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Serum Calcium Level and Functional Atherosclerosis in Relation to Human T-Cell Leukemia Virus 1 Infection in Older Individuals

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Abstract: Serum calcium levels are known to influence vascular function. Cells infected with human T-cell leukemia virus 1 (HTLV-1) impact serum calcium levels and also affect the endothelium. Since a damaged endothelium causes functional atherosclerosis, serum calcium levels in HTLV-1 carriers may be positively associated with functional atherosclerosis. This cross-sectional study enrolled 1694 Japanese individuals aged 60 to 89 years. Functional atherosclerosis was defined as a cardio-ankle vascular index (CAVI) $\geq$ 9.0. Logistic regression analysis was used to evaluate the relationship between HTLV-1 carrier status and both serum calcium level and functional atherosclerosis. Even after adjusting for known confounding factors, the serum calcium level was significantly positively associated with functional atherosclerosis only in asymptomatic HTLV-1 carriers. The fully adjusted odds ratio and 95% confidence interval of functional atherosclerosis in relation to a one-standard-deviation increment of serum calcium level (0.4 mg/dL in both men and women) were 1.54 (1.18, 2.01) for HTLV-1 carriers (n = 322) and 0.99 (0.87, 1.13) for HTLV-1 non-carriers (n = 1372), respectively. The serum calcium level was positively associated with functional atherosclerosis only among older carriers of HTLV-1 infection. This finding can help effectively estimate the risk of functional atherosclerosis in asymptomatic HTLV-1 carriers.

Keywords: atherosclerosis; calcium; CAVI; HTLV-1

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1. Introduction

Aggressive endothelial repair causes both structural arterial stiffness, which is evaluated based on carotid intima-media thickness (CIMT), and functional arterial stiffness, which is determined using the cardio-ankle vascular index (CAVI). In contrast, deficient endothelial repair results in functional but not structural arterial stiffness [1]. In this article, arterial stiffness is defined as a continuous variable and atherosclerosis as a binary variable.

The majority of human T-cell leukemia virus 1 (HTLV-1) carriers remain asymptomatic throughout their lives [2–5], although asymptomatic carrier status is positively associated with the progression of periodontitis [6,7]. Since the severity of periodontitis is associated with CAVI values [8], asymptomatic HTLV-1 infection could be associated with functional atherosclerosis defined as CAVI $\geq$ 9.0 [9].

HTLV-1 spreads though parenteral, sexual, and vertical (mother-to-child) routes [10] because HTLV-1 infection occurs mainly through cell-to-cell contact [11]. Therefore, the majority of HTLV-1 carriers might be infected with HTLV-1 at a young age.

HTLV-1 is known to enhance inflammation [12,13], possibly by activating the nuclear factor-kappa B (NF-κB) pathway [14], leading to structural atherosclerosis [15]. Therefore, HTLV-1 carriers often develop both structural and functional atherosclerosis. However, chronic aggressive endothelial repair eventually hinders the repair process due to the consumption and reduced number of CD34-positive hematopoietic stem cells. Under such conditions, structural atherosclerosis does not develop, but functional atherosclerosis does [1,16].

Endothelial cells are known to engage in heterocellular communication with hematologic malignant cells, such as adult T-cell leukemia cells [17]. HTLV-1-infected endothelial cells exhibit altered biological characteristics [18,19], including dysfunctional intercellular communication, which might increase the risk of endothelial dysfunction. Furthermore, HTLV-1 infection of CD34-positive cells causes cell cycle arrest [20]. Since a deficiency of CD34-positive cells causes functional but not structural atherosclerosis [1,21,22], HTLV-1 infection of these cells is likely to result in functional atherosclerosis.

HTLV-1-infected cells elevate serum calcium levels by inducing the transcription of parathyroid hormone-related protein (PTHrP) [23,24]. Therefore, serum calcium levels might reflect the activity of HTLV-1-infected cells. In asymptomatic HTLV-1 carriers, elevated serum calcium levels may indicate HTLV-1 activity and, therefore, exhibit a positive association with functional atherosclerosis.

