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TWELVE-MONTH CONSUMPTION OF A POLYPHENOL EXTRACT FROM OLIVE (*OLEA EUROPAEA*) IN A DOUBLE BLIND, RANDOMIZED TRIAL INCREASES SERUM TOTAL OSTEOCALCIN LEVELS AND IMPROVES SERUM LIPID PROFILES IN POSTMENOPAUSAL WOMEN WITH OSTEOPENIA

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Abstract: *Objectives:* Osteoporosis is a skeletal disorder characterized by impaired bone turnover and compromised bone strength, thereby predisposing to increased risk of fracture. Preclinical research has shown that compounds produced by the olive tree (*Olea europaea*), may protect from bone loss, by increasing osteoblast activity at the expense of adipocyte formation. The aim of this exploratory study was to obtain a first insight on the effect of intake of an olive extract on bone turnover in postmenopausal women with decreased bone mass (osteopenia). *Design and setting:* For that, a double blind, placebo-controlled study was performed in which participants were randomly allocated to either treatment or placebo groups. *Participants:* 64 osteopenic patients, with a mean bone mineral density (BMD) T-score between –1.5 and –2.5 in the lumbar spine (L2–L4) were included in the study. *Intervention and measurements:* Participants received for 12 months daily either 250 mg/day of olive extract and 1000 mg Ca (treatment) or 1000 mg Ca alone (placebo). Primary endpoints consisted of evaluation of bone turnover markers. Secondary endpoints included BMD measurements and blood lipid profiles. *Results:* After 12 months, the levels of the pro-osteoblastic marker osteocalcin were found to significantly increase in the treatment group as compared to placebo. Simultaneously, BMD decreased in the placebo group, while remaining stable in the treatment group. In addition, improved lipid profiles were observed, with significant decrease in total- and LDL-cholesterol in the treatment group. *Conclusion:* This exploratory study supports preclinical observations and warrants further research by showing that a specific olive polyphenol extract (Bonolive®) affects serum osteocalcin levels and may stabilize lumbar spine BMD. Moreover, the improved blood lipid profiles suggest additional health benefits associated to the intake of the olive polyphenol extract.

Key words: Oleuropein, osteocalcin, bone mineral density, lipid profile.

Introduction

In developed countries life expectancy is increasing together with the growing prevalence of age related chronic diseases, particularly those involving significant disability such as osteoporosis (1). This is a skeletal disorder that affects bone density and strength and increases the susceptibility to spontaneous fractures (2). In a healthy individual, bone architecture is maintained by a constant remodelling process: osteoclasts remove bone tissue (i.e. bone resorption), whereas bone is renewed thanks to osteoblastic activity (3,4). Osteocalcin, secreted by osteoblasts, is a key molecule in this regeneration process, and is therefore often used as a marker for bone building process (5). With aging, the balance between bone formation and bone-breakdown shifts to a negative turnover, leading to a net bone loss of about 0.3% to 0.5% per year. In women, the decrease in estrogens associated with menopause, accelerates this net bone loss by about 10-fold in about 5 to 7 years (4,6).

According to the World Health Organization (WHO), osteoporosis causes more that 9 million fractures per year worldwide (6). This, together with the increase in aging population, confirms the medical and socioeconomic burden of osteoporosis, particularly postmenopausal osteoporosis (7,8). Currently, most treatments aim to dramatically decrease bone resorption, leading to a higher net bone mineral density (BMD) (9). Nevertheless, although effective, the expenses associated with the prescription of these drugs are substantial, they have recognized side effects and they do not include preventive methods. That is why health professionals and the WHO strongly advocate the development of new strategies with proven clinical value for prevention, thereby delaying the onset of osteoporosis (6).

In light of these facts, over the past 30 years, research in nutrition has led to an exciting progress supporting the hypothesis that, by modulating specific target functions in the body, dietary intervention can help to reduce the risk of disease and may thus offer an innovative way to deal osteoporosis and its associated health costs. Within Europe, the lowest incidence of osteoporosis is found in the Mediterranean areas, and the

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consumption of olive oil phenols typically associated with Mediterranean diet, has been suggested to play an important role (10). Oleuropein, hydroxytyrosol and tyrosol, the main phenolic compounds present in olive oil, are formed by enzymatic removal of glucose from the polar parent-compound oleuropein-glycoside (11, 12). Interestingly, several animal studies showed that olives, olive oil and its main phenolics exert a protective effect in models of ovariectomy/inflammation-induced bone loss (13-17) and ameliorate arthritis in experimentally-induced arthritis (18,19).

BMD measurement is a widely accepted method for the diagnosis of osteoporosis (20), and the low BMD levels observed upon bone mass loss are associated with a decrease in the number of osteoblasts in the bone marrow (21). This change is accompanied by an increase in the number of adipocytes, thereby leading to continuous increase in the volume of marrow adipose tissue (21, 22). Interestingly, previous ex vivo studies studied the effect of oleuropein on the differentiation of mesenchymal stem cells (MSCs); these are the progenitor cells for both osteoblasts and adipocytes. It was shown that oleuropein was able to inhibit the differentiation of MSCs into adipocytes, and to enhance differentiation into osteoblasts (23). Moreover, oleuropein and hydroxytyrosol have been shown to inhibit adipocyte differentiation in 3T3-L1 cells (24). Altogether, these results suggest that oleuropein may positively influence the number of osteoblasts at the expense of adipocyte formation and thereby could contribute to the prevention of age-related bone loss and osteoporosis. In light of these findings, the present study was designed as a first exploratory study to provide clinical data on the effects of olive polyphenols in a small human clinical cohort. By using an olive-leaf extract (Bonolive®), standardized on oleuropein content, effects of olive polyphenols on both parameters which are commonly linked to bone function (bone turnover markers and BMD) and lipid metabolism (lipid profile) were investigated in postmenopausal subjects receiving calcium supplementation.

