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Posted Date: 31 October 2023

doi: 10.20944/preprints202310.2094.v1

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Effect of the Consumption of Vitamin C, D3, Ca Supplements and Olive Paste Enriched with Mountain Tea on Bone Metabolism Indices in Postmenopausal Women with Increased Osteoporosis' Risk: A Prospective Interventional Study

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Abstract: (1) Background: Low dietary intake of calcium, vitamin D, vitamin C and magnesium has been associated with increased risk of osteoposrosis. The purpose was to evaluate changes in biomarkers of bone metabolism, and cardiometabolic health in postmenopausal women, with high risk of osteoporosis, followed by a nutritional intervention program. (2) Method: It was a prospective randomized controlled trial. A sample of 115 apparently healthy postmenopausal women(45 -75 years of age) randomized into four groups: (I) nutritional intervention group(n=40) received daily 1000mg vitamin C, 500mg vitamin D_3, 500mg calcium(Ca) and 300mg magnesium (Mg); (II) control group(n=42) received daily 500mg vitamin D_3, 500mg Ca and 300mg Mg; (III) nutritional intervention group (n=18) received daily 150mg bisphosphonates, 500mg vitamin D_3, 500mg Ca and 300mg Mg; and (IV) nutritional intervention group (n=15) received daily about 364 mg polyphenols via an innovative functional food (50g olive paste enriched with mountain tea extract) along with 500mg vitamin D_3, 500mg Ca and 300mg Mg. Groups I -III received supplementation for a year whereas group IV for 5 months. Changes in calciregulatory hormone parathormone (PTH) were evaluated at the beginning of the study as well as at 5 and 12 months intervals. Blood levels of vitamin D, vitamin C, Ca, Mg and the lipid profile were assessed at the beginning and at the end of the study. Anthropometric indices (weight, body fat(Kg), Body Mass Index(BMI) and bone mineral density(BMD) were evaluated at the beginning and at the end of the study. Statistical analysis was performed with IBM-SPSS Statistics-21. The level of statistical significance was set at $p<0.05$. (3) Results: 25(OH)D3 levels were improved, in groups II, III and IV (+3,71% and +1.45% and +5.62% respectively). PTH levels were increased at the end of the intervention period, for groups I and IV. Significant positive changes recorded, in total BMD, in all four study groups. Significant beneficial changes for total cholesterol was observed in group IV (-2.07%, $P<0.05$) and positive changes in group I for HDL biomarker (+61.62%, $P<0.05$) (4) Conclusions:. Additional larger-scale clinical trials and intervention studies are considered essential, in order to fully investigate and elucidate associations between dietary components and biochemical indices of bone metabolism.

Keywords: micronutients; functional foods; olive paste; bone mineral density; bone metabolism biomarkers; postmenopausal women

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1. Introduction

According to the international Osteoporosis Foundation (IOF), just under nine million fractures a year are attributed to osteoporosis. Osteoporosis is an extremely debilitating ailment characterised by reduced bone density and progressive weakening of bones, associated with an increased risk of bone fractures. Osteopenia is considered the precursor of osteoporosis and as defined by World Health Organisation (WHO) is characterised by decreased bone mineral density (BMD) with a T-score between 1 up to 2.5 (-2.5 ≤ T-score ≤ -1.0) while a T-score below 2.5 indicates osteoporosis (T-score < -2.5) (1).

Although osteoporosis is more prevalent in women, it has been shown that gradual loss of bone density occurs in both men and women, with ageing. According to WHO the elderly populations is estimated to reach 12 billion by 2025 (1), whereas aging has been associated with numerous chronic diseases including sleep disorders, malnutrition, osteoporosis as well as increased risk of falls (2, 3). Osteoporosis, however, is the most common metabolic disease amongst the elderly poplation (4) whilst increases the risk of fractures. Osteoporotic fractures are associated with chronic pain, decreased quality of life, hospitalization, disability and increased mortality (5). In the United States, it is estimated that more than forty million people have been diagnosed with osteoporosis, or are at high risk of developing the disease, due to progressive bone loss (6). In addition, according to IOF it has been estimated that more than 200 million people, worldwide, are affected by osteoporosis, with approximately 6.3% men and 21.2% women, over the age of 50 (6) with one in three women and one in five men over the age of 50 to sufer osteoporotic fractures. The prevalence of osteoporosis in postomenopausal women, however, is higher compared with the general population, primarily due to hormonal changes associated with menopause (6-8).

The proportion of osteoporotic patients, above the age of 50, who received treatment, increased from 1.67 % in 2001 to 9.1 % in 2009 but subsequently declined to 8.2 % in 2011 (1). Bone metabolism in osteoporosis is characterised by an imbalance between bone formation and bone resorption, two fundamental processes that are involved in the continous remodeling of bone tissue. Osteoblasts are speciallised cells, responsible for bone formation, while osteoclasts are cells, primarily responsible for bone resorption. Osteocytes are mature bone cells, derived from osteoblasts, and are rsponsible for the maintenance of bone health. The balance of bone formation and resorption ensures that bone tissue is continually renewed maintaining the amount of bone tissue and the integrity of the bone structure. This equilibriium is regulated by hormones, including parathyroid hormones, calcitonin as well as calcium and vitamin D, while abnormal bone metabolism is caused by various factors, including hormonal imbalances such as menopause, nutritional deficiences, chronic inflammation, ageing, endocrine disorders and certain cancer (2).