Against this background, we hypothesized that serum calcium levels may be positively associated with functional atherosclerosis in HTLV-1 carriers but not HTLV-1 non-carriers. Clarifying the association between serum calcium levels and functional atherosclerosis in HTLV-1 carriers may make it possible to effectively estimate the risk of developing functional atherosclerosis in daily clinical practice. To test our hypothesis, we conducted a cross-sectional study of older Japanese individuals.

2. Materials and Methods

2.1. Study Desgin and Population

This was a cross-sectional study of 1694 Japanese individuals aged 60–89 years. We initially included 1774 participants (644 men and 1130 women) from Goto City in western Japan, who received general health check-ups from 2016 to 2018. These checkups were required to include an arterial health assessment that evaluated structural arterial stiffness (via carotid intima-media thickness (CIMT), a continuous variable) and functional arterial stiffness (via CAVI, also a continuous variable). The annual check-up program was conducted by the local government and directed by the Ministry of Health, Labor, and Welfare of Japan. Participants who had missing data on tartrate-resistant acid phosphatase 5b (TRACP-5b) (n = 6) or bone-specific alkaline phosphatase (BAP) (n = 74) were excluded. The remaining participants were enrolled in this study, which comprised 589 men and 1105 women, with a mean age of 72.8 (standard deviation (SD), 7.0) years among men and 72.0 (SD, 7.0) years among women.

Written consent forms were made available to ensure that the participants understood the study objectives. Informed consent was obtained from all participants. This study was approved by the ethics committee of the Nagasaki University Graduate School of Biomedical Sciences (project registration number, 14051404-13). All study procedures involving human participants were performed in accordance with the ethical standards of the institutional research committee and the 1964 Helsinki Declaration and its later amendments.

2.2. Blood Pressure

Anti-hypertensive medication use was ascertained by specially trained interviewers. After at least 5 min of rest, blood pressure (systolic and diastolic) was measured in a seated position using a blood-pressure-measuring device (HEM-907; Omron, Kyoto, Japan).

Hypertension was defined as systolic blood pressure $\geq$ 140 mmHg, diastolic blood pressure $\geq$ 90 mmHg, or anti-hypertensive medication use.

2.3. Laboratory Measurments

Bone metabolism, including osteoclast and osteoblast activity, and phosphate metabolism influence serum calcium levels and can, thereby, affect the development of functional atherosclerosis [25,26]. Levels of TRACP-5b (an osteoclast marker), BAP (an osteoblast marker), serum phosphate (a calcification marker), serum calcium, and serum creatinine were measured using standard laboratory procedures by SRL, Inc. (Tokyo, Japan). Estimated glomerular filtration rate (eGFR) was calculated using a method recently established by a working group of the Japanese Chronic Kidney Disease Initiative [27]: eGFR (mL/min/1.73 m^2) = 194 $\times$ (serum creatinine (enzyme method))$^{-1.094}$ $\times$ (age)$^{-0.287}$ $\times$ (0.739 for women).

2.4. Detection of Human T-Cell Leukemia Virus 1 (HTLV-1)

To detect HTLV-1 infection, a chemiluminescent enzyme immunoassay (CLEIA) kit (Fujirebio Inc., Tokyo, Japan) was used at SRL, Inc., instead of confirmatory tests involving real-time reverse transcription polymerase chain reaction using a hydrolysis probe and Western blotting assays. Our previous study revealed that the CLEIA method had a low false-positive rate (1.2%) compared with the traditional confirmatory tests by using real-time reverse transcription polymerase chain reaction with the hydrolysis probe and Western blotting assays [28].

2.5. Cardio-Ankle Vascular Index (CAVI) Measurement

Functional atherosclerosis was defined as CAVI $\geq$ 9.0, as in our previous study [9]. CAVI was determined with a VaSera VS-1000 vascular screening system (Fukuda Denshi, Tokyo, Japan) in the supine position. The underlying principles of CAVI were previously described by Yambe et al. [29]. The brachial–ankle pulse wave velocity (PWV) is generally used to evaluate functional arterial stiffness. Since PWV measurements can be strongly affected by blood pressure [30], CAVI was recently developed in Japan to avoid this interference [31].

