

# Positive Association between Circulating CD34-Positive Cells and Urinary Sodium Excretion in Elderly Japanese Men: The Nagasaki Islands Study

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## Abstract

Increasing evidence points to involvement of circulating Endothelial Progenitor Cells (EPCs) in maintenance of the vasculature. On the other hand, daily salt intake is reportedly associated with vascular damage such as seen in atherosclerosis and arteriosclerosis. However, no study has investigated the association between circulating endothelial progenitor cells and salt intake in relation to vascular status. To investigate the association between EPCs (CD34-positive cells) and estimated total volume of daily urinary sodium excretion, which is known to be a marker of daily salt intake, we conducted a cross-sectional study of 94 elderly Japanese men aged 60-89 years who underwent general health checks. There was a slightly but significantly positive association between number of circulating CD34-positive cells and daily urinary sodium excretion especially for the participants without a history of Cardiovascular Disease (CVD). A simple analysis of the relationship between these two variables yielded  $r=0.21$  ( $P=0.039$ ) for total subjects,  $r=0.29$  ( $P=0.009$ ) for participants without and  $r=0.18$  ( $P=0.538$ ) for participants with a history of CVD. This suggests that vascular damage due to a high daily intake of salt may stimulate EPC production in healthy elderly Japanese men especially in those without a history of CVD.

**Keywords:** Circulating CD34-positive cells; Urinary sodium excretion; Japanese men

## Introduction

Endothelial progenitor cells (EPCs), including CD34-positive cells, have been shown to contribute to maintenance of the vasculature [1]. Bone marrow-derived endothelial stem cells can differentiate into mature vascular endothelial cells and participate in vascular repair [2]. However findings concerning the relationship between the number of endothelial progenitor cells and atherosclerosis are contradictory. One Japanese study reported finding a strong inverse association between the number of circulating CD34-positive cells and frequency of cerebral infarction, but no relationship between the number of EPCs and degree of atherosclerosis [3]. However, another study reported that the number of circulating endothelial progenitor cell numbers was significantly higher in patients with atherosclerotic intracranial artery stenosis [4].

Vascular endothelium is reportedly damaged by salt overload [5], with one study stating that endothelial function as assessed by Flow Mediated Dilatation (FMD) is impaired by high salt intake [6]. However, no studies have been reported on the possible association between the number of circulating CD34-positive cells and estimated total volume of urinary sodium excretion which is a marker of daily salt intake.

Since contentious vascular damage requires contentious vascular repair, we speculated that the number of circulating endothelial progenitor cells is positively associated with the estimated total volume of urinary sodium excretion, especially for individuals without a history of Cardiovascular Disease (CVD) because the endothelial progenitor cell productivity of those with a history of CVD may be lower, thus resulting in a reduced capability for vascular repair.

To investigate the validity of this hypothesis, we conducted a cross-sectional study of elderly Japanese men aged 60-89 years.

## Materials and Methods

### Subjects

The survey population comprised 101 60-89-year-old men, who were residents in the western rural communities of Hisaka and Kisyuku located in the Goto Islands and who participated in this study in 2013. Seven individuals with missing data were excluded, and the remaining 94 men with a mean age of 71.3 years (standard deviation (SD):  $\pm 6.7$ ; range: 60-89) were enrolled in this study, which was approved by the Ethics Committee for Human Use of Nagasaki University (project registration number 0501120073).

### Data collection and laboratory measurements

Body weight and height were measured with an automatic body composition analyzer (BF-220; Tanita, Tokyo, Japan) when blood samples were obtained, and body mass index (BMI;  $\text{kg}/\text{m}^2$ ) and body surface area (BSA) were calculated with the Fujimoto formula [7]:  $\text{BSA} (\text{m}^2) = \text{weight} (\text{kg})^{0.444} \times \text{height} (\text{cm})^{0.663} \times 0.008883$ . Systolic blood pressure and diastolic blood pressure were recorded at rest.

