



# Effect of Collagen Tripeptide on Atherosclerosis in Healthy Humans

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**Aim:** Collagen tripeptide (CTP) is a functional food with a high content of Gly-X-Y tripeptides derived from collagen. The objective of this study was to evaluate the effect of CTP administration on the development of atherosclerosis in healthy individuals.

**Methods:** The present study was conducted in the form of an open-label, single-dose trial for 6 months. All subjects ingested CTP twice daily: at breakfast and supper (total intake per day: 16 g). The effect of CTP on atherosclerosis was verified by measuring several indices, including serum lipid levels, toxic advanced glycation end-products (TAGE), and the cardio-ankle vascular index (CAVI), at baseline and 6 months.

**Results:** The low-density lipoprotein cholesterol (LDL-C) to high-density lipoprotein cholesterol (HDL-C) ratio (LDL-C/HDL-C ratio) was significantly reduced in patients with an initial ratio of  $\geq 2.5$  ( $p=0.025$ ). A significant reduction in TAGE was observed in all the subjects ( $p=0.031$ ) and in the high-risk group ( $p=0.024$ ). A significant reduction in CAVI was observed in all the subjects (right side:  $p=0.048$ , left side:  $p=0.047$ ). As a result of multiple regression analysis, a significant relationship between the change in CAVI and that in each factor was not observed. No adverse events were observed during the study period.

**Conclusions:** The results of the present study indicate that CTP contributes to the prevention and treatment of atherosclerosis in healthy humans (UMIN000018525).

**Key words:** CTP, Atherosclerosis, LDL-C/HDL-C ratio, CAVI, TAGE

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## Introduction

Two distinct mechanisms are believed to underlie the pathogenesis of atherosclerosis: one is atherosclerosis, i.e., the deposition of lipids in the arterial intima, which may lead to thrombotic occlusion and subsequent severe symptoms caused by the rupture of vulnerable plaques in the vascular intima, and the other

one is sclerosis, i.e., the hardening of arterial walls. A fragmentation of elastin results in an increased number of collagen fibers and smooth muscle cells, which cause hardening of blood vessel walls by inducing calcification<sup>1, 2</sup>. Because atherosclerosis progresses slowly with no symptoms, patients typically notice only when they present with severe symptoms such as infarcts or ischemia. Therefore, the risk of serious cardiovascular diseases should be reduced by evaluating arteriosclerosis by morphological and functional methods at an early stage prior to the development of risk factors associated with the development of atherosclerosis, such as dyslipidemia, diabetes, and hypertension.

Several indicators of premature atherosclerosis

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have been described. The low-density lipoprotein cholesterol (LDL-C) to high-density lipoprotein cholesterol (HDL-C) ratio (LDL-C/HDL-C ratio) indicates the balance between the two cholesterol types associated with cardiovascular events. The LDL-C/HDL-C ratio reportedly has utility as a predictor of carotid intima-media thickness (IMT)<sup>3</sup>. Furthermore, the plaque area rapidly increases when the LDL-C/HDL-C ratio becomes  $>2.5$ <sup>4</sup>. In healthy persons, correlations between the LDL-C/HDL-C ratio and risk factors for atherosclerosis have been observed<sup>5</sup>.

The measurement of advanced glycation end-products (AGEs) has utility as an indicator of atherosclerotic progression caused by impaired glucose metabolism. The measurement of hemoglobin A1c, the glycation product of hemoglobin, is currently used as a therapeutic indicator of diabetic complications. A recent clinical study evaluated the effects of toxic AGEs (TAGE), i.e., AGEs derived from glyceraldehyde. TAGE is reportedly associated with the onset and progression of atherosclerosis via a receptor for AGEs<sup>6,7</sup>. In addition, TAGE is associated with vascular inflammation and plaque progression in patients with acute coronary syndromes<sup>8,9</sup>. These studies have demonstrated the utility of serum TAGE level measurements in the early diagnosis of atherosclerosis and in the assessment of diabetes treatment efficacy.

