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ORIGINAL ARTICLE

Multiple vitamin deficiencies additively increase the risk of incident fractures in Japanese postmenopausal women

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Abstract

Summary The associations of multiple vitamin deficiencies on incident fractures were uncertain, the relationships between serum vitamin markers and incident bone fractures were investigated in Japanese postmenopausal women. The number of deficiencies was additively associated with incident fracture after adjustment for possible confounding factors including the treatment of osteoporosis.

Introduction To evaluate the associations of multiple vitamin deficiencies on incident fractures, the relationships between serum vitamin markers and incident bone fractures were investigated in Japanese postmenopausal women.

Methods This analysis used a subset of the ongoing cohort maintained by a primary care institution. Inclusion criteria of the present study were postmenopausal women aged ≥ 50 years, without vitamin supplementation and secondary osteoporosis. Baseline serum concentrations of 25-hydroxyvitamin D (25(OH)D), undercarboxylated osteocalcin (ucOC), and homocysteine (Hcy) were measured to assess vitamin D, vitamin K, and vitamin B, respectively. Since 25(OH) D positively relates to vitamin D, ucOC and Hcy negatively relate to vitamin K and vitamin B nutrients, respectively, the subjects with lower (25(OH)D) or higher (ucOC or Hcy) values than each median value was defined as subjects with the corresponding vitamin deficiency. Subjects were divided into four groups according to the number of deficiency: no deficiency, single deficiency, double deficiencies, and triple deficiencies. Relationships between the vitamin deficiencies and incident fractures were evaluated by Cox regression analysis.

Results A total of 889 subjects were included in this analysis; their mean and SD age was 68.3 ± 9.5 years, and the follow-up period was 6.3 ± 5.1 years. The numbers of subjects in the four groups were 139 (15.6%), 304 (34.2%), 316 (35.5%), and 130 (14.6%) for the groups with no, single, double, and triple deficiencies, respectively. Incident fractures were observed in 264 subjects (29.7%) during the observation period. The number of deficiencies was significantly associated with incident fracture (hazard ratio 1.25, 95% confidence interval 1.04–1.50, $P = 0.018$) after adjustment for possible confounding factors including the treatment of osteoporosis.

Conclusion Accumulation of vitamin deficiencies was related to incident fractures.

Keywords Fracture · Vitamin B · Vitamin D · Vitamin K

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Introduction

Osteoporosis is recognized as a major public health problem because it lowers health state utility with socio-economic burdens [1–3]. The implementation of preventive and therapeutic interventions for osteoporosis has become a challenge in an aging society. Although several therapeutic regimens for the prevention of osteoporotic fractures are available, nutritional improvement in osteoporosis has been believed to be important, because lifestyle interventions such as adequate nutritional intake or training for maintaining physical activity were found to be effective to increase bone mineral density or prevent fragility fractures in elderly populations [4, 5]. Among the various nutritional factors, vitamins have been considered to play an important role in bone health.

Vitamin D combined with calcium intake occupies a central role in bone health. Sufficiency or deficiency of vitamin D was evaluated by the measurement of serum concentrations of 25-hydroxy (OH) vitamin D (25(OH)D) [6–8], and low 25(OH) D levels were associated with higher vertebral fracture risks and mortality in Japanese female populations [9, 10]. In addition, 25(OH) D is a leading risk factor for long bone fracture, comparable to BMD in Japanese postmenopausal women [11].

A low level of vitamin K1 has been observed in osteoporotic patients with fractures [12, 13], and low dietary intake of vitamin K1 was associated with low bone mineral density (BMD) and an increased risk of hip fracture [14]. Osteocalcin is synthesized by osteoblasts and is activated by post-translational modification in its molecule, namely γ carboxylation on glutamic acid (Glu) to γ carboxyglutamic acid (Gla). This process is activated by vitamin K, and Gla-containing osteocalcin has the ability to bind hydroxyapatite in bone, leading to resistance against bone resorption [15]. Since vitamin K is a vitamin that activates the blood coagulation system, which is critical to the sustenance of life, vitamin K is preferentially metabolized in the liver. Therefore, vitamin K deficiency or insufficiency in bone is frequently observed in humans [16]. Vitamin K deficiency in bone has been evaluated by measurement of undercarboxylated osteocalcin (ucOC) [17]. Furthermore, it has been reported that high levels of ucOC are associated with both lower BMD and an increased risk of hip fracture [18, 19]. Vitamin B6, vitamin 12, and folate are nutrients that may have impacts on osteoporosis, since they tightly regulate serum levels of homocysteine (Hcy) [20]. The serum level of Hcy is associated with incident fractures independently of the BMD level [21–23].