Fasting blood samples were collected in an EDTA-2K tube and a siliconized tube. Samples from EDTA-2K were used for determining

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numbers of CD34-positive cells. Circulating CD34-positive cells in 50  $\mu$ L of peripheral blood were quantified with ProCOUNT™ according to the manufacturer's protocol (Beckton Dickinson Biosciences, Franklin Lakes, NJ). To minimize intersample variations in measurements of CD34-positive cells, several methods were used: a nucleic acid dye was added as a threshold reagent; a no-wash technique was performed to eliminate cell loss, followed by reverse pipetting; an internal reference particle was added for determination of absolute cell numbers; and an isotope control, matched for the concentration of anti-CD34 antibody and the fluorochrome-to-protein ratio, was included. The BD FACSCant™ II flow cytometer (Beckton Dickinson Biosciences) was used for all measurements. Serum samples were separated. Concentrations of HDL-cholesterol, triglyceride(TG), hemoglobin A1c (HbA1c(NGSP)), sodium (Na), potassium (K), alkaline phosphatase (ALP), calcium (Ca), phosphorus (P), serum creatinine, urinary sodium, and urinary creatinine were measured with standard laboratory procedures. The glomerular filtration rate (GFR) was estimated by means of an established method with three variations recently proposed by a working group of the Japanese Chronic Kidney Disease initiative [8]. According to this adapted version,  $GFR (ml/min/1.73 m^2) = 194 \times [\text{serum creatinine (enzyme method)}]^{-1.094} \times (\text{age})^{-0.287}$ .

We also obtained urine samples for measurements of concentrations of creatinine and sodium with standard laboratory procedures. Creatinine clearance (Ccr) from individual BSA was calculated and GFR was adjusted with the following equation:  $Ccr (mL/min) = GFR (mL/min/1.73m^2) \times BSA (m^2)/1.73m^2$ . Moreover, since Ccr can be calculated as  $Ccr (mL/min) = \text{urinary creatinine (mg/dL)} / \text{serum creatinine (mg/dL)} \times \text{urine volume (mL/min)}$ , and urine volume per day (mL/day) =  $GFR (mL/min/1.73m^2) \times BSA (m^2)/1.73m^2 \times \text{serum creatinine (mg/dL)} / \text{urinary creatinine (mg/dL)} \times 60 \times 24$ , the estimated total volume of urinary sodium excretion in a day (mEq/day) =  $\text{urine volume per day (mL/day)} \times \text{urinary sodium (mEq/L)} / 1000 (mL)$ . We used Eq/day as the unit for analysis of the association between the numbers of CD34-positive cells and estimated total volume of daily urinary sodium excretion because the unit of mEq/day is not suitable for this purpose.

### Statistical analysis

Characteristics of the study participants are expressed in the form of an age-adjusted model. Simple correlation analysis of the numbers of CD34-positive cells and other variables were performed as well as multiple linear analysis to evaluate the number of circulating CD34-positive cells and other parameters adjusted for confounding factors (age, systolic blood pressure, diastolic blood pressure, BMI, HDL-cholesterol, TG, HbA<sub>1c</sub>(NGSP), serum Na, serum K, serum ALP, serum Ca, serum P, and serum creatinine). We also investigated correlations for participants without history of CVD.

All statistical analyses were performed with the SAS system for Windows (version 9.3; SAS Inc., Cary, NC). All p-values for statistical tests were two-tailed, and values of <0.05 were regarded as statistically significant.