The cardio-ankle vascular index (CAVI) allows non-invasive, quantitative evaluation of the stiffness of the aorta, femoral artery, and tibial artery, independently of systemic blood pressure<sup>10</sup>. It has been reported that there is a positive association of the CAVI score with vascular structure and function parameters such as IMT, the central augmentation index (CAIx), and the pulse wave velocity (PWV). Moreover, the ability of the CAVI score to predict carotid atherosclerosis is similar to that of PWV<sup>11</sup>. In contrast to PWV that essentially depends on the blood pressure at the time of measurement, CAVI is unaffected. Therefore, CAVI may be a good marker for arterial stiffness, reflecting coronary risk control, including hypertension treatment<sup>12</sup>. Patients with dyslipidemia have increased CAVI values than healthy individuals<sup>13</sup>. In a study followed for several years, in 1003 patients with diabetes, hypertension, and dyslipidemia, future cardiovascular events increased as CAVI became higher<sup>14</sup>. CAVI increases with the progression of atherosclerosis, with approximately half of the patients with CAVI  $>9.0$  developing atherosclerosis of the cerebral or coronary arteries<sup>15</sup>. Because CAVI is able to detect small changes such as those related to aging, it is also able to detect minor atherosclerotic progression in healthy individuals<sup>16</sup>.

Although dietary management and exercise

remain the mainstays of preventing atherosclerosis in healthy individuals, there has been increasing interest in the modulation of functional food intake in recent years. Collagen tripeptide (CTP), a functional food derived from collagen, contains a high concentration of tripeptides with a Gly-X-Y sequence. The oral administration of CTP to Kurosawa and Kusanagi-hypercholesterolemic (KHC) rabbits decreases atherosclerotic plaque area, serum total cholesterol levels, and number of macrophages and smooth muscle cells in atherosclerotic plaques<sup>17</sup>. CTP has been shown to inhibit the proliferation and migration of cultured aortic smooth muscle cells (AoSMCs) *in vitro*<sup>18</sup>. Furthermore, Gly-Pro-Hyp, the main component of CTP, reportedly inhibits dipeptidyl peptidase-IV activity, indicating that CTP may have potential utility in the prevention of diabetes<sup>19,20</sup>.

The various beneficial effects of ingesting gelatin and collagen hydrolysate are believed to be attributable to the physiological activities of circulating short peptide metabolites. Serum levels of peptide metabolites are preponderantly higher after CTP ingestion than after collagen peptide (macromolecule peptide) ingestion, with CTP being shown to be more efficiently absorbed<sup>21</sup>. In addition, the tripeptide component of CTP has been found to be selectively absorbed into connective tissues according to whole-body autoradiography with single-dose administration of tritium-labeled Gly-Pro-Hyp<sup>22</sup>. According to the results of these reports, CTP is expected to have utility as a functional food for the prevention and treatment of atherosclerosis.

In the present study, we conducted an open-label, single-dose trial of CTP in healthy subjects for 6 months. We then verified the effect of CTP on atherosclerosis by measuring several indices, including serum lipid levels, TAGE, and CAVI.

## Aim

To evaluate the effects of CTP administration on the development of atherosclerosis in healthy individuals.

## Methods

### Test Substance

CTP containing  $>15\%$  tripeptide components (Gly-X-Y sequences) was purchased from Jellice Co., Ltd., Miyagi, Japan.

### Subjects

In conformity with the Declaration of Helsinki, the content and methodology of the present study

were fully explained to all human subjects. Written informed consent was obtained from all the subjects prior to the initiation of the present study. We further obtained the approval of the clinical research ethics review committee of Kanazawa Medical University (clinical study number: 285, date of approval: June 17, 2013, date of the additional approval: March 22, 2013). The protocol was registered in the UMIN Clinical Trial Registry as UMIN000018525.

### Study Design

The subjects included in the present study were 32 healthy volunteers (16 males and 16 females) who had not previously received treatments for cardiovascular disease or diabetes. The present study was conducted in the form of an open-label, single-dose trial for 6 months. CTP dissolved in beverages or soup that they drank daily, such as water, warm water, miso soup, green tea, coffee, or a similar beverage, was administered twice daily at breakfast and supper. The total intake per day was 16 g (tripeptide equivalent of 2.4 g per day). It was confirmed that CTP does not interact with polyphenols contained in beverages, such as coffee and green tea, and does not interact with other proteins (data not shown). The subjects were instructed to perform normal activities of daily living to maintain their soundness without any changes, such as drinking and eating too much, going on a diet, and ingesting other healthy foods.

