg/mg increase in β-cryptoxanthin /cholesterol, 60 ng/ml, 95% confidence interval –CI- 29 to 91, p=0.0006;) whereas for P1NP, both cholesterol adjusted β-cryptoxanthin and lycopene were found to be significant predictors (change in P1NP for every 1 μg/ml increase in β-cryptoxanthin /cholesterol 97 ng/ml, 95% CI, 24 to 170, p=0.013; for every 1μg/ml increase in lycopene -96 ng/ml, 95% CI, -196 to 4.5, p=0.067;). For CTx, only lycopene showed a trend (change for every 1 μg/ml increase in lycopene -0.004 pg/ml, 95% CI, -0.0025 to 0.0005, p=0.087).

On introducing vitamin A, E, D and carotenoids (model 2), cholesterol-adjusted β-cryptoxanthin remained the only analyte to significantly predict the levels of osteocalcin in serum (change of serum osteocalcin for every 1 μg/mg increase in β-cryptoxanthin /cholesterol, 56 ng/ml, 95% CI, 21 to 92, p=0.003). For P1NP, cholesterol-adjusted β-cryptoxanthin and lycopene were found significant (estimate, 79 ng/ml, 95% CI, -5 to 173, p=0.072, for β-cryptoxanthin; estimate, -126 ng/ml, 95% CI, -239 to -14, p=0.032, for lycopene) while for CTx, a slight negative trend was observed for lycopene (estimate, -1.08 pg/ml, 95% CI, -2.4 to 0.21, p=0,10). In the regression analysis, β-cryptoxanthin alone could explain 28% of the variation of osteocalcin (r2= 0.28; B= 0.60; p=0.001).

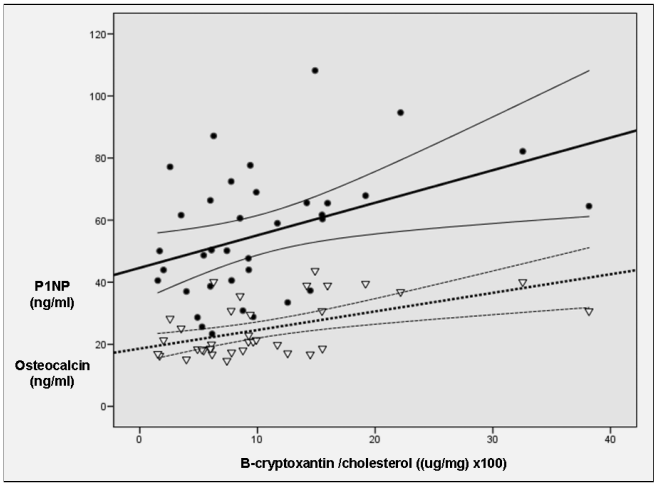

Figure 1. Scatter plot between cholesterol-adjusted β-cryptoxanthin and bone formation markers in post-menopausal women. Lines represent mean and 95%CI. R2= 0.167 for P1NP (black dots); R2 = 0.278 for osteocalcin (triangles)

Discussion

The present results showed a significant positive relationship between serum levels of β-cryptoxanthin and markers of bone formation in a sample of post-menopausal women. In this sense, our findings are consistent with in vitro, animal and human studies showing that β-cryptoxanthin displays a unique anabolic effect on bone calcification (6) and modulates bone remodeling markers (18). Despite being observed in a small group of post-menopausal women, the associations were only found for β-cryptoxanthin and bone formation markers. The most robust association was for osteocalcin with coefficients similar to those observed for serum carotenoids that are simultaneously present and ingested from foods (i.e. lutein and β-carotene). Interestingly, while the association between bone formation markers (osteocalcin and P1NP) and serum β-cryptoxanthin need to be confirmed, it should be noticed that this association was observed in winter, a season in which serum 25-OH-vitamin D show the lowest mean values on a population

11 Granado-Lorencio/c/p_04 LORD_c 27/04/12 16:36 Page36

levels. In this sense, it may become relevant given the complementary seasonal distribution of both analytes and the potential "compensating" anabolic effect of β-cryptoxanthin in women with osteoporosis, as previously suggested (21).

Statistical associations, however, should be cautiously interpreted as they may only reflect parallel but independent and non-causal effects rather than related phenomena. Additionally, correlation between β-cryptoxanthin and bone formation markers does not necessarily indicate an effect on bone metabolism since β-cryptoxanthin may be a marker of other co-ingested bioactive phytochemicals. In this sense, polyphenols (i.e. flavonoids) have been suggested as potential beneficial components from citrus fruits in disease prevention (22, 23). Nevertheless, we found a positive relationship between β-cryptoxanthin with bone formation markers and a negative trend between serum lycopene and CTx (a resorption marker) coinciding with an anabolic role for β-cryptoxanthin and the antiresorptive effect of lycopene (7, 19).

Oxidative stress may increase bone resorption through activation of nuclear factor-kappa β, and it has been pointed out that oxidative stress can activate bone resorption and deactivate bone formation (9). Also, increased serum 8-iso-PGF2α levels (a marker of in vivo oxidative stress) has been associated with a lower bone mass and reduced bone alkaline phosphatase and osteocalcin concentrations, supporting a possible role for oxidative stress in the development of lower bone mass (8). In accordance, dietary intervention with antioxidants (i.e. lycopene) have been shown to reduce oxidative stress and bone resorption markers in post-menopausal women, supporting the potential role of dietary components in bone health (7).

Finally, our results suggest that β-cryptoxanthin or β-cryptoxanthin containing foods may be beneficial for bone health. In this manner, the present findings are consistent with previous observations regarding the beneficial effects of dietary patterns that suggest that vitamin C and β-cryptoxanthin intake may be associated with radial BMD and may provide benefit to bone health in post-menopausal women (12, 17). Thus, dietary advice regarding the consumption of β-cryptoxanthin-rich foods (i.e. citrus fruits) may be a safe and sustainable approach to improve bone health.

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