Fruits and vegetables are rich sources of vitamins and biologically active phytochemicals, which may protect against diabetes mellitus through their antioxidant properties. Some epidemiologic studies have suggested that a higher intake of fruits and vegetables may decrease the risk of type 2 diabetes (1–3), but evidence obtained from other studies (4, 5) and meta-analyses (6–9) is inconclusive. There are several reasons for the inconclusiveness. Fruit and vegetable intake has often been assessed using self-reporting methods, which has an inclination to misclassification and reporting bias (10, 11). A high intake of green leafy vegetables that are rich in antioxidative nutrients such as vitamin (V) C and β-carotene, rather than vegetables in general, may protect against the risk of type 2 diabetes (4, 7–9).

Moreover, measurement of the circulating nutrient concentrations can indicate the nutrient status more directly than the intake values, and VC and carotenoid concentrations are recognized as good biomarkers for the intake of fruits and vegetables (12–15).

It is important to detect the nutrient-disease association in the target population as there are differences among races in the types of fruits and vegetables consumed, cooking methods, and possibly the absorption and bioavailability of nutrients. However, the relationship between these nutrients and diabetes has received little attention in Asia. Some Western studies on VC (16–19) or carotenoid (19–22) concentrations show an inverse association of the nutrient status with the risk of type 2 diabetes in free-living populations. The combination of VC and β-carotene representing the intake of a mixed fruit and vegetable diet demonstrated a strong inverse association with incidence of diabetes in an EPIC-Norfolk study conducted in Europe (19). For VC, South Asian individuals at a increased risk of diabetes were reported to show lower plasma VC levels compared to white Europeans (17). For β-carotene, Korean (20) and Japanese (21) people who were already diabetic showed decreased serum β-carotene levels.

Although the risk of diabetes is significantly higher among Asian people than among Caucasians (23), there are very few Asian studies on antioxidative vitamin concentrations and dysglycemia. In the present study, we focused on the nutrient-disease association and investigated the relationship between serum anti-
oxidative vitamin concentrations and type 2 diabetes in Japanese subjects.

SUBJECTS AND METHODS

Subjects. A total of 999 subjects (506 men and 493 women) who first underwent anti-aging health checks at Tokai University Tokyo Hospital between fiscal year 2006 and 2017 were enrolled in the study. The present diabetes treatment status was surveyed by asking whether the individual had been treated for diabetes, using self-administered questionnaires and interviews by doctors. Subjects who were prescribed pharmacological amounts of vitamins were excluded, but who used dietary supplements or health foods were included. To exclude unreported uses of medications or excessive uses of supplements, we eliminated 37 subjects whose concentrations of each vitamin exceeded more than triple of the upper limits given by the laboratory testing service (BML, Inc., Tokyo, Japan) as described below. Therefore, a data set of 962 subjects was subjected to statistical analysis. All subjects gave written informed consent to the use of their health records for analysis. This study was approved by the Ethics Committee of Tokai University (No. 11R-125), and was conducted in accordance with the Declaration of Helsinki.

Measurements. Anthropometry and blood drawing were performed after overnight fasting. Among the measurements included in the anti-aging health check examinations (24, 25), the following parameters were used for analysis: age, BMI, fasting plasma glucose (FPG), HbA1c, and serum concentration of vitamin A (VA) (reference range 27.2–102.7 μg/dl), β-carotene (reference range <96.4 μg/dl), VC (reference range 4.7–17.8 μg/ml), α-tocopherol (reference range 4.9–13.8 μg/ml), VB12 (reference range 233–914 pg/ml), folate (reference range 3.6–12.9 ng/ml), and homocysteine (reference range 6.31–18.9 nmol/ml for men and 5.1–11.7 nmol/ml for women). Serum samples for measuring vitamin concentrations were treated properly to prevent degradation until measurement and measured at BML, Inc. VA, β-carotene, VC, α-tocopherol and homocysteine were measured by high-performance liquid chromatography (HPLC). VB12 and folate were measured by chemiluminescent immunoassay (CLIA).