## Results

### Characteristics of the study population

Characteristics of the study population are shown in Table 1. The mean age was 71.3 years ( $\pm 6.7$  SD) for total subjects, 71.1 years ( $\pm 6.7$  SD) for participants without, and 72.1 years ( $\pm 6.9$  SD) for participants with a history of CVD. For the latter, the age-adjusted mean estimated

Parameters	Total subjects	History of cardiovascular disease		P
		(-)	(+)	
No of participants	94	80	14	
Age, years	71.3 $\pm$ 6.7	71.1 $\pm$ 6.7	72.1 $\pm$ 6.9	
Systolic blood pressure, mmHg	137	137	137	0.991
Diastolic blood pressure, mmHg	81	81	84	0.335
Body mass index, kg/m <sup>2</sup>	23.4	23.4	23.7	0.739
Body surface area (BSA), m <sup>2</sup>	1.61	1.61	1.63	0.687
Serum HDL-cholesterol, mg/dL	56	57	46	0.011
Serum triglycerides, mg/dL	105	104	109	0.808
HbA1C, %	5.7	5.7	5.7	0.622
Serum sodium (Na), mEq/L	145	145	145	0.998
Serum potassium (K), mEq/L	4.4	4.4	4.4	0.745
Serum alkaline phosphatase (ALP), U/L	241	242	233	0.638
Serum calcium (Ca), mg/dL	9.4	9.4	9.4	0.677
Serum phosphorus (P), mg/dL	3.3	3.3	3.3	0.918
Serum creatinine, mg/dL	0.85	0.84	0.89	0.446
Glomerular filtration rate (GFR), mL/min/1.73m <sup>2</sup>	71.7	72.0	70.0	0.674
Urinary creatinine, mg/dL	114.9	114.2	118.8	0.784
Urinary sodium, mEq/L	125	124	129	0.750
Estimated total daily urinary sodium excretion, Eq/day	0.11	0.10	0.14	0.033
Circulating CD34 positive cells, cells/ $\mu$ L	1.5	1.5	1.3	0.605

Age values show mean  $\pm$  standard deviation. History of cardiovascular disease: history of ischemic heart disease and / or stroke. P: p factor for history of cardiovascular disease

**Table 1:** Age adjusted clinical characteristic of the study population

total daily urinary sodium excretion was significantly higher than that of participants without a history of CVD. As for the age-adjusted numbers of CD34 positive cells, the statistical power of the association did not reach significance but our finding showed that the number for participants with a history of CVD was lower than that for participants without a history of CVD.

### Association between the number of CD34-positive cells and the estimated total daily urinary sodium excretion and serum ALP

Simple linear regression analysis was used for determining the correlations between the number of CD34-positive cells and the estimated total daily urinary sodium excretion. The results were  $r=0.21$  ( $p=0.039$ ) for total subjects,  $r=0.29$  ( $p=0.009$ ) for participants without a history of CVD, and  $r=0.18$  ( $p=0.538$ ) for those with a history of CVD. The corresponding values for serum ALP were  $r=0.21$  ( $p=0.039$ ),  $r=0.22$  ( $p=0.048$ ) and  $r=0.17$  ( $p=0.573$ ) (Table 2) (Figures 1 and 2). Multivariable linear regression analysis adjusted for confounding factors showed that the correlations between the number of CD34-positive cells and the estimated total daily urinary sodium excretion were  $\beta=6.03$  (95% CI: 0.47, 11.60) for total subjects and  $\beta=11.69$  (95% CI: 3.58, 19.81) for participants without a history of CVD and serum ALP  $\beta=7.93 \times 10^{-3}$  (95% CI:  $2.00 \times 10^{-3}$ ,  $13.85 \times 10^{-3}$ ) for total subjects and serum ALP  $\beta=8.02 \times 10^{-3}$  (95% CI:  $1.22 \times 10^{-3}$ ,  $14.81 \times 10^{-3}$ ) for participants without a history of CVD (Table 3).

