

A Mediterranean-like dietary pattern with vitamin D₃ (10 µg/day) supplements reduced rate of bone loss in older Europeans with osteoporosis at baseline: results of a one year randomised controlled trial

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Abbreviations list: NU-AGE, new dietary strategies addressing the specific needs of the
elderly population for healthy ageing in Europe; MD, Mediterranean diet; BMD, bone
mineral density; BMI, body mass index; DXA, dual energy X-ray absorptiometry; fPYD, free
pyridinoline; fDPD, free deoxypyridinoline; LC-MS/MS, liquid chromatography-mass
spectrometry; 25(OH)D, 25-hydroxyvitamin D; PTH, parathyroid hormone.

Clinical Trial Registry number and website: The NU-AGE trial was registered at
clinicaltrials.gov as NCT01754012.

1 **Abstract**

2 **BACKGROUND:** The Mediterranean diet (MD) is widely recommended for the prevention of
3 chronic disease, but evidence for a beneficial effect on bone health is lacking.

4 **OBJECTIVE:** To examine the effect of a Mediterranean-like dietary pattern (NU-AGE diet) on
5 indices of inflammation with a number of secondary endpoints, including BMD and
6 biomarkers of bone and collagen degradation in a 1-y multi-center randomised controlled
7 trial (RCT) (NU-AGE) in elderly Europeans.

8 **DESIGN:** A RCT was undertaken across 5 European centers. Subjects in the intervention
9 group consumed the NU-AGE diet for 1-y by receiving individually tailored dietary advice,
10 coupled with supplies of foods such as wholegrain pasta, olive oil and a vitamin D₃
11 supplement (10 µg/day). Participants in the control group were provided with leaflets on
12 healthy eating available in their country.

13 **RESULTS:** 1294 participants (mean age 70.9 ± 4.0 y, 44% male) were recruited to the study
14 and 1142 completed the 1-y trial. The Mediterranean-like dietary pattern had no effect on
15 BMD (site specific or whole body); including compliance to the intervention in the statistical
16 model did not change the findings. There was also no effect of the intervention on the
17 urinary biomarkers, free pyridinoline or free deoxypyridinoline. Serum 25(OH)D significantly
18 increased and PTH decreased (p<0.001) in the MD compared with the control group. Sub-
19 group analysis of individuals with osteoporosis at baseline (site specific BMD T-score ≤ -2.5
20 SD) showed that the MD attenuated the expected decline in femoral neck BMD (n=24 MD
21 group, n=30 control group, p = 0.04) but had no effect on lumbar spine or whole body BMD.

22 **CONCLUSIONS:** A 1-y intervention of the Mediterranean-like diet together with vitamin D₃
23 supplements (10 µg/day) reduced the rate of loss of bone at the femoral neck in individuals
24 with osteoporosis but had no effect on those with BMD in the normal range.

25 The NU-AGE trial is registered at clinicaltrials.gov as [NCT01754012](https://clinicaltrials.gov/ct2/show/study/NCT01754012).

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42 INTRODUCTION

43 A Mediterranean dietary pattern (MD), widely recommended for the prevention of chronic
44 disease, is characterized by a high intake of fruits, vegetables, nuts, unrefined cereals and
45 olive oil, a moderately high intake of fish, a low-to-moderate intake of dairy products, a low
46 intake of meat, and a moderate intake of alcohol (1, 2). Data from prospective cohort
47 studies show that greater adherence to a MD is associated with a significant improvement
48 in health status, including reduced total mortality (2) and reduced incidence of
49 cardiovascular disease, cancer, Parkinson's and Alzheimer's disease (3). Randomised
50 controlled trials confirm that the MD may protect against vascular disease, although the
51 quantity and quality of evidence available is limited and highly variable (4).

52

53 There are relatively few studies examining the association between a MD and bone health
54 (bone mineral density and/or fracture incidence) and the available data are conflicting (5). A
55 review of population-based studies, which focussed on fracture as an outcome (6),
56 suggested that one of the modifiable risk factors for bone health is adherence to a MD. This
57 conclusion was based on post-hoc analysis of longitudinal data from 93,676 women aged
58 50-79 y at the start of the Women's Health Initiative study which reported that higher
59 adherence to a MD was associated with a lower risk for hip fractures (7). Data from
60 randomized controlled trials investigating the effect of the MD on measures of bone health
61 are sparse due to the difficulties of undertaking a dietary intervention that is long enough
62 (i.e. minimum one year's duration) to be able to detect changes in BMD.