These facts mentioned above indicate that vitamin deficiencies seem to be causative factors leading to high susceptibility to fractures. However, meta-analyses examining the effect of vitamin supplementation on fracture prevention have been controversial. Several meta-analyses reported that the use of vitamin D with or without calcium was not associated with the reduction of fracture risk among community-dwelling older adults [24, 25]. There are meta-analyses that were uncertain about the clinical efficacy of vitamin K supplements [26, 27]. A recent meta-analysis reported that vitamin K2 plays a kind of role in the maintenance of vertebral BMD and the prevention of fractures [26]. Another meta-analysis reported that vitamin K may reduce bone loss, while it reduced fracture risk only in a Japanese population [27]. Two large-scale, randomized, controlled studies to evaluate the efficacy of vitamin B, which can reduce Hcy, have been reported [28, 29]. However, folic acid, vitamin B6, vitamin B12, and vitamin B12 plus folic acid supplementation were found to have no effect on osteoporotic fracture incidence. Moreover, a recent meta-analysis reported that vitamin B supplementation might not be effective in preventing fractures and improving bone turnover [30].

Taken together, although the vitamin deficiencies such as vitamin D, vitamin K, and the vitamin B group have a close relationship with incident fractures, vitamin supplementation to correct the vitamin deficiency could not be confirmed to prevent fractures.

The possible explanation of these contradictions may be the low power of single-vitamin supplementation for 1 to 2 years to prevent incident fractures in an intervention study. On the other hand, observations to evaluate the effects of vitamin deficiency on fracture incidence in epidemiological studies have been carried out for more than 5 years. Thus, it is expected that the exposure duration for vitamin deficiency or replacement may be important. Another possible explanation is the habitual dietary habits of the people. Since people usually take foods deficient in various vitamins, single-vitamin deficiency may be rare, but a multiple vitamin-deficient diet might be usual. Therefore, the associations of multiple vitamin deficiencies in a participant on incident fractures were examined. In the present study, serum levels of 25(OH) D, ucOC, and Hcy were measured to assess vitamin D, vitamin K, and vitamin B deficiencies at baseline, respectively, and the subjects, Japanese postmenopausal women, were followed up for incident bone fractures.

Methods

Participants

This analysis was performed using the data of the Nagano cohort study, which is an ongoing registration study of outpatients at a primary care institute in Nagano Prefecture, Japan, since 1993 [10, 12, 31]. The subjects of this analysis were selected from over 3000 patients who were registered from 1993 to July 2016. Inclusion criteria for this analysis were age ≥ 50-year-old postmenopausal women whose serum concentrations of 25(OH) D, ucOC, and Hcy were measured simultaneously. Subjects who were treated by native or active vitamin D or menatetrenone for osteoporosis were excluded. Patients treated with vitamin B supplementation for any reason were also excluded. Subjects who did not undergo follow-up observation were excluded. Finally, a total of 889 subjects were selected for the present study.

Measurements

At baseline, age and information on alcohol consumption and tobacco use were obtained by interview. Body weight and height were measured, and the body mass index (BMI) was calculated. Bone mineral density (BMD) was measured at the lumbar spine and femoral neck using dual-energy X-ray absorptiometry (DXA) in fast-scan mode (Prodigy, GE Lunar,

Madison, WI, USA). A quality assurance test was performed on every measurement to detect machine drift.