Research studies have primarily focused in calcium and vitamin D for the prevention of osteoporosis and bone health. In recent years consideration has been given to additional nutrients and food constituents including magnesium, potassium, vitamin C, vitamin K, several B vitamins, carotenoids and polyphenols in maintaining a healthy BMD, preventing bone loss and fractures. More specifically population studies have shown that magnesium and potassium contributes to bone strength through bone mineralisation (9). Carotenoids and vitamin C have shown to protect BMD by reducing the effects of oxidative stress (10, 11), while vitamin K intake has been associated with a 65% reduced risk of hip fracture (12). Ascorbic acid has gained attention in recent years and several research studies have found a positive correlation between vitamic C supplementation and BMD (13). Recent studies have also shown an indirect inflience of B vitamins in bone turnover while B-complex vitamins are required as cofactors in metabolic reactions stimulate osteoblast activity and bone formation, whilst increased risk fracture and osteoporosis has been associated with pernisious anemia (14). Moreover, recent studies have shown the effects of various functional foods and bioactive ingredients have been studied, such as polyphenols, as factors that may affect bone metabolism (10). A recent Greek study, with the aim to assess vitamin D status in adults population, comparing serum levels with dietary intake, sun exposure and other factors found a high prevalence in vitamin D deficiency, with a total of 64.8% of the adult population to have either deficient or insuficient serum concentration levels (<20ng/ml) (13). Moreover, dietary calcium deficiency is

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known to be associated with low BMD, which in long-term results in osteopenia and osteoporosis (15). Calcium supplementation, however, is widely considered to be the most important for the prevention of osteoporosis, in postmenopausal women (16). The combination of calcium with vitamin D supplements, it is also known to reduce the risk of fracture, especially in populations with low dietary intakes (17, 18). According to the study of Albani & Petrou (19), the combination of calcium and vitamin D supplementation was associated with the prevention of osteoporotic fractures, specifically reduing total fractutes and hip fractures, by enhancing the dynamic process of bone formation (19).

Although there is no universal definition for the term "functional foods", foods are considered functional when in addition to their nutritional value, provide specific health promoting effects when are consumed as part of a typical diet. Funtional foods typically contain bioactive compounds such as vitamins, minerals, antioxidants, probiotics and/or other phytochemicals like carotenoidfs or polyphenols. Functional foods are developed with specific health promoting properties, as for example foods fortified with vitamins aiming to address nutrient deficiences or with the potential to reduce risk of certain diseases like cardiovascular (19, 20). In recent years, numerous epidemiological and clinical studies have consistently demonstrated that adopting of a diet rich in conventional functional foods, such as fruits, vegetables, raw cereals and fish can contribute to a significant reduction of chronic diseases, including cardiovascular diseases, cancer, obesity, osteoporosis and others (21, 22). Olive paste and olive oil, two well-known traditional Mediterrannean food products, are considered functional due to their potential health benefits and the presence of bioactive compounds, such as oleic acid and plyphenols (23). A range of health benefits are known to include cardioprotective effects, reduce inflammation and thus protecting against chronic diseases, supporting gut health, enhancing bone health and reduing the risk of osteoporosis (23). Mountain tea (*Sideritis sp.*), also known as Greek mountain tea, and orange juice are also important conventional functional foods with potential health benefits, beyond basic nutrition, as they both contain a number of bioactive compounds, including antioxidants like flavonoids, carotenoids and vitamin C which help to reduce oxidative stress and inflammation acting as free redical scavengers (24, 25). Mountain tea has been widely recognised for its high antioxidant potential mainly due to its natural content of antioxidants, as well as its ability to enhance the action of certain enzymes like catalase, particularly in the liver (26, 27). Tea and herbal infusions contribute to the major source of phenolic compounds in our diet (28).

The purpose of the present study was to investigate the effects of micronutrients supplementation including calcium, magnesium, vitamin C and vitamin D and a novel functional enriched food rich in polyphenols, on bone metabolism biomarkers as well as in BMD in postmenopausal women with an increased risk of osteoporosis. The role of vitamin C and polyphenols supplementation, on bone metabolism, has been little studied, while the novelty of the study lays in the use of the innovative functional food supplement. The main objective of the current study was to examine the changes that occured during the intervention program, aiming to maintain a health BMD and therefore preventing osteoporosis through diet supplementation and physical activity.

2. Materials and Methods

2.1. Recruitment process

The prospective randomized controlled trial was carried out at the Human Nutrition Unit (HNU), a reasearch facility based within the University of the Aegean, Department of Food Science aiming to evaluate the effects of multiple nutritional supplementation on bone heath, cardiometabolic and diabetes type 2 biochemical markers, in postmenopausal women, with high risk of osteoporosis,. Ethics approval was granted by the University of the Aegean Ethics Committee (No. 7505, 20 October 2019), provided that all procedures and processed were followed in accordance with the ethical standards of the Declaration of Helsinki. Phase I recruitment took place from March 2019 to June 2021 and women between 45 to 75 years of age, from Lemnos, Attiki area and Tripoli city in Pelloponnese area, were

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invited to participate in the study, via private clinics. A total number of subjects n=130 was recruited and all participants were thoroughly informed with printed forms about the study objectives, methods, anticipated benefits confidentiality of the data and the voluntary nature of participation. The subjects were assured that their contribution to the study would be completely anonymous and signed a consent form. Following the consent forms, medical history, recent biochemical blood tests (<15 days old) and data regarding their total bone density were recorded. Inclusion criteria were age 45 to 75 years old (y.o.) and screening for osteoporosis (t-score ≤ - 1 normal bone density, t-score ≤ 2.5 osteoporosis) and osteopenia of the femur strength or spine (t-score between -1 and -2.5). Subjects who were age > 75 y.o. and <45 y.o. and have been diagnosed with chronic diseases including cancer, diabetes, coronary heart disease and stroke were excluded from the study. Subjects who did not meet the aforementioned t-scores were also excluded from the study. All volunteers (n=130) were offerred with a free of charge service in order to be able to provide their biochemical tests and their t-scores as part of the research protocol. The HNU research group provided the participants with the contact details of certain diagnostic clinics and private doctors, they were collaborating with, depending the city and place of residence of the volunteers, so they were able to undertake their blood tets and DEXA scan.