Statistical analysis. Data was analyzed using SPSS Statistics (Version 22.0; SPSS Inc.). Results are shown as median and 25–75%tile. Since none of the clinical parameters were normally distributed, non-parametric analyses were used. The correlations among the parameters were determined by Spearman’s correlation coefficients. Statistical significance for comparisons among the clinical parameters was analyzed using the Mann-Whitney U test or Kruskal-Wallis test where appropriate. Multiple linear regression analysis was performed to find significant determinants of FPG or HbA1c, including VA, β-carotene, VC, α-tocopherol, VB12 and folate as independent variables. Prior to the multiple linear regression analysis, we confirmed the absence of high multicollinearity among the independent variables. Variable selection in the multiple linear regression analysis was made by a stepwise procedure. Multiple logistic regression analysis was performed including the same variables as those used in the multiple linear regression analysis to calculate the odds ratios (ORs) for a diabetic state (HbA1c ≥6.5% and/or under diabetes treatment) using the non-diabetes group as the reference. All p-values were two-tailed, and p<0.05 was considered significant.

RESULTS

The clinical characteristics of the subjects are shown in Table 1. The median age was 62.0 y. The median BMI (22.3) of the subjects was within the normal range. Since serum α-tocopherol level can be affected
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by lipid concentration (26), we used α-tocopherol/total cholesterol (TC) values as well as absolute α-tocopherol values. Men and women were included almost equally.

The percentage of subjects under diabetes treatment was 4.7%.

The associations of FPG and HbA1c with serum vitamin concentrations are shown in Table 2. Both FPG and HbA1c were significantly positively correlated with age and BMI. FPG and HbA1c showed a strong positive correlation. Both FPG and HbA1c were significantly negatively correlated with β-carotene and VC. In the subjects without diabetes, the results of the correlation analysis were almost identical to the results presented in Table 2 (data not shown). In the subjects with diabetes, no associations were found between FPG/HbA1c and serum vitamin concentrations (data not shown).

Next, the clinical parameters were compared with or without diabetes. Compared with the non-diabetes subjects, the diabetes subjects showed significantly higher age, BMI, FPG and HbA1c, and significantly lower serum concentration of β-carotene and VC (Table 3). We then examined the β-carotene and VC levels stratified by the HbA1c values (HbA1c, 6.0%, 6.0–6.5%, >6.5% and under diabetes treatment). There was a statistical difference in the β-carotene levels between A1c <6.0 and A1c ≥6.5% groups, and between A1c <6.0 and under diabetes treatment groups. The serum VC level of the A1c ≥6.5% group was significantly lower than that of the HbA1c <6.0% group.

Table 3. Comparison of clinical parameters between non-diabetes and diabetes groups.

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetes (n=917)</th>
<th>Diabetes (n=45)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (25–75%tile)</td>
<td>Median (25–75%tile)</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>62.0 (53.0–69.0)</td>
<td>68.0 (59.3–73.5)</td>
<td>0.004</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.3 (20.3–24.3)</td>
<td>24.0 (22.1–27.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FPG (mg/dL)</td>
<td>96.0 (91.0–103.0)</td>
<td>132.5 (114.3–151.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.2 (4.9–5.5)</td>
<td>6.7 (6.1–7.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.74 (0.64–0.90)</td>
<td>0.79 (0.70–0.90)</td>
<td>0.582</td>
</tr>
<tr>
<td>Vitamin A (µg/dL)</td>
<td>52.4 (43.1–62.5)</td>
<td>49.8 (42.3–55.6)</td>
<td>0.180</td>
</tr>
<tr>
<td>β-Carotene (µg/dL)</td>
<td>51.9 (30.8–83.1)</td>
<td>29.3 (14.1–55.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin C (µg/mL)</td>
<td>9.6 (7.8–11.4)</td>
<td>8.2 (6.4–9.8)</td>
<td>0.002</td>
</tr>
<tr>
<td>α-Tocopherol (µg/mL)</td>
<td>13.4 (11.1–16.2)</td>
<td>14.7 (11.8–16.6)</td>
<td>0.333</td>
</tr>
<tr>
<td>α-Tocopherol/TC</td>
<td>0.612 (0.538–0.725)</td>
<td>0.654 (0.551–0.774)</td>
<td>0.066</td>
</tr>
<tr>
<td>Vitamin B12 (pg/mL)</td>
<td>544.0 (427.0–733.8)</td>
<td>648.0 (451.0–938.0)</td>
<td>0.053</td>
</tr>
<tr>
<td>Folate (ng/mL)</td>
<td>12.3 (8.2–15.0)</td>
<td>10.9 (8.1–14.1)</td>
<td>0.454</td>
</tr>
<tr>
<td>Homocysteine (nmol/mL)</td>
<td>8.3 (7.0–10.4)</td>
<td>9.5 (7.8–11.4)</td>
<td>0.028</td>
</tr>
</tbody>
</table>

BMI, body mass index; FPG, fasting plasma glucose; TC, total cholesterol.