2.6. Carotid Intima-Media Thickness (CIMT) Measurement

Experienced vascular technicians measured CIMT using a LOGIQ Book XP device with a 10 MHz transducer (GE Healthcare, Chicago, WI, USA). The maximum CIMT values for the left and right common carotid arteries were calculated using semi-automated digital edge-detection software (IntimaScope; Media Cross, Tokyo, Japan), according to a previously described protocol [32]. Structural atherosclerosis was defined as CIMT $\geq$ 1.1 mm, as in our previous study [6].

2.7. Statistical Analysis

Patient characteristics and serum calcium levels stratified by HTLV-1 infection status were expressed as mean $\pm$ SD for continuous variables. The tertile values and 1 SD increments of serum calcium levels were evaluated by sex since these levels are known to be sex-specific.

To evaluate the statistical significance of subject characteristics categorized by HTLV-1 carrier status, Student's *t*-test was used for continuous variables, and the chi-squared test was used for prevalence. For serum calcium levels (tertiles) stratified by HTLV-1 carrier status, a trend test was performed by analysis of variance for continuous variables and the chi-squared test for prevalence.

Hypertension, which is reported to be positively associated with serum calcium levels [33], should reduce endothelial repair due to consumption of CD34-positive cells [34]. Since deficient endothelial repair causes functional atherosclerosis [1,21,22], hypertension may play an important role in the association between serum calcium levels and functional atherosclerosis.

Therefore, logistic regression models were used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) to determine the associations between serum calcium levels and both hypertension and functional atherosclerosis stratified by HTLV-1 carrier status. In addition, the ORs and 95% CIs between hypertension and functional atherosclerosis stratified by HTLV-1 carrier status were calculated as a sub-analysis. Logistic regression models were also used to evaluate *p*-values for the associations between tertiles of serum calcium levels and both hypertension and functional atherosclerosis to confirm the linearity of these associations. Adjustment for confounding factors was performed using three models. The first model (Model 1) was adjusted only for sex and age. The second model (Model 2) was adjusted for sex, age (years), serum phosphate (mg/dL), TRACP-5b (mU/dL), BAP (μg/dL), and eGFR (mL/min/1.73 m^2). Since bone turnover and renal reabsorption are common mechanisms for regulating serum calcium and phosphate levels [25,26], several parameters could act as confounding factors in the present analyses, including serum phosphate level, markers of bone turnover, such as TRACP-5b and BAP, and markers of renal function, such as eGFR. Therefore, in addition to the factors in Model 2, CIMT (mm) was added as a confounding factor in the third model (Model 3). Aggressive endothelial repair progresses both structural and functional arteriosclerosis, while deficient endothelial repair increases functional but not structural arterial stiffness [1]. Therefore, structural arterial stiffness could also act as a confounding factor in the present study because deficient endothelial repair might cause HTLV-1-related functional atherosclerosis.

A logistic regression model was also used to examine the effect of HTLV-1 infection on the association between serum calcium levels and functional atherosclerosis. A sensitivity analysis was performed to determine the sex-specific association between serum calcium levels and functional atherosclerosis stratified by HTLV-1 carrier status. The *p*-value for interaction of HTLV-1 infection between serum calcium levels and functional atherosclerosis was calculated by including the product term in the multivariable models.

All statistical analyses were performed with SAS for Windows (version 9.4; SAS Inc., Cary, NC, USA). As in previous studies [35,36], $p < 0.05$ was considered to be statistically significant for the main effects, and $p < 0.2$ was considered to be statistically significant for the interactions.

3. Results

3.1. Characteristics of the Study Population

Of the 1694 participants in this study, 322 were HTLV-1 carriers, and 654 had functional atherosclerosis. Table 1 shows the clinical characteristics of the study population by HTLV-1 carrier status. Compared with individuals without HTLV-1 infection (non-carriers), those with HTLV-1 infection (carriers) had a significantly lower serum calcium level and significantly higher CIMT and CAVI values.