2.2. Intervention study design

Eligible participants were 115 postmenopausal women, with an increased risk of osteoporosis (t-score -1.0 to -2.5) and osteopenia (t-score -1 and -2.5). The subjects were randomized into 4 groups: (I) nutritional intervention group (n=40) received daily vitamin C (1000 mg), vitamin D_3 (500 mg), calcium (Ca) (500 mg) and magnesium (Mg) (300 mg); (II) control group (n=42) received daily vitamin D_3 (500 mg), Ca (500 mg) and Mg (300 mg); (III) nutritional intervention group (n=18) received daily bisphosphonates (150 mg), vitamin D_3 (500 mg), Ca (500 mg) and Mg (300 mg); and (IV) nutritional intervention group (n=15) received daily 364 mg polyphenols via 50 g of an innovative functional food (Kalamata olive paste with mountain tea rich in polyphenols, (total phenolics 7,28±3.11 gallic acid/g) along with vitamin D_3 (500 mg), Ca (500 mg) and Mg (300 mg). Groups I -III received supplementation for a year (October 2020 to September 2021) whereas group IV received supplementation for 5 months (October 2020 to February 2021). The supplements and the functional food was receiving from the participants by post per 3 months.

2.2.1. Questionnaires

Nutritional assessment on function foods consumption was evaluated using a semi-quantitative food frequency questionnaire (FFQ) (29), modified to include additional natural functional foods, without alterations to the type of questions (30). Modified categories included dairy products (low-fat, enriched-fortified etc), meat (semi-dough etc), superfoods, sweet (low-fat, sugar free), fruit juices, fruit and vegetables, cereals (vegetable fiber etc.) beverages (stew, without alcohol, with sweeteners), oil (olive oil, dried fruits, fish oil). Participants were informed on the suggested portion sizes for each food included on the list and were asked to record their frequency of consumption. Frequency of consumption was recorded as “everyday”, “3–6 times per week”, ‘2 times per week”, “once a week”, “1–2 times per month” and” seldom/never”. Moreover participants were asked to complete a questionnaire assesing the adherence to Mediterranean-style diet (MedDiet Score questionnaire) (31-33). The final score provides three levels of compliance categorizing as: low 0-20, moderate 21-35 and high 36-55 adherence refering to the weekly consumption of 9 food groups (non-refined cereals, fruit, vegetables, legumes, potatoes, fish, meat and meat products, poultry, full fat dairy products). Higher values of this diet score indicate greater adherence to the Mediterranean diet.

A general self-reported sociodemographic questionnaire was used to obtain data on age, gender, occupation, years of education, income etc. Participants also self-reported their medical history answering a standard questionnaire on several diseases in the form of Yes-or-No questions. In addition, self-reported data were collected for their weight and heigh (31-34).

Physical activity levels were evaluated using a 3-day questionnaire via personal interview. Women were asked to report the time spent, alone or with a companion, in various physical activities

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on two weekdays and one weekend day. The questionnaire used classifies all activities during work, at the gym, and during leisure time into four (4) categories related to the average intensity of each activity and its effects on the cardiovascular and musculoskeletal systems (from low to high) (30). Questionnaires were designed to assess frequency of physical activity (months/year, weeks/month, days/week), duration (hours) and intensity (moderate to high). Based on these data, the total time spent in organized (including all activities performed regularly each week, usually in the gym under the guidance of a trainer) and unorganized activities that promote bone mass was calculated. The total weekly hours found to be spent in such activities were defined as moderate-intensity and intense physical activity. Physical activity was assessed only at the beginning of the study.

2.2.2. Anthropometric measurements

Body mass index (BMI) (kg/m^2) was calculated by dividing weight (kg) with standing height squared (m^2). Participants were classified as underweight, normal weight, overweight and obese according to the BMI criteria for adult population (WHO 2021) (35). More specifically participants with a BMI < 18.5 kg/m^2 were classified as underweight, normal weight with BMI between 18.5-24.9 18.5 kg/m^2, overweight ranged between 25.0-29.9 kg/m^2, and obese classified with BMI >30 kg/m^2. Body composition analysis was performed assessing body fat (kg), muscle mass (kg), body water (kg) and bone mass (kg) with a segmental body composition analyzer (TANITA SC300) according to the body composition procedures manual. Height was measured with a stadiometer (seca 222) and accuracy ±0.5 cm. Waist and hip ration were measured with a measuring tape (seca). Waist to hip ratio (WHR) was estimated by dividing the waist circumference to hip circumference (WHR = Waist Circumference/Hip Circumference). It is generally used as an indicator of body fat distribution and as a predictor of metabolic health risks including insulin resistance and impaired lipid profile (31). A WHR > 0.9 for men and > 0.85 for women indicates an increased risk for metabolic diseases including cardiovascular diseases, diabetes type 2 and other.

Bone density was assessed by Dual Energy X-ray Absorption (DEXA). DEXA scan included total bone density t-scores (t-score ≤ - 1 normal bone density, t-score ≤ 2.5 indicated osteoporosis) and bone density of the femur strength or spine (t-score between -1 and -2.5 indicated osteopenia). DEXA scans were performed at baseline and at the end of the study.

2.3. Biomarkers

The procedure for testing volunteers' blood for biomarkers was performed after a 12-hour overnight fast, and blood samples were collected early in the morning (8:30-10:00 AM). The volunteers visited the clinics after a scheduled appontment and performed the corresponding biochemical tests. The participants provided the test results, to the research team, no later than 15 days after the sheduled appointment. Serum levels of 25-OH vitamin D3, vitamin C, calcium (Ca), magnesium (Mg), parathyroid hormone (PTH), triglycerides, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, glucose, glycated hemoglobin HbA1c were then recorded.