Materials and methods

Ethics statement

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, approved by the ethics committee of the Institute of Agricultural Medicine and registered under NCT00789425 at the NIH clinical trial register (clinicaltrials.gov). Informed written consent was obtained from all subjects.

Study design

The study was designed as a randomized, parallel group, double blind, 12-month study and was conducted from December 2008 to March 2010 at the Osteoporosis Outpatient Department of the Institute of Agricultural Medicine (IAM) in Lublin, Poland. The study population was either randomly assigned to a placebo group, receiving an oral dose of 1 g calcium daily (400 mg elemental calcium), or to a treatment group receiving a daily dose of 250 mg of an olive-leaf extract, containing a minimum of 40% polyphenols, plus 1 g of calcium (400 mg elemental calcium). A total of 6 clinic visits were scheduled, consisting of a first screening visit, an introductory visit (V1), a visit at start of treatment (baseline; V2), and follow-up visits after 3, 6 and 12 months of treatment, respectively referred to as visits (V)3, 4 and 5.

Participants and eligibility criteria

Patients were recruited among women who reported to the study facility (IAM) in Lublin, Poland to undergo a prophylactic densitometric examination. A total of 103 females were assessed for eligibility between November 2008 and March 2009. Of these, 64 patients aged between 49 and 68 years-old were randomized to two groups. The inclusion criteria were: at least 24 months after cessation of menses or oophorectomy and having a BMD T-score between –1.5 and –2.5 (surely osteopenic) as measured by DEXA (dual energy X-ray absorptiometry; LUNAR Prodigy Advanced, LUNAR Corp., USA) in the lumbar spine (L2–L4), Furthermore, daily calcium intake was assessed by means of a self-administered Food Frequency Questionnaire (FFQ) (25,26) and inclusion was allowed when daily intake was in line with reference calcium intake for the Lublin region (27) (300-600 mg/d) and when participants confirmed willingness to be trained in maintaining such average daily calcium intake. The criteria for exclusion were major diseases affecting bone tissue: clinical or laboratory evidence of renal or hepatic disorders, dysfunction of the parathyroid or adrenal glands, etc. Other exclusion criteria included current or recent (during past 12 months) therapy with bisphosphonates, selective estrogen receptor modulators (SERMs), estrogens, corticosteroids, or any other drugs that may seriously interfere with bone metabolism. As this study was designed as a first exploratory study, no specific control of dietary habits, other than calcium intake, and exercise was included in this study.

Test products and interventions

The olive extract (Bonolive®, consisting of a mixture of polyphenols derived from olive leaf, standardized for its oleuropein content (>40%)), was supplied by BioActor BV (Maastricht, The Netherlands). As placebo, maltodextrin (Lycatab, Roquette, Lestrem, France) was used. The study medication was provided as gelatin capsules (250 mg of active or placebo, 2.5 mg Magnesium stearate and 2.5 mg Silicium dioxide), contained in 90-day packs (90 capsules per container plus 15 extra). Additionally, 400 mg elemental calcium in the form of 1000 mg of Calcium carbonate was supplied as a separate commercial product (Calperos 1000, Pliva OTC, Poland). The daily dose was set to one capsule with the study medication or placebo and one calcium tablet per day, to provide the daily recommended dose of calcium. Patients were

instructed to ingest the test products 30 min before the first meal with a glass of water and to not take antacids containing calcium, aluminum, iron, and/or magnesium 2 hours prior to and 2 hours after dosing. Patients were not allowed to take any other calcium and vitamin supplements or parapharmaceuticals during the study period.

Randomization, blinding and sample size

Randomization was performed using block randomization with 50% of patients assigned to each treatment arm. For allocation of the participants, a computer-generated list of random numbers was used, created by a person not involved in the execution of the study or analysis of the results. The result of the randomisation was unknown to the investigator who allocated the participants to consecutive study numbers from the list. Similarly, neither the patient nor the investigator were aware of the allocation to either treatment or placebo group, as both study products had identical appearance and were only numbered with the participant's study number. This ensures the complete double-blind nature of the study. The needed total sample size was calculated based on a 15% difference in osteocalcin levels between groups: for a type I error α of 0.05 and 90% power, 32 subjects for each group were found.

Outcomes

The primary efficacy endpoint was the difference between groups in individual change (in percentage) from baseline in serum levels of two biochemical markers of bone metabolism: osteocalcin (OC) and C-terminal cross-linking telopeptide of type I collagen (CTX), which are markers of bone tissue formation and resorption, respectively. The secondary efficacy endpoints were the difference between groups in mean change from baseline in lumbar spine (L2–L4) and femur neck BMD (as assessed by DEXA) and in the levels of other bone-formation markers: bone alkaline phosphatase (BALP) and amino-terminal pro-peptide (PINP) of type 1 collagen. Additional secondary endpoints were the difference between groups in relative change from baseline in serum lipid profiles (total cholesterol, HDL-C, LDL-C and triglycerides). Levels of serum inflammatory markers - high-sensitivity C-reactive protein (hs-CRP) and interleukin-6 (IL-6) - were measured in order to screen for infections and inflammatory processes.

Sample collection, tests and assays

Assays on serum samples. Samples for OC and CTX determination were collected at visits 2, 3, 4 and 5. After 12 hours fasting, serum samples were collected between 9 and 11 am at the study facility and stored at -70 °C until further analysis. The assessment of bone turnover markers was performed in the Clinical Laboratory of the study facility. For OC, the N-MID Osteocalcin ELISA assay was used (IDS, Boldon, Nordic Bioscience A/S, Denmark). This assay measures total OC, consisting of both full length OC and the N-terminal mid fragment. Concentrations of BALP were measured by monoclonal antibody immunoenzymatic assay using the Ostase BAP kit (IDS EURL, Paris). Serum PINP was determined by competitive radioimmunoassay (RIA), using a polyclonal rabbit anti-PINP antibody (Uniq PINP RIA; Orion Diagnostica Oy, Finland). Serum levels of CTX were measured using the Serum CrossLaps One Step ELISA assay (IDS).