Fig. 1. Comparison of β-carotene and vitamin C levels stratified according to the HbA1c values (HbA1c, 6.0%, 6.0–6.5%, >6.5% and under diabetes treatment). DMTx: under diabetes treatment. There was a statistical difference in the β-carotene levels between A1c <6.0 and A1c ≥6.5% groups, and between A1c <6.0 and under diabetes treatment groups. The serum VC level of the A1c ≥6.5% group was significantly lower than that of the HbA1c <6.0% group.
Tables 4, 5 and 6 show the results of multivariate analyses to find significant determinants of FPG or HbA1c, including VA, β-carotene, VC, α-tocopherol, VB12 and folate as independent variables. Stepwise multiple linear regression analysis was performed and we found that β-carotene and VC were significantly negatively associated with FPG (Table 4). VC also showed a significantly negative correlation with HbA1c (Table 5). Based on the values of the variance inflation factor (VIF), we confirmed that multicollinearity had almost no effect on the results of multiple linear regression analysis. Next, we performed multiple logistic regression analysis for the diabetic state (HbA1c $\geq 6.5\%$ and/or under diabetes treatment) and found that the serum levels of β-carotene and VC were significantly decreased (Table 6). Table 6 shows the results obtained by a simultaneous forced entry procedure, but a stepwise procedure gave the same results. When α-tocopherol/TC was used as substitute for α-tocopherol, we obtained the same results in these multivariate analyses (data not shown). These multivariate analyses suggested that low levels of β-carotene and VC were factors related to diabetes.

**DISCUSSION**

In the present study, we focused on nutrient-disease association and evaluated the relationship between serum antioxidative vitamin concentrations and type 2 diabetes in Japanese subjects. We ascertained that a low serum concentration of β-carotene and VC was significantly associated with a high FPG and HbA1c. Diabetic subjects showed significantly decreased β-carotene and VC levels, and multivariate analyses suggested that low levels of β-carotene and VC were factors related to diabetes. To our knowledge, the present study is one of the few that explore the relationship between antioxidative vitamin concentrations and dysglycemia in the community-dwelling population in Asia. In Japan, the number of elderly people is expanding year after year, reaching to a super-aging society.
Anti-aging checkups focus on early signs of aging-associated changes such as oxidative stress-induced, vascular, hormonal and body compositional changes, and to help people reduce age-related risks through specialists in anti-aging medicine (24, 25). More than 2,100 examinees have undergone anti-aging checkups at Tokai University Tokyo Hospital since its foundation in 2006. Our anti-aging health check-ups provide an evaluation of lifestyle-related diseases and antioxidative vitamin concentrations, and include advice on lifestyle modifications or supplement intake if necessary (27).

Hyperglycemia promotes the generation of free radicals, and oxidative stress has been considered to play an important role in the pathophysiology of diabetes. Insulin resistance and β-cell dysfunction are caused by oxidative stress, leading to the development of impaired glucose tolerance and type 2 diabetes (28, 29). The human body maintains enzymatic antioxidant defense systems to scavenge reactive oxygen species as well as non-enzymatic nutritional antioxidants (30, 31). VC and β-carotene are nutritional antioxidants and are able to deactivate free radicals. VC is present in aqueous solutions (e.g. plasma, cytosol and body fluids) and can scavenge superoxides, hydrogen peroxide, hydrochlorite, and other radicals (32). β-Carotene acts as a quencher of singlet molecular oxygen (1O2) in the lipophilic membranes (32). Since humans cannot synthesize these nutrients, the serum concentrations of β-carotene and VC are recognized as good biomarkers for the intake of fruits and vegetables (12–15).

Epidemiologic studies have suggested that a higher intake of fruits and vegetables may decrease the risk of type 2 diabetes (1–3), but the evidence is inconclusive (4–9).