63 The aim of the present study was to examine the effect of the MD on BMD and biomarkers
64 of bone and collagen degradation, as pre-specified secondary outcome measures, in a 1-y

65 multi-center randomized controlled trial (NU-AGE) in elderly Europeans. The trial was
66 designed to assess the effects of consuming a Mediterranean-like dietary pattern for 1-y on
67 markers of inflammation as the primary outcome and a series of secondary health-related
68 outcomes, which include BMD and biomarkers of bone and collagen degradation. The
69 Mediterranean-like dietary pattern was tailored individually to complement habitual dietary
70 patterns to maximise compliance.

71

72 **STUDY DESIGN AND METHODS**

73 The NU-AGE trial was conducted in five European centres (Bologna in Italy, Norwich in the
74 United Kingdom, Wageningen in the Netherlands, Warsaw in Poland and Clermont Ferrand
75 in France). A detailed description of the European Commission-funded NU-AGE project has
76 been reported elsewhere (8).

77

78 **Ethics approval**

79 Local ethical approval was provided by the Independent Ethics Committee of the
80 Sant'Orsola-Malpighi Hospital Bologna (Italy), the National Research Ethics Committee –
81 East of England (UK), the Wageningen University Medical Ethics Committee (Netherlands),
82 the Bioethics Committee of the Polish National Food and Nutrition Institute (Poland) and
83 South-East 6 Person Protection Committee (France). All study procedures were in
84 accordance with the ethical standards of the Helsinki Declaration. All participants gave
85 informed consent before participating. The trial was registered at clinicaltrials.gov
86 (NCT01754012).

87

88 Participants

89 Recruitment and selection criteria have been reported previously (9). Briefly, 1294
90 participants aged 65–79 y were recruited through local advertisements, media publicity, and
91 general practitioner surgeries between April 2012 and January 2014 at the five recruitment
92 centres. Study participants were free living and responsible for their own dietary choices.
93 Ineligibility criteria included any clinically diagnosed chronic disease, use of corticosteroids
94 or insulin medications, recent use of antibiotics or vaccinations, recent change in habitual
95 medication, presence of food allergy or intolerance necessitating a special diet, presence of
96 frailty according to the Fried criteria (10) or malnutrition (defined as BMI < 18.5 kg/m² or
97 >10% weight loss in the previous six months). Participants were randomly allocated to the
98 intervention or control group (1:1 allocation ratio) after stratification by gender, age, frailty
99 status (pre-frail or non-frail) and BMI. Randomization was performed by entering the
100 described variables of a subject into a computer program that automatically allocates and
101 generates a unique ID-code. Participants were informed about their group after
102 randomization. Technicians performing laboratory analysis were blinded to the group
103 assignment, but researchers carrying out BMD measurements were not blinded because of
104 practical impossibilities, including the fact that the participants themselves knew which
105 group they were in and were in a position to discuss this with researchers whilst undergoing
106 measurements.

107

108 Dietary intervention

109 Participants randomised to the intervention group received individually tailored
110 standardised dietary advice in order to meet the study dietary requirements, as described
111 previously (9). The NU-AGE food based dietary guidelines were based on nutrient reference
112 values and food-based dietary recommendations for older adults from each of the five
113 countries where the intervention took place, the modified MyPyramid for Older Adults, and
114 nutrient requirements from the European Commission and the Institute of Medicine (9).
115 The individually tailored dietary advice, either given face-to-face or by telephone by a
116 trained dietician or research nutritionist, was administered nine times during the year and
117 supported by mail or e-mail. To aid compliance participants in the intervention group
118 received commercially available foods to help them meet the dietary guidelines including
119 wholegrain pasta, olive oil, low-fat low-salt cheese, and high-MUFA and high-PUFA
120 margarine in all centres and frozen vegetable soup (in Italy only) and vitamin D₃
121 supplements. Participants completed 3-day food diaries and returned unused vitamin D₃
122 supplements at months four and eight to evaluate follow-up adherence and use of the
123 provided foods. Participants randomised to the control group were asked to continue with
124 their usual diet for the year and only received a generally available leaflet with national
125 dietary guidance.

126

127 Compliance to the study protocol in both the intervention and control groups was evaluated
128 with seven-day food diaries at the start and end of the one-year intervention. A scoring
129 system was developed to measure adherence to the diet; sixteen dietary components were
130 included, 12 for which the highest intakes were ideal (fruits, vegetables, legumes, low-fat
131 dairy and cheese, fish, lean meat and poultry, nuts, eggs, olive oil, fluids and vitamin D

132 supplements), two for which moderate intake was ideal (wholegrains and alcohol) and two
133 for which low intakes were ideal (salt and sweets). Each component was scored
134 proportionally from zero to 10 and contributed equally to the final score, which ranged from
135 0 to 160, with a higher score representing better adherence to the diet. High compliers
136 were defined as participants whose change in the NU-AGE Index was ranked in the top two
137 quintiles and low compliers were those in the lowest two quintiles.

