

disruption, leading to bone fragility and an increased susceptibility to fractures [2]. Nutrition is an important modifiable factor in the development and maintenance of bone health, and numerous studies on nutrition and bone health have been conducted [3, 4]. Recent epidemiological studies have shown an association between fruit and vegetable intake and bone mineral density (BMD) in both young and elderly subjects [5–10].

Fruits and vegetables are rich sources of antioxidant vitamins and carotenoids, which have been shown to contribute to the body's defense against reactive oxygen species [11, 12].

Recent animal experiments and *in vitro* studies have shown that reactive oxygen species and free radicals are involved in osteoclastogenesis, in apoptosis of osteoblasts and osteocytes and therefore also in bone resorption [13–15]. Furthermore, recent epidemiological studies have shown a relationship between oxidative stress and BMD or osteoporosis [16–18]. These previous findings in epidemiological and experimental studies suggest that antioxidant micronutrients may provide benefits to bone metabolism against oxidative stress. In fact, recent epidemiological studies have reported inverse associations of antioxidant vitamin and carotenoid intake and/or serum level with low BMD, risk of fracture, and/or osteoporosis [19–24]. Very recently, we found that serum concentrations of carotenoids such as  $\beta$ -cryptoxanthin and  $\beta$ -carotene were weakly but positively associated with radial BMD in post-menopausal female subjects [25]. Therefore, antioxidant vitamins and carotenoids may be beneficial to the maintenance of bone health.

With regard to antioxidant vitamins and carotenoids, most studies have focused on a single antioxidant and examined the relationship between antioxidant intake and/or serum level and the status of bone health. However, these common approaches may not adequately account for the complicated interactions of these antioxidants because people consume diets consisting of a variety of foods with complex combinations of antioxidants rather than single antioxidant. Furthermore, it is unclear whether the beneficial effects of these antioxidants on bone health are synergistic or additive. To answer such questions, the identification of dietary patterns using factor analysis has been widely used to elucidate the relationship between diet and disease. This type of statistical analysis allows the development of appropriate recommendations for overall dietary habits.

The objective of this study was to identify the dietary patterns of antioxidant vitamin and carotenoid intake associated with radial BMD in post-menopausal Japanese female subjects and to investigate the association of interactions of these antioxidants with bone health. The dietary patterns of antioxidant vitamin and carotenoid intake with radial BMD were evaluated cross-sectionally.

## Subjects and methods

### Subjects

In this survey, study subjects were recruited from participants in an annual health check-up program conducted by the local government of the town of Mikkabi, Shizuoka Prefecture, Japan in April 2005. Mikkabi is located in western Shizuoka, and about 40% of its residents work in agriculture. Fruit trees are the key industry in Mikkabi, which is an important producer of mandarin orange in Japan. A total of 1,891 males and females were subjects for the annual health check-up program. In total, 1,369 males and females (72.4% of total subjects), ranging in age from 30 to 70 years, had received the health check-up through the program.

Participants were recruited for this study, and informed consent was obtained from 699 subjects (222 males and 477 females). The response rate was 51.1%. This study was approved by the ethics committees of the National Institute of Fruit Tree Science and the Hamamatsu University School of Medicine. For the present study, we used the data of post-menopausal female subjects because, in our previous study, we had found inverse associations of serum antioxidant carotenoids with risk for low BMD in post-menopausal female subjects [25].

### Bone mineral density measurement

The radial BMD was measured using dual-energy X-ray absorptiometry (DXA) of each participant's nondominant forearm with an osteometer (model DCS-600EX-III, ALOKA Co., LTD., Tokyo, Japan). This osteometer automatically measured the forearm length from the styloid process on the ulna, and DXA scan was automatically placed on the radial centered 1/3 of the forearm length. Calibration of the machine was performed daily, and quality assurance was performed by measuring the manufacturer's phantom. The CV of the radial BMD measurement was within 0.5%. In this study, the measurement of the radial BMD of each participant was performed by well-trained clinical technologists of the Seirei Preventive Health Care Center (Shizuoka, Japan).

### Self-administered questionnaire

A self-administered questionnaire was used to collect information about a subject's history of osteoporosis, medications and/or hormone use, and lifestyle, including tobacco use (current smoker, ex-smoker, or non-smoker), exercise (1+ times per week), regular alcohol intake (1+ time per week), dietary supplement use (non-user, occasional-user, and current-user), and dietary habits. Diet

was assessed with a modified validated simple food-frequency questionnaire (FFQ) developed especially for the Japanese [26, 27]. In this FFQ, Wakai et al. selected a total of 97 foods and dishes through a two-step procedure, first by ranking food items according to the contribution to the population intake of energy and nutrients and second by stepwise multiple regression analysis of individual food items as the independent variables and of total nutrient intake as the dependent variable. For simplicity, questions on portion sizes were not included except for a few selected food items, resulting in short time to complete the questionnaire. They validated this FFQ for food groups by referring to four 4-day dietary records (DRs), and correlation coefficients between FFQ and DRs were larger than 0.4 for most food groups. Information about alcohol consumption and the daily intake of 18 nutrients was estimated from the monthly food intake frequencies with either standard portion size (for most types of food) or subject-specified usual portion size (for rice, bread, and alcoholic and non-alcoholic beverages) using FFQ analysis software package for windows (Food-Frequency Questionnaire System, System Supply Co., LTD., Kanagawa, Japan). This FFQ analysis software computes an individual's food and nutrient intake from FFQ data based on "Standard tables of food composition in Japan" [28, 29].

The dietary carotenoid intakes of each individual were computed to obtain the amount of six carotenoids, lycopene,  $\alpha$ -carotene,  $\beta$ -carotene, lutein,  $\beta$ -cryptoxanthin, and zeaxanthin using a published database of the carotenoid composition of fruit and vegetables [30, 31]. In our survey, we calculated an individual's carotenoid intake from important sources of carotenoids. In this data analysis, the dietary carotenoid intakes were calculated from the FFQ data of individual food items not dishes [32].

The dietary intakes of total energy, calcium, potassium, magnesium, vitamins C, D, and E, preformed retinol, lycopene,  $\alpha$ -carotene,  $\beta$ -carotene, lutein,  $\beta$ -cryptoxanthin, and zeaxanthin of each subject were used in this report.

### Statistical analyses

For this study, the following subjects were excluded from the data analyses: (1) those who reported a history of osteoporosis or taking medications for bone metabolism in the self-administered questionnaire ( $n=14$ ); (2) those for whom the self-administered questionnaire data were incomplete ( $n=1$ ); and (3) those for whom blood samples for serum-carotenoid analysis were not collected ( $n=1$ ). As a result, a total of 293 post-menopausal female subjects were included in further data analysis.

Intakes of preformed retinol, vitamins C, D, and E, and six carotenoids were skewed toward the higher concentrations. These values were loge (natural)-transformed to

improve the normality of their distribution. All variables were presented as an original scale. The data are expressed as means (standard deviation), geometric mean (95% confidence interval), range, or percent.

A principal component analysis was used to derive the dietary patterns on the basis of the intakes of nine antioxidant vitamins and carotenoids obtained from the FFQ. To identify the number of factors to be retained, we used the criterion of eigenvalues  $>1.0$ , the most widely used criterion in factor analysis. Finally, we decided to retain three factors for further analysis. We applied a varimax rotation to the factor-loading matrix to achieve a simpler structure with greater interpretability. After the varimax rotation, the factor scores for each subject were saved from the principal component analysis. The factor-loading matrix represents correlation coefficients between individual antioxidants and dietary patterns. The percentage of variance explained by each factor was calculated by dividing the sum of the squares of the respective factor loadings by the number of variables.

Participants were divided into three categories according to tertiles of factor scores. Low radial BMD was defined as the lowest quartile of the value among study participants, i.e., equal to or less than  $0.501 \text{ g/cm}^2$  in post-menopausal female subjects. To assess the relationship between dietary patterns and low radial BMD, logistic regression analyses were performed using three models. In model 1, we adjusted for age, weight, and height. Model 2: Years since menopause, current tobacco use, regular alcohol intake, exercise habits, supplement use, and total energy intake were further adjusted. Model 3: Intakes of calcium, magnesium, potassium, and vitamin D were further adjusted. The goodness-of-fit for logistic regression model was evaluated by Hosmer–Lemeshow Goodness-of-Fit test, and then, we calculated odds ratios.

For dietary intake of each antioxidant vitamin and carotenoid, participants were further divided into three categories according to tertiles of antioxidant vitamin and carotenoid intake, and logistic regression analyses were performed to assess the relationship between antioxidant vitamin and carotenoid intake with low radial BMD.

All statistical analyses were performed using a statistical software package for Windows (SPSS ver. 17.0, SPSS Inc., Chicago, IL, USA) on a personal computer.