Samples for analysis of serum lipid profiles, inflammation markers and vitamin D (25OHD) were collected at visits 2, 4 and 5. Serum levels of 25OHD were measured using the 25OHD EIA assay (IDS). For the quantitative determination of human IL-6 and hs-CRP, high sensitivity ELISA test kits were used (Quantikine HS IL-6, Quantikine HS Human CRP; R&D Systems Minneapolis, USA).

Concerning OC, intra- and inter-assay variations were 5.4%-6.8% and 2.8%-6.8%, respectively; sensitivity was 0.5 ng/ml. For CTX intra- and inter-assay variations were 4.7%-4.9% and 5.4%-8.1%, respectively; sensitivity was 92 pM/l. For 25OHD intra- and inter-assay variations were below 8% and 10%, respectively; sensitivity was 5 nmol/l. For human IL-6 and hs-CRP intra-assay coefficients of variation (CVs) were both below 5%, and inter-assay CVs were below 7%. For BALP, intra- and inter-assay variations were 2.9%-6.5% and 5.8%-6.4%, respectively; sensitivity was 0.7 µg/l. For PINP sensitivity was 2 µg/l; intra- and inter-assay precisions were 6.5%-10.2% and 6%-7%, respectively.

Serum calcium, phosphate, creatinine, urea, total protein, alkaline phosphatase (ALP, EC 3.1.3.1) concentrations and lipid profile (total cholesterol, triglycerides, direct HDL-cholesterol and direct LDL-cholesterol) were analyzed using routine laboratory methods (Express Plus Analyzer, Chiron Diagnostics, USA). Basic hematological analysis was performed with the use of automatic system (ACT5 Diff Haematology Analyzer, Beckman Coulter Inc, USA). Urine analysis was performed using dipstick (Multistix 10 SG; Bayer Diagnostics, UK) and microscopic analysis of sediment.

DEXA measurements. The lumbar spine (L2-L4) and femur neck BMD of all subjects was examined in the anterior-posterior position using DEXA (LUNAR Prodigy Advanced, LUNAR Corp., USA) at the Densitometric Laboratory of the study facility, at the baseline visit (V2), and after 12 months (V5). DEXA data were analyzed for consistent instrument performance throughout the study. Mean percentage coefficient of variance (%CV) in the Densitometry Laboratory in BMD for single hip scans was approximately 0.65% for the total hip and 1.65% for the femoral neck. Mean %CV for spine BMD (L2-L4) was approximately 1.10%. Scan printouts were assessed independently by 2 research scientists in the Department of Bone Metabolic Diseases at the study facility.

Standardization of dietary calcium intake. Dietary calcium intake assessment was provided by a self-administered FFQ (25,26). The bias and precision of the FFQ were assessed by comparing the intake of nutrients estimated from the FFQ, 24 hour recall and 4-day food records. A dietary calcium intake of 300-600 mg/day was defined as "standard" for the study, based

on previous observations of local intake levels (27). At the screening visit, all the subjects were trained in how to keep their dietary calcium intake within this range throughout the duration of the study. This was performed by providing dietary recommendations for the intake of calcium-containing foods in line with the information the participants provided in the FFQ.

Safety measures. All adverse events (AE's) were recorded at all visits. Their severity and implications to the treatment were evaluated by the investigators and medical experts, and reported to the respective authority. Basic physical examination, vital signs, hematology, biochemistry, and basic urine analysis were conducted at all study visits.

Statistical analysis

Statistical analyses were performed in SAS version 9.2 and SPSS version 18. Summary statistics of all variables at baseline in the treatment and the placebo groups were calculated for each subject. P-values for the Wilcoxon rank-sum were calculated to test for differences in baseline values between placebo and treatment groups. To investigate the significance of the treatment effect, statistical analysis on all endpoints, with time as continuous covariate, was performed. A linear model accounting for repeated measures was fitted within the subjects, using residual maximum likelihood estimation (REML), with unstructured covariance. The model included three covariates: treatment (linear term), week (quadratic term) and the treatment by week interaction. In addition, two-tailed paired or unpaired (two-sample with unequal variance) t-tests were used to assess within and between group differences, respectively. The t-test was done for both absolute values and for the relative change over time as expressed as percentage from baseline (Microsoft Excel 2011, Microsoft inc, Redmond, WA, USA). For all tests a level of $p<0.05$ was considered to be statistically significant.

Results

Report of adverse events and enrolled subjects

A total of 103 female patients were contacted, of which 64 enrolled the study (32 per group) (see Supplementary information Fig. S1 for flow diagram of the study). Overall incidence of adverse events (AE's) was similar across the two study groups. No serious adverse events (SAE's) were related to the treatment. However, two treatment unrelated SAE's occurred, leading to withdrawal from the study in the placebo group: a right forearm fracture and an incorrect mammography result which raised the suspicion of breast cancer. To sum up, a total of 12 women did not finish the study due to consent withdrawal (including the two reported SAE's), and 4 women were excluded from the study due to poor cooperation during the study or/and loss of follow up. This resulted in a total of 11 subjects being excluded from the placebo group after V4 (6 months) and 5 subjects excluded from the treatment group after V3 (3 months) (Table 1).