Non-fasting serum and urine samples were collected at baseline, and the levels of serum bone-derived alkaline phosphatase (BAP) and urinary excretion of type I collagen cross-linked N-telopeptides (NTX; Osteomark, Creative Diagnostic, Shirley, NY, USA) were measured. Serum levels of PTH were measured by the intact PTH CLEIA kit (LSI Medience Corporation, Tokyo, Japan). Urinary levels of pentosidine were measured by high-performance liquid chromatography (HPLC) [32]. Serum levels of 25(OH) D were measured using a competitive protein-binding assay after extraction and purification of the samples using HPLC (Teijin Bio Science Laboratories, Tokyo, Japan, and LSI Medience, Tokyo, Japan) [33]. Serum levels of ucOC were measured using a new electrochemiluminescence immunoassay (Sanko Junyaku, Ibaraki, Japan) [34]. Levels of Hcy were measured by an HPLC system [35]. The assay coefficient of variations for 25(OH) D, ucOC, and Hcy were less than 12.5%, 8%, and 5.2%, respectively [9]. The estimated glomerular filtration rate (eGFR) was calculated by the following formula: $194 \times \text{serum creatinine}^{-1.094} \times \text{age}^{-0.287} \times 0.739$. The above laboratory measurements were made once at the beginning of the observation period.

Diagnosis of osteoporosis

If the BMD was less than − 2.5 T-score at any bone site, or if the subjects had non-traumatic prevalent osteoporotic fractures, osteoporosis was diagnosed [36].

Fracture

Radiographs were taken at baseline, during the follow-up period annually, and when a patient complained of fracture-related symptoms. Prevalence at baseline and incident fragility fractures during follow-up were evaluated as major osteoporotic fractures that occurred at four sites (vertebra, femur, forearm, humerus) and at other sites. Traumatic fracture was excluded. Vertebral fracture was classified into the four grades (0 to 3) by semi-quantitative method [37]. Incident vertebral fracture was evaluated by the increase of the grade. Fracture incidences were investigated until May 2017. The period of follow-up for each participant was calculated as the time from inclusion in the study to the first fracture, death, referral to the other hospital, institutionalization, missing, or to the end of 2017, whichever occurred first.

Ethical issues

The entire protocol of the Nagano cohort study was reviewed and approved by the ethics committee of the Research Institute and Practice for Involutional Diseases, and comprehensive written informed consent was obtained from all the subjects before registration.

Statistical analysis

Numerical data at baseline are shown as means and standard deviation (SD). Relationships between the baseline characteristics and the incidence of fractures were assessed. First, the median values of 25(OH) D, ucOC, and Hcy were calculated; they were 20.0 ng/mL, 3.4 ng/ml, and 8.6 nmol/mL, respectively. Vitamin D, K, or B deficiency was defined by the level of vitamin markers, namely serum 25(OH) D less than 20 ng/ml for vitamin D deficiency, ucOC 3.4 ng/ml or more for vitamin K deficiency, and Hcy 8.6 nmol/ml or more for vitamin B deficiency. According to the number of "deficiencies," the subjects were classified into four groups: "single deficiency," "double deficiencies," "triple deficiencies," and "no deficiency." Baseline characteristics were evaluated by ANOVA or the chi-squared test. Kaplan-Meier curves were plotted to describe the incidence of fractures over the observation period, and the log-rank test was used to examine the significance of differences between the groups. To elucidate the independent risk factors for incident fractures adjusted for confounders, a Cox proportional hazard model was used. Selected confounders were age, femoral neck BMD, eGFR, PTH, NTX, BAP, pentosidine, prevalent fracture, and treatment of osteoporosis. All tests were two-sided, and $P < 0.05$ was considered significant. The statistical analysis was carried out using JMP version 13.0 (SAS Institute, Cary, NC, USA).

Results

Levels of the three vitamin markers were measured in a total of 1019 subjects. Among them, 130 subjects were excluded, because 48 subjects were aged less than 50 years, 78 subjects were given vitamin supplementation, and 4 subjects were not evaluated for incident fractures. A total of 889 subjects were included in this analysis, and the mean and SD age was 68.3 ± 9.5 years, BMI was 22.3 ± 3.2 kg/m^2, and the follow-up period was 6.3 ± 5.1 years (Table 1). A total of 337 participants were diagnosed as having osteoporosis, and they were treated with a bisphosphonate ($n = 227$, 25.5%), SERM ($n = 103$, 11.6%), or estrogen ($n = 7$, 0.8%). The remaining 552 subjects (62.1%) were followed without any treatment, which may affect bone health. Lost to follow-up was observed in 153 subjects (17.2%) such as death, missing, moving, referred to the other hospital, institutionalized.