### Measurement of Outcomes

Body weight, body mass index (BMI), and blood pressure were measured at baseline and 6 months. Blood sampling was performed at baseline and at 6 months. Venous blood samples were collected and centrifuged at  $1,600 \times g$  for 10 min at  $4^{\circ}\text{C}$  to obtain supernatant serum samples. The following parameters were measured: total cholesterol (TC), HDL-C, LDL-C, and triglyceride (TG) levels; red blood cell (RBC) and white blood cell (WBC) counts; hemoglobin level; hematocrit; mean cell volume (MCV); mean cell hemoglobin (MCH); mean cell hemoglobin concentration (MCHC); platelet count; and total protein, albumin, total bilirubin, lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP),  $\gamma$ -glutamyl trans peptidase ( $\gamma$ -GTP), electrolyte (Na, K, Cl, and Ca), serum ion, inorganic phosphorus, blood urea nitrogen (BUN), ferritin, unsaturated iron binding capacity (UIBC), and creatinine levels (measured by SRL Inc., Tokyo, Japan). LDL-C was calculated using the Friedewald formula. The LDL-C/HDL-C ratio was calculated from LDL-C and HDL-C values. Stratification analysis was performed by dividing the sub-

**Table 1.** Subjects' demographics

Number of subjects	30 (male 15, female 15)
Age (years)	$53.7 \pm 7.2$ (male $58.1 \pm 6.0$ , female $49.3 \pm 5.5$ )

Values are expressed as the mean  $\pm$  standard deviation.

jects into two groups, i.e., the  $\geq 2.5$  group (high-risk group) and the  $< 2.5$  group (low-risk group), based on the initial LDL-C/HDL-C ratio values. In addition, changes in the health status were evaluated from blood test values. HDL-C, LDL-C, and TG levels were evaluated on the basis of the lipid management target of the Japan Atherosclerosis Society (JAS) Guidelines for Prevention of Atherosclerotic Cardiovascular Diseases 2012<sup>23</sup>). TC was evaluated on the basis of the sum of the target value of non HDL-C and HDL-C according to the JAS guideline. TAGE was measured from supernatant serum samples using competitive enzyme-linked immunosorbent assay (ELISA) with an anti-TAGE polyclonal antibody<sup>24</sup>). Stratification analysis was performed by dividing the subjects into two groups on the basis of the average value of 10.0 (precisely 9.95) in the initial TAGE levels: the  $\geq 10$  units/mL group (high-TAGE group) and the  $< 10$  units/mL group (low-TAGE group). CAVI was calculated for the left and right sides of the body at baseline and 6 months using Vasera VS-1500A (Fukuda Denshi, Tokyo, Japan).

### Statistical Analysis

All data are expressed as the mean  $\pm$  standard deviation. Statistical analyses were performed using Stat Flex for Windows, version 5.0 (Artec, Osaka, Japan). The paired *t*-test was used to assess significant differences between samples. Multivariate analysis was performed by multiple regression analysis. *P* values of  $< 0.05$  were considered statistically significant.