138

139 **Outcome assessment**

140 At baseline and after 1-y, trained nurses or researchers measured whole body BMD with the
141 use of DXA according to standard protocols and training (Hologic Discovery Wi , Hologic,
142 Bedford, MA (UK); Lunar iDXA, GE Health Care Madison, WI, USA, enCORE™ 2011 software
143 version 13.6 (Bologna, Italy); Discovery QDR®, Hologic Inc., USA, software version 3,
144 (Clermont-Ferrand, France); Lunar Prodigy, GE Health Care, Madison, WI, USA, enCORE™
145 2011 software version 13.6 (Wageningen, the Netherlands and Warsaw, Poland.
146 Additionally, at three of the intervention sites (Italy, UK and Poland) BMD was assessed at
147 predefined anatomical regions, including the lumbar spine (L1 to L4) and proximal femur
148 (including total hip and femoral neck BMD). Osteoporosis was defined as a T-score of ≤ -2.5
149 SD below peak bone mass (11).

150

151 **Measurements of urine fPYD and fDPD**

152 Free pyridinium crosslinks in urine were measured by liquid chromatography tandem mass
153 spectrometry (LC-MS/MS), as described elsewhere (12). In brief, the LC-MS/MS method

154 quantified free pyridinoline (fPYD) and free deoxypyridinoline (fDPD) simultaneously from a
155 single sample analysis. fPYD and fDPD were calibrated using commercial standards
156 (Immundiagnostik, Bensheim, Germany), and acetylated pyridinoline as internal standard.
157 Prior to LC-MS/MS analysis, a solid phase extraction procedure was carried out on urine
158 samples pre-treated with hydrochloric acid. The acidified samples were extracted using
159 cellulose packed columns and eluted with 0.2% heptafluoro-butyric acid (HFBA) in water.
160 The inter- and intra-assay coefficient of variation (CV) were $\leq 9.9\%$ between the assay
161 working range of 2-200 nmol/L. fPYD and fDPD results obtained from LC-MS/MS analysis
162 were adjusted against urine creatinine measurements, which was performed on the COBAS®
163 C501 analyser (Roche, Burgess Hill, UK). The inter- and intra-assay CV was $\leq 3.1\%$ across the
164 assay working range (375-55000 $\mu\text{mol/L}$).

165

166 **Measurement of serum 25-dihydroxyvitamin D and parathyroid hormone**

167 Concentrations of total 25-hydroxyvitamin D (25(OH)D) [i.e. 25(OH)D₂ plus 25(OH)D₃] in all
168 serum samples were measured at the laboratory of the Cork Centre for Vitamin D and
169 Nutrition Research using a slightly modified version of the LC-MS/MS method that has been
170 described in detail elsewhere (13) and is certified by the Centers for Disease Control and
171 Prevention's (CDC) Vitamin D Standardization Certification Program (14). The modifications
172 were effected so as to reduce the total run time per sample from 10 mins in our existing
173 method to 7 mins in the current method thereby increasing our efficiency of analysis of the
174 sample loads (see **Supplemental Table 1** for details of gradient and multiple reaction
175 monitoring (MRM) parameters). The 3-epimer of 25-hydroxyvitamin D₃ was
176 chromatographically resolved from 25(OH)D₃, and the isotopically labelled *d*₃-3-epi-

177 25(OH)D₃ was used as an internal standard to verify retention time and separation of 3-epi-
178 25(OH)D₃ and 25(OH)D₃ in each sample run. The mean intra-and inter-assay CVs of the
179 methods were 3.9% and 6.5%, respectively, for 25(OH)D₃ (using low, medium and high
180 concentrations of 33.5, 49.2 and 86.2 nmol/L, respectively). The mean intra-assay and inter-
181 assay CVs of the method were 12% and 7.1%, respectively, for 25(OH)D₂ (using low, medium
182 and high concentrations of 1.10, 6.57 and 13.9 nmol/L, respectively).

183

184 Serum parathyroid hormone (PTH) concentrations were measured at the Cork Centre for
185 Vitamin D and Nutrition Research in all serum samples with the use of an ELISA (intact PTH;
186 MD Biosciences Inc.) Intra-assay and inter- assay CVs were 3.0% and 5.1%, respectively (at a
187 concentration of 47.7 and 52.6 pg/ml, respectively).