The numbers of subjects in the four groups were 139 (15.6%), 304 (34.2%), 316 (35.5%), and 130 (14.6%) for the groups with no, single, double, and triple deficiencies, respectively. The differences in the baseline characteristics among these four groups are indicated in Table 1. Age, femoral neck

Table 1 Baseline characteristics divided by vitamin deficiency category

		Vitamin deficiency category				
	Total ($n = 889$)	No deficiency ($n = 139$)	Single deficiency ($n = 304$)	Double deficiencies ($n = 316$)	Triple deficiencies ($n = 130$)	*P*
Age, y	68.3 ± 9.5	65.8 ± 8.8	67.4 ± 9.2	69.2 ± 9.6	70.9 ± 9.8	< 0.001
BMI, kg/m^2	22.3 ± 3.2	22.0 ± 3.1	22.2 ± 3.1	22.4 ± 3.3	22.4 ± 3.4	0.694
Lumbar BMD, g/cm^2	0.902 ± 0.191	0.902 ± 0.190	0.908 ± 0.184	0.904 ± 0.197	0.883 ± 0.196	0.633
Femoral neck BMD, g/cm^2	0.739 ± 0.130	0.777 ± 0.122	0.746 ± 0.128	0.732 ± 0.128	0.701 ± 0.137	< 0.001
eGFR, mL/min/1.73 m^2	68.5 ± 18.0	71.4 ± 17.2	70.8 ± 17.9	66.8 ± 17.4	64.0 ± 19.2	< 0.001
Serum PTH, pg/mL	40.0 ± 18.3	35.8 ± 12.5	36.9 ± 13.6	40.3 ± 16.6	50.9 ± 29.5	< 0.001
Serum NTX, nM BCE/mM Cr	53.6 ± 30.7	47.9 ± 24.1	52.3 ± 28.8	53.5 ± 29.2	62.7 ± 41.2	0.001
Serum BAP, U/L	31.2 ± 11.7	28.9 ± 10.2	30.8 ± 11.4	31.3 ± 11.3	34.3 ± 14.1	0.009
Urine pentosidine, pM/mgCr	48.9 ± 29.8	41.2 ± 15.2	46.3 ± 30.9	51.9 ± 32.5	55.9 ± 30.0	< 0.001
Serum 25(OH) D, ng/mL	20.8 ± 6.1	25.8 ± 4.7	22.6 ± 5.7	19.0 ± 5.6	15.7 ± 2.9	< 0.001
Serum ucOC, ng/mL	4.5 ± 3.7	2.2 ± 0.7	3.3 ± 2.6	5.4 ± 4.0	7.6 ± 4.1	< 0.001
Serum Hcy, nM/mL	9.4 ± 3.5	7.0 ± 1.1	8.5 ± 2.7	10.2 ± 3.6	12.3 ± 4.0	< 0.001
Observation period, y	6.3 ± 5.1	6.2 ± 4.8	6.6 ± 5.0	6.3 ± 5.2	5.8 ± 5.1	0.530
Prevalent fracture: yes, %	35.4	30.2	36.8	36.1	36.2	0.572
Treatment of osteoporosis: yes, %	37.9	44.6	39.5	35.1	33.8	0.180

Values are means ± SD or %, *P* values indicate differences between categorical groups

BMI body mass index, *BMD* bone mineral density, *eGFR* estimated glomerular filtration rate, *PTH* parathyroid hormone, *NTX* cross-linked N-telopeptide of type I collagen, *BAP* bone alkaline phosphatase, *25(OH)D* 25-hydroxyvitamin D, *ucOC* undercalboxylated osteocalcin, *Hcy* homocysteine

BMD, eGFR, serum PTH, NTX, BAP, and urinary pentosidine were significantly different among the groups. However, the lumbar BMD and BMI were not different among the groups. Prevalent fractures were observed in 315 subjects (35.4%) at baseline. The percentages of subjects having prevalent fractures were not significantly different among the four groups. The mean observation period, alcohol consumption, and tobacco use were not significantly different among the groups.