The most commonly occurring AE's during the study were upper pulmonary tract infections (including bronchitis and influenza: one in the treatment group and one in the placebo group) and dyspepsia (one in the treatment group and one in the placebo group), increase of systolic blood pressure (two in the treatment group) and back pain (two in the placebo group). Dyspepsia was usually mild and it occurred among patients who had previous gastrointestinal disease or among NSAID's users and the symptoms disappeared within a few days. Therefore, dyspepsia did not generally lead to discontinuation from the study. Increase of systolic blood pressure was also mild and occurred episodically in subjects who had a history of arterial hypertension. Similarly, back pain occurred in those patients who had previous history of lumbar spine discopathy. No clinically relevant treatment related events were recorded during the study.

No differences between groups were detected before start of treatment

The clinical cohort consisted of a group with a mean age of 59.5±4.9, and a mean body mass index (BMI) of 26.9±4.4. Baseline characteristics of the study population are shown in Table 2. None of the examined parameters differed at baseline between treatment and placebo groups (Table 2). Baseline levels of osteocalcin were 11.51±4.13 and 11.99±3.75 μg/L in the treatment and placebo groups, respectively; CTX was 0.57±0.20 and 0.61±0.23 μg/L; lumbar spine BMD was 0.99±0.06 and 1.00±0.06 g/cm2 and femur neck BMD was 0.83±0.05 and 0.84±0.07 g/cm2 (Table 2).

Compliance with the treatment regimen was similar as calculated from the number of study products dispensed to the

Table 1

Number of subjects engaged at each stage of the study and final number of individuals reported after withdrawal from the study. The starting number of patients per group was 32. However, 2 Serious Adverse Events were reported and 14 patients withdraw the study at different stages. Thus, the final number of subjects that completed the study was 21 and 27 in the placebo and treatment groups, respectively

	Baseline (V2)		3-months (V3)		6-months (V4)		12-months (V5)
Group	**Drop-out**	**N**	**Drop-out**	**N**	**Drop-out**	**N**	**Final N**
Placebo	-5	27	-4	23	-2	21	21
Treatment	-1	31	-4	27	0	27	27

Table 2

Baseline parameters of the study population. Differences in baseline values between the treatment and placebo groups were assessed using the non-parametric Wilcoxon test. No statistically significant differences were found. While routine parameters were analyzed on all individuals (first part of table), specific analyses were only performed for those subjects which remained part of the study for either at least 3 months (primary endpoints OC and CTX, inflammation markers and vitamin D status) or for the complete duration of the study (secondary endpoints BALP and PINP)

	Treatment					Placebo					
Parameter	**Mean**	**SD**	**Min**	**Max**	**N**	**Mean**	**SD**	**Min**	**Max**	**N**	**p-value**
Parameters measured at V2 (baseline)											
Age	59.72	4.39	53.00	69.00	32	59.35	5.55	50.00	69.00	32	0.77
Body weight (kg)	66.22	12.11	40.00	98.00	32	68.71	10.92	52.00	104.00	32	0.32
Body height (cm)	159.75	5.04	150.00	170.00	32	158.03	5.95	142.00	168.00	32	0.41
BMI (kg/m^2)[d]	25.90	4.25	14.87	36.44	32	27.52	4.01	19.33	38.20	32	0.28
Postmenopausal age (y)	10.35	5.36	2.00	24.00	32	10.59	7.99	3.00	32.00	32	0.08
Total cholesterol (mg/dL)	236.16	51.95	148.00	371.00	32	249.06	35.13	193.00	324.00	32	0.14
HDL-C (mg/dL)	48.22	10.17	34.00	69.00	32	50.09	9.71	36.00	70.00	32	0.43
LDL-C (mg/dL)	167.15	51.24	88.00	299.8	32	174.43	37.62	108.6	235.80	32	0.29
Triglycerides (mg/dL)	98.84	42.92	40.00	241.00	31[a]	122.72	59.57	44.00	257.00	32	0.20
DEXA L2-L4 (g/cm^2)	0.99	0.06	0.90	1.14	25[b]	1.00	0.07	0.89	1.12	21b	0.53
DEXA Femur neck (g/cm^2)	0.83	0.05	0.74	0.94	30[b]	0.84	0.07	0.72	1.06	31b	0.93
Parameters measured for subjects that engaged until V3 (3 months)											
OC ($\mu g/L$)	11.51	4.13	4.98	23.99	31	11.99	3.75	7.03	20.25	27	0.68
CTX (µg/L)	0.57	0.20	0.27	1.11	31	0.61	0.23	0.33	1.39	27	0.54
hs-CRP (mg/L)	2.83	1.16	0.93	5.42	31	3.24	1.57	1.08	6.65	27	0.58
IL-6 (pg/mL)	2.04	2.14	0.43	9.11	31	1.80	1.40	0.50	6.09	27	0.96
25(OH)D (ng/mL)	60.65	25.10	23.79	112.63	31	60.23	21.67	21.22	98.57	27	1.00
Parameters measured for subjects that engaged until V4 (6 months)											
BALP (µg/L)	12.19	4.98	5.23	24.14	24[c]	14.08	6.23	6.45	34.49	20c	0.28
PINP (µg/L)	56.45	25.69	15.63	105.36	24[c]	64.05	19.18	21.05	99.13	20c	0.27

a. One patient was removed from this group because of extreme outlier value (possibly an experimental error); b. Not all DEXA scans were eligible for analysis due to poor quality, and so were excluded from the report; c. For some blood samples these assays have failed and so no value has been obtained for these parameters; d. BALP: bone alkaline phosphatase; BMI: Body mass index; CTX: C-terminal cross-linking telopeptide of type I collagen; DEXA: Dual-energy X-ray absorptiometry; HDL-C: High-density lipoprotein cholesterol; hs-CRP: high-sensitivity C-reactive protein; IL-6: interleukin-6; LDL-C: Low-density lipoprotein cholesterol; 25(OH)D: vitamin D; OC: osteocalcin; PINP: amino-terminal pro-peptide of type 1 collagen