2.4. Olive paste enriched with mountain tea

Homogenised olive pastes, enriched with mountain tea, were supplied by two accredited Greek olive oil and olive products companies, Arcadian Taste and GAEA. The fortified olive pastes, contained 7.5 g of extra virgin olive oil, 3.75 g of salt, 3.75 g of grated oregano, 3.75 g of grated pepper, 7.5 g of grated garlic, 6.25 g of orange juice, 65 g of Kalamon olives without seeds and 3 g of mountain tea and weighted 100 gr per portion size, as well. The nutritional composition (per 100 g), of the fortified homogenates of olive pastes mix, are described in the table below.

Table 1. Nutritional composition of fortified homogenates of olive paste mix, per portion size.

Nutritional Composition (per 100 g)	
Energy (kcal)	157

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Carbohydrates (g)	6,6
Fat, total (g)	12,4
Protein (g/kg)	2,7
Saturated fat (g)	1,1
Sugar , total (g)	2,7
Total phenolic ingredients (μg Gallic Acid)	728±311
Total Antioxidant activity (μmol FeSO4)	956±33

In vitro studies were performed at the HNU laboratory aiming to test the phenolic components and the antioxidant activity of the fortified olive pastes mix with Folin-Ciocalteau and FRAP (Ferric reducing/Antioxidant power assay) assays, according to relevant studies (36-45). The results showed that the novel olive paste with mountain tea had Total phenolic content 7,28±3,11 μg Gallic Acid/g and Total Antioxidant activity 9,56±0,33 μmol FeSO4/g.

2.5. Statistical Analysis

All data are presented as means±SD and as percentages (%) for differences observed at baseline and at the end of the study. The normal distribution of continuous variables was tested via Kolmogorov-Smirnov test. Changes in subjects` characteristics at baseline were estimated with one-wat ANOVA. Repeated measures ANOVA was used in order to define significant differences in all variables tested for each study groups, at baseline and at the end of the study. Repeated measures ANOVA was used to estimate significant differences amongst the four study groups, at baseline and at the end of the study. Statistical analysis was performed with the IBM-SPSS Statistics (version 21.0 IBM Corp, Armonk, NY). Level of statistical significance was at $P<0.05$.

3. Results

Descriptive data, at baseline, are shown in Table 1 for each study group. There were no differences that were statistical significant across Groups, indicating a homogeneity of the subjects within all groups at the beginning of the study.

Table 1. Descriptive characteristics per study group at baseline.

Characteristics	**Group I (n=40)**	**Group II (n=42)**	**Group III (n=18)**	**Group IV (n=15)**	***P*-value**
Age (years)	45 ± 8	56 ± 8	56 ± 8	45 ± 7	-
Height (cm)	154 ± 36	162 ± 9	159 ± 6.5	162 ± 8.4	-
Weight (kg)	74 ± 15.4	76 ± 17.4	73 ± 13	69 ± 10	-
BMI (kg/m^2)	28.34 ± 5.95	29.11 ± 6.45	28.79 ± 3.86	26.7 ± 0.82	-
BMI overweight category (kg/m^2)	28.34 ± 5.95	29.11 ± 6.45	28.79 ± 3.86	26.7 ± 0.82	-
Body fat (Kg)	36.23 ± 10.87	35.91 ± 8.2	39.17 ± 13.32	35.0 ± 10.74	-
Lean body mass (Kg)	-	-	-	-	-
Muscle mass (Kg)	44.88 ± 7.22	44.21 ± 8.05	43.16 ± 6.40	43.17 ± 7.01	-
Total body water (Kg)	47.01 ± 5.48	44 ± 6.13	44.86 ± 5	44.84 ± 5.20	-

All data are presented as means±SD. Level of significance determined at P<0.05.

Table 2 shows the differences observed in bone metabolism biomarkers tested, at the beginning and at the end of the study, including vitamin D (25(OH)D3) and parathyroid hormone (PTH). There were no differences that were statistical significant in either vitamin D (25(OH)D3) or PTH between the first and the second measurement.

Table 2. Changes in the levels of bone metabolism biomarkers.

	Beginning of study	End of study	% change	*P-value**
25(OH)D3 (ng/ml)				
Group I	27.42 ± 12.12	26.48 ± 7.96	- 3.43 %	-
Group II	23.15 ± 8.37	24.01 ± 8.68	3.71 %	-
Group III	28.21 ± 8.84	28.62 ± 7.78	1.45 %	-
Group IV	26.69 ± 6.83	28.19 ± 6.44	5.62 %	-
PTH (pg/ml)				
Group I	62.63 ± 27.00	76.44 ± 36.45	22.05 %	-
Group II	58.95 ± 23.96	56.71 ± 23.85	- 3.80 %	-
Group III	69.01 ± 17.82	52.21 ± 17.87	- 24.34 %	-
Group IV	48.84 ± 19.49	55.6 ± 19.63	13.84 %	-

Values presented at baseline and at the end of the study are presented as means±SD. Level of significance for differences observed within the same group, between the two measurements.

Statistical significant differences were recorded, between the two measurements, for total cholesterol, trglycerides and HDL (Table 3). More specifically, a significant increase of serum cholesterol was noted for group III, in addition to a significant decrease observed in total cholesterol (-2.07%, P=0.034) for group IV. Elevated triglyceride levels, that were statistical significant, were also recorded between the two measurements, for both groups II and IV at +17.02%, (P= 0.034) and +16.32% (P=0.025) respectively. Moreover, an inctrease HDL that was statistical significant was recorded for group I (+61.62%, P=0.047). This generated an additional significant difference in HDL, across groups, at the end of the study period (P=0.032) (not shown in Table 3). There were no other differences that were statistical significant.