It is suggested by further analyses that a specific group of vegetables, rather than vegetables as a whole, may protect against the risk of type 2 diabetes. A high intake of green leafy vegetables, which are rich in antioxidative nutrients such as VC and β-carotene, have been shown to play a protective role against type 2 diabetes (4, 7–9). Excessive fruit intake is not recommended for people at a higher risk of diabetes due to the high sugar content. On the other hand, green leafy vegetables are good for people at a higher risk of diabetes; not only are they a rich source of VC and β-carotene, but they are also rich in dietary fiber and low in calories. Direct measurement of VC or β-carotene concentrations has been able to indicate the nutrient status more directly (16–22). Only one study has used the measurement of both VC and β-carotene (19). According to the results obtained by the Epic-Norfolk study, a combination of biomarkers representing the intake of fruit and vegetable was inversely associated with incidence of diabetes (19). In the present study, we ascertained that a low serum concentration of β-carotene and VC was significantly associated with a high FPG and HbA1c. Diabetic subjects showed significantly decreased β-carotene and VC levels, and multivariate analyses suggested that β-carotene and VC were factors related to diabetes. Our findings support the usefulness of measuring the circulating vitamin concentrations in studies of diet-disease associations and emphasize the importance of taking sufficient β-carotene and VC for Asian people at risk of diabetes.

The effect of the supplementation of antioxidative nutrients including VC and β-carotene has been evaluated by randomized control trials. However, no beneficial effect for diabetes has been reported (33–36). The supplement intake value does not always mean that nutrients can play their expected role. Rather, measurement of the circulating nutrient concentrations can indicate the nutrient status more directly. Dietary supplements including health foods can be beneficial for covering the shortcomings of nutrition from meals, and adequate supplement use is reasonable as long as it is not used to replace the balanced variety of foods. We included subjects who use supplements in the present study, and then showed that low levels of β-carotene and VC were factors related to diabetes. Even if we had excluded those who used supplement pills, we cannot completely exclude the possibility of additional vitamin intake from commercially available items such as fortified foods. Cooper et al. gave another explanation for the discrepancy between interventional trials using dietary supplements and observational studies (high intake of green leafy vegetables or high blood concentrations of VC and β-carotene) describing that fruits and vegetables are also a primary source of other bioactive phytochemicals as well as having a high fiber content and low energy density, properties which are almost impossible to mimic in pill form as used in vitamin supplements (19).

Although the functional role of each nutrient is difficult to justify because various other nutrients are simultaneously taken through mixed meals, a combination measurement of antioxidative nutrients is considered to be a good marker for many other poorly defined or unknown phytochemicals. For the purpose of demonstrating the antioxidant capacity of all dietary antioxidants, the total antioxidant capacity estimated with the ferric-ion-reducing antioxidant power (FRAP) method (37) may be more useful and will be considered in a future study.

Our study has several limitations. The sample size was not large enough for conclusions with regard to marginally insignificant p-values. Our subjects, who undergo anti-aging health checks at Tokai University Tokyo Hospital, are considered to be more health-conscious than the average person. The single measurement of nutrients may only reflect an instantaneous equilibrium at one point, and may not represent the habitual levels. Not only measurement of serum nutrient concentrations, but also evaluation of dietary intake may be more convincing to support the findings obtained from the present study. Finally, the cause-effect relationship of our results is unclear because of the cross-sectional nature.

In conclusion, the present study evaluated the relationship between serum antioxidative vitamin concentrations and type 2 diabetes in Japanese subjects. It
was shown that low levels of β-carotene and VC were significantly associated with dysglycemia. Diabetic subjects showed significantly decreased β-carotene and VC levels, and multivariate analyses suggested that low levels of β-carotene and VC were factors related to diabetes. Taken together, low levels of β-carotene and VC are significantly related to dysglycemia/type 2 diabetes, and encouraging people at a higher risk of diabetes to take more green vegetables may be useful as a dietary intervention to improve the antioxidative vitamin status and dysglycemia.

**Authorship**
Research conception and design: CY and YN; data collection: CY, NK and NU; statistical analysis of the data: CY, YM, and ST; interpretation of the data: MK, MT, AK and NI; writing of the manuscript: CY and YN. All authors edited and approved the final draft of the manuscript.

**Disclosure of state of COI**
The authors declare no conflict of interest.

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**REFERENCES**


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