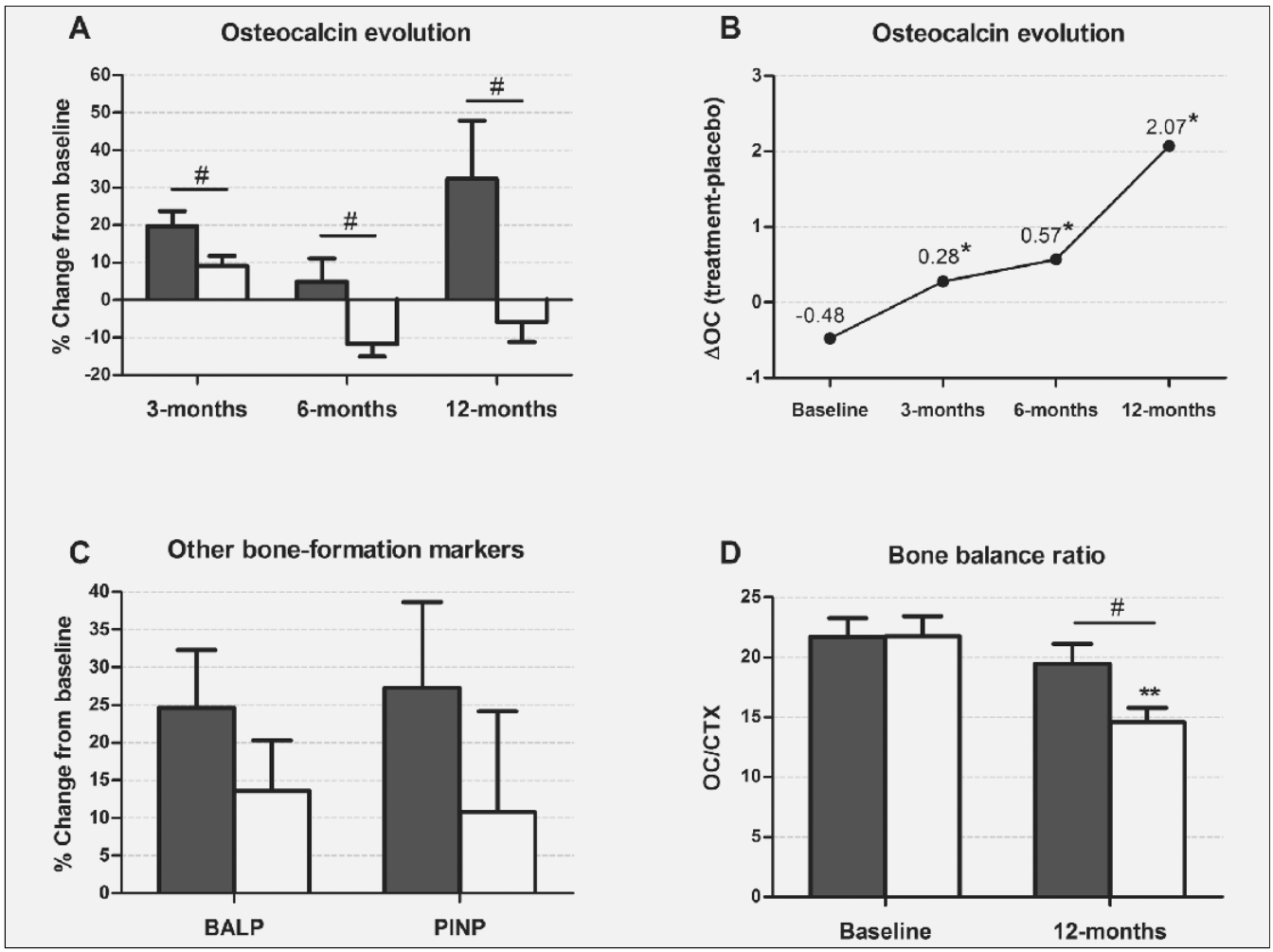
subjects and the number of study products returned to the study site.

The olive extract increases osteocalcin levels and sustains BMD in osteopenic women

None of the serum levels of the investigated bone-formation or bone-resorption markers significantly differed between the treatment and the placebo groups at the start of the study (Table 2). In contrast, OC serum levels decreased by 6% in the placebo group, whereas a 32% increase was observed in the treatment group 12 months after the start of the study (Fig. 1A). In fact, the difference between the treatment and placebo groups developed gradually throughout the study, resulting in a significant treatment effect on the evolution of serum OC levels (Fig. 1B), as well in a significant difference between placebo and treatment conditions at each time point (3, 6 and 12 months) (Fig. 1A). The results for BALP and PINP were inconclusive, however also for these markers, a trend towards increasing levels from the start to the end of the study was observed in the treatment group (25% and 27% increase, respectively for BALP and PINP vs 14% and 11% in the placebo group) (Fig. 1C). CTX levels increased significantly during the treatment period in both treatment and placebo groups, with a slightly smaller increase in the treatment group (37%) when compared to the placebo (41%), yet no significant differences between groups were observed for this marker. The bone balance ratio (OC/CTX), a good indicator of bone-turnover balance (28), was also calculated for each subject and the mean ratio at baseline and at 12 months plotted (Fig. 1D). The OC/CTX ratio significantly decreased in the placebo group, whereas no significant change was observed in the treatment group. This suggests an increased bone-resorption activity in the placebo group, thereby also suggesting that the olive extract has a stabilizing and balancing effect on bone-turnover.