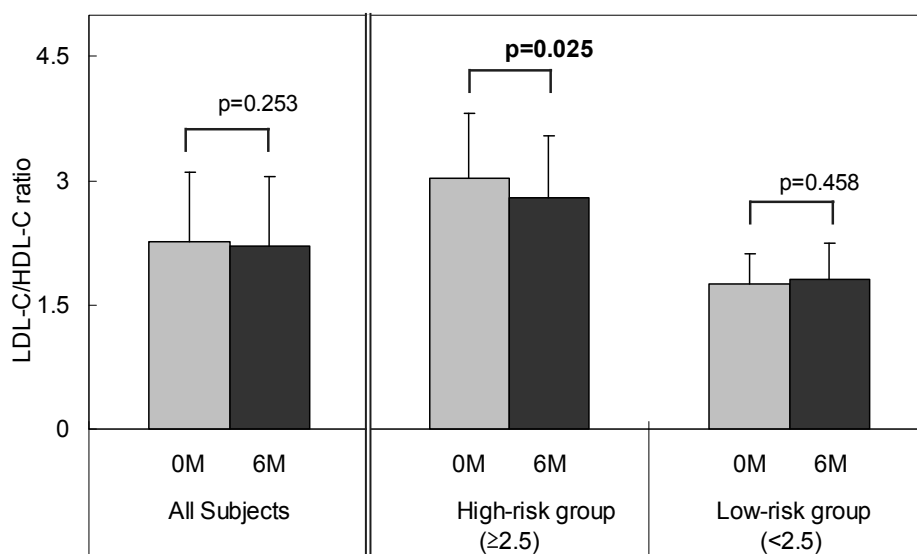
## Results

Of the 32 subjects, one subject complained that the taste of the test food was unpalatable and another subject withdrew from the study after initiating treatment for heart disease. As a result, a total of 30 subjects were included in the final analysis (**Table 1**). **Table 2** shows height, weight, BMI, blood pressure, and blood lipid levels at baseline and 6 months. Weight, BMI, and blood pressure were almost unchanged for 6 months. TC, HDL-C, LDL-C, and TG levels were within the normal range at baseline and 6 months in all the subjects. However, HDL-C and TG levels were significantly higher at 6 months

**Table 2.** Changes in height, weight, BMI, blood pressure, and blood lipid levels in all subjects

	Baseline	After 6 months	<i>p</i>
Height (cm)	162.7 ± 8.1	163.3 ± 8.8	0.316
Weight (kg)	61.5 ± 13.4	61.0 ± 13.3	0.911
BMI	22.9 ± 3.3	22.7 ± 3.4	0.055
Systolic blood pressure (mmHg)	118.6 ± 14.6	115.6 ± 14.3	0.209
Diastolic blood pressure (mmHg)	76.3 ± 10.0	75.1 ± 9.7	0.435
Total-C (mg/dL)	206.8 ± 39.1	214.0 ± 32.2	0.400
TG (mg/dl)	91.6 ± 42.4	103.1 ± 42.3	<b>0.033</b>
LDL-C (mg/dL)	128.6 ± 35.7	132.1 ± 30.5	0.229
HDL-C (mg/dL)	59.8 ± 15.8	63.7 ± 15.4	<b>0.007</b>

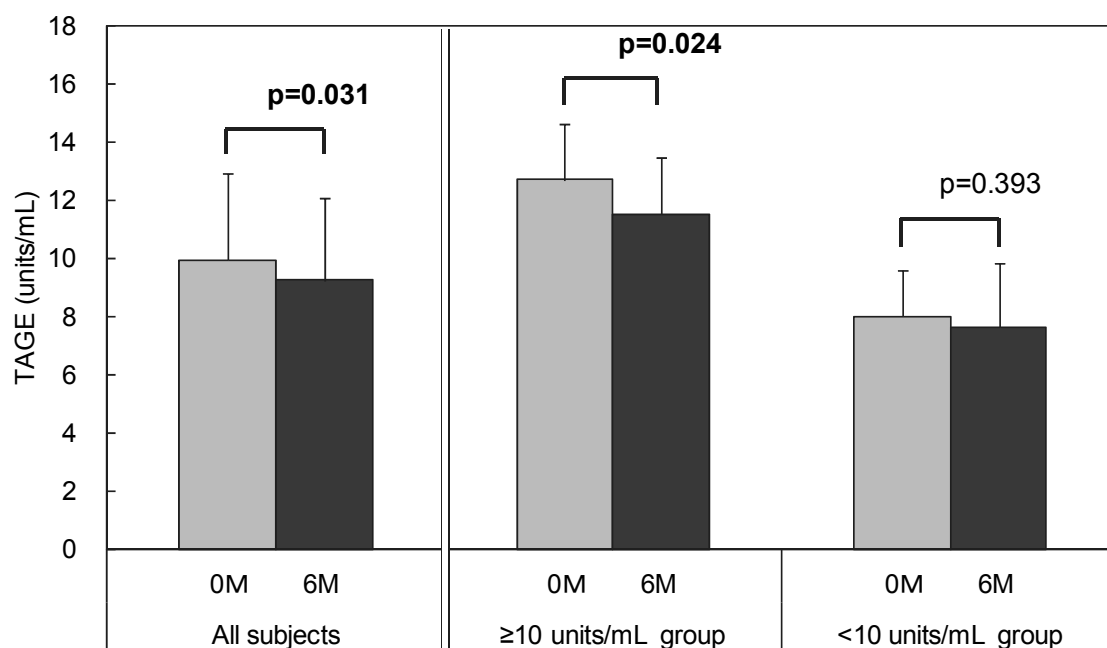
Values are expressed as the mean ± standard deviation. Bold indicates statistically significant differences.