Table 3. Changes in blood lipid profile.

	Beginning of study	End of study	% change	*P-value**
Total cholesterol (mg/dl)				
Group I	210.82 ± 30.17	207 ± 29.32	- 1.81 %	-
Group II	200.1 ± 33.14	197.85 ± 36.96	- 1.12 %	-
Group III	192.89 ± 29.46	197.17 ± 13.72	2.22 %	0.034
Group IV	185.14 ± 34.17	181.31 ± 32.21	- 2.07 %	0.034
P-value†	-	-	-	-
Triglycerides (mg/dl)				
Group I	105.54 ± 52.71	106 ± 9.89	0.44 %	-
Group II	115.37 ± 63.89	135 ± 73.25	17.02 %	0.034
Group III	198.29 ± 10.24	160.8 ± 93.28	- 18,91 %	-
Group IV	107.56 ± 35.48	125.11 ± 35.48	16.32 %	0.025
P-value†	**0.008**	-	-	-
LDL (mg/dl)				
Group I	125.5 ± 28.12	125.71 ± 39.51	0.17 %	-
Group II	111.27 ± 22.12	107.93 ± 24.32	- 3.002 %	-
Group III	107.14 ± 25.12	110.8 ± 16.04	3.42 %	-
Group IV	108.96 ± 19.63	104.13 ± 18.65	- 4.43 %	-
P-value†	**0.04**	-	-	-
HDL (mg/dl)				
Group I	56.57 ± 10.46	91.43 ± 69.69	61.62 %	0.047
Group II	62.01 ± 13.12	61.81 ± 15.16	- 0.32 %	-
Group III	56.29 ± 13.48	58.6 ± 10.33	4.10 %	-
Group IV	52.19 ± 10.74	51.75 ± 12.39	- 0.84 %	-

Values presented at baseline and at the end of the study are presented as means±SD.*Level of significance for differences observed within the same group, between the two measurements.

Additional blood biomarkers were tested and differences that were statistically significant were noted for glucose, glycosylated hemoglobin (HbA1c), vitamin C, calcium (Ca) and magnesium (Mg) for group IV, only. (Table 4). More specifically significantly increased values were recorded for glucose (+2.33%, P=0.048) and HbA1c (+1.56%, P=0.0027), whereas decreased values with statistical significance were obserevd for both Ca (-0.42%, P=0.041) and Mg (-20.19%, P=0.01).

Table 4. Changes in additional blood biomarkers of importnace.

	Beginning of study	End of study	% change	*P-value**
Glucose (mg/dl)				
Group I	91.82 ± 8.93	93.33 ± 4.62	1.64 %	-
Group II	96.15 ± 15.27	99.56 ± 17.68	3.55 %	-
Group III	108 ± 16.97	109.5 ± 14.85	1.40 %	-
Group IV	93.81 ± 8.98	96 ± 11.74	2.33 %	0.048
P-value†	0.136	0.29	2.23 %	-
HbA1c (%)				
Group I	5.64 ± 0.39	5.77 ± 0.42	2.30 %	-
Group II	5.74 ± 0.53	5.72 ± 0.55	- 0.35 %	-
Group III	5.71 ± 0.67	5.76 ± 0.8	0.88 %	-
Group IV	5.76 ± 0.81	5.85 ± 0.83	1.56 %	0.027
Vitamin C (μmol/l)				
Group I	61 ± 19.78	60 ± 18.8	- 1.64 %	-
Group II	-	-	-	-
Group III	-	-	-	-
Group IV	-	-	-	-
Ca (mg/dl)				
Group I	9.37 ± 0.42	9.63 ± 0.29	2.77 %	-
Group II	9.5 ± 0.38	9.49 ± 0.37	- 0.11 %	-
Group III	9.34 ± 0.36	9.54 ± 0.36	2.14 %	-
Group IV	9.44 ± 0.5	9.4 ± 0.26	- 0.42 %	0.041
Mg (mmol/l)				
Group I	2.07 ± 0.17	2 ± 0	- 3.38 %	-
Group II	2.1 ± 0.25	2.09 ± 0.27	- 0.48 %	-
Group III	1.98 ± 0.01	2 ± 0.01	1.01 %	-
Group IV	2.13 ± 0	1.7 ± 0.52	- 20.19 %	0.01

Values presented at baseline and at the end of the study are presented as means±SD. *Level of significance for differences observed within the same group, between the two measurements.

Differences that were statistically significant were recorded for total bone density, at the end of the intervention period for all four groups (Table 5). More specifically the highest increase in total bone density (+34.06%, P=0.027) was observed for group I, whereas the lowest increase was noted for group II (+1.55%, P=0.036%). In addition for groups III and IV a similar increase in total bone density was recorded at +12.23% (P=00.43) and +11.98% (P=0.003) respectively.

Table 5. Changes in bone density.

	Beginning of study	End of study	% change	*P-value**
Total bone density (g/cm²)				
Group I	1.38 ± 0.49	1.85 ± 0.5	34.06 %	0.027
Group II	1.29 ± 0.45	1.31 ± 0.47	1.55 %	0.036
Group III	1.39 ± 0.5	1.56 ± 0.51	12.23 %	0.043
Group IV	1.67 ± 0.48	1.87 ± 0.35	11.98 %	0.003
P-value	0.298	0.187	14.96 %	0.027

Values presented at baseline and at the end of the study are presented as means±SD. *Level of significance for differences observed within the same group, between the two measurements.