Concerning the lumbar spine BMD, this decreased throughout the study for both groups (Fig. 2A). However, whereas the lumbar spine BMD values decreased 1.9% in the

Figure 1
Individual evolution of bone-formation markers throughout the study and final bone balance ratio



In all graphs treatment is depicted as black bars and placebo as white bars. Panel A depicts the individual change (in %) from baseline of osteocalcin (OC) levels measured after 3, 6 and 12 months of Bonolive intake; panel B shows the difference in absolute OC levels at each time point between treatment and placebo. The significance of this evolution was assessed by REML analysis with time as continuous covariate (*, $p<0.05$). Panel C depicts the individual change (in %) from baseline of bone alkaline phosphatase (BALP) and amino-terminal propeptide of type I collagen (PINP) levels measured after 12 months; Panel D depicts the individual bone balance ratio (OC over C-terminal cross-linking telopeptide of type I collagen (CTX)) at baseline and after 12 months. In panels A, C and D, statistically significant differences between groups (within each time point) were tested by two-tailed two-sample t-test with unequal variance and are noted with a (#). Statistically significant differences within groups (baseline vs visit) are highlighted with a (*) and were evaluated by two-tailed paired t-test. (#) or (*) represent significances at $p<0.05$; (**) represents significance at $p<0.01$.

placebo group, with a significant within group difference, they have only decreased 0.9% in the treatment group (not significant). In contrast, femur neck BMD did not significantly change within the study duration and for the included study population (Fig. 2B).

The lipid profile is ameliorated by the olive extract after 12 months

A significant treatment effect was observed on the serum lipid profiles (Table 3). Although both total cholesterol and LDL-cholesterol levels decreased in both groups after 12 months, an effect which may be associated with increased awareness for healthy food consumption, they were significantly lower in the treatment group when compared to the placebo by the end of the study. In contrast, triglyceride levels significantly increased in the placebo group, whereas a small decrease was detected in the treatment group (Table 3).

The effects of the olive extract on hematological parameters are neglectable

The standard hematology parameters analyzed can be seen in Table 4. Except for phosphate levels, none of the parameters was statistically different between the two groups after 12 months. In addition, no significant differences were observed

Table 3

Blood lipid profiles at baseline and after 6 and 12 months of treatment. Values are expressed in mg/dL. At baseline (V2), 32 subjects were included in each group; at 6 months (V4) 23 subjects were engaged in the placebo group and 27 in the treatment group; at 12 months (V5) the number of subjects engaged were 21 and 27 for the placebo and treatment groups, respectively

		Baseline		6-Months		12-Months		Relative change	
Parameter	**Group**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**End-Start**	**p-value**
Total Cholesterol	Placebo	249.06	35.13	220.70	32.51	233.38	32.60	-15.68	0.01*
	Treatment	236.16	51.95	219.00	41.32	209.96	35.51	-26.2	
HDL-C [b]	Placebo	50.09	9.71	51.13	6.01	51.38	9.31	1.29	0.37
	Treatment	48.22	10.17	51.59	8.11	53.85	8.98	5.63	
LDL-C	Placebo	174.43	37.62	143.44	30.00	154.06	30.51	-20.37	0.02*
	Treatment	167.15	51.24	145.45	38.58	132.49	29.70	-34.66	
Triglycerides	Placebo	122.72	59.57	130.91	60.14	141.62	77.39	18.9	0.01*
	Treatment[a]	98.84	42.92	104.58	59.97	94.69	37.48	-4.15	

a. One patient was removed from this group because of extreme outlier value (possibly an experimental error); *Significance ($p<0.05$) of relative change over time was assessed by REML analysis with time as continuous covariate; results are shown as exact statistic; b. HDL-C, High-density lipoprotein cholesterol; LDL-C, Low-density lipoprotein cholesterol

Table 4

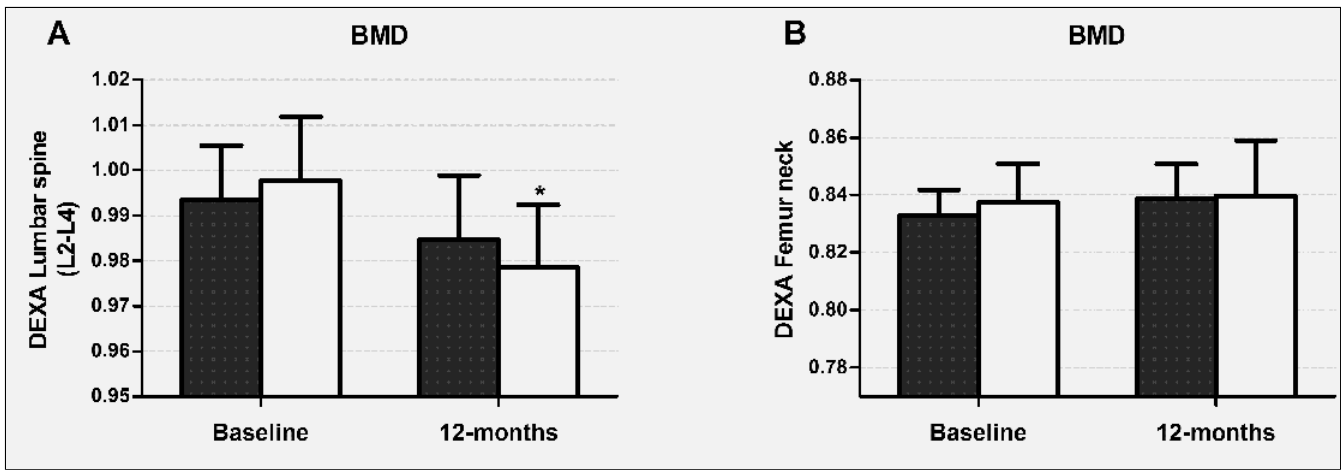
Hematology parameters for both study groups at baseline and at the end of study (12 months)