HTLV-1carrier status-specific clinical characteristics of the study population in relation to the serum calcium levels (tertiles) are shown in Table 2. For both HTLV-1 non-carriers and HTLV-1 carriers, anti-hypertensive medication use was positively associated with serum calcium. In HTLV-1 non-carriers, the serum calcium level was positively associated with TRACP-5 and the serum phosphate level, and it was inversely associated with age and eGFR. A significant positive association between serum calcium and functional atherosclerosis was observed in HTLV-1 carriers.

3.2. Association between Serum Calcium Level and Hypertension by HTLV-1 Carrier Status

Table 3 shows the association between the serum calcium level and hypertension by HTLV-1 carrier status. A significantly positive association between the serum calcium level and hypertension was observed in both HTLV-1 carriers and non-carriers. This association remained after adjusting for potential confounding factors.

Table 1. Characteristics of the study population by HTLV-1 carrier status.

	Human T-Cell Leukemia Virus Type-1 (HTLV-1)		*p*
	(−)	(+)	
No. of participants	1372	322	
Men, %	35.8	30.2	0.070
Age, year	72.0 ± 6.9	73.8 ± 7.0	<0.001
Hypertension, *n* (%)	875 (63.8)	198 (61.5)	0.444
Anti-hypertensive medication use, *n* (%)	464 (33.8)	107 (33.2)	0.840
TRACP-5b, mU/dL	318 ± 159	304 ± 160	0.183
BAP, μg/dL	14.8 ± 5.7	14.4 ± 5.8	0.261
Serum calcium, mg/dL	9.5 ± 0.4	9.4 ± 0.4	0.003
Serum phosphate, mg/dL	3.4 ± 0.4	3.5 ± 0.5	0.074
eGFR, mL/min/1.73 m^2	70.2 ± 14.9	68.6 ± 14.5	0.074
Structural atherosclerosis, *n* (%)	457 (33.3)	120 (37.3)	0.177
Functional atherosclerosis, *n* (%)	517 (37.7)	137 (42.5)	0.107
CIMT, mm	0.94 ± 0.21	0.97 ± 0.23	0.035
CAVI	8.7 ± 1.2	8.8 ± 1.3	0.025

TRACP-5b: tartrate-resistant acid phosphatase 5b. BAP: bone-specific alkaline phosphatase. eGFR: estimate glomerular filtration rate. CIMT: carotid intima-media thickness. CAVI: cardio-ankle vascular index.

Table 2. HTLV-1 carrier status-specific characteristics of study population in relation to serum calcium levels.

	Human T-Cell Leukemia Virus Type-1 (HTLV-1)							
	(−)				(+)			
	Serum Calcium			*p* for Trend	Serum Calcium			*p* for Trend
	T1 (Low)	T2	T3 (High)		T1 (Low)	T2	T3 (High)	
No. of participants	464	484	424		144	103	75	
Men, %	34.9	33.5	39.4	0.160	30.6	24.3	38.7	0.120
Age, year	73.2 ± 7.0	71.8 ± 6.9	70.8 ± 6.9	<0.001	74.1 ± 6.8	73.7 ± 6.9	73.6 ± 7.8	0.860
Hypertension, *n* (%)	259 (55.8)	311 (64.3)	305 (71.9)	<0.001	77 (53.5)	63 (61.2)	58 (77.3)	0.003
Anti-hypertensive medication use, *n* (%)	114 (24.6)	164 (33.9)	186 (43.9)	<0.001	35 (24.3)	38 (36.9)	34 (45.3)	0.005
TRACP-5b, mU/dL	290 ± 155	324 ± 153	339 ± 166	<0.001	282 ± 151	326 ± 171	317 ± 159	0.081
BAP, μg/dL	14.8 ± 5.8	14.7 ± 5.5	14.9 ± 5.7	0.872	14.9 ± 6.5	14.2 ± 5.5	13.8 ± 4.5	0.418
Serum phosphate, mg/dL	3.3 ± 0.4	3.4 ± 0.4	3.5 ± 0.5	<0.001	3.4 ± 0.5	3.5 ± 0.5	3.5 ± 0.5	0.190
eGFR, mL/min/1.73 m^2	71.7 ± 15.7	69.9 ± 14.1	69.0 ± 14.8	0.025	69.1 ± 14.6	68.8 ± 14.4	67.3 ± 14.5	0.655
Structural atherosclerosis, *n* (%)	164 (35.3)	156 (32.2)	137 (32.3)	0.520	53 (36.8)	40 (38.8)	27 (36.0)	0.918
Functional atherosclerosis, *n* (%)	192 (41.4)	173 (35.7)	152 (35.8)	0.130	54 (37.5)	42 (40.8)	41 (54.7)	0.046
CIMT, mm	0.95 ± 0.20	0.94 ± 0.21	0.94 ± 0.21	0.787	0.96 ± 0.24	1.00 ± 0.24	0.95 ± 0.18	0.302
CAVI	8.7 ± 1.0	8.7 ± 1.2	8.7 ± 0.65	0.646	8.7 ± 1.0	8.9 ± 1.3	9.1 ± 1.6	0.100