Incident fractures were observed in 264 subjects (29.7%), involving the vertebra ($n = 202$), femur ($n = 18$), forearm ($n = 15$), humerus ($n = 1$), and other sites ($n = 29$), as shown in Table 2. The incidence rate of major osteoporotic fractures increased in accordance with the number of deficiencies. The time-dependent fracture course by deficiency group is shown in Fig. 1 using Kaplan-Meier plots. A significantly higher incidence was observed according to the number of vitamin deficiencies. A clear difference in the fracture-free survival rate among the groups was observed after 5 years of observation.

The hazard ratio for incident fractures by the Cox proportional model is shown in Table 3. The number of deficiencies contributed significantly to incident fracture susceptibility after adjustment for age, femoral neck BMD, eGFR, PTH, NTX, BAP, pentosidine, prevalent fracture, and treatment of osteoporosis (hazard ratio 1.25, 95% confidence interval 1.04–1.50, $p = 0.018$).

Discussion

Japanese life expectancy is becoming the longest in the world. People believe that Japanese foods might play an important

Table 2 Type and number of incident fractures

Type of incident fracture	No deficiency	Single deficiency	Double deficiencies	Triple deficiencies
None	110 (79.1)	221 (72.7)	218 (69.0)	76 (58.5)
Major osteoporotic fracture	22 (15.8)	74 (24.3)	90 (28.5)	50 (38.5)
Vertebrae	18 (13.0)	64 (21.1)	77 (24.4)	43 (33.1)
Femur	0 (0.0)	7 (2.3)	6 (1.9)	5 (3.9)
Forearm	4 (2.9)	2 (0.7)	7 (2.2)	2 (1.5)
Humerus	0 (0.0)	1 (0.3)	0 (0.0)	0 (0.0)
Other sites of fracture	7 (5.0)	9 (3.0)	8 (2.5)	4 (3.1)

Values are indicated as numbers and (%)

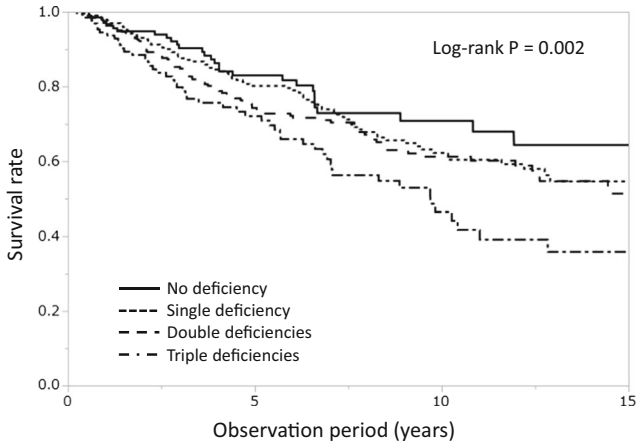

Fig. 1 Kaplan-Meier plots of incident fractures with different numbers of deficient vitamins

role in the achievement of the longest life expectancy. The dietary habits of Japanese people have recently been changed to three major patterns. The first is a traditional Japanese diet, which includes salty pickles and regular fish and soy products. The second one is a prudent/healthy dietary pattern, which is characterized by a high intake of vegetables, fruits, fish, poultry, whole grains, and low-fat dairy products. The third one is a modern Westernized food pattern, characterized by increased intake of meat, bread, and dairy products. The majority of the Japanese food intake pattern has been changed from the first type to the second or third types in accordance with the economic success after World War II [38]. The diet pattern may affect vitamin sufficiency, because the traditional type may contain less vitamin B12 and vitamin K and relatively higher intake of vitamin D from fish. On the other hand, the second type of diet pattern may result in intake of more folic acid and vitamin K from green leafy vegetables. The third type of diet pattern may result in intake of more vitamin B12 and calcium.