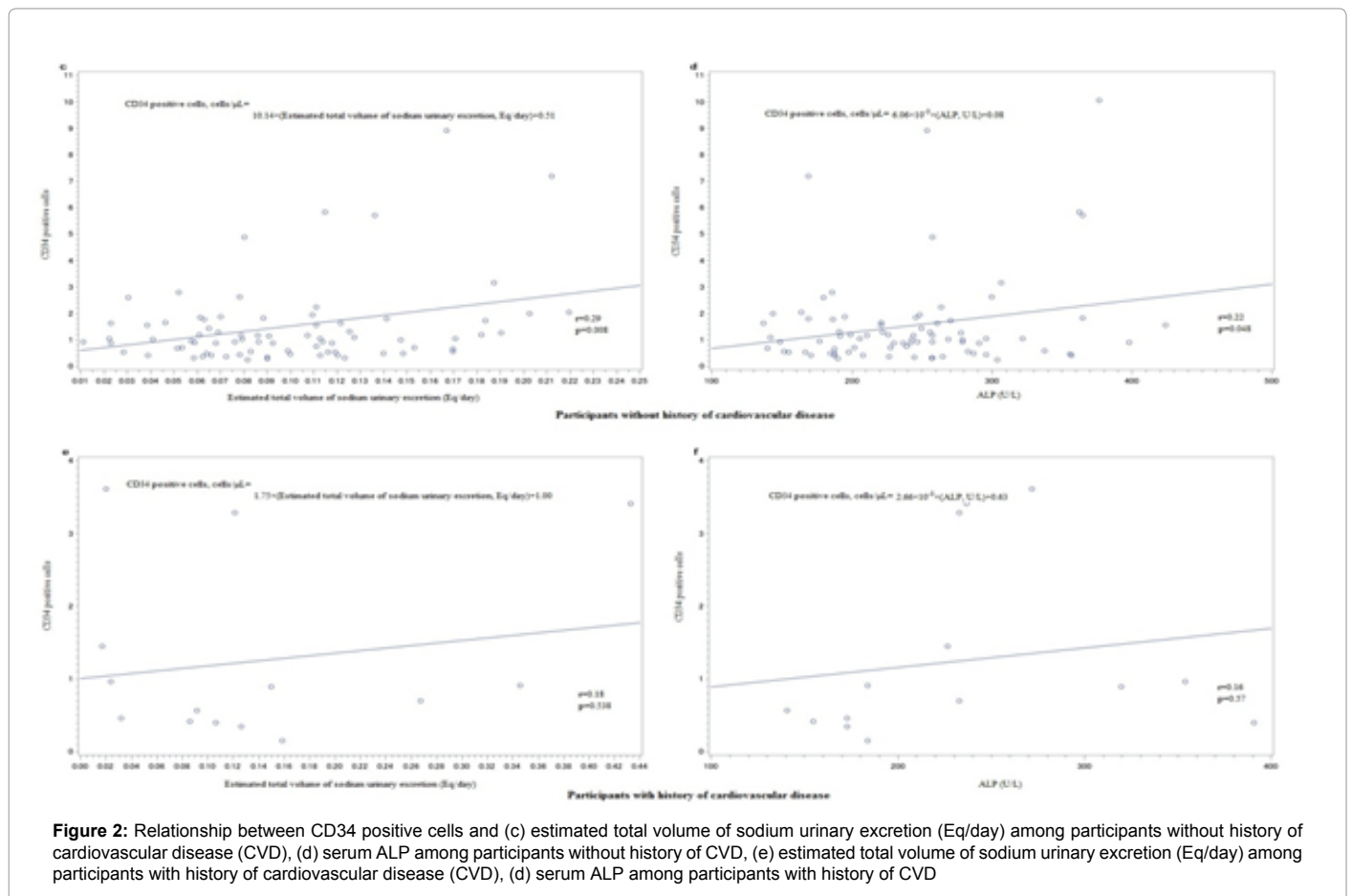
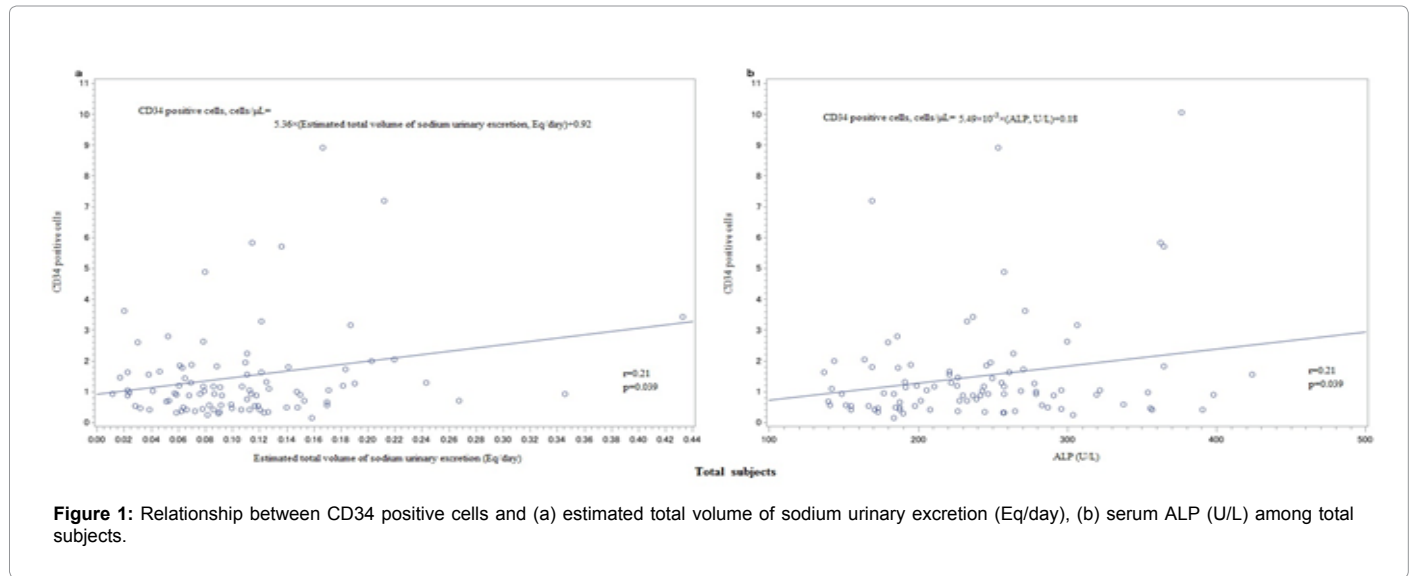
Parameters	Total subjects		Participants			
	r	p	History of cardiovascular disease (-)		History of cardiovascular disease (+)	
			r	p	r	p
No of participants	94		80		14	
Age	-0.134	0.198	-0.156	0.168	0.070	0.812
Systolic blood pressure	-0.057	0.584	-0.074	0.513	0.148	0.614
Diastolic blood pressure	0.103	0.324	0.125	0.269	-0.004	0.990
Body mass index	-0.045	0.667	-0.065	-0.569	0.119	0.687
Serum HDL-cholesterol	0.009	0.928	0.003	0.980	-0.127	0.666
Serum triglycerides	0.081	0.437	0.083	0.467	0.080	0.786
HbA1C	0.099	0.342	0.105	0.353	-0.041	0.889
Serum sodium (Na)	-0.090	0.415	-0.106	0.347	0.177	0.546
Serum potassium (K)	0.052	0.619	0.060	0.594	-0.026	0.931
Serum Alkaline Phosphatase (ALP)	0.214	0.039	0.221	0.048	0.165	0.573
Serum calcium (Ca)	-0.077	0.461	-0.047	0.676	-0.269	0.353
Serum phosphorus (P)	-0.117	0.260	-0.082	0.472	-0.386	0.173
Serum creatinine	-0.037	0.726	-0.039	0.729	0.019	0.949
Estimated total daily urinary sodium excretion	0.214	0.039	0.291	0.009	0.180	0.538