TRACP-5b: tartrate-resistant acid phosphatase 5b. BAP: bone-specific alkaline phosphatase. eGFR: estimate glomerular filtration rate. CIMT: carotid intima-media thickness. CAVI: cardio-ankle vascular index. Serum calcium levels (tertiles) for men are <9.3 mg/dL for T1 (low) and 9.3–9.5 mg/dL for T2, and 9.6 mg/dL ≤ for T3 (high) for both men and women.

Table 3. Odds ratios and 95% confidence intervals between serum calcium and hypertension by HTLV-1 carrier status.

	Serum Calcium			*p*	1 SD Increment of Serum Calcium
	T1 (Low)	T2	T3 (High)		
HTLV-1 (+)					
No. of participants	144	103	75		
No. of cases (%)	77 (53.5)	63 (61.2)	58 (77.3)		
Model 1	Ref	1.48 (0.86, 2.52)	3.32 (1.72, 6.44)	<0.001	1.79 (1.37, 2.33)
Model 2	Ref	1.43 (0.82, 2.51)	3.63 (1.81, 7.29)	<0.001	1.79 (1.36, 2.37)
Model 3	Ref	1.39 (0.79, 2.45)	3.67 (1.82, 7.38)	<0.001	1.78 (1.35, 2.35)
HTLV-1 (−)					
No. of participants	464	484	424		
No. of cases (%)	259 (55.8)	311 (64.3)	305 (71.9)		
Model 1	Ref	1.59 (1.21, 2.08)	2.43 (1.81, 3.25)	<0.001	1.54 (1.36, 1.75)
Model 2	Ref	1.56 (1.18, 2.05)	2.37 (1.75, 3.21)	<0.001	1.53 (1.34, 1.74)
Model 3	Ref	1.57 (1.19, 2.07)	2.38 (1.76, 3.23)	<0.001	1.53 (1.35, 1.74)

Ref: reference. SD: standard deviation. Serum calcium levels (tertiles) for men are <9.3 mg/dL for T1 (low) and 9.3–9.5 mg/dL for T2, and 9.6 mg/dL ≤ for T3 (high) for both men and women. The 1 SD increments of serum calcium are 0.4 mg/dL for both men and women. Model 1: adjusted only for sex and age. Model 2: adjusted for factors included in Model 1 and tartrate-resistant acid phosphatase 5b (TRACP-5b), bone-specific alkaline phosphatase (BAP), serum phosphate, and estimate glomerular filtration rate (eGFR). Model 3: adjusted for factors included in Model 2 and carotid intima-media thickness (CIMT).

3.3. Association between Serum Calcium Level and Functional Atherosclerosis by HTLV-1 Carrier Status

Table 4 shows the association between the serum calcium level and functional atherosclerosis by HTLV- 1 carrier status. A significant positive association between the serum calcium level and functional atherosclerosis was observed in HTLV-1 carriers but not in HTLV-1 non-carriers. This association remained after adjusting for potential confounding factors.