**Fig. 1.** LDL-C/HDL-C ratios

The paired *t*-test was performed to compare the LDL-C/HDL-C ratio between baseline and 6 months among all subjects and between the high-risk group (≥2.5 group, 12 subjects) and low-risk group (<2.5 group, 18 subjects). Error bars represent standard deviation. Bold indicates statistically significant differences.

than at baseline. **Fig. 1** shows the LDL-C/HDL-C ratio at baseline and 6 months. No significant difference in the LDL-C/HDL-C ratio was observed over the study period among all the subjects or within the low-risk group (18 subjects). In contrast, the LDL-C/HDL-C ratio was significantly decreased in the high-risk group (12 subjects). **Fig. 2** shows serum TAGE levels at baseline and 6 months. TAGE levels were significantly lower at 6 months than at baseline in all the subjects. Because a difference was observed between high-risk and low-risk groups in the LDL-C/HDL-C ratio, high-TAGE subjects and low-TAGE subjects divided on the basis of the average value at baseline were also compared. TAGE levels were significantly

decreased from baseline in the high-TAGE group only. Accordingly, the significant decrease in the TAGE levels from baseline observed in all the subjects was considered to be attributable to the significant decrease in the high-TAGE group. **Table 3** shows CAVI values in all the subjects at baseline and 6 months. CAVI values were decreased in the left and right sides of the body by approximately 0.2 points at 6 months. **Table 4** shows the result of single correlation analysis between the right-side CAVI value and each factor. CAVI was significantly correlated with systolic blood pressure, TG, HDL-C, TAGE, age, and sex at baseline. However, the indicators that significantly correlated at 6 months were only age and sex, and the change of



**Fig. 2.** Serum TAGE levels

The paired *t*-test was performed to compare TAGE levels between baseline and 6 months among all subjects and between the high-TAGE ( $\geq 10$  units/mL group, 12 subjects) and low-TAGE groups ( $< 10$  units/mL group, 18 subjects). Error bars represent standard deviation. Bold indicates statistically significant differences.

CAVI in 6 months did not correlate with the change in each factor. CAVI was defined as an objective variable, and six factors (systolic blood pressure, TG, HDL-C, TAGE, age, and sex) showing single correlation with CAVI at baseline were defined as explanatory variables in multiple regression analysis. The results of analysis are shown in **Table 5**. Only age was extracted as an independent contributing factor at baseline and 6 months. A significant relationship between the change in CAVI and that in each factor was not observed.

No significant differences in the RBC and WBC counts; hemoglobin level; hematocrit; MCV; MCH; MCHC; platelet count; and total protein, albumin, total bilirubin, LDH, AST, ALT, ALP,  $\gamma$ -GTP, electrolyte (Na, K, Cl, and Ca), serum ion, inorganic phosphorus, BUN, ferritin, UIBC, or creatinine levels were observed between baseline and 6 months, with all the values remaining within the normal range. No adverse events were observed during the study period.