### Results

Clinical, biochemical, and nutrient intake profiles of study subjects

Table 1 shows the characteristics of the study subjects. The mean radial BMD in post-menopausal Japanese female

**Table 1** Characteristics of the study subject

	Post-menopausal female	
Number study subjects	293	
Age (years)	60.2	(6.2)
Body height (cm)	152.0	(5.5)
Body weight (kg)	51.9	(7.6)
Body mass index (kg/m <sup>2</sup> )	22.5	(3.0)
Bone mineral density (g/cm <sup>2</sup> )	0.561	(0.084)
Range	0.366–0.820	
Intake		
Total energy including ethanol (MJ/day)	8.20	(2.01)
Total energy excluding ethanol (MJ/day)	8.15	(2.00)
Calcium (mg/day)	651	(256)
Potassium (mg/day)	2910	(967)
Magnesium (mg/day)	281	(81)
Retinol (μg/day) <sup>a, b</sup>	281	(259–305)
Vitamin C (mg/day) <sup>b</sup>	170	(161–179)
Vitamin D (μg/day) <sup>b</sup>	6.4	(5.9–6.9)
Vitamin E (mg/day) <sup>b</sup>	8.1	(7.8–8.4)
Lycopene (mg/day) <sup>b</sup>	0.15	(0.13–0.17)
α-Carotene (mg/day) <sup>b</sup>	0.25	(0.23–0.27)
β-Carotene (mg/day) <sup>b</sup>	1.86	(1.74–1.99)
Lutein (mg/day) <sup>b</sup>	2.06	(1.92–2.20)
β-Cryptoxanthin (mg/day) <sup>b</sup>	0.62	(0.52–0.73)
Zeaxanthin (mg/day) <sup>b</sup>	0.67	(0.61–0.73)
Current tobacco use (%)	1.7	
Exercise habits (%) <sup>c</sup>	21.5	
Regular alcohol intake (%) <sup>c</sup>	11.0	
Current supplement use (%)	9.6	

Data are mean (standard deviation), geometric mean (95% confidence interval)

<sup>a</sup> Preformed retinol

<sup>b</sup> These variables were represented as original scale after analysis by log

<sup>c</sup> ≥1 time per week

subjects was 0.561 g/cm<sup>2</sup>. The percent of subjects with osteoporosis whose radial BMD was less than 70% of that of the young adult mean was 9.2% [33]. The mean daily intakes of calcium, potassium, magnesium, preformed retinol, and vitamins C, D, and E were at least comparable to the recommended dietary allowance. Of the six carotenoids analyzed, that with the highest intake was lutein; the second was β-carotene, and the lowest was lycopene. In our survey, 9.6% of study subjects used supplements, but most used multivitamin supplements. The rate of supplement users among study subjects for vitamin C and D, β-carotene, and calcium were 3.1%, 0.3%, 0.7%, and 5.5%, respectively. Therefore, we think that specific quantitative intakes of vitamin, carotenoid, and mineral from supplement were negligible compared with those from foods.

### Principal component analysis of dietary patterns of antioxidant vitamin and carotenoid intake

The factor-loading matrices for the three retained factors are shown in Table 2. The high positive loadings indicate strong associations between given antioxidants and dietary patterns. Factor 1 had heavy loadings on β-carotene, α-carotene, lutein, lycopene, and vitamins E and C. This pattern was especially heavily loaded on carotenoids and was labeled the “Carotene” pattern. Factor 2 had heavy loadings on preformed retinol, zeaxanthin, vitamin E, lutein, vitamin C, and β-carotene. This pattern, heavily loaded on preformed retinol, zeaxanthin, and vitamin E, was labeled the “Retinol” pattern. Factor 3 had heavy loadings on β-cryptoxanthin, vitamin C, β-carotene, lutein, and vitamin E. This pattern, heavily loaded on β-cryptoxanthin and vitamin C, was labeled the “β-cryptoxanthin” pattern. Overall, the three dietary patterns accounted for 73.1% of the variance in antioxidant vitamin and carotenoid intake.

### Odds ratio of low radial BMD in the highest group of factor scores of each dietary pattern

The odds ratios of low radial BMD associated with the tertiles of factor scores of each of the three dietary patterns after adjustments for confounding factors are shown in Table 3. The odds ratios for the risk of low radial BMD in the highest tertile of factor scores against the lowest tertile used for the reference group were calculated. In the

**Table 2** Factor-loading matrix for the three dietary patterns of antioxidant vitamins and carotenoid intakes identified among 293 post-menopausal Japanese female subjects

	Factor 1: carotene	Factor 2: retinol	Factor 3: β- cryptoxanthin
Retinol <sup>a</sup>		0.825	
Vitamin C	0.435	0.285	0.773
Vitamin E	0.464	0.711	0.258
Lycopene	0.633		
α-Carotene	0.788		
β-Carotene	0.852	0.257	0.369
Lutein	0.740	0.447	0.270
β-Cryptoxanthin			0.920
Zeaxanthin		0.712	
Percentage of variance (%)	30.3	22.8	20.1

Data for 293 subjects from the self-administered food-frequency questionnaire. Absolute values <0.25 were excluded from the table for simplicity

<sup>a</sup> Preformed retinol

**Table 3** The odds ratios (and 95% confidence intervals) of tertiles of three dietary patterns on low bone mineral density in post-menopausal Japanese female subjects

Dietary patterns	Factor score	Number	Model 1			Model 2			Model 3		
			OR	95% CI	<i>P</i> for trend	OR	95% CI	<i>P</i> for trend	OR	95% CI	<i>P</i> for trend
Factor 1: carotene	Lowest (Q1)	97	1.00			1.00			1.00		
	Middle (Q2)	98	0.83	(0.40–1.72)		0.94	(0.44–2.00)		1.14	(0.51–2.54)	
	Highest (Q3)	98	1.31	(0.65–2.64)	0.370	1.38	(0.66–2.89)	0.340	2.30	(0.93–5.70)	0.064
Factor 2: retinol	Lowest (Q1)	97	1.00			1.00			1.00		
	Middle (Q2)	98	1.16	(0.55–2.45)		1.35	(0.61–2.98)		1.08	(0.47–2.47)	
	Highest (Q3)	98	2.02	(0.99–4.09)	0.041	3.09	(1.28–7.47)	0.009	2.31	(0.90–5.89)	0.059
Factor 3: $\beta$ -cryptoxanthin	Lowest (Q1)	97	1.00			1.00			1.00		
	Middle (Q2)	98	0.55	(0.26–1.16)		0.54	(0.25–1.18)		0.53	(0.24–1.17)	
	Highest (Q3)	98	0.26	(0.11–0.59)	0.001	0.22	(0.09–0.54)	0.001	0.30	(0.11–0.77)	0.017

Model 1: Age, weight and height were adjusted. Model 2: Years since menopause, current tobacco use, regular alcohol intake, exercise habits, supplement use, and total energy intake were further adjusted. Model 3: Intakes of calcium, magnesium, potassium, and vitamins D were further adjusted

“Carotene” pattern, there was no significant association between the factor score and low radial BMD. In the “Retinol” pattern, a significantly higher odds ratio was observed in the highest tertile of factor score after adjustments for age, years since menopause, weight, height, current tobacco use, regular alcohol intake, exercise habits, use of dietary supplements, and total energy. However, this significant association was not observed after further adjustments for intake of calcium, magnesium, potassium, and vitamin D. On the other hand, in the “ $\beta$ -cryptoxanthin” pattern, a significantly lower odds ratio was observed in the highest tertile of factor scores after multivariate adjustment.

Odds ratios of low radial BMD in the highest group of antioxidant vitamin and carotenoid intake

The odds ratios for the risk of low radial BMD associated with the tertiles of daily intakes of each antioxidant vitamin and carotenoid after adjustments for confounding factors are shown in Table 4. A significantly higher odds ratio was observed in the highest tertile of preformed retinol intake after adjustments for age, weight, and height. This significant association was also observed after multivariate adjustments. Similarly, a significantly higher odds ratio was observed in the highest tertile of zeaxanthin intake after adjustments for age, years since menopause, weight, height, current tobacco use, regular alcohol intake, exercise habits, use of dietary supplements, and total energy, but this significant association was not observed after further adjustments for intakes of calcium, magnesium, potassium, and vitamin D. In contrast, a significantly lower odds ratio was observed in the highest tertile of vitamin C intake after

multivariate adjustments. Also, a significantly lower odds ratio was observed in the highest tertile of  $\beta$ -cryptoxanthin intake after adjustments for age, years since menopause, weight, height, current tobacco use, regular alcohol intake, exercise habits, use of dietary supplements, and total energy, but this significant association was not observed after further adjustments for intakes of calcium, magnesium, potassium, and vitamin D.