3.4. Effect of HTLV-1 Infection on the Association between Serum Calcium Level and Functional Atherosclerosis

An investigation of the effect of the interaction between HTLV-1 carrier status and a one SD increment of the serum calcium level (0.4 mg/dL for both men and women) on functional atherosclerosis revealed a significant value ($p = 0.005$) for all models (Model 1, Model 2, and Model 3).

3.5. Sex-Specific Analysis of the Association bewteen Serum Calcium Level and Functional Atherosclerosis by HTLV-1 Carrier Status

We also performed a sex-specific analysis of the association between the serum calcium level and functional atherosclerosis, and the results are shown in Table 5. The same associations were observed in men and women.

Table 4. Odds ratios and 95% confidence intervals between serum calcium level and functional atherosclerosis by HTLV-1 carrier status.

	Serum Calcium			*p*	1 SD Increment of Serum Calcium
	T1 (Low)	T2	T3 (High)		
HTLV-1 (+)					
No. of participants	144	103	75		
No. of cases (%)	54 (37.5)	42 (40.8)	41 (54.7)		
Model 1	Ref	1.29 (0.73, 2.27)	2.36 (1.25, 4.45)	0.010	1.47 (1.14, 1.89)
Model 2	Ref	1.37 (0.77, 2.45)	2.62 (1.36, 5.06)	0.005	1.54 (1.18, 2.01)
Model 3	Ref	1.36 (0.76, 2.44)	2.62 (1.36, 5.06)	0.005	1.54 (1.18, 2.01)
HTLV-1 (−)					
No. of participants	464	484	424		
No. of cases (%)	192 (41.4)	173 (35.7)	152 (35.8)		
Model 1	Ref	0.91 (0.68, 1.22)	0.98 (0.71, 1.32)	0.856	0.98 (0.87, 1.12)
Model 2	Ref	0.92 (0.69, 1.24)	0.99 (0.72, 1.35)	0.927	0.99 (0.88, 1.13)
Model 3	Ref	0.92 (0.69, 1.24)	0.99 (0.72, 1.35)	0.906	0.99 (0.87, 1.13)

Ref: reference. SD: standard deviation. Serum calcium levels (tertiles) for men are <9.3 mg/dL for T1 (low) and 9.3–9.5 mg/dL for T2, and 9.6 mg/dL $\leq$ for T3 (high) for both men and women. The 1 SD increments of serum calcium are 0.4 mg/dL for both men and women. Model 1: adjusted only for sex and age. Model 2: adjusted for factors included in Model 1 and tartrate-resistant acid phosphatase 5b (TRACP-5b), bone-specific alkaline phosphatase (BAP), serum phosphate, and estimate glomerular filtration rate (eGFR). Model 3: adjusted for factors included in Model 2 and carotid intima-media thickness (CIMT).

Table 5. Sex-specific ratios and 95% confidence intervals between serum calcium level and functional atherosclerosis by HTLV-1 carrier status.

	Human T-Cell Leukemia Virus Type-1 (HTLV-1)		Interaction
	(−)	(+)	
Men			
No. of participants	491	98	
No. of cases (%)	253 (51.5)	55 (56.1)	
Model 1	0.97 (0.80, 1.18)	1.50 (0.95, 2.35)	0.067
Model 2	0.98 (0.83, 1.19)	1.50 (0.93, 2.41)	0.073
Model 3	0.98 (0.80, 1.19)	1.50 (0.93, 2.42)	0.073
Women			
No. of participants	881	224	
No. of cases (%)	264 (30.0)	82 (36.6)	
Model 1	0.99 (0.85, 1.16)	1.46 (1.08, 1.99)	0.033
Model 2	1.01 (0.86, 1.20)	1.59 (1.15, 2.19)	0.042
Model 3	1.02 (0.86, 1.20)	1.60 (1.16, 2.22)	0.049

Interaction: *p*-value for effect of HTVL-1 carrier on the association between serum calcium and functional atherosclerosis. Model 1: adjusted only for sex and age. Model 2: adjusted for factors included in Model 1 and tartrate-resistant acid phosphatase 5b (TRACP-5b), bone-specific alkaline phosphatase (BAP), serum phosphate, and estimate glomerular filtration rate (eGFR). Model 3: adjusted for factors included in Model 2 and carotid intima-media thickness (CIMT).