A positive association was observed between intense physical activity and bone density for groups I, II and III at the end of the intervention period. The mean total bone density was slightly higher for group II (2.83 ± 0.38) compared to groups I (2.65 ± 0.48) and and III (2.61 ± 0.50).

The use of questionaries about functional food consumption and Mediterranean diet adherence did not show differences between the baseline and the end of the study in all intervention groups (data not shown).

4. Discussion

This study was designed to examine the effects of micronutrient supplementation in biomarkers of bone formation as well as in bone mineral density, in postmenopausal women, with high of osteoporosis, after one year (groups I, II and III) and six months (group IV) intervention. According to the findings of the present study, 25(OH)D3 levels were improved, in groups II, III and IV (+3,71% and +1.45% and +5.62% respectively), as shown in Table 2. It was also observed that PTH levels were increased at the end of the intervention period, for groups I and IV, regardless the improved levels of serum 25(OH)D3 recorded for the same groups (Table 2).

PTH increase was higher in group I (+22.5%) compared with group IV (+13.84%). Similar data have been observed and presented by other research groups but the exact mechanism which regulates PTH levels has not fully explained (46). On the contrary PTH levels were decreased in groups II and III (-3.80% and 24.34%, respectively) at the end of the intervals, showing an inverse association between PTH and serum 25(OH)D3 levels, as it has been also similarly observed, by previous researchers (22). The inverse association between decreased serum 25(OH)D3 levels and PTH is widely known as this relationship plays an important role in calcium homeostasis and bone health. Declined serum 25(OH)D3 lead to impaired calcium absorption and concequently low calcium levels in the blood trigger the release of PTH. However, the exact threshold at which serum 25(OH)D3 is evident to affect PTH levels to start rising still remains controversial eventhough some studies have provided evidence of this inverse association reporting that when 25(OH)D levels are between 20 and 30 ng/mL PTH levels progressively increase (23). In addition, as the exact dose of calcium supplementation necessary, to inhibit PTH secretion, has yet to be defined, as well (24) one could assume that the elevated PTH levels observed for groups I and IV, in the present study, were possibly due to insufficient supplementation either because of low adherence and/or becasuse of insufficient dose. It has also been suggested that the decreased PTH levels may be a result of increased serum 25(OH)D3 levels especially when combined with high calcium intake (>800 mg) (24). This could further explain the decreased PTH levels recorded in group IIs and III were improved elevated levels of serum 25(OH)D3 were observed (Table 2). Nevertheless, additional investigation is considered necessary to examine and elucidate the metabolic response of PTH in postmenopausal women during calcium and vitamin D supplementation. Overall, changes in 25(OH)D3 were obsreved to be beneficial for groups II, III and IV as serum levels were increased at the end of the intervention period (Table 2). Only for group I a decrease in 25(OH)D3 was recorded. Given that no significant changes observed, in none of the study groups, one could assume that the amount of 25(OH)D3 administered to the subjects was not sufficient enough to elevate serum 25(OH)D3 levels, and/or the duration of the study was short, especially for group IV (5 months intervention). In addition for group IV low serum 25(OH)D3 levels may have been recorded, as a result of seasonality effect, as the end of the intervention run from October (2020) to February (2021). This finding has been confirmed by other research groups as well, who have presented a decrease in serum 25(OH)D3 levels during the winter months (25) as it seems that the effect of seasonality on serum 25(OH)D3 levels excels vitamin D supplementation. Suprisingly research data have demonstrated that vitamin D deficiency is more common in elderly population living in Mediterranean countries (26), including Greece, Italy and Spain, compared with northern European countries such as with less sunlight exposure. The high consumption of fish, the fortification of widely consumed foods and the higher use of vitamin D supplements in these countries could explain the lower prevalence rates in vitamin D deficiency compared with southern European countries.

It is widely accepted that the effectiveness of intervention programmes which entail the administration of calcium and/or vitamin D supplementation investigating bone metabolism, can be evaluated much better considering the resulting changes in bone mineral density (total and/or site-specific) rather than the biomarkers of bone metabolisn (15). According to DEXA measurements taken, during the present study, it was observed that significant positive changes recorded, in total BMD, in all four study groups (Table 5). The highest significant increase was noted for group I (+34.06, P=0.027), followed by group III, Group IV and group II, in descending order. In accordance with the results presented above similar findings have been obsereved by other researchers, reporting an increase in total BMD in postmenopausal Caucasian women after calcium supplementation (1600 mg/d) for a year (16). Increased BMD was also observed to be positively associated with intense exercise (Table 6, P<0.05) for groups I, II and III as it has been similarly observed by other studies in postmenopausal women, as well (17). Moreover, experimental pre-clinical data have shown similar findings when using a polyphenol-rich olive extract, maintaining BMD levels in postmenopausal women with high risk in osteoporosis (47). In addition, the highest increase in BMD, recorded for group I, may be possibly associtaed with the vitamin C supplemetation, as it is known that vitamin C plays an important role in bone health as foods rich in vitamin C, including fruits and vegetables have been positively associated with bone health by other research groups. More specifically, it has been shown that intakes of dietary vitamin C were associated with higher bone mineral density (BMD) in postmenopausal women (20) as vitamin C it is known to affect bone turnover by enhancing collagen synthesis and osteoblast genesis (27). Moreover, previous studies have shown an inverse relationship between vitamin C intake and the risk of fracture or osteoporosis (46,15).

Table 6. Correlation of physical activity with total bone density.

Physical activity levels	Total bone density (rho)	*P*-value
moderate		
Group I	-	-
Group II	-	-
Group III	-	-
Group IV	3.20 ± 0.28	-
P-value	-	-
intense		
Group I	2.65 ± 0.48	0.032
Group II	2.83 ± 0.38	0.032
Group III	2.61 ± 0.50	0.032
Group IV	-	-
P-value	-	-

*Correlation is significant at the 0.05 level.