History of cardiovascular disease: history of ischemic heart disease and / or stroke

**Table 2:** Simple correlation analysis of circulating CD34 positive cells and other variables

Parameters	β	95%CL	p value
Total subjects			
No. of participants	94		
Age	-0.04	-0.10, 0.02	0.218
Systolic blood pressure	-0.02	-0.05, 0.01	0.120
Diastolic blood pressure	0.04	-0.01, 0.10	0.136
Body mass index	-0.02	-0.15, 0.11	0.744
Serum HDL-cholesterol	0.003	-0.023, 0.029	0.812
Serum triglycerides	0.001	-0.005, 0.007	0.765
HbA <sub>1c</sub>	0.50	-0.105, 1.098	0.104
Serum sodium (Na)	-0.01	-0.25, 0.22	0.905
Serum potassium (K)	0.18	-0.92, 1.29	0.744
Serum Alkaline Phosphatase (ALP)	7.93×10 <sup>-3</sup>	2.00×10 <sup>-3</sup> , 13.85×10 <sup>-3</sup>	0.009
Serum calcium (Ca)	-0.56	-1.91, 0.79	0.415
Serum phosphorus (P)	-0.29	-1.14, 0.55	0.489
Serum creatinine	1.22	-1.00, 3.44	0.278
Estimated total daily urinary sodium excretion	6.03	0.47, 11.60	0.034
Participants without history of cardiovascular disease			
No. of participants	80		
Age	-0.04	-0.10, 0.03	0.278
Systolic blood pressure	-0.04	-0.07, -0.01	0.025
Diastolic blood pressure	0.07	0.01, 0.14	0.037
Body mass index	-0.02	-0.17, 0.13	0.758
Serum HDL-cholesterol	-0.003	-0.031, 0.026	0.860
Serum triglycerides	0.001	-0.006, 0.008	0.801
HbA <sub>1c</sub>	0.40	-0.24, 1.05	0.213
Serum sodium (Na)	-0.09	-0.34, 0.17	0.501
Serum potassium (K)	-0.02	-1.20, 1.15	0.969
Serum Alkaline Phosphatase (ALP)	8.02×10 <sup>-3</sup>	1.22×10 <sup>-3</sup> , 14.81×10 <sup>-3</sup>	0.022
Serum calcium (Ca)	-0.84	-2.42, 0.73	0.287
Serum phosphorus (P)	0.01	-0.96, 0.99	0.287
Serum creatinine	1.15	-1.27, 3.56	0.348
Estimated total daily urinary sodium excretion	11.69	3.58, 19.81	0.005

**Table 3:** Multiple linear regression analysis of CD34 positive cells and relevant factors adjusted for confounding factors



## Discussion

The major finding of the study presented here was that the number of circulating CD34-positive cells is positively associated with the estimated total daily volume of urinary sodium excretion in elderly healthy Japanese men, especially for those without a history of CVD.

Endothelial progenitor cells (EPCs), including CD34-positive cells, have been shown to contribute to maintenance of the vasculature, not only as a pool of EPCs but also as a source of growth and a factor in angiogenesis [1]. Several studies have reported that accumulation of smooth muscle cells plays a major role in atherosclerosis [9-11], while Miyamoto et al. reported stem cell factor protein was produced in both human aortic endothelial cells and smooth muscle cells [12].

Furthermore, hematopoietic stem cells, which had differentiated into vascular cells, were found to participate in the pathogenesis of atherosclerosis [13]. These findings indicate that atherosclerotic status might be positively associated with the number of CD34-positive cells.

Furthermore, a previous study with 16 healthy normotensive subjects who consumed a meal with added salt and controls who consumed a low-salt meal showed that endothelial function evaluated by Flow Mediated Dilation (FMD) was significantly more impaired after consumption of the meal with added salt than of the low-salt meal, but no significant differences in blood pressure were observed between the two groups [6]. Another study found that salt reduction improves endothelial-dependent vasodilation in normotensive subjects independently of any changes in measured resting clinical blood pressure [14]. Finally, our study showed there is a positive association between the numbers of CD34-positive cells and estimated total volume of urinary sodium excretion, which indicates daily salt intake is independent from blood pressure.