## Discussion

In the present study, we examined the effects of CTP on the development of atherosclerosis in healthy individuals. The LDL-C/HDL-C ratio has been reported to be approximately 2.02 in healthy individ-

**Table 3.** Changes in CAVI values in all subjects

	Baseline	After 6 months	<i>p</i>
Right side	7.79 $\pm$ 0.83	7.60 $\pm$ 0.97	<b>0.048</b>
Left side	7.79 $\pm$ 0.80	7.61 $\pm$ 0.90	<b>0.047</b>

Values are expressed as the mean  $\pm$  standard deviation. Bold indicates statistically significant differences.

uals<sup>5)</sup>, with the risk of cardiovascular disease being increased in individuals with an LDL-C/HDL-C ratio of  $> 2.5$ <sup>4)</sup>. We then compared its effects in the high-risk group (initial LDL-C/HDL-C  $\geq 2.5$ ) with those in the low-risk group (initial LDL-C/HDL-C  $< 2.5$ ). The result that the LDL-C/HDL-C ratio significantly decreased from baseline in the high-risk group indicates that CTP has efficacy only in the plaque-prone condition, with an imbalance of the two cholesterol levels. The administration of CTP has been shown to decrease the atherosclerotic plaque area in KHC rabbits<sup>17)</sup>. Furthermore, TC levels decreased in the KHC rabbit study in response to CTP; however, such a decrease was not observed in the present study. In addition, HDL-C levels significantly increased in the present study, a finding not reported in the KHC rabbit study. These discrepancies between the present study and the KHC rabbit studies are likely attribut-

**Table 4.** Single correlation between CAVI and each factor

	Baseline		After 6 months		Change in 6 months	
	R	<i>p</i>	R	<i>p</i>	R	<i>p</i>
Weight (kg)	0.085	0.198	0.093	0.780	-0.169	0.373
BMI	0.102	0.719	0.090	0.629	-0.032	0.862
Systolic blood pressure	0.245	<b>0.049</b>	0.301	0.061	0.089	0.640
Diastolic blood pressure	0.020	0.207	0.022	0.257	0.010	0.960
Total-C	-0.250	0.101	-0.084	0.517	0.027	0.889
TG	0.457	<b>0.027</b>	0.375	0.072	-0.064	0.735
LDL-C	-0.240	0.128	-0.016	0.815	-0.059	0.755
HDL-C	-0.309	<b>0.043</b>	-0.264	0.101	0.102	0.590
L/H ratio	0.049	0.751	0.201	0.354	-0.082	0.664
TAGE	0.321	<b>0.038</b>	0.268	0.152	0.116	0.542
Age	0.760	<b>0.018</b>	0.660	<b>0.020</b>	-0.100	0.615
Sex (1: male, 2: female)	-0.350	<b>&lt;0.001</b>	-0.340	<b>&lt;0.001</b>	0.010	0.947

“R” is the correlation coefficient. Bold indicates statistically significant differences.

able to differences between healthy humans and an animal model of hypercholesterolemia.

TAGE levels significantly decreased in the high-TAGE group only, suggesting that CTP has efficacy in conditions where vascular wall damage is more likely to occur. Because there are no reports on normal values and risk values of TAGE, it was divided on the basis of the average value of 10 units/mL. It should be noted that the serum TAGE level of the highest inflammation group was  $9.63 \pm 2.42$  units/mL in the study on the association between TAGE and vascular inflammation; the high-value group in the present study was higher than that in the abovementioned previous study<sup>8</sup>). We did not directly evaluate the influence of blood glucose, plasma insulin, and HbA1c in the present study. However, TAGE generation is promoted by glyceraldehyde, the metabolic intermediate of glucose and fructose, in intracellular compartments. TAGE accumulation results in cell damage, and TAGE leak into the blood; thus, TAGE levels in circulating fluids are considered to increase. Therefore, it is considered that blood TAGE levels are not always related to blood glucose, plasma insulin, and HbA1c<sup>25</sup>).