188

189 **Statistical analysis**

190 The power calculation for the estimation of the required sample size for this trial was based
191 on a change in CRP (as the primary outcome measure) of 0.6 mg/L (SD 4), which required a
192 sample size of 1000 participants (two-sided, 80% power and 0.05 alpha). We increased this
193 number to 1250 to account for an anticipated dropout rate of 20%. A previous study
194 examining the effect of a dietary intervention and consumption of fortified dairy products
195 for 12 months on spine BMD in postmenopausal women observed changes of -0.045 g/cm²
196 in the control group, 0.008 g/cm² in the calcium supplemented group and 0.053 g/cm² in the
197 dietary intervention group (15). Based on these data we would need 36 participants (18 per
198 group) to observe an effect on spinal BMD (two-sided, 99% power and 0.05 alpha). This

199 indicated that we had sufficient osteoporotic participants (see **Table 2**) in our study to
200 conduct stratified analysis on the effect of MD on BMD.

201

202 The normality of the data for each variable was tested using Kolmogorov-Smirnov Test and
203 the Shapiro-Wilk Test. Baseline characteristics are presented as mean SD or n (%) for
204 categorical variables, and baseline between-group differences were assessed using
205 independent sample t-tests or χ^2 tests. The effect of the intervention on changes in BMD
206 and bone biomarkers was assessed using linear mixed-effect models with participant
207 included as random effect, time, treatment group, time x treatment group interaction, and
208 the explanatory variables study centre, age, sex, baseline BMI, baseline calcium intakes and
209 baseline 25(OH)D were included. Where we observed a significant time*treatment
210 interaction we also tested if there was a study centre effect by including a three way
211 time*treatment*study centre interaction term in the model. For each variable, values <3 or
212 >3 SDs from the mean were considered outliers and removed. As data were not normally
213 distributed, the models were fitted on a log-transformed scale. To account for multiple
214 testing we applied a Bonferroni correction, with eight tests per group (three BMD measures
215 and five biomarkers). We calculated the site-adjusted mean difference in intake of dietary
216 components associated with bone health using ANCOVA. Data were analysed using Stata
217 version 14 (Stata Corp., College Station, TX, USA).

218

219 **RESULTS**

220 Of the 1294 participants recruited to the NU-AGE study n=1142 completed (11.7% drop out
221 rate) (**Supplemental Figure 1**). Of these completers, n=562 in the control group and n=555

222 in the intervention group had whole-body DXA scans at baseline and follow-up and
223 complete covariate data (97.8%). There were no significant differences in baseline
224 characteristics between the two groups (**Table 1**). Osteopenia (defined as a lumbar spine T-
225 score of <-1.5 SD below peak bone mass) was present in 37%, and osteoporosis (defined as
226 a lumbar spine T-score of <-2.5 SD below peak bone mass) in 8% of participants at baseline.

227

228 After 1-y dietary intervention, there was no effect on BMD at any bone site (**Table 2**) or on
229 the concentrations of urinary fDPD and fPYD or the fDPD: fPYD ratio (**Table 3**). There was a
230 significant ($p<0.001$) time x treatment interaction in change in serum 25(OH)D over the 12
231 months (**Table 3**), where the mean concentration significantly increased in the intervention
232 group (4.5 ng/mL; 95% CI 3.9, 5.1) but was unchanged in the control group (0.5 ng/mL;
233 95%CI -0.1, 1.0) ($p<0.01$). There was a significant ($p<0.001$) time x treatment interaction in
234 change in serum PTH over the 12 months, where the mean concentration increased in the
235 control group (3.9 pg/mL 95% CI 2.1, 5.6) but no significant change in the intervention group
236 (-1.4 pg/mL 95% CI -3.1, 0.4) ($p<0.001$)(Table 3). There was no effect of study centre for
237 serum 25(OH)D ($P=0.049$) or serum PTH ($P=0.755$).

238

239 When examining the sub-group of participants diagnosed with osteoporosis at baseline
240 ($n=54$) there was a 0.9% difference between the groups in the change in femoral neck BMD
241 (Table 2); BMD increased in the intervention group (0.008 g/cm² 95% CI -0.001,0.018) and
242 decreased in the controls (-0.009 g/cm² 95%CI -0.018,-0.001) $P=0.04$. No effect of study
243 centre was observed ($P=0.415$). The intervention had no effect on BMD measured at the
244 lumbar spine or the whole body.

245

246 When examining changes in specific dietary components associated with bone health we
247 observed a significant increase in intakes of olive oil, low fat dairy and calcium in the
248 intervention group relative to controls (**Figure 2**).