Next, study subjects were divided into two groups by median values of vitamin C and/or  $\beta$ -cryptoxanthin intake. And then, all subjects were ranked into four groups as follows: group 1: lower intake of vitamin C (47–169 mg/day) with lower intake of  $\beta$ -cryptoxanthin (0–0.96 mg/day); group 2: lower intake of vitamin C (47–169 mg/day) with higher intake of  $\beta$ -cryptoxanthin (0.97–7.91 mg/day); group 3, higher intake of vitamin C (170–625 mg/day) with lower intake of  $\beta$ -cryptoxanthin (0–0.96 mg/day); group 4, higher intake of vitamin C (170–625 mg/day) with higher intake of  $\beta$ -cryptoxanthin (0.97–7.91 mg/day). In both groups of higher intake of vitamin C with lower intake of  $\beta$ -cryptoxanthin and/or lower intake of vitamin C with higher intake of  $\beta$ -cryptoxanthin, significantly lower odds ratios were not observed against the lower intake group of both of them used for the reference group. In contrast, a significantly lower odds ratio was observed in the higher intake group of both of them after adjustments for age, years since menopause, weight, height, current tobacco use, regular alcohol intake, exercise habits, use of dietary supplements, and total energy (Table 5). However, this significant lower odds ratio became insignificant after further adjustments for intakes of calcium, magnesium, potassium, and vitamin D (data not shown).

**Table 4** The odds ratios (and 95% confidence intervals) of tertiles of antioxidant intakes on low bone mineral density in post-menopausal Japanese female subjects

Dietary intake	Number	Range (mg/d) or (μg/d)	Model 1			Model 2			Model 3			
			OR	95% CI	<i>P</i> for trend	OR	95% CI	<i>P</i> for trend	OR	95% CI	<i>P</i> for trend	
Retinol <sup>a</sup>	Lowest (Q1)	97	(29–213)	1.00			1.00			1.00		
	Middle (Q2)	98	(218–383)	1.30	(0.61–2.75)		1.65	(0.74–3.69)		1.28	(0.56–2.94)	
	Highest (Q3)	98	(386–3531)	2.37	(1.16–4.85)	0.014	3.22	(1.38–7.51)	0.007	2.52	(1.03–6.14)	0.031
Vitamin C	Lowest (Q1)	96	(47–139)	1.00			1.00			1.00		
	Middle (Q2)	99	(140–214)	1.15	(0.55–2.40)		1.02	(0.47–2.22)		1.03	(0.45–2.36)	
	Highest (Q3)	98	(215–625)	0.35	(0.15–0.80)	0.004	0.25	(0.10–0.66)	0.001	0.25	(0.07–0.82)	0.010
Vitamin E	Lowest (Q1)	101	(3.2–7.2)	1.00			1.00			1.00		
	Middle (Q2)	97	(7.3–9.1)	0.61	(0.29–1.27)		0.56	(0.25–1.25)		0.49	(0.21–1.14)	
	Highest (Q3)	95	(9.2–30.9)	0.61	(0.29–1.27)	0.244	0.45	(0.16–1.31)	0.176	0.43	(0.14–1.36)	0.193
Lycopene	Lowest (Q1)	121	(0.00–0.06)	1.00			1.00			1.00		
	Middle (Q2)	76	(0.15–0.15)	1.02	(0.48–2.15)		1.10	(0.51–2.35)		1.06	(0.48–2.34)	
	Highest (Q3)	96	(0.36–1.78)	1.55	(0.79–3.04)	0.177	1.72	(0.85–3.47)	0.117	1.60	(0.75–3.38)	0.201
α-Carotene	Lowest (Q1)	95	(0.03–0.23)	1.00			1.00			1.00		
	Middle (Q2)	97	(0.24–0.37)	0.73	(0.36–1.48)		0.79	(0.38–1.66)		0.90	(0.42–1.95)	
	Highest (Q3)	101	(0.38–1.27)	0.77	(0.38–1.57)	0.522	0.78	(0.36–1.67)	0.551	1.05	(0.45–2.45)	0.882
β-Carotene	Lowest (Q1)	97	(0.34–1.52)	1.00			1.00			1.00		
	Middle (Q2)	99	(1.53–2.36)	0.61	(0.29–1.27)		0.63	(0.29–1.35)		0.74	(0.32–1.70)	
	Highest (Q3)	97	(2.37–8.19)	0.75	(0.37–1.53)	0.586	0.69	(0.31–1.55)	0.487	0.93	(0.33–2.62)	0.981
Lutein	Lowest (Q1)	98	(0.49–1.68)	1.00			1.00			1.00		
	Middle (Q2)	97	(1.70–2.58)	1.56	(0.74–3.28)		1.84	(0.83–4.06)		2.10	(0.89–4.93)	
	Highest (Q3)	98	(2.59–10.01)	1.25	(0.59–2.62)	0.762	1.39	(0.60–3.23)	0.698	1.94	(0.69–5.48)	0.339
β-Cryptoxanthin	Lowest (Q1)	98	(0.00–0.30)	1.00			1.00			1.00		
	Middle (Q2)	101	(0.31–1.21)	0.52	(0.25–1.10)		0.47	(0.22–1.01)		0.49	(0.22–1.10)	
	Highest (Q3)	94	(1.22–7.91)	0.46	(0.21–1.00)	0.099	0.40	(0.17–0.92)	0.068	0.53	(0.22–1.28)	0.295
Zeaxanthin	Lowest (Q1)	95	(0.08–0.46)	1.00			1.00			1.00		
	Middle (Q2)	100	(0.47–0.96)	1.73	(0.82–3.65)		1.95	(0.89–4.27)		1.71	(0.75–3.87)	
	Highest (Q3)	98	(0.97–6.09)	1.96	(0.93–4.13)	0.104	2.65	(1.11–6.31)	0.038	2.51	(0.99–6.33)	0.061

Model 1: Age, weight and height were adjusted. Model 2: Years since menopause, current tobacco use, regular alcohol intake, exercise habits, supplement use, and total energy intake were further adjusted. Model 3: Intakes of calcium, magnesium, potassium, and vitamins D were further adjusted

<sup>a</sup> Preformed retinol

**Table 5** The odds ratios (and 95% confidence intervals) of four groups stratified by dietary intakes of vitamin C and  $\beta$ -cryptoxanthin on low bone mineral density in post-menopausal Japanese female subjects

		$\beta$ -Cryptoxanthin intake					
		Low intake (0–0.96mg/d)			High intake (0.97–7.91mg/d)		
		Number	OR	95%CI	Number	OR	95%CI
Vitamin C intake	Low intake (47–169 mg/d)	113	1.00	(Reference)	34	0.73	(0.27–1.99)
	High intake (170–625 mg/d)	36	0.52	(0.18–1.52)	110	0.42	(0.19–0.93)

Age, weight, height, years since menopause, current tobacco use, regular alcohol intake, exercise habits, supplement use, and total energy intake were adjusted

## Discussion

The objective of this study was to investigate the associations of dietary patterns of antioxidant vitamin and carotenoid intake with radial BMD in post-menopausal Japanese female subjects. The results indicate that radial BMD was significantly associated with a dietary pattern heavily loaded on  $\beta$ -cryptoxanthin and vitamin C. Furthermore, we found that a high intake of vitamin C with  $\beta$ -cryptoxanthin was inversely associated with a low radial BMD. This investigation is the first reported cross-sectional study to examine the association of dietary patterns of antioxidant vitamin and carotenoid intake with BMD. Numerous antioxidant vitamins and carotenoids are contained in fruits and vegetables, and several recent epidemiological reports have shown inverse associations of antioxidant vitamin and carotenoid intake or serum level with low BMD, risk of fracture, and/or risk of osteoporosis [16–18]. However, the association of BMD with dietary patterns of antioxidant vitamin and carotenoid intake has not been thoroughly studied. Our findings further support the hypothesis that high intakes of fruits and vegetables rich in antioxidant vitamins and carotenoids, especially vitamin C and  $\beta$ -cryptoxanthin, may be beneficial to bone health in post-menopausal women.

On the other hand, some epidemiological studies have reported that excessive intake of retinol may have adverse effects on BMD [34–36]. In our study, a positive association between the factor score of the “Retinol” pattern and low radial BMD was observed after adjustments for age, years since menopause, weight, height, current tobacco use, regular alcohol intake, exercise habits, use of dietary supplements, and total energy. For dietary antioxidants, a significantly higher odds ratio was observed in the highest tertile of preformed retinol intake against the lowest tertile used for the reference group after multivariate adjustments. The recommended daily intake of retinol activity equivalents is 600  $\mu$ gRE/day for Japanese women, with a tolerable upper intake of 3,000  $\mu$ gRE/day [37]. In the highest tertile of preformed retinol intake, all of them consumed more than the recommended dietary allowance

for Japanese adult females (600  $\mu$ gRE/day) although most subjects consumed less than 3,000  $\mu$ gRE/day. The effect of the dietary amount of preformed retinol on bone metabolism in Japanese female subjects has not been studied in detail, but a high intake of preformed retinol may be associated with the risk for low radial BMD. Further study is required.

In our data analyses, we identified three dietary patterns of antioxidant vitamin and carotenoid intake from the principal component analysis. Although all dietary patterns were heavily loaded on vitamin C intake, the highest positive loading between vitamin C, and dietary pattern was observed in the “ $\beta$ -cryptoxanthin” pattern. On the other hand, an association between  $\beta$ -cryptoxanthin and dietary pattern was observed in only the “ $\beta$ -cryptoxanthin” pattern, which had an extremely high factor loading. Vitamin C and  $\beta$ -cryptoxanthin are especially concentrated in citrus fruits such as Japanese mandarin orange. Therefore, a high intake of citrus fruit may be inversely associated with low BMD. In fact, in our previous study, we found that fruit intake was inversely associated with low radial BMD [25].