CAVI values significantly decreased in all the subjects from baseline at 6 months, indicating the recovery of blood vessel elasticity following CTP ingestion. Improvements in either one or both sides of the body were observed in 20 of 30 subjects, with a trend toward overall improvement regardless of the initial values (data not shown). Many clinical studies have reported that CAVI shows a high value in patients with coronary risk factors and the improvement of risk factors decreases CAVI<sup>12</sup>). The subjects in the present

study were healthy individuals and not patients, but it was expected that the decrease of CAVI by CTP administration would reduce future atherosclerotic risk. Although CAVI is improved by weight loss<sup>26</sup>), antihypertensive agents, diabetes medications<sup>27-29</sup>), hypercholesterolemia medications<sup>30, 31</sup>), eicosapentaenoic acid, and smoking cessation<sup>32</sup>), none of these factors applied to any subject included in the present study. Meanwhile, some reports have shown that catechin or soy foods prevent lifestyle-related diseases and atherosclerosis. These effects are due to long-term intake<sup>33</sup>) or high intake<sup>34-36</sup>). In addition, the effects of green tea have not been observed in healthy individuals<sup>36, 37</sup>). Therefore, it was considered that catechin and soy in the daily meal did not affect the test.

Because CAVI increases by approximately 0.5 points for every additional 10 years of age<sup>16</sup>), the 0.2-point decrease at 6 months observed in the present study indicates a significant preventive effect of CTP. We performed single regression analysis and multiple regression analysis between CAVI and other factors to verify which factors reduced CAVI. Several factors showing single correlation with CAVI at baseline no longer correlated at 6 months. Results of the comparison of change in 6 months showed that CAVI did not relate with any factor, except for age, which is consistent with the report on the correlation with CAVI<sup>16</sup>). These results may indicate that CTP directly acts on the vascular wall. Moreover, CTP has been shown to inhibit the proliferation and migration of AoSMCs and to accelerate the fibrillogenesis of type I collagen and promote the expression of type IV collagen as the extracellular matrix around AoSMCs *in vitro*<sup>18</sup>). Future clinical studies are required to validate the effi-

**Table 5.** Correlation between CAVI and the six indicators by multiple regression analysis

	Baseline		After 6 months		Change in 6 months	
	$\beta$	<i>p</i>	$\beta$	<i>p</i>	$\beta$	<i>p</i>
Explanatory variables						
Systolic blood pressure	-0.106	0.488	0.014	0.939	0.083	0.691
TG	0.231	0.209	0.108	0.620	-0.098	0.651
HDL-C	0.150	0.510	0.196	0.440	0.060	0.778
TAGE	0.099	0.517	0.076	0.656	0.164	0.467
Age	0.811	<b>&lt;0.001</b>	0.641	<b>0.006</b>	-0.035	0.895
Sex	0.113	0.612	-0.093	0.715	-0.154	0.565
R <sup>2</sup>	0.649	<b>&lt;0.001</b>	0.493	<b>0.013</b>	0.053	0.968

" $\beta$ " is the standardized regression coefficient. "R<sup>2</sup>" is the coefficient of determination. Bold indicates statistically significant differences.

cacy of long-term CTP ingestion in treating advanced atherosclerosis and to compare between the effects with and without the administration of CTP.

As mentioned above, CTP administration resulted in significant improvements in several predictors of atherosclerosis. More than one risk factor is typically present even in healthy persons if they neglect appropriate nutrition and exercise habits. Accordingly, multifunctionality is a notable feature of CTP. In addition, CTP is expected to have efficacy in treating advanced atherosclerosis in subjects with greater risk factors for atherosclerosis. The absorption of CTP into the blood has been shown to be relatively higher than that of other collagen-containing foods, with C<sub>max</sub> of Gly-Pro-Hyp being 10 nmol/mL in healthy individuals after ingesting 4 g/50 kg body weight of CTP-rich food (50% content)<sup>21</sup>. Accordingly, blood levels approximately half of those in the cases ingesting CTP-rich food were expected to be obtained twice daily in the present study. Because the typical progression of atherosclerosis is slow and asymptomatic, the majority of cases presented with severe cardiovascular disease. The findings of the present study indicate that the ingestion of functional foods such as CTP is likely to become an increasingly important component of preventive medicine.

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### Conflict of Interest

Naohisa Tomosugi is a board member of Medical Care Proteomics Biotechnology Co., Ltd. and holds

shares of more than 5% of Medical Care Proteomics Biotechnology Co., Ltd.; Shogo Katsuda is a board member of Kanazawa University; Yasuo Sakai was a board member of Jellice Co., Ltd.; and Shoko Yamamoto and Noriaki Numata are employees of Jellice Co., Ltd.

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