		Baseline			12-months			Individual % change	
Parameter	**Group**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N[b]**	**% from BL**	**p-value**
Hemoglobin (g/L)	Placebo	137.76	7.58	32	134.75	6.34	20	-1.9	0.30
	Treatment	135.41	7.50	32	134.41	7.45	27	-0.6	
Hematocrit (%)	Placebo	39.44	2.08	32	40.15	1.78	20	2.0	0.12
	Treatment	38.77	2.32	32	40.39	2.58	27	4.3	
RBC (T/L) [c]	Placebo	4.58	0.30	32	4.63	0.30	20	1.5	0.14
	Treatment	4.51	0.35	32	4.66	0.35	27	3.6	
WBC (G/L)	Placebo	5.92	2.14	32	5.65	1.88	20	-0.9	1.00
	Treatment	5.89	1.70	32	5.60	1.47	26	-0.9	
Platelets (G/L)	Placebo	284.48	60.51	32	269.70	78.65	20	-5.4	0.90
	Treatment	301.15	82.02	32	278.85	64.94	27	-4.7	
MCV (fl)	Placebo	86.34	4.67	32	86.94	3.66	20	0.6	0.48
	Treatment	85.89	3.82	32	85.10	9.54	27	-0.9	
ALP (U/L)	Placebo	76.38	19.98	32	70.15	20.66	20	-4.6	0.91
	Treatment	66.78	17.05	32	62.78	15.56	27	-3.9	
Creatinine (mg/dL)	Placebo	0.76	0.16	32	0.82	0.11	20	12.3	0.44
	Treatment	0.81	0.12	32	0.85	0.10	27	7.1	
TP (g/L)	Placebo	7.11	0.31	32	6.89	0.43	20	-2.9	0.38
	Treatment	6.99	0.43	32	6.89	0.39	26	-1.2	
Ca (mg/dL)	Placebo	8.94	0.36	32	9.05	0.18	20	1.6	0.40
	Treatment	9.05	0.33	32	9.09	0.17	27	0.6	
25(OH)D (ng/mL)	Placebo	60.23	21.67	27	66.26	26.85	21	10.0	0.27
	Treatment	60.65	25.10	31	71.12	24.89	27	17.3	
P (mEq/L)	Placebo	1.26	0.16	32	1.27	0.06	19	1.4	0.02*
	Treatment	1.18	0.15	32	1.29	0.07	27	11.3	
hs-CRP (mg/L)	Placebo	3.24	1.57	27[a]	2.47	1.91	21	-14.9	0.07
	Treatment	2.83	1.16	31[a]	3.17	2.27	27	25.5	
IL-6 (pg/mL)	Placebo	1.80	1.40	27[a]	1.91	1.20	21	37.5	0.93
	Treatment	2.04	2.14	31[a]	2.04	2.24	27	34.7	

a. Parameters measured for subjects that engaged until V3 (3 months); b. For one blood sample from the placebo group the assay on the blood parameters failed; *The statistical significance (between groups) of the individual relative change from baseline was tested using a two-tailed two-sample t-test with unequal variance ($p<0.05$); results are shown as exact statistic; c. ALP , alkaline phosphatase; BL, baseline; Ca, calcium; hs-CRP, high-sensitivity C-reactive protein; IL 6, interleukin 6; MCV, mean cell volume; 25(OH)D, vitamin D; P, phosphate; RBC, red blood cells; TP, total protein; WBC, white blood cells.

Figure 2

Bone mineral density (BMD) at baseline and after 12 months of Bonolive intake by osteopenic women



In both graphs treatment is depicted as black bars and placebo as white bars. Panel A, Dual-energy X-ray absorptiometry (DEXA) lumbar spine (L2-L4) BMD (g/cm^2); panel B, DEXA femur neck BMD (g/cm^2). Statistically significant differences between groups (within each time point) were not found as tested by two-tailed two-sample t-test with unequal variance. Statistically significant differences within groups (baseline vs 12-months) are highlighted with a (*) and were evaluated by two-tailed paired t test. (*) $p<0.05$.

for the inflammatory markers hs CRP and IL-6 (Table 4). Nonetheless, hs-CRP showed a relative increase in the treatment group (25.5%), which in fact was caused by only two subjects. This could indicate an unrelated infection. Plasma 25(OH)D levels were monitored throughout the study and did not significantly change in any of the groups (Table 4). Finally, serum calcium levels were not modified by the study and no differences were observed between the placebo and treatment groups at the end of the study (Table 4). These results exclude an effect mediated by Vitamin D and/or calcium intake.

Discussion

Given the complexity of factors involved in the onset of osteoporosis, preventive strategies aimed to lower the risks of developing this disease need to be established and implemented. Definitely, there is an overwhelming body of evidence emphasising the important role of calcium as bone building blocks. Nevertheless, because osteoporosis is a multifactorial disease and because calcium supplementation may not necessarily offset bone loss, a recent interest in other natural ingredients, particularly food phenolics, has significantly increased. Therefore, we have focused our research in polyphenols derived from olives, which are usually studied for their anti-inflammatory and free radical-scavenging properties (29). In the present study, we aimed to obtain first initial insight in the potential effect of a polyphenol-rich olive extract on markers associated with bone metabolism after a 12-month administration to postmenopausal women with osteopenia. For that, 250 mg/day of olive extract were ingested, thereby providing with a dose of 100 mg oleuropein per day. For the sake of evaluating whether this dose as such can be considered nutritional, i.e. whether it is possible to consume such levels through dietary means, we can refer to the fact that some olive oils are particularly rich in those micronutrients: 238 mg/kg up to 1 g/kg (30,31). Of course, although this suggests sufficient intake is possible through consumption of very specific olive products, it is unrealistic to assume this to be sufficient over long time periods, as a typical daily consumption of standard olive oil only provides with 10-25 mg of polyphenols (30). According to previous clinical trials targeting oil-derived-phenolics antioxidant properties, it seems that the efficient dose of oleuropein is at least 98 mg per day (32). In addition, to avoid potential confounding effects related to deficiency in calcium, a dose of 1 g of calcium was systematically given to the enrolled subjects to account for the total daily calcium requirements (33).