Normal ranges for TG levels were observed for groups I, II and IV (<150 mg/dl) and borderline high levels for group III (150 – 199 mg/dl) at baseline and at the end of the sudy. Normal levels were also recorded for total cholesterol (<200 mg/dl) in groups II, III, IV and borderline levels in group I (200-239 mg/dl), while normal ranges were recorded for LDL (100-129 mg/dl) and HDL (>60mg/dl) in all four study groups both at base line and at the end of the study, as well. The results presented above are in agreement with previous studies, investigating the calcium and vitamin D co-supplementation, showing no associations with serum LDL levels (28). In addition, significant beneficial changes for total cholesterol was observed in group IV (-2.07%, P<0.05) and positive changes in group I for HDL biomarker (+61.62%, P<0.05) similarly with other reserachers showing a positive effect of vitamin D and calcium supplementation on total cholesterol and HDL. However the lack of consistency in the above results presenetd for all four study groups highlights the need of larger-scale well designed intervention trials to clarify the effects of micronutrient supplementation on lipid profile markers. Yet, the significant reduction of total cholesterol in group IV obsreved along with the statistical increase in BMD recorded, highlights a positive development of the current intervention study as similar findings have emphasised a negative association between total

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cholesterol and BMD in previous studies (18). Similarly to the present study, physiological ranges in serum lipid profile were recorded for total cholesterol, TG, LDL and HLD-cholesterol by Filip et. al., after administering a combination of polyphenol rich olive extract (250 mg/d) and calcium (1000 mg/d) (47). Both the present study and Filip et al. published data, demonstrate a novel positive influence on blood lipids profile suggesting additional health benefits associated with olive polyphenols intake. Nevertheless no firm conclusions can be drown from the present study with regards to the effect of micronutrient supplementation on lipid profile biomarkers in postmenopausal women, and therefore further investigation is warranted.

Serum calcium levels detected within normal ranges for all four study groups at the end of the intervention period. In addition, improved calcium levels were observed for groups I and III, possibly, as a result of calcium supplementation. No other positive changes observed for either serum caclium or magnesium levels, possibly attributed to low adherence of supplementation, during the intervention period and/or short-term follow-up. It is widely accepted that optimal dietary calcium intake (approx. 1000-12000 mg/d) is essential for bone health in adults and older adults for the prevention of bone loss and osteoporosis (48). Similarly magnesium has gained a lot of interest in recent years and studies have demonstrated a positive correlation between magnesium and BMD (19). According to the Institute of Medicine of the National Academy of Sciences (IOM), the recommended daily allowance for calcium is 1000 mg for adults (men and women) and 1200 mg for people over 65 years of age, adolescents and those with osteoporosis. Optimal magnesium intake with food is 320 mg/d foor women and 420 mg/d for men but higher requirements may be needed in physical conditions such as ageing (49).

Given the complexity of the factors involved in the development of osteoporosis, preventive strategies with the aim to reduce the risk of developing the disease must be defined and implemented. Certainly, there is an overwhelming body of evidence that emphasizes the important role of calcium as a building block of bone. However, because osteoporosis is a multifactorial disease and calcium supplements may not necessarily compensate for bone loss, a recent interest in natural components such as polyphenols, has grown significantly. In the Filip et. al. study the potential effect of an olive extract rich in polyphenols on bone metabolism biomarkers was investigated, following a 12 month administration in postmenopausal women with osteopenia. The polyphenol-rich olive extract provided a dose of 100 mg of eleuropein per day. To assess whether this dose can be considered nutritious, i.e. whether it is possible to consume such amounts through diet, the research group relied on the fact that some olive oils are particularly rich in polyphenols containing 238 mg/kg up to 1 g/kg. In addition, to avoid possible confounding effects related to calcium deficiency, a dose of 1 g of calcium was administered daily to the enrolled subjects, in order to ensure optimal daily calcium intake. When evaluating the different biomarkers between the treatment group and the control group, a significant increase in osteocalcin was observed throughout the study in the treatment group, and the final levels of osteocalcin were significantly higher in this group compared to the placebo group, despite the fact that the original intended sample size of 32 subjects per group was not achieved due to dropouts (47).

Polyphenols derived from olives, are usually studied for their anti-inflammatory and free radical scavenging properties whilst these natural compounds have beneficial effects in preventing the development of chronic disease caused by oxidative stress, including osteoporosis. Oxidative stress and inflammation can damage bones and lead to diseases such as osteoporosis, which increases the risk of fractures. Polyphenols promote bone health by reducing oxidative stress and inflammation, while supporting bone density through the growth of new bone cells (50, 51). Polyphenols have shown promising results in inhibiting osteoclast activity and suppressing bone resorption. The mechanisms by which these natural compounds exhibit such positive health effects has not been fully been elucidated however research studies have shown that inhibition of pro-inflammatory cytokines involved in enhancing osteoclast activation and bone resorption possesing anti-inflammatory properties (50, 51). Understanding and controlling osteoclast activation is essential for managing various bone-related conditions and diseases, for the development of therapies in conditions like osteoporosis. Similarly to the present study, researchers warrant additional research and continue to investigate ways to modulate osteoclast activation, promoting bone health and treat bone disorders.