However, a previous Japanese study reported finding a strong inverse association between number of circulating CD34-positive cells and frequency of cerebral infarction. This study also could find no association between the number of EPCs and degree of atherosclerosis [3]. This is partly compatible with our result that showed estimated total daily urinary sodium excretion is significantly higher but the association did not reach significant levels, possibly because the number of CD34 positive cells tended to be lower for participants with than for those without history of CVD. There might thus be another mechanism underlying those mechanisms.

Because osteoblasts (whose activity can be evaluated by bone-type ALP expression [15,16]) regulate the production of hematopoietic stem cells in bone marrow, and previous studies also reported that bone marrow cells give rise to smooth muscle cells as observed in atherosclerotic plaques [8], serum ALP concentration may be correlated with vascular homeostatic activity [17-19]. In our study we found a significantly positive association between CD34-positive cells and serum ALP concentration, while previous studies reported that ALP is positively associated with cardiovascular risk factors and independent from blood pressure [20,21]. This suggests that ALP may be positively associated with bone marrow activity. Furthermore, one study found that bone marrow cells, including hematopoietic stem cells, contribute not only to the healing process of injured organs, but also to pathological remodeling [13]. This study indicated that participants with a low number of EPCs (CD34-positive cells) may suffer from insufficient vascular repair whereas those with a high number of EPCs (CD34-positive cells) may suffer from excessive vascular remodeling such as seen in atherosclerosis. Therefore, both low and high numbers of CD34-positive cells in peripheral blood may constitute risks of cardiovascular disease. In this connection, the finding of a previous study of ours of a U-shaped association between ALP and incidence of stroke among Japanese [22] seems to be compatible with the aforementioned mechanisms.

Some potential limitations of this study warrant further consideration. First, since we did not have access to FMD data we could not determine the possible effect of FMD on both the number of CD34-positive cells and the estimated total daily urinary sodium excretion. Second, it has been reported that side population hematopoietic stem cells in bone marrow decrease as individuals age [23,24], and this decline may be associated with an increase in the frequency of anemia and other hematopoietic disorders that are seen in the elderly [25]. Compared studies of younger subjects, those of elderly subjects may

therefore involve less bone marrow action for vascular maintenance which might limit the power of statistical analysis of this relationship. However, even though our analyses concerned elderly participants, significant positive associations were found between the number of circulating CD34-positive cells and estimated total volume of daily urinary sodium excretion for total subjects and participants without a history of CVD. Third, although a previous study of ours reported that alcohol consumption strongly affects serum ALP, which is associated with stroke [22] and hypertension [26] especially for Japanese men, the limited number of participants in this study prevented us from conducting a drinking-status specific analysis. However, a significant positive association was observed between number of CD34-positive cells and serum ALP. Because of the limited number of participants with a history of CVD, no multiple linear regression analysis could be performed for them. However, the simple correlation analysis indicated the values for participants with and without a history of CVD were different. Finally, because this was a cross-sectional study, we could not establish any causal relationships. However, we found that the estimated total daily urinary sodium excretion for participants with a history of CVD was significantly higher than that for those without a history of CVD, while such an association was not observed for serum sodium levels. This could be partly explained by the fact that the total daily urinary sodium excretion represents total daily salt intake and that a high salt intake constitutes a risk for CVD. Furthermore, when the analysis was limited to participants without a history of CVD, it showed a significant association between the numbers of circulating CD34 positive cells and estimated total daily urinary sodium excretion. This indicates that the number of CD34 positive cells increases as a result of active vascular damage which is induced by high salt intake.

## Conclusions

Circulating CD34-positive cells are positively associated with estimated total volume of daily urinary sodium excretion. This finding suggests that a high daily intake of salt may activate EPC production because of the presence of vascular damage in healthy elderly Japanese men especially for those without a history of CVD.

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