While it is obvious that the ultimate therapeutic potential of the studied olive extract depends on the stabilizing effect on BMD or even on reduced fracture rates, the goal of this study was to obtain a first insight in the effectivity of the product in humans in a prospective intervention study, i.e. with a relatively limited population size and, in terms of bone remodeling, relatively short duration. For that reason, the well-described biomarkers for bone metabolism, OC and CTX were selected as primary endpoints instead of BMD and the study duration was limited to 12 months. Changes in actual BMD and in additional bone turnover biomarkers were included as secondary endpoints. The obtained information can then be used for larger-scale follow up human intervention studies, with BMD as primary endpoint.

Upon evaluation of the different biomarkers between the treatment and control groups, a significant increase in osteocalcin was observed throughout the study in the treatment group, and the final osteocalcin levels were significantly higher in this group when compared to the placebo group (despite the

fact that the initially projected sample size of 32 individuals per group was not reached due to dropouts). This finding was supported by the simultaneous increase in BALP and PINP levels in the treatment group, albeit not being significant. Furthermore, while the bone balance ratio (OC/CTX) significantly decreased over time in the placebo group, this ratio remained constant in the treatment group. Altogether, these results are consistent with preclinical effects observed with the olive extract and are in agreement with recently published data (34) showing that, in an intervention trial carried out in 127 elderly men for 2 years, consumption of a Mediterranean diet enriched with olive oil was associated with increased plasma osteocalcin levels.

Although the authors are fully aware of the small size of the study population and the difficulty to make strong conclusions based on this prospective study, our results support further research to confirm the protective effect of the olive polyphenol extract on lumbar spine BMD. Indeed, while this parameter did not significantly change in the treatment group, a significant decrease in BMD was observed in the placebo group. Although these changes were not statistically different between groups, BMD values decreased significantly in the placebo group, whereas the within-group change in the treatment group did not alter significantly. This seems to suggest a larger bone loss in the placebo group when compared to the treatment group. These findings would be in line with previous in vivo studies performed in a rodent model for osteoporosis showing that olive polyphenols had a bone sparing effect in ovariectomized rodents (13,15,16). Again acknowledging the need for final scientific confirmation, this result may partly explain why epidemiological studies have shown that the incidence of osteoporosis in Europe is lower in the Mediterranean basin (35,36), where a high consumption of olive oil is regarded as the hallmark of the traditional Mediterranean diet (37). Additionally, no significant differences between groups have been found for calcium and vitamin D levels between the groups by the end of the treatment (12 months), thereby excluding a direct role of these parameters in the results obtained.

From a mechanistic point of view, the observed higher levels of the bone-formation marker osteocalcin may suggest that the treatment either increases the number of osteoblasts, or potentiates the activity of the existing cells (38). In fact, previous ex vivo studies with oleuropein have shown a marked effect of olive polyphenols on the differentiation of isolated MSCs (23). An increase in the number of osteoblasts in cell culture has been observed. Similarly, increased calcium deposition by cultured osteoblasts and inhibition of osteoclast activity by olive polyphenols were reported before (16). Taken together, these preclinical studies indicate that olive polyphenols have an anabolic effect on bone metabolism and may protect from impaired bone-turnover by stimulating bone formation and thereby balancing the dynamic bone renewal process.

On the other hand, since osteoblasts and adipocytes are derived from a common MSC progenitor, stimulation of osteoblast formation by olive polyphenols may also affect bone marrow adipocyte formation and ultimately change lipid profiles in humans (23). Interestingly, previous studies (39) have shown that the polyphenol genistein is able to concurrently activate osteogenesis or adipogenesis by activating two different transcription factors, the Estrogen receptor (ER) and the Peroxisome proliferator-activated receptor gamma (PPAR-γ). At high concentrations (>1 μmol), genistein acts as a ligand for PPAR-γ, leading to up-regulation of adipogenesis and down-regulation of osteogenesis, whereas osteogenesis is increased at lower doses at the expense of adipogenesis. In fact, it has been proposed that the close relationship between these lineages underlies the reciprocal relationship between the increase in adipocyte accumulation and decrease in bone-formation that occurs during aging (40,41).

The combination of this potential mode-of-action and the strong and significant effect which was observed in this prospective study on serum lipid profiles, is therefore also an interesting starting point for further research. The serum lipid profiles of subjects in the treatment group improved significantly compared to the placebo group, such that total cholesterol, LDL-cholesterol and triglycerides were decreased. Further research is therefore needed to substantiate this promising effect of olive polyphenols on blood lipid profiles and the potential link with bone metabolism.

Conclusion

In the light of the increasing incidence in osteoporosis in developed countries, the results from this first prospective 12-month human intervention study show that olive polyphenolic compounds may have a promising biological activity towards maintenance of a balanced bone-turnover process and improved blood lipid profile. Prevention by dietary means or food supplements is of special relevance, as nowadays, the WHO recommendations focus on primary prevention, with the goal of finding agents, other than calcium and vitamin D, which have preventive effects on bone-turnover (3). Based on the results of this limited-scale prospective study, targeted larger human intervention studies can now be set up to come to an in-depth understanding on the ultimate therapeutic potential of olive polyphenols.

Ethical standards: The intervention study described in this manuscript complies with the current laws of the country in which it was performed (Poland), approved by the ethics committee of the Institute of Agricultural Medicine (Lublin, Poland) and registered under NCT00789425 at the NIH clinical trial register (clinicaltrials.gov).

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Davicco reports a license agreement with BioActor. • Dr. Coxam reports a license agreement with BioActor.

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