In animals, an experimentally induced deficiency of vitamin C led to impairments in bone mass, cartilage, and connective tissues [38, 39]. The protein in the bone matrix is over 90% collagen [40]. Vitamin C is an essential cofactor for the formation of collagen and the synthesis of hydroxyproline and hydroxylysine [41]. Therefore, vitamin C is an important micronutrient for the maintenance of bone health. Furthermore, it is well known that vitamin C reduces oxidative stress by scavenging singlet oxygen and peroxy radicals. The relationship between oxidative stress and BMD or osteoporosis has recently been reported [16–18]. From the finding of osteoporosis in mice lacking NF- $\kappa$ B1 and NF- $\kappa$ B2, Iotsova et al. reported that NF- $\kappa$ B proteins are important for osteoclastogenesis [42]. NF- $\kappa$ B is activated by the exposure of cells to oxidative stress [43]. Therefore, it seems that reactive oxygen species enhance osteoclastogenesis and bone resorption. In fact, some studies have implicated reactive oxygen species in bone regulation [44, 45]. Furthermore, in epidemiological studies, it was reported that oxidative stress levels were

negatively associated with BMD and that antioxidant levels were lower in osteoporotic patients [19–24]. These previous findings in epidemiological and experimental studies suggest that antioxidant micronutrients may provide benefits to bone metabolism against oxidative stress. Therefore, it seems that vitamin C is an important micronutrient for the maintenance of bone health through its biological action on cofactors for collagen formation, the synthesis of hydroxyproline and hydroxylysine, and antioxidant activity.

Carotenoids, as antioxidants, may also play an important role in the prevention of oxidative stress-related osteoclastogenesis and bone resorption. Very recently, Yamaguchi et al. reported the beneficial effects of  $\beta$ -cryptoxanthin on bone metabolism [46–48]. Through *in vitro* and *in vivo* studies, they found that  $\beta$ -cryptoxanthin stimulated bone formation and inhibited bone resorption. Their results support the idea that  $\beta$ -cryptoxanthin may have a direct stimulatory effect on bone formation and an inhibitory effect on bone resorption. Recent epidemiological studies have shown an association of serum  $\beta$ -cryptoxanthin with bone health. Yang et al. examined serum-carotenoid concentrations in postmenopausal American female subjects and found that the serum concentrations of  $\beta$ -cryptoxanthin and lycopene were significantly lower in osteoporotic subjects than in non-osteoporotic subjects [22]. Furthermore, we found that serum  $\beta$ -cryptoxanthin was significantly but partially associated with radial BMD [25]. The results of these experimental and epidemiological studies strongly support the hypothesis that the development of osteoporosis may be reduced by  $\beta$ -cryptoxanthin intake.

In our data analysis, significantly lower odds ratios in the highest tertiles of vitamin C and  $\beta$ -cryptoxanthin intakes were observed, but these significant associations were not observed after adjusting for  $\beta$ -cryptoxanthin and/or vitamin C intakes, respectively (OR, 0.36; CI, 0.12–1.11 for vitamin C and OR, 0.70; CI, 0.27–1.90 for  $\beta$ -cryptoxanthin). These results indicate that a combined intake of vitamin C and  $\beta$ -cryptoxanthin may be associated with radial BMD. Next, we examined the association of low radial BMD with the combined intake of vitamin C and  $\beta$ -cryptoxanthin. A significantly lower odds ratio was observed in the high-intake group for both of vitamin C and  $\beta$ -cryptoxanthin than in the low-intake group for both nutrients after adjustments for age, years since menopause, weight, height, current tobacco use, regular alcohol intake, exercise habits, use of dietary supplements, and total energy. However, this significantly lower odds ratio became insignificant after further adjustments for intakes of calcium, magnesium, potassium, and vitamin D (data not shown). For this reason, we think that these micronutrients might be more relevant factors for BMD rather than vitamin C and  $\beta$ -cryptoxanthin, or there is no denying the possibility of multicollinearity among these nutrients because these

micronutrients were also rich in fruit and vegetables. From these results, we concluded that the intakes of vitamin C and  $\beta$ -cryptoxanthin may be significantly but partially associated with radial BMD, and these associations may be caused by a combination of vitamin C and  $\beta$ -cryptoxanthin. To our knowledge, there has been no experimental or epidemiological study of the combined effect of vitamin C and carotenoid on bone metabolism. It is conceivable that, rather than vitamin C alone, vitamin C intake combined with the intakes of other antioxidants such as carotenoids may yield an important dietary pattern conducive to the maintenance of bone health. Further studies on the complicated interactions of antioxidants on bone metabolism are required.

This study had some limitations. First, the data obtained here cross-sectional; therefore, only limited inferences can be made regarding temporality and causation. Furthermore, the sample size was limited, and thus further large-scale studies are required. Second, in our survey, portion size questions were not included for most items. Absolute nutrient intake could not be estimated from FFQ without portion size questions. Third, we evaluated radial BMD at 1/3 of the forearm length measured from the styloid process on the ulna. Therefore, an analysis of the association of serum carotenoids with BMD in cancellous bone, such as the femoral neck or lumbar spine, is required. Lastly, we could not evaluate the dietary patterns of other antioxidants such as flavonoids. Some studies have shown a beneficial effect of bioactive flavonoids on bone metabolism [49, 50].

In conclusion, dietary patterns heavily loaded on  $\beta$ -cryptoxanthin, and vitamin C are associated with radial BMD in post-menopausal Japanese female subjects. A high intake of vitamin C with  $\beta$ -cryptoxanthin is inversely associated with low radial BMD and may be beneficial to bone health. To determine whether antioxidant vitamins and carotenoids are beneficial to bone health, further cohort or intervention studies are required.

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**Conflicts of interest** None.

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## 2. サルコペニアの疫学

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しもかた ひろし あんどう ふじこ

- サルコペニアは40歳以上の地域在住男性の25.0%、女性の24.2%に認められる。
- 加齢とともに男女で筋力は年間約1%低下する。筋量は男性のみで低下し、女性では筋量の変化は少なく、筋肉の質的な変化があるものと思われる。
- 一般の高齢者では、筋力や筋量には遺伝子多型よりも生活習慣などの影響のほうが大きい。
- サルコペニアの主な危険因子は運動不足と低栄養、特に蛋白摂取の不足、カロテノイドやビタミンDの不足である。
- 適度な運動と適切な栄養摂取に心がけることで筋量や筋力の低下を防ぐことは十分可能であると考えられる。

### Key Words

サルコペニア, 疫学研究, カロテノイド, ビタミンD, DXA,

サルコペニアは高齢者のADLを低下させ、健康長寿実現の大きな障害となる。しかし、老化に伴う筋量減少の実態は明らかでなく、また日常生活機能との関連もはっきりはしていない。特に一般地域住民での日常生活機能と骨格筋量、筋力との関連についての疫学研究は日本ではほとんどない。高齢者のサルコペニアや脆弱を予防していくためには、日本におけるサルコペニアの実態を明らかにするとともに、サルコペニアの危険因子を明らかにするような観察研究や介入研究などの疫学的研究が重要である。

### □ サルコペニアの実態

サルコペニアの頻度はその定義により異なるが、米国でのNew Mexico Elderly Health Studyからの報告<sup>1)</sup>では、二重エネルギーX線吸収法(DXA)を用いた診断で、70歳未満では20%程度であるが、80歳以上になると50%以上がサルコペニアとなるとしている。

われわれは平成9年から、無作為抽出された地域住民を対象としたコホート研究「国立長寿医療研究センター・老化に関する長期縦断疫学研究(NILS-LSA)」を実施しており<sup>2)</sup>、第5次調査の参加者、40~88歳の中老年者2,419名(男性1,200名、女性1,219名)を対象としてサルコペ

ニアの頻度について検討を行った。DXA(QDR 4500, Hologic)装置をと用いて四肢除脂肪・除骨重量測定し、これを四肢筋量とした。前述のNew Mexico Elderly Health Studyの方法<sup>1)</sup>に準じ、四肢筋量(kg)を身長(m)の二乗で除した値をskeletal muscle index(SMI)とし、サルコペニアの指標とした。その判定基準には同じDXAのHologic QDR 4500で測定したSanadaら<sup>3)</sup>によるYAM(young adult mean:18~40歳の若年成人平均値)から標準偏差の2倍を引いた男性6.87kg/m<sup>2</sup>、女性5.46kg/m<sup>2</sup>を基準値としてサルコペニアの有無を判定した。その結果、この基準値から40歳以上の男性の25.0%が、女性の24.2%がサルコペニアに分類された(図1)。

運動神経線維のうち、筋線維を支配して実際の筋収縮に関与するα運動ニューロンは、加齢とともに50%も低下するといわれる。特に下肢では軸索が長くなって障害を受けやすい。また筋の増殖に必要な骨格筋組織特異的幹細胞であるサテライト細胞も数が減少することが知られている。食欲の低下や運動不足、性ホルモンの分泌低下、炎症反応の増大などサルコペニアを引き起こすさまざまな要因が、加齢に伴って増加する<sup>4,5)</sup>。