During the last decade, functional foods have been attracted much attention and have been broadly studied, using *in vitro* models, aiming to determine their total antioxidant capacity (TAC) as well as their total phenolic content (TPC) and content in additional bioactive extracts. In the present study, the total antioxidant and phenolic content of olive paste enriched with mountain tea, was evaluated. Other research studies examining the antioxidant capacity and the phenolic content in similar products have detected, a wide range of values.These differences may well be attributed to the different sample preparation and extraction methods used, to the different variety of the analysed sample used (52), as well as to the different climatic and soil conditionsof the samples of origin (REF 53). Similar values for the TPC (6.4–180.5 mg GA/g) were determined for Thai plants (extracts with 95% ethanol), traditional Chinese medicinal plants (1.1–52.3 mg GA/g in extracts with 80% methanol), culinary herbs and spices from Finland (18.5–147.0 mg GA/g in water extracts). Correlations ranging between 0.87 and 0.98 were reported for the TAC FRAP and TPC values in extracts from medicinal plants. These results show that medicinal herbs with high TAC are characterized by high levels of TPC, while it was obsereved that changes in the extraction solvents (methanolic and water extracts) had no significant impact on the TPC. It was therefore assumed, that mountain tea can be used as a potential source of antioxidants, either by consuming it on its own or in the form of fortified food enriched with the extract, presenting the same benefits to humans` health as well as considering reducing the risk of osteoporosis (54).

Previous researchers, have used similar functional foods as part of the Mediterranean diet, and have detected a possitive association with BMI as well as with reducing the risk of osteoporosis (54). Similarly, our findings have shown a possitive association between BMD and the consumption of the polyphenol-rich olive sample as in group IV (+11.98%, P=0.027), and a healthy weight maintaing a BMI within physiological ranges (18.5 – 24.9 Kg/m^2), at the end of the intervention period. These results demonstrate a positive scientific advancement of the current study and the use of the innovative functional food when administering in postmenopausal women, with high risk in osteoporosis. Nevertheless, consumers` awareness of how foods can contribute to their health and wellbeing has been shown to be low (55). It is therefore considered essential for the consumers to be properly informed about the health benefits of certain foods as this may help them realise the health promotig effects of such foods, when consumed on a regular basis, as part of their normal diet. Innovative research studies like the present one, highlight the importance of developing novel functional foods with potential health promoting effects, providing benefits beyond basic nutrition. There is an increasing demand in functional foods globally as consumers` needs have shown sustainable food trends towards traditional, more nutritious food products (21), similar to the novel funtional food used in the curret study. Reed olives and olive oil could also help address environmental problems, reusing industrial waste pollutants in innovative food products. In addition, the development of processed new bio-functional foods with nutritional claims and health-promoting properites can help create more healthier options for consumers. Investment in research and development can lead to the discovery of new traditional functional foods, contributing to innovative food formulations and/or novel applications in food processing. The use of traditional natural functional foods, such as olives, olive oil and herbs is a promising concept for the food industry which can help develop alternative new products that cater to evolving consumer preferences and contribute to healthier, more sustainable food options (41).

Limitations of the study included fairly small sample of volunteers even though the participants were equally screened from both Limnos and Athens. In addition, a number of volunteers did not agree to provide to the research team their health record and blood tests. A wider screening of volunteers from additional regions within the country, such as urban, rural and island, would be desirable, so that more reliable conclusions could be drawn for the Greek population. Moreover, the larger sample size and bigger intervention period would increase precision, would be more likely to detect true effect or differences and reduce sampling bias, epxecvially in group Iv wheer the intervention period was limited to five (5) months interval, due to difficulties on the total amount of

 doi:10.20944/preprints202310.2094.v1

food produvtion. Finally, due to the pandemic, significant challenges were were posed such limited face to face meetings with the volunteers, while trying to maintain an effective communication and engagement during the intervention period.

5. Conclusions

The positive significant changes observed in BMD could be attributed to the micronutrient and innovative functional food supplementation. Intense physical activity is positively correlated with significant increase in BMD. Supplentation even after 5 months intervention was observed to increase BMD and maintain higher physiological levels of serum calcium, 25(OH)D3, vitamin C and magnesium, in postmenopausal women with high risk in osteoporosis. Additional larger-scale clinical trials and intervention studies are considered essential, in order to fully investigate and elucidate associations between dietary components and biochemical indices of bone metabolism. A future forecast is the cooperation with the food companies producing olive oil and olive products, such as olive paste for the implementation and promotion of novel food products in the market.

Supplementary Materials: No Supplementary Materials.

Author Contributions: Conceptualization, Melina Konstantinidi and Antonios Koutelidakis; Data curation, Melina Konstantinidi and Stavroula Stoupi; Formal analysis, Stavroula Stoupi; Funding acquisition, Antonios Koutelidakis; Investigation, Melina Konstantinidi, Stavroula Stoupi, Ioannis Anastasiou, Vlachos Nikolaos and George Tsakotos; Methodology, Melina Konstantinidi and Antonios Koutelidakis; Project administration, Antonios Koutelidakis; Resources, Melina Konstantinidi, Ioannis Anastasiou, Vlachos Nikolaos and George Tsakotos; Software, Melina Konstantinidi; Supervision, Antonios Koutelidakis; Validation, Stavroula Stoupi, Ioannis Anastasiou, Vlachos Nikolaos and George Tsakotos; Visualization, Stavroula Stoupi, Ioannis Anastasiou, Vlachos Nikolaos, George Tsakotos and Antonios Koutelidakis; Writing – original draft, Melina Konstantinidi and Stavroula Stoupi; Writing – review & editing, Antonios Koutelidakis. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially funded by AGRICA II, network of excellence in agri-food research and innovation of the northern Aegean, project 80462, ESPA 2020.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics and deontology committee of the University of the Aegean (protocol no. No. 7505, 20 October 2019).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

Data Availability Statement: New data is not available.

Acknowledgments: Authors would like to thank every individual, postmenopausal women, who participated to the study for her cooperation. The authors also thank a lot the companies Arcadian Taste and GEAE for the production of the innovative food.

Conflicts of Interest: The authors declare no conflict of interest.

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