249

250 **DISCUSSION**

251 In one of the first long-term intervention studies examining the effect of the MD on BMD,
252 we have found that consuming a MD with 10 µg/day vitamin D₃ reduces the rate of femoral
253 neck bone loss, but not total body or spinal BMD loss, in elderly people with osteoporosis.
254 There were no beneficial changes in BMD in individuals with BMD in the normal range at
255 baseline.

256

257 There is conflicting evidence from cross-sectional studies examining the association
258 between the MD and BMD, and a lack of consistency regarding the BMD sites which are
259 most affected. In Chinese adults aged 40-75y, higher scores for adherence to a MD, adapted
260 for China, were positively and dose-dependently associated with higher BMDs at whole
261 body, lumbar spine, total hip, femur neck, trochanter, intertrochanter, but not Ward's
262 triangle area (2.41–3.96% higher, quintile 5 vs. quintile 1, all P-values < 0.001), after
263 adjusting for age and gender (16). Higher intakes of whole grains, fruits, and nuts and a
264 lower intake of red and processed meat were independently associated with higher levels of
265 BMD at several bone sites, but vegetables, legumes, fish, monounsaturated fat/saturated
266 fat ratio, and moderate alcohol consumption showed no independent associations with

267 BMD in this study. In Finnish women aged 65-71 y (17) lumbar spine, femoral neck and total
268 BMD were not significantly different across the Baltic sea diet (BSD) or MD quartiles. Also,
269 there were no significant associations of BSD and MD quartiles in the subgroup with
270 osteoporosis. A study of 220 Greek women (mean age 48 ± 12 y) found no link between
271 adherence to a MD and bone mass, but when Principal Components Analysis was used to
272 differentiate 10 dietary patterns, a high consumption of fish and olive oil and low intake of
273 red meat was positively associated with lumbar spine BMD (18). A study in 200 pre- and
274 post-menopausal Spanish women showed that a higher habitual intake of fruits, vegetables
275 and nuts was associated with higher total body BMD in post-menopausal women (19). A
276 smaller study in 87 Italians aged 70.1 ± 4.9 y also showed that adherence to the MD was
277 associated with a higher BMD (T score assessed by calcaneal quantitative ultrasound of the
278 mid-calcaneus) with lowest adherence observed in the 15% osteoporotic subjects (20).

279

280 Although it is not possible to draw conclusions about cause and effect from cross-sectional
281 data, an association between the MD, or some components of the diet, and bone health is
282 reported in some studies. To our knowledge, no previous dietary intervention studies in
283 elderly people have reported the effect of the MD on BMD, therefore the NU-AGE
284 randomized controlled trial provides an important opportunity to clarify the relationship.
285 Our multi-center trial results show that consuming a MD (together with vitamin D
286 supplements) for a year had no effect on BMD (whole body or site specific) in older people,
287 and even when we included the degree of compliance to the dietary change in the statistical
288 model this did not change the findings (**Supplemental Table 2**). Sub-group analysis,
289 however, showed a significant beneficial effect of the MD plus supplemental vitamin D₃ on

290 femoral neck (but not lumbar spine or whole body) BMD in subjects identified at baseline as
291 having osteoporosis.

292

293 Phenolic compounds, as found in virgin olive oil, are suggested as one of the components of
294 the MD responsible for the effect on bone; the proposed mechanism is modulation of the
295 proliferative capacity and cell maturation of osteoblasts through increased alkaline
296 phosphatase activity and deposition of calcium ions in the extracellular matrix (21). A
297 randomized controlled trial reported that the consumption of a MD enriched with virgin
298 olive oil for 2 y was associated with an increase in the bone biomarkers for bone formation,
299 serum osteocalcin and procollagen 1 N-terminal propeptide (P1NP) concentrations, in
300 elderly men (22), indicating that the MD increases bone formation rather than decreasing
301 resorption. In our dietary intervention, we provided virgin olive oil to the intervention group
302 to encourage subjects to consume more olive oil. Baseline olive oil intake was highest in
303 Italy (9.6 ± 0.4 g/d) and lowest in France (2.1 ± 0.4 g/d) and although there were no
304 significant differences in intake between the countries, the greatest changes in intakes were
305 observed in France (6.0 g/d) and the lowest in Italy (1.0 ± 0.5 g/d). A reduction in sodium
306 intake, as undertaken in the DASH diet study (23), may be one of the consequences of
307 consuming a MD (with reduced processed meat intake, and increased intakes of fruits and
308 vegetables), and this has been reported to have beneficial effects on bone health through a
309 reduction in urinary calcium excretion (24). However, knowing the difficulties of accurately
310 measuring sodium intake, we did not attempt to evaluate the effect of sodium intake on
311 BMD. Similarly, for other dietary components that may impact on bone turnover, such as

312 vitamin K, we were unable to include them in our model due to the lack of reliable intake
313 data.