3.6. Association bewteen Hypertension and Functional Atherosclerosis by HTLV-1 Carrier Status

Table 6 shows the association between hypertension and functional atherosclerosis by HTLV-1 carrier status. These factors showed a significant positive association among HTLV-1 non-carriers, which persisted after adjusting for potential confounding factors, but there was no association among HTLV-1 carriers.

Table 6. Odds ratios and 95% confidential intervals between hypertension and functional atherosclerosis by HTLV-1 carrier status.

	Hypertension		*p*
	(−)	(+)	
HTLV-1 (+)			
No. of participants	124	198	
No. of cases	44 (35.5)	93 (47.0)	
Model 1	Ref	1.08 (0.65, 1.81)	0.765
Model 2	Ref	1.08 (0.64, 1.83)	0.771
Model 3	Ref	1.08 (0.63, 1.83)	0.786
HTLV-1 (−)			
No. of participants	497	875	
No. of cases	141 (28.4)	376 (43.0)	
Model 1	Ref	1.48 (1.14, 1.93)	0.003
Model 2	Ref	1.53 (1.17, 1.99)	0.002
Model 3	Ref	1.51 (1.16, 1.97)	0.002

Ref: reference. Model 1: adjusted only for sex and age. Model 2: adjusted for factors included in Model 1 and tartrate-resistant acid phosphatase 5b (TRACP-5b), bone-specific alkaline phosphatase (BAP), serum phosphate, and estimate glomerular filtration rate (eGFR). Model 3: adjusted for factors included in Model 2 and carotid intima-media thickness (CIMT).

4. Discussion

The major finding of this study was that the serum calcium level was positively associated with functional atherosclerosis, but only in HTLV-1 carriers and not in HTLV-1 non-carriers.

The majority of older HTLV-1 carriers were initially infected with HTLV-1 at a young age. However, there was subsequently much greater awareness of the hygiene necessary to prevent HTLV-1 infection; thus, fewer individuals were infected, and the current population of HTLV-1 carriers is relatively older. However, age might not have influenced the main results of this study because no biologically relevant correlation between age and the serum calcium level was observed in HTLV-1 carriers. The sex-adjusted partial correlation coefficient (r) and (p) was r = −0.14 ($p < 0.001$).

Atherosclerosis can be categorized as structural or functional from the viewpoint of endothelial repair [1,21,22]: aggressive endothelial repair progresses both structural and functional atherosclerosis, while endothelial repair deficiency progresses only functional atherosclerosis [1]. Independent of structural arterial stiffness evaluated by CIMT, this study demonstrated a significant positive association between the serum calcium level and functional atherosclerosis, but only in HTLV-1 carriers.

Figure 1 shows the potential mechanism that underlies the present results. A reduction in the number of CD34-positive cells due to consumption resulted in insufficient endothelial repair. Since HTLV-1 infection induces aggressive endothelial repair that causes structural atherosclerosis [37], HTLV-1 carriers may have a higher risk of insufficient endothelial repair than HTLV-1 non-carriers.

Calcium regulates smooth muscle tone [38]. A previous study on humans evaluated the effects of intravenous calcium infusion on blood pressure and total peripheral vascular resistance, and it revealed that a higher serum calcium level was correlated with elevated blood pressure (both systolic and diastolic) and total peripheral vascular resistance [33]. Increased serum calcium induces calcium influx into arterial smooth muscle, which increases cytosolic calcium and leads to arterial vasoconstriction, thereby elevating blood pressure. Our finding that the serum calcium level was significantly positively associated with hypertension among both participants with and without HTLV-1 infection (Table 3) (a, b in Figure 1) was in line with prior results. Since hypertension greatly damages the endothelium, the serum calcium level may serve as a marker of endothelial injury. In the present study, however, only HTLV-1 carriers demonstrated a positive association between the serum calcium level and functional atherosclerosis. We address this issue below.