SMIは男性年間約0.3%低下するが、女性ではSMIの低下はほとんどない。一方、筋力は男女

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とも40歳以降、握力も下肢筋力も年間約1%ずつ減少する。このことは女性では筋肉の量的な変化よりも質的な変化が問題になっていることを示している。男性ではどの年代においても女性よりも筋力は強く、80代の男性の筋力は40代の女性の筋力にはほぼ等しい(図2)。もともと女性は男性よりも筋力が弱いために、加齢による筋力の低

下は女性により大きな影響を与える。

### □ サルコペニアの危険因子

ヨーロッパにおける老年学、栄養学などの学会によるワーキンググループである The European Working Group on Sarcopenia in Older People (EWGSOP)<sup>6)</sup>ではサルコペニアを、加齢以外に明らかな誘因がない原発性サルコペニア(primary sarcopenia)と、加齢以外の何らかの要因がサルコペニアを引き起こす二次性サルコペニア(secondary sarcopenia)に分類している。さらに二次性サルコペニアを、ベッド上安静、運動しない生活スタイル、廃用、無重力状態などが原因となる身体活動性サルコペニア(activity-related sarcopenia)、高度な臓器障害、炎症性疾患、悪性腫瘍に伴う悪液質、内分泌疾患などによる疾患性サルコペニア(disease-related sarcopenia)、吸収不良、胃腸疾患、食思不振を引き起こす薬剤の使用に伴うエネルギーおよび蛋白質摂取不足などによる栄養性サルコペニア(nutrition-related sarcopenia)の3つに分けている(表1)。

これまでに報告されているサルコペニアの危険因子には遺伝的素因、性別、加齢、身長、体重、BMI、閉経、エストロゲン、テストステロン、

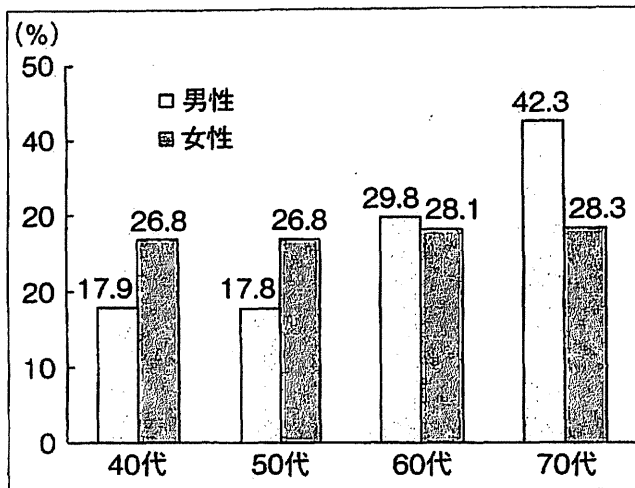


図1 年齢・性別にみたサルコペニアの頻度

DXA法により性別の若年成人平均値(YAM)の-2SDを基準としてサルコペニアの判定を行った。女性では年齢による変化はなかったが、男性では年代上昇で割合が有意に上昇していた(Cochran-Mantel-Haenszel検定でp trend<0.001)。

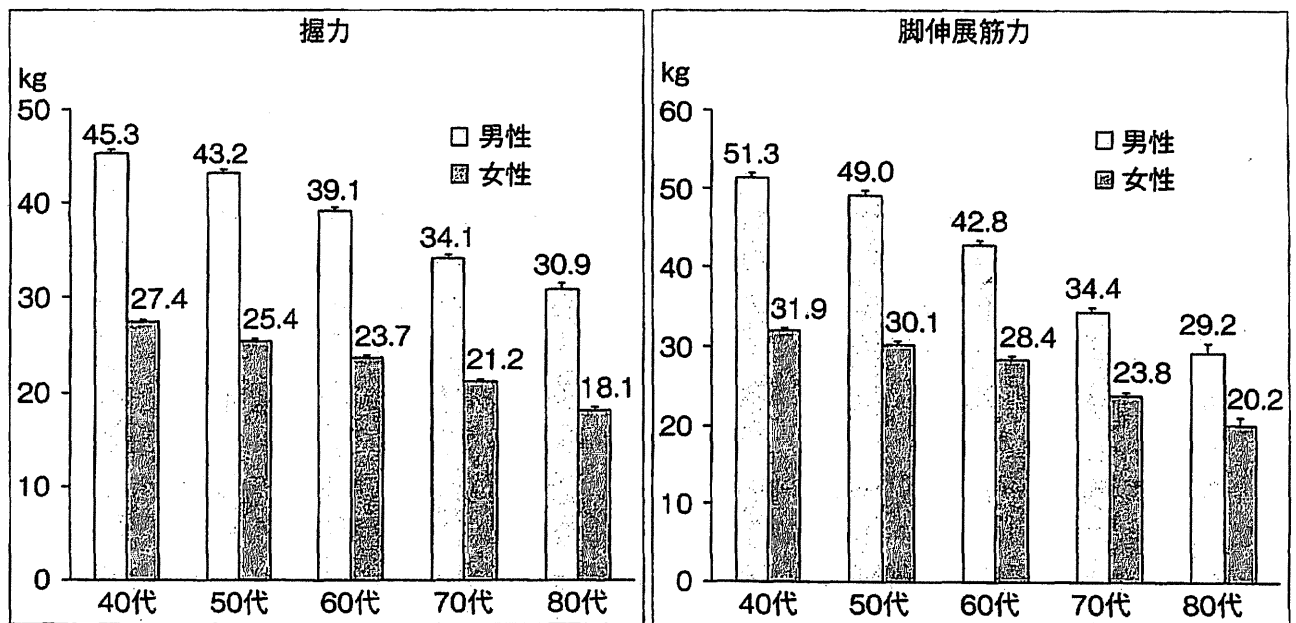


図2 年齢・性別にみた筋力

利き手の握力および脚伸展筋力(大腿四頭筋筋力)の年代別平均値および標準誤差。握力、脚伸展筋力は男女ともに、年代上昇に伴い減少していた(p trend<0.001)。しかし、男性では握力は80代でも女性の40代よりも大きく、また男性の脚伸展筋力は80代でも女性の40代とほぼ同じ値であった。

表1 サルコペニアの分類

分類	原因
原発性サルコペニア	
加齢性サルコペニア	加齢以外の原因がない
二次性サルコペニア	
身体活動性サルコペニア	ベッド上安静, 運動しない生活スタイル, 廃用, 無重力状態
疾患性サルコペニア	高度な臓器障害 (心臓, 肺, 肝臓, 腎臓, 脳), 炎症性疾患, 悪性腫瘍, 内分泌疾患
栄養性サルコペニア	吸収不良, 胃腸疾患, 食思不振を引き起こす薬剤の使用に伴うエネルギー, 蛋白質摂取不足

(Cruz-Jentoft AJ, et al. : Age Ageing 39, 412-423, 2010. より引用)<sup>6)</sup>

総体脂肪量, 身体活動, カロテノイド, ビタミンD, 分岐鎖アミノ酸および蛋白質摂取量などがある。遺伝的な素因としては, myostatin の Lys153Arg 多型, alpha-actinin 3 の R577X 多型が筋量や筋力に関連しているとの報告がある<sup>7,8)</sup>。しかし, レジスタンストレーニングを行うスポーツ選手ではこうした遺伝子多型の影響があるとしても, 一般の高齢者では遺伝子多型よりもむしろ生活習慣のほうが筋力や筋量への影響が大きいと思われる。

#### □ 身体活動とサルコペニア

運動不足による筋量や筋力の低下はどの年代にも起きうる。しかし高齢者では筋の再生・増殖機能が低下しており, いったん減少した筋量は回復が難しい。筋量が低下し筋力が低下すれば, 運動が困難になり, さらに筋量が低下するという悪循環に陥りやすい<sup>1)</sup>。一方, 筋力トレーニングを中心とした運動介入により高齢者でも筋力や筋量が増加するという報告は多く<sup>4,6)</sup>, 身体活動はサルコペニア予防の重要な要素であるともいえる。

#### □ 性ホルモンとサルコペニア

閉経により内臓脂肪は増加し, 骨密度が低下し, 筋量および筋力が低下する。一方, エストロゲンの投与はこれらの変化を予防する効果があるとされる。テストステロンの筋増殖効果はよく知られている<sup>9)</sup>。高齢男性のテストステロンの低下と筋量, 筋力の低下が報告されている。加齢に伴い, 性ホルモン結合グロブリン (SHBG) が増加し, 生体作用を持つ遊離テストステロンが大きく低下

する。テストステロンは蛋白合成を促進する。テストステロンの低下は蛋白合成能の低下をきたし, 筋肉を萎縮させる。さらにテストステロンの低下は筋サテライト細胞数の低下を引き起こし, 筋肉の再生・増殖能を低下させるといわれている<sup>10)</sup>。