314

315 Although osteoporosis is assumed to be a risk factor for bone fracture, the evidence for a
316 protective effect of the MD on risk of fracture is conflicting. Post-hoc analysis of longitudinal
317 data (median follow-up time of 15.9 y) from the US Women's Health Initiative reported a
318 lower risk for hip (but not total) fractures with higher adherence to a Mediterranean diet in
319 women 50-79 y (7). Conversely, a smaller population-based study of shorter duration (8 y) in
320 France found that greater adherence to the MD was not associated with a decreased risk of
321 fractures in men and women aged 67 y on recruitment (25). In a prospective study in
322 European men and women (EPIC) with a mean age of 48.6 y, followed for a median of 9 y,
323 increased adherence to MD protected against hip fracture occurrence, particularly among
324 men (26). In the PREDIMED trial, an observational cohort study nested in the main trial,
325 found that a higher consumption of extra-virgin olive oil was associated with a lower risk of
326 osteoporosis related fractures in Mediterranean men and women, aged 55-80 y, at high
327 cardiovascular risk (21). As with the cross-sectional studies cited above, the effect of the MD
328 appears to be mediated through particular dietary components, such as virgin olive oil.

329

330 In our study, subjects in the intervention group were given vitamin D₃ supplements (10
331 µg/day), which significantly increased serum total 25(OH)D and reduced parathyroid
332 hormone concentrations in the whole intervention group (but not in the osteoporosis sub-
333 group) compared with the control group (Table 3). This may be a question of insufficient
334 power as the osteoporotic sub-group was small. In this combined intervention design it is

335 not possible to disentangle the relative influence of the MD and/or vitamin D on femoral
336 neck BMD in osteoporotic subjects. However, it is likely that the daily dose of vitamin D₃ (10
337 µg) was too low to have a significant impact on bone loss. MacDonald et al (27) found that
338 hip bone loss was attenuated when vitamin D₃ supplements of 1000 IU (25µg) were given
339 daily for 1-y to postmenopausal women, but 400 IU (10 µg) had no effect. A systematic
340 review of vitamin D supplementation and risk of fractures concluded that vitamin D
341 supplements of 700-800 IU (17.5-20 µg) per day appears to reduce the risk of hip and any
342 non-vertebral fractures in ambulatory or institutionalized elderly persons, but that a vitamin
343 D dose of 400 IU (10 µg) per day is not sufficient for fracture prevention (28). It is also
344 worth noting that the baseline serum 25(OH)D of the participants in this RCT at ~25 ng/ml,
345 exceeded that suggested by the Institute of Medicine (i.e., 20 ng/mL) as covering the needs
346 of nearly all individuals from a bone health perspective (29). The Endocrine Society,
347 however, have suggested that to maximize the effect of vitamin D on calcium, bone, and
348 muscle metabolism, the circulating 25(OH)D should be above 30 ng/ml (30). This latter
349 threshold was only achieved in just under half of the intervention group in the present RCT
350 (mean serum 25(OH)D at endpoint, 29 ng/ml).

351 The strength of this study is that it was a long-term (one year) RCT carried out in a relatively large
352 number (over 1,000) of European men and women, designed to examine the effects of a
353 Mediterranean-like diet on various health parameters, including bone health. One of the limitations
354 is the relatively small size of the sub-group with osteoporosis, and the significant and interesting
355 findings of differences in response between individuals with BMD in the normal range and those
356 with osteoporosis needs to be verified in a future study.

357 In conclusion, our study showed that a 1-y intervention of the MD together with vitamin D₃
358 supplements (10µg/day) reduced the rate of loss of bone at the femoral neck in individuals
359 with osteoporosis but had no effect on those with BMD in the normal range.

360

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362 The authors' responsibilities were as follows: SFT, CF conceived the study; AB, LdG designed
363 the dietary intervention; AJ, RG, AB, BP, EW, RO carried out the intervention study; JT, WDF,
364 KGD, GLJH, KDC were responsible for the biochemical analysis; AJ, RG, AB, GB were
365 responsible for imaging, DXA assessment and analysis; AJ, RG, AB, RO, AS were responsible
366 for data collection; AS coordinated the NU-AGE data collection across centers; AJ was
367 responsible for data analysis; SFT and AJ wrote the first draft of the manuscript; all authors
368 were accountable for all aspects of the work in ensuring that questions related to the
369 accuracy or integrity of any part of the work were appropriately investigated and resolved,
370 critically revised the manuscript for important intellectual content, and agreed on the final
371 draft of the manuscript. None of the other authors reported any conflicts of interest related
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Table 1: Baseline characteristics of the NU-AGE study participants according to intervention group