Table 3 Hazard ratio for incident fracture risk by Cox proportional model

Item	HR	(95% CI)	*P*
Age, + 5 years	1.22	1.10–1.35	< 0.001
Femoral neck BMD, + 0.1 g/cm^2	0.76	0.66–0.88	< 0.001
eGFR, + 10 mL/min/1.73m^2	1.04	0.95–1.13	0.391
Serum PTH, + 1 pg/mL	1.00	0.99–1.01	0.777
Serum NTX, + 10 nM BCE/mM Cr	0.98	0.93–1.04	0.580
Serum BAP, + 5 U/L	0.93	0.87–1.00	0.063
Urine pentosidine, + 10 pM/mgCr	1.06	1.02–1.09	0.002
Prevalent all fracture, no/yes	2.33	1.64–3.33	< 0.001
Treatment of osteoporosis, no/yes	1.38	0.99–1.94	0.058
Number of deficiency, 0/1/2/3	1.25	1.04–1.50	0.018

HR hazard ratio, *CI* confidence interval, *BMD* bone mineral density, *eGFR* estimated glomerular filtration rate, *NTX* cross-linked N-telopeptide of type I collagen, *BAP* bone alkaline phosphatase

Therefore, the dietary pattern may be highly predictive of vitamin intakes.

Instead of a food-frequency questionnaire (FFQ) analysis, serum concentrations of vitamin markers were measured to directly assess vitamin deficiency. Since measurement of serum vitamin markers may include the rate of utilization of each vitamin, the present method may have an advantage over the FFQ method, which cannot assess utilization of vitamins.

In the present analysis, the relationships between levels of 25(OH) D, ucOC, and Hcy, and incident fractures were evaluated in Japanese postmenopausal women. After adjusting the age, BMD, prevalent fracture, and urinary pentosidine, which are previously reported risk factors [22], a significantly higher incidence of fracture was observed according to the number of vitamin deficiencies. Moreover, the lowest fracture incidence was observed in the group with a deficiency of none of the three vitamins (no deficiency group). Therefore, vitamin deficiency assessed by 25(OH) D, ucOC, and Hcy may additively contribute to incident fracture risk. Vitamin deficiency contributed to the incidence of major osteoporotic fracture sites, but not other sites (Table 2). Figure 1 shows the survival rate of fracture-free subjects among the groups. The clearly high fracture incidence in the group with triple deficiencies was observed after 5 years of observation. Therefore, a longer period of vitamin deficiencies may contribute to incident fractures.

Although the clinical efficacy for preventing osteoporotic fractures of supplementation with vitamin D, K, or B has been reported in meta-analyses and randomized trials [24–30], no clear consistent result was obtained. The reason why supplementation with a relevant vitamin did not show a beneficial effect may be explained by the lower power of each vitamin to prevent fractures. It is possible to say that single-vitamin replacement does not cancel out the effects of deficiencies of other vitamins.

It has been reported that treatment with folic acid plus vitamin B12 was not associated with the risk of hip fracture. However, a high dose of vitamin B6 was associated with a slightly increased risk of hip fracture in two large-scale RCTs [39].

Vitamin D has a beneficial role for not only bone health, but also other organs, such as muscle function [40, 41]. Lower vitamin K status assessed by the ucOC concentration was reported to be associated with the risk of vascular calcification [42, 43]. A higher level of Hcy is related to reduced muscular strength and gait speed [20, 43–45]. These reports indicated that the deficiency of three vitamins could contribute to frailty leading to incident fracture.

One limitation of the present analysis was performed using the data from observational study. Therefore, causal relationships between deficiencies of vitamins and incident fracture were not evaluated prospectively. However, a relationship between vitamin deficiencies and incident fracture was shown

after adjusting for many confounding factors. Therefore, the results of the present study should be confirmed by a prospective, randomized study of a multivitamin supplement in the future. A second limitation was the study population. The study population of the present study was subjects who visited a primary care institution, and there may be selection bias. However, our previous study indicated that the subjects did not differ from another community-dwelling population in terms of serum 25(OH) D levels and confounders [11]. Thus, selection bias may be minimal.

In conclusion, vitamin deficiency measured by 25(OH) D, ucOC, and Hcy may additively increase the risk of incident fractures. Long-term multiple vitamin optimization may be considered to prevent osteoporotic incident fractures.

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Compliance with ethical standards

Conflicts of interest H.O. received lecture fees from Pfizer. M.S. received consulting fees from Asahi Kasei Pharma and Teijin Pharma. T.K. is an employee of Asahi Kasei Corporation. K. U. and M. S. have no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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