#### □ カロテノイドとサルコペニア

高齢者の筋力低下, 身体機能低下はフリーラジカルによる酸化ストレスが原因の1つとなっている可能性が指摘されている。酸化ストレスは骨格筋のDNAを傷つけ, 蛋白質や脂質に障害を与える<sup>11,12)</sup>。

抗酸化作用を持つカロテノイドが不足すると, 高齢者では筋力低下や歩行障害をきたすことが, いくつかの疫学的研究で報告されている。米国の Women's Health and Aging Studies では, 年齢, 人種, 喫煙, 心血管性疾患, 関節炎, 血清IL-6を調整して検討したところ, 血清総カロテノイドの低下は握力, 腰や脚の筋力の低下と有意に関連していた<sup>13)</sup>。イタリア, トスカーナ州キャンティ地区の地域在住高齢者での研究でも,  $\beta$ カロテン摂取量が高齢者の脚伸展筋力と相関していた<sup>14)</sup>。

NILS-LSA のデータでは血清カロテノイドと体力・運動やADLとの関係が示されている。外出に不安がある人, 階段の昇降や長距離の歩行が困難である人では, 血清カロテノイドが低値を示した。一方, 筋力や余暇活動時間, 一日平均歩数は血清カロテノイドと正の相関を示し, 特に日常活動量を示す一日平均歩数はすべての血清カロテノイドで正の関連を示していた。摂取エネルギー

で調整したβカロテン摂取量も正の関連を示したことから、単に「元気な人がたくさん食べている」のではなく、多く摂取する人が体力的にも健康であり、また、ADLの低下している人ではカロテノイドが不足している状況が明らかになった。

## □ ビタミンDとサルコペニア

血中の25-OHビタミンDレベルは経口摂取、あるいは皮膚で産生されたビタミンDの量を反映する指標である。25-OHビタミンDは老化とともに低下することが知られている。ビタミンDはカルシウム代謝に関連するビタミンであり、摂取量の不足は骨粗鬆症などの骨疾患の要因となる。このビタミンDが筋肉とも関連することが明らかになってきた。

ビタミンD受容体は筋肉中に存在し、ビタミンDが低下することで筋肉の蛋白同化作用が下がってしまう。またビタミンD受容体の遺伝子多型が高齢者のサルコペニアの要因の1つであることも報告されている<sup>15)</sup>。ビタミンDの低下が、高齢者の転倒や身体機能障害の要因であるとの報告がある<sup>16,17)</sup>。アムステルダム縦断加齢研究(Longitudinal Aging Study Amsterdam)での3年間の追跡研究ではベースラインの25-OHビタミンDが低値の場合には、高値の場合に比べて3年後にサルコペニアとなるオッズ比は2.57(95%信頼区間1.40~4.70)であった。このような結果からビタミンDの摂取の不足が、高齢者のサルコペニアを引き起こす可能性があると思われる<sup>18)</sup>。

## □ 蛋白質、アミノ酸とサルコペニア

筋肉は蛋白質からなっており、蛋白質摂取量、アミノ酸摂取量が低下すれば、筋量は当然低下する。1食あたりの蛋白質量が20~25gであるときが筋蛋白質の合成がもっとも高いとされている<sup>19,20)</sup>。食事摂取基準では、健康な70歳以上者に必要な蛋白質量は1.06g/体重kgであり、男性60g、女性50g以上が必要とされている。しかし、エネルギー消費量が減少し、食事量全体が少なくなってしまう高齢者の食事では、必要な蛋白質摂取を維持することが難しい場合もある。

体内で合成できない必須アミノ酸のうちロイシン、イソロイシン、バリンは炭素骨格が分岐した構造をもつことから分岐鎖アミノ酸と呼ばれる。これらの分岐鎖アミノ酸は筋肉をつくる主な蛋白質であるアクチンとミオシンの主成分である。十分な分岐鎖アミノ酸を摂取することで筋肉の消耗を防ぐことができる可能性がある<sup>21,22)</sup>。分岐鎖アミノ酸は肉類、乳製品、レバーなどに多く含まれるが、高齢者ではこうした食品は敬遠されることが多いことも、高齢者のサルコペニアの要因となっている可能性がある。

## まとめ

サルコペニアは40歳以上の地域在住男性の25.0%、女性の24.2%に認められ、多くの方がサルコペニアの状態にある。サルコペニアは特に高齢者に多くみられ、健康長寿達成の大きな障害となる。サルコペニアの予防が、高齢化がさらに進んでいく今後の日本にとって重要な課題である。サルコペニアの主な危険因子は運動不足と低栄養、特に蛋白質摂取の不足、カロテノイドやビタミンDの不足である。筋力や筋量の低下には、老化による避けがたい生理的な要因もあるが、適度な運動と適切な栄養摂取に心がけることで、筋量や筋力の低下を防ぐことは十分可能である。

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## Seminar

# 2. 虚弱の危険因子

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### KEY WORD

- 虚弱
- 高齢者
- 危険因子
- 体力
- 老化

### SUMMARY

高齢者の虚弱は年齢が高いほど割合が高くなり、また男性よりも女性で割合が高い。虚弱の要因としては、低栄養、喫煙、慢性疾患への罹患、慢性炎症、性ホルモンの減少などの身体的要因に加えて、世帯の年収や教育歴、人種、生活空間など社会的な因子も重要である。高齢者ではこれらの多くの要因が重積し、虚弱を引き起こすものと考えられる。高齢者の虚弱に対しての運動や栄養による介入研究が数多く行われているが、その効果ははっきりしていない。運動介入や栄養の単独の介入では虚弱の予防は難しく、生活全般のサポートでの対応が望まれる。

### はじめに

日本人の平均寿命は年々長くなり、高齢者、特に後期高齢者の人口が急増している。しかし、高齢になるほど虚弱な高齢者は増加する。一方で少子化が進み、今後は若い労働力が不足していくことが予想される。そのような状況で、介護のために若い人材の労力が費やされるようでは、日本の国が成り立っていかなくなってしまうだろう。高齢化する日本の社会で、介護や支援を要するような虚弱な高齢者を減らし、健康長寿を達成することは急務となっている。本稿では、高齢者の虚弱の要因を明らかにし、その予防法を探る。

### 性別、加齢と虚弱

虚弱は男性よりも女性に多い。日本では平均寿命は女性の方が男性よりも7歳近く長い。寝たきりの期間も女性の方が長く、虚弱の女性の数は男性よりも多い。しかし年齢を調整しても

虚弱のリスクは男性よりも女性の方が高い<sup>1)</sup>。また、虚弱な女性の死亡率は男性よりも高いという報告もある<sup>2)</sup>。

虚弱の定義にもよるが、虚弱は75歳以上の20~30%に認められ、高齢になるほどその割合は高くなる<sup>3)</sup>。多くの研究で、加齢は虚弱の最も強い危険因子の1つに挙げられている。しかし、加齢そのものが虚弱の要因なのか、加齢に伴って生じる様々な障害や疾病が虚弱の要因であって、これらの要因をすべて除いても加齢が虚弱の要因であるかどうかについては、まだ十分には明らかにされていない。

### 生活習慣と虚弱

高齢者では一般に身体活動量が減り、また歯の脱落、嗅覚や味覚の低下、消化機能の低下など生理学的な要因に加えて、抑うつなどの精神的な要因のため食欲が低下する。こうした生活習慣の変化が高齢者の虚弱を引き起こす可能性が高い。虚弱の栄養学的要因として低栄養、瘦

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せが重要である。特に摂取エネルギー、蛋白質や必須アミノ酸摂取の低下、ビタミンやミネラル、特にビタミンD、カロテン、ビタミンB<sub>12</sub>、葉酸の摂取不足は高齢者の虚弱と関連が深いといわれている<sup>4)</sup>。

虚弱における栄養の指標としてアルブミン、コレステロールが使われてきた。横断的な解析では、低アルブミン血症(血清アルブミン3.5 g/dL未満)は地域在住高齢者の身体機能やADL障害に関連していた<sup>5,6)</sup>。縦断的研究では、3.8 g/dL以下の低アルブミン血症が3年後の身体機能低下と関連していたが、7年後の身体機能低下とは関連をしていなかった。170 mg/dL未満の低コレステロール血症は死亡のリスクにはなっていたが、虚弱のリスクにはなっていなかった<sup>7)</sup>。コレステロールとアルブミンを組み合わせた縦断的な検討では、血清総コレステロールが5.2 mmol/L(201 mg/dL)以下で女性での虚弱危険因子となっていたが、血清アルブミンが4.3 g/dL以下での判定では男女とも虚弱危険因子とはならなかった。しかし、コレステロールとアルブミンの両方を組み合わせたところ、男性での虚弱危険因子となった<sup>8)</sup>。HDLコレステロールについても施設入所の高齢者の2年間の追跡で、身体機能低下の重要なリスクファクターになっていることが示されている<sup>9)</sup>。虚弱の栄養指標は、単独では虚弱を見逃してしまうこともある。いくつかの指標を組み合わせることも重要であろう。