Characteristic	Intervention diet		Control diet		P=
	n	Mean (SD)	n	Mean (SD)	
Sex, female	n=632	363 (57.4)	n=644	356 (55.3)	0.437
Age, y	n=632	70.7 ± 4.1	n=643	71.1 ± 3.9	0.046
Body mass index, kg/m ²	n=633	26.9 ± 4.2	n=643	26.7 ± 3.8	0.492
Calcium intakes, g/d	n=618	618 ± 912	n=622	895 ± 347	0.361
Lumbar spine BMD, g/cm ²	n=377	1.1 ± 0.2	n=379	1.1 ± 0.2	0.553
Femoral neck BMD, g/cm ²	n=379	0.8 ± 0.1	n=385	0.8 ± 0.1	0.328
Whole body BMD, g/cm ²	n=616	1.1 ± 0.1	n=621	1.1 ± 0.1	0.963
Osteoporosis, yes	n=377	27 (7.2)	n=380	37 (9.7)	0.370
Free Pyridinoline (fPYD), creatinine adjusted nmol/mmol	n=612	24.0 ± 7.3	n=620	24.3 ± 7.5	0.489
Free Deoxypyridinoline (fDPD), creatinine adjusted nmol/mmol	n=612	6.1 ± 1.9	n=619	6.2 ± 2.0	0.636
deoxypyridinoline to Pyridinoline ratio	n=612	0.3 ± 0.1	n=619	0.3 ± 0.1	0.180
Parathyroid hormone, pg/ml	n=483	44.3 ± 26.5	n=479	42.4 ± 23.6	0.223
25-hydroxyvitamin D, ng/ml	n=613	24.6 ± 9.1	n=619	24.8 ± 8.9	0.745

Values are mean ± SD or n= (%)

Table 2: Mean difference in bone mineral density after 1-y of follow-up in the intervention and control diet groups

	Intervention	Control	P
All participants			
Lumbar spine BMD, g/cm ²	n=338	n=325	
<i>Baseline</i>	1.060 (1.042,1.078)	1.045 (1.026,1.063)	
<i>1-y</i>	1.065 (1.047,1.084)	1.049 (1.030,1.067)	
<i>Change</i>	0.005 (0.002,0.009)	0.004 (0.000,0.007)	1.000
Femoral neck BMD, g/cm ²	n=342	n=326	
<i>Baseline</i>	0.820 (0.807,0.833)	0.809 (0.796,0.822)	
<i>1-y</i>	0.816 (0.804,0.829)	0.804 (0.791,0.817)	
<i>Change</i>	-0.004 (-0.006,-0.001)	-0.005 (-0.008,-0.002)	1.000
Whole body BMD, g/cm ²	n=551	n=557	
<i>Baseline</i>	1.099 (1.090,1.107)	1.092 (1.084,1.101)	
<i>1-y</i>	1.098 (1.089,1.106)	1.091 (1.082,1.099)	
<i>Change</i>	-0.001 (-0.003,0.000)	-0.002 (-0.003,0.000)	1.000
Osteoporosis subgroup²			
Lumbar spine BMD, g/cm ²	n=25	n=33	
<i>Baseline</i>	0.770 (0.743,0.797)	0.768 (0.745,0.791)	
<i>1-y</i>	0.782 (0.755,0.810)	0.779 (0.755,0.802)	
<i>Change</i>	0.012 (0.001,0.024)	0.011 (0.001,0.021)	1.000
Femoral neck BMD, g/cm ²	n=24	n=30	
<i>Baseline</i>	0.649 (0.624,0.673)	0.635 (0.614,0.656)	

<i>1-y</i>	0.657 (0.633,0.681)	0.625 (0.605,0.646)	
<i>Change</i>	0.008 (-0.001,0.018)	-0.009 (-0.018,-0.001)	0.040
Whole body BMD, g/cm ²	n=20	n=22	
<i>Baseline</i>	0.883 (0.867,0.899)	0.856 (0.841,0.870)	
<i>1-y</i>	0.885 (0.869,0.901)	0.860 (0.846,0.875)	
<i>Change</i>	0.002 (-0.004,0.008)	0.005 (-0.001,0.011)	1.000

Values are mean (95% CI) adjusted for study centre, age, sex, calcium intakes, use of vitamin D supplements, 25-hydroxyvitamin D levels and BMI (all measured at baseline). Participants were excluded from the analysis if outcome values were <3 or >3 SDs from the mean; ¹P = Bonferroni corrected p values for the time x treatment interaction. ²Osteoporosis was defined as femoral neck BMD T-score <2.5 SD.