高齢者の虚弱についての大規模な縦断研究として、米国の40,657人の65~79歳の女性を対象とした3年間の追跡研究Women's Health Initiative Observational Study (WHI-OS)がある<sup>10)</sup>。WHI-OSではベースライン調査で16.3%が虚弱と判断され、さらに3年間の追跡で14.8%が新たに虚弱となった。虚弱の要因として生活習慣についても詳細な調査が行われているが、その結果では喫煙は虚弱の危険因子であるが、飲酒は少量ならばむしろ虚弱を予防するという結果が出ている。また、体重は低体重も肥満もともに正常体重に比べて虚弱の要因となっていた。

## 慢性疾患と虚弱

WHI-OSの報告では慢性疾患やうつ症状が虚弱の要因であり、一方、自覚的健康度が高いことは虚弱を防ぐ要因であった。虚弱との関連が認められた慢性疾患は、冠動脈疾患、脳血管障害、糖尿病、高血圧症、大腿骨頸部骨折、慢性閉塞性肺疾患(COPD)、転倒、抑うつ、関節炎であった<sup>10)</sup>。さらに認知症や認知機能障害が、高齢者の虚弱と関連しているとする報告もある<sup>11,12)</sup>。

慢性の炎症も虚弱の要因となる。IL-6が3.8 pg/mLを超える場合、CRPが2.65 mg/Lを超える場合には、3年間の追跡で有意に身体機能が低下していた<sup>7)</sup>。男性ホルモンの低下についても、高齢男性の虚弱の要因であるとの報告がある。米国での1,469名の65歳以上高齢男性の検討では、血清テストステロン濃度が低いほど虚弱の割合が多く、4年間の縦断的追跡でも血清テストステロン濃度が低いほど虚弱となるリスクが高かった<sup>13)</sup>。男性高齢者の場合、アンドロポーズと呼ばれる加齢に伴う男性ホルモンの低下が、虚弱の要因として重要である。副腎や性腺で産生される男性ホルモンの1種であるデヒドロエピアンドロステロン(DHEA)も低値であることが、高齢男女で虚弱と関連していた<sup>14)</sup>。これら様々な慢性疾患や病態が重積することで、さらに虚弱の危険が増加する。

## 社会経済的要因と虚弱

同じ定義を用いても、虚弱高齢者の分布には地域差があるといわれている。ヨーロッパ10カ国の調査では、65歳以上の虚弱高齢者の割合はスイスの5.8%からスペインの27.3%までと異なっており、同じヨーロッパでも概して南欧は北欧よりも虚弱な高齢者が多いと結果であった<sup>15)</sup>。この地域差には教育など社会経済的な要因が関与しているという。

米国のWHI-OSでは社会経済的要因として、世帯年収が高いほど、教育が長いほど、白人に比べむしろ黒人やアジア人でリスクが低かつ



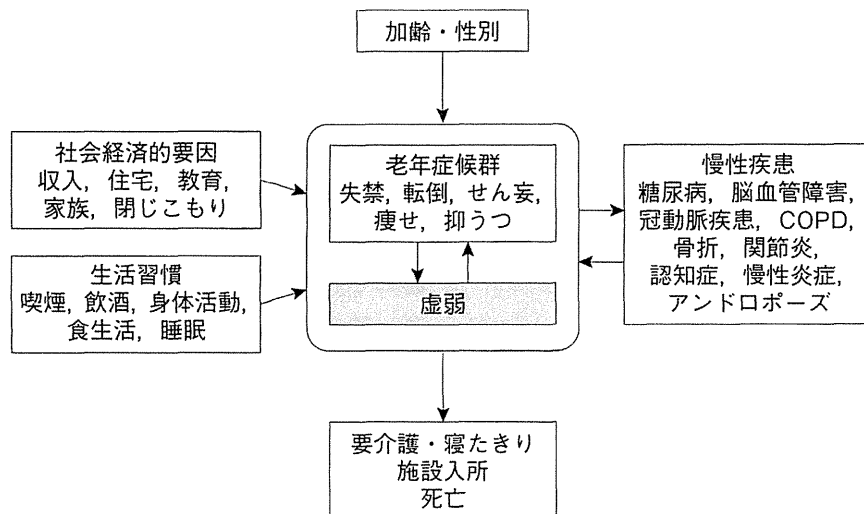


図1 高齢者の虚弱的要因と老年症候群

た<sup>10)</sup>。また、一人暮らしは虚弱となるリスクを20%下げている。一人暮らしは、他の家族に依存できず自立が必要なためと思われる。一方で、3年間にわたる縦断的研究で、外出頻度が少ない、いわゆる「閉じこもり」で虚弱の発生率が高かったとの報告もある<sup>16)</sup>。

### 虚弱高齢者への介入研究

虚弱の予防を目指しての介入研究が繰り返し行われている。1994年に『New England Journal of Medicine』に掲載された Fiatarone らによる虚弱高齢者への古典的な介入研究がある<sup>17)</sup>。施設入所中の高齢者に対する無作為割付研究で、筋肉トレーニングにより虚弱の有意な改善が認められている。運動による介入の虚弱の改善効果については、ほかのよくデザインされた研究でも認められているが<sup>18)</sup>、否定的な結果の研究もある<sup>19)</sup>。

栄養での介入でも虚弱の改善効果ははっきりしない。Fiatarone らによる無作為割付研究でのビタミン、ミネラル、蛋白質、脂質、炭水化物による栄養介入では、虚弱の改善効果は認められなかった<sup>17)</sup>。必須アミノ酸であるバリン、ロイシン、イソロイシンの3つを分岐鎖アミノ酸という。筋肉を構成している必須アミノ酸の約35~40%がこの分岐鎖アミノ酸であり、筋肉

の蛋白質分解を抑制する。高齢者の筋量維持、増加にこの分岐鎖アミノ酸が有効だとする報告は多い<sup>20)</sup>。しかし、実際に無作為割付研究を行っても、ロイシンをサプリメントとして3カ月間にわたって高齢男性に投与した介入試験では、筋肉量や筋力への影響はなかったという<sup>21)</sup>。このほかにもビタミンDの投与による栄養介入の研究などもあるが<sup>19)</sup>、虚弱の改善効果は認められていない。

### おわりに

老年症候群は高齢者に特有のあるいは高頻度にみられる諸症状であり、高齢者のADLやQOLを阻害する。老年症候群には、めまい、息切れ、痩せ、食欲不振、抑うつ、転倒、関節痛、視力低下、聴力低下などが含まれる。これらの老年症候群の諸症状は、高齢者虚弱との関わりが強い。図1に示すように、加齢、性別は高齢者の虚弱の要因として重要であるが、世帯年収や教育、住宅環境、家族構成などの社会経済的要因、喫煙、飲酒、身体活動、食生活などの生活習慣、糖尿病、脳血管障害、冠動脈疾患、COPD、骨折、関節炎、認知症、慢性炎症、アンドロポーズなどの慢性疾患や慢性的な病態が、高齢者の虚弱を引き起こす。これらの要因は同時に様々な老年症候群の要因にもなる。さらに

高齢者の虚弱が老年症候群の原因ともなる。そして要介護や寝たきり、施設入所、最終的には死に至る。このように、多くの要因が重積し虚弱を引き起こす。運動介入や栄養の単独の介入では虚弱の予防は難しい。高齢者に対しての生活全般のサポートによる対応が望まれる。

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## ENABLING TECHNOLOGIES

# CDK4 and cyclin D1 allow human myogenic cells to recapture growth property without compromising differentiation potential

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*In vitro* culture systems of human myogenic cells contribute greatly to elucidation of the molecular mechanisms underlying terminal myogenic differentiation and symptoms of neuromuscular diseases. However, human myogenic cells have limited ability to proliferate in culture. We have established an improved immortalization protocol for human myogenic cells derived from healthy and diseased muscles; constitutive expression of mutated cyclin-dependent kinase 4, cyclin D1 and telomerase immortalized human myogenic cells. Normal diploid chromosomes were preserved after immortalization. The immortalized human myogenic cells divided as rapidly as primary human myogenic cells during the early passages, and underwent myogenic, osteogenic and adipogenic differentiation under appropriate culture conditions. The immortalized cells contributed to muscle differentiation upon xenotransplantation to immunodeficient mice under conditions of regeneration following muscle injury. We also succeeded in immortalizing cryopreserved human myogenic cells derived from Leigh disease patients following primary culture. Forced expression of the three genes shortened their cell cycle to <30 h, which is similar to the doubling time of primary cultured human myogenic cells during early passages. The immortalization protocol described here allowed human myogenic cells to recapture high proliferation activity without compromising their differentiation potential and normal diploidy. *Gene Therapy* (2011) **18**, 857–866; doi:10.1038/gt.2011.44; published online 14 April 2011

**Keywords:** muscle satellite cell; CDK4; telomerase; immortalization; replicative senescence; growth arrest

## INTRODUCTION

Skeletal muscle stem cells of adult muscle are known as muscle satellite cells because they are located adjacent to the plasma membrane of myofibers beneath the basement membrane. The postnatal growth, repair and maintenance of skeletal muscle rely on muscle satellite cells that proliferate and then fuse together to form myotubes. Actually, phenotypic analysis of Pax7-deficient mice strongly suggests that the loss of satellite cells abolishes the regenerative capacity of skeletal muscle.<sup>1,2</sup> The decrease of regenerative capacity of muscle results in muscle dysfunction during both normal aging and progression of muscle-regenerative diseases, such as muscular dystrophies.