Table 3: Mean difference in bone biomarkers after 1-y of follow-up in the intervention and control diet groups

	Intervention	Control	P¹
Free Pyridinoline, nmol/mmol	n=551	n=563	
<i>Baseline</i>	23.1 (22.6,23.7)	23.6 (23.0,24.1)	
<i>1-y</i>	23.6 (23.1,24.2)	23.6 (23.1,24.2)	
<i>Change</i>	0.5 (0.0,1.0)	0.1 (-0.4,0.6)	1.000
Free Deoxypyridinoline, nmol/mmol	n=551	n=560	
<i>Baseline</i>	5.88 (5.74,6.01)	6.02 (5.88,6.15)	
<i>1-y</i>	5.99 (5.85,6.12)	5.93 (5.80,6.07)	
<i>Change</i>	0.1 (0.0,0.2)	-0.1 (-0.2,0.0)	0.208
Free Deoxypyridinoline: free pyridinoline ratio	n=554	n=563	
<i>Baseline</i>	0.26 (0.25,0.26)	0.25 (0.25,0.26)	
<i>1-y</i>	0.25 (0.25,0.26)	0.25 (0.25,0.26)	
<i>Change</i>	0.00 (-0.01,0.00)	0.00 (-0.01,0.00)	1.000
Parathyroid hormone, pg/ml	n=468	n=467	
<i>Baseline</i>	40.7 (38.7,42.8)	38.5 (36.5,40.5)	
<i>1-y</i>	39.4 (37.3,41.4)	42.4 (40.2,44.5)	
<i>Change</i>	-1.4 (-3.1,0.4)	3.9 (2.1,5.6)	0.000
25-hydroxyvitamin D, ng/ml	n=548	n=562	
<i>Baseline</i>	24.6 (24.0,25.3)	24.1 (23.5,24.8)	
<i>1-y</i>	29.1 (28.4,29.8)	24.6 (24.0,25.2)	

<i>Change</i>	4.5 (3.9,5.1)	0.5 (-0.1,1.0)	0.000
Osteoporosis subgroup²			
Free Pyridinoline, nmol/mmol	n=24	n=30	
<i>Baseline</i>	24.0 (21.7,26.4)	23.9 (21.8,26.0)	
<i>1-y</i>	25.6 (23.2,28.0)	24.9 (22.8,27.0)	
<i>Change</i>	1.6 (-0.6,3.8)	1.0 (-1.1,3.1)	1.000
Free Deoxypyridinoline, nmol/mmol	n=24	n=30	
<i>Baseline</i>	6.69 (5.96,7.43)	6.30 (5.66,6.94)	
<i>1-y</i>	6.44 (5.72,7.16)	6.76 (6.10,7.43)	
<i>Change</i>	-0.3 (-0.9,0.4)	0.5 (-0.2,1.1)	1.000
Free Deoxypyridinoline: free pyridinoline ratio	n=24	n=30	
<i>Baseline</i>	0.28 (0.25,0.30)	0.26 (0.24,0.28)	
<i>1-y</i>	0.26 (0.23,0.28)	0.27 (0.25,0.29)	
<i>Change</i>	-0.02 (-0.05,0.00)	0.01 (-0.01,0.03)	0.192
Parathyroid hormone, pg/ml	n=19	n=24	
<i>Baseline</i>	44.4 (33.9,54.9)	43.0 (34.0,52.0)	
<i>1-y</i>	44.4 (34.2,54.5)	49.0 (39.4,58.5)	
<i>Change</i>	0.0 (-11.0,11.0)	6.0 (-2.4,14.4)	1.000
25-hydroxyvitamin D, ng/ml	n=23	n=29	
<i>Baseline</i>	23.9 (20.9,27.0)	24.3 (21.6,27.1)	
<i>1-y</i>	29.2 (25.8,40.2)	28.1 (25.2,31.1)	
<i>Change</i>	5.2 (1.7,8.8)	3.8 (0.7,6.9)	1.000

Values are mean (95% CI) adjusted for study centre, age, sex, calcium intakes, use of vitamin D supplements, 25-hydroxyvitamin D levels and BMI (all measured at baseline). Participants were excluded from the analysis if outcome values were <3 or >3 SDs from the mean;

P^1 = Bonferroni corrected p values for the time x treatment interaction. 2 Osteoporosis was defined as femoral neck BMD T-score <2.5 SD.

Figure legend

Figure 1. Mean difference in intake of dietary components associated with bone health after 1-y of follow-up in the intervention and control diet groups.