Most of the data on the regulation of proliferation and differentiation of muscle satellite cells and their descendant progenitor cells have been obtained from primary cultured chick myogenic cells or mouse myogenic cell lines.<sup>3–5</sup> However, several previous studies strongly suggest that animal myogenic cells do not always use the same pathways to control proliferation and differentiation as human myogenic cells.<sup>6,7</sup> Although animal cell models certainly contribute to understanding the mechanisms of human myogenesis and muscle diseases, the precise and detailed analysis of human myogenic cells is essential for fundamental and therapeutic investigation. Unfortunately, progres-

sively compromised differentiation potential, as well as proliferation potential, is seen in cultured human myogenic cells.<sup>8,9</sup> The limited proliferation capacity and progressive alterations of characteristics of human myogenic cells do not allow us to carry out both qualitative and quantitative analyses with high reproducibility.

Previous attempts have been made to extend the replication capacity of human myogenic cells using viral oncogenes such as simian virus 40 large T antigen and/or the reverse transcriptase component of human telomerase (hTERT).<sup>10</sup> However, no reliable model of immortalized human myogenic cells that exhibit differentiation potential had been established until our previous study.<sup>9</sup> We previously reported that constitutive expression of hTERT and human papillomavirus type 16 gene E7 immortalizes a primary normal human myogenic cell clone designated Hu5. The immortalized human myogenic cell clone Hu5/E18 largely preserves the myogenic phenotype represented by parental Hu5 cells, but their doubling time is approximately 12 h longer than that of primary human myogenic cells during early passages. E7 is an oncogene that inactivates the retinoblastoma protein pRb,<sup>11</sup> and does not transform human myogenic cells. However, we cannot exclude a possibility that E7 also affects other biological functions, including transformation-related pathways.

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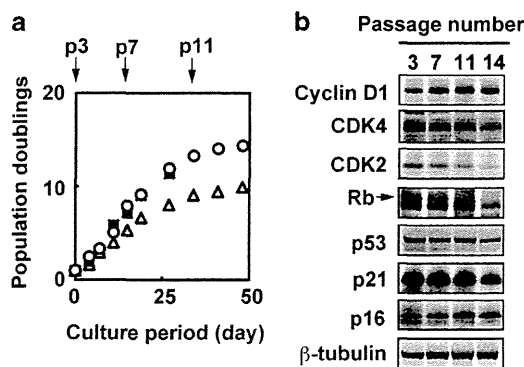
Cellular stress activates a pathway of the cyclin-dependent kinase inhibitor p16<sup>INK4a</sup>, resulting in premature cell cycle arrest before telomere attrition,<sup>12</sup> probably due to the activation of Rb. The forced expression of wild-type cyclin-dependent kinase 4 (CDK4) enabled hTERT to immortalize primary human myogenic cells, presumably because cdk4 sequesters the increased p16 exclusively when stimulated with dexamethasone and hepatocyte growth factor.<sup>13</sup> In addition, the co-expression of hTERT and Bmi-1, which suppresses p16<sup>INK4a</sup> expression, failed to immortalize human myogenic cells.<sup>9,14</sup> These results indicate that combining the expression of hTERT and sequestration of p16<sup>INK4a</sup> is insufficient to immortalize human myogenic cells, or that the p16<sup>INK4a</sup> pathway is incompletely suppressed under these conditions.

In the present study, to block the p16<sup>INK4a</sup>-Rb pathway and enhance cell cycle progression, without the use of oncoprotein E7, expressions of hTERT and both mutant CDK4 (CDK4R24C) and cyclin D1 were induced in human myogenic cells. Combined expression of the three genes efficiently immortalized normal human myogenic cells. The immortalized cells still retained multipotentiality and a doubling time similar to that of primary cultured human myogenic cells. The established normal human myogenic cell clones in the present study are the human equivalents of mouse cell lines such as C2 (ref. 3) and Ric10.<sup>5,15</sup> In addition, we succeeded in immortalization of diseased muscle-derived primary human myogenic cells that showed the prolonged doubling time. The newly established method for immortalization of primary human myogenic cells will open new avenues for mechanistic and therapeutic research on human muscle diseases.

## RESULTS

### p16<sup>INK4a</sup>-Rb pathway is activated upon growth arrest of primary cultured human myogenic cells

Proliferation capacity of primary cultured human myogenic cells severely declined during serial passages under the present culture condition (Figure 1a). The doubling time of the cells became longer as they were serially succeeded (Supplementary Figure 1). Constant or



**Figure 1** Growth properties of primary cultured human myogenic cells. (a) Life span plots of primary cultured human myogenic cells Hu20 (filled square), Hu23 (triangle) and Hu26 (circle) between passages 3 and 13. Arrows show the timing of passages 3, 7 and 11. Day 0 of culture period represents the day when the cells were plated for passage 3. (b) Expression patterns of growth-related proteins in primary cultured human myogenic cell H23 during serial passages. Fifteen micrograms of total proteins were subjected to immunoblotting analysis with antibodies against proteins shown in the left panels. Similar expression patterns of the proteins were obtained in Hu20 and Hu26. An arrow represents the position of hyperphosphorylated Rb protein.

high level expression of cyclin D1, CDK4, cyclin-dependent kinase inhibitor p21<sup>cip1</sup> and p53 was observed in primary human myogenic cells even upon growth arrest (Figure 1b). In contrast, the amount of the cell cycle inhibitor p16<sup>INK4a</sup> increased along with the culture period, whereas the amount of hyperphosphorylated form of Rb declined. The amount of another cell cycle-driving kinase CDK2 decreased following the disappearance of hyperphosphorylated form of Rb. The results indicate that the p16<sup>INK4a</sup>-Rb pathway is activated before growth arrest of primary cultured human myogenic cells, suggesting that their cell cycle arrest is due to the activation of Rb. The disappearance of hyperphosphorylated Rb seems unlikely to depend on the downregulation of either CDK2 or CDK4 that are kinases relevant for phosphorylation of Rb.

### E7 promotes nuclear progression in terminally differentiated myotubes

The primary human myogenic progenitor cell clone Hu5 was obtained from a healthy muscle of a non-dystrophic woman.<sup>4</sup> Hu5 cells have limited ability to proliferate but can be immortalized by constitutive expression of both telomerase and the E7 gene from human papillomavirus type 16.<sup>9</sup> E7 inactivates Rb but is also suspected to affect other cellular functions. To determine whether constitutive expression of E7 transforms human myogenic cells, the Hu5-derived myogenic cell clone Hu5/E18 (ref. 9), immortalized by constitutive expression of both hTERT and E7, was transplanted into cardiotoxin-injected TA muscles of immunodeficient NOD/Scid mice (Figures 2a and b). Before transplantation, E18 cells were infected with a lentivirus vector encoding green fluorescent protein Venus (kindly provided by Dr Miyoshi). Transplanted cells were identified by the fluorescence of Venus and antibodies specific for green fluorescent protein. Transplanted E18 cells ( $2.5 \times 10^6$  cells per TA) gave rise to myofibers labeled with green fluorescence. No tumor was formed in the transplanted TA muscles. Soft agar assays also showed that E18 was unable to grow in an anchorage-independent way (Supplementary Figure 2). The results indicate that E18 cells did not show any oncogenic potential either *in vivo* or *in vitro*.

In the next experiment, effects of the immortalization on cell cycle exit during terminal muscle differentiation were analyzed *in vitro*. E18 cells undergo myogenic terminal differentiation under the myogenic differentiation-inducing condition.<sup>9</sup> Primary cultured human myogenic progenitor cells exited the cell cycle and gave rise to terminally differentiated myotubes (Figures 2c-f). In contrast, the nuclei of E18 myotubes synthesized DNA and also contained the proliferation marker protein Ki-67, although neither nuclear nor cellular division was observed in the myotubes (Figures 2g-j). The results suggest that E7 promotes nuclear progression in terminally differentiated myotubes that have lost mitogenic potential. In addition, the doubling time of the Hu5-derived immortalized cells is approximately 35 h,<sup>9</sup> whereas primary cultured human myogenic cells divided at 20–29 h intervals (Supplementary Figure 3). Taken together with the results here, the expression of hTERT and E7 immortalizes human myogenic cells without the loss of their differentiation potential but also affects their cell cycle properties during the terminal myogenic differentiation.

### Cell cycle drivers efficiently immortalize primary cultured human myogenic cells

E7 promotes nuclear progression in myotubes, perhaps, because it accelerates the degradation of Rb family proteins including Rb, p130 and p107. To inactivate Rb directly and avoid unusual promotion of nuclear progression in myotubes, Hu5 cells were infected with recombinant lentiviruses encoding hTERT, CDK4R24C and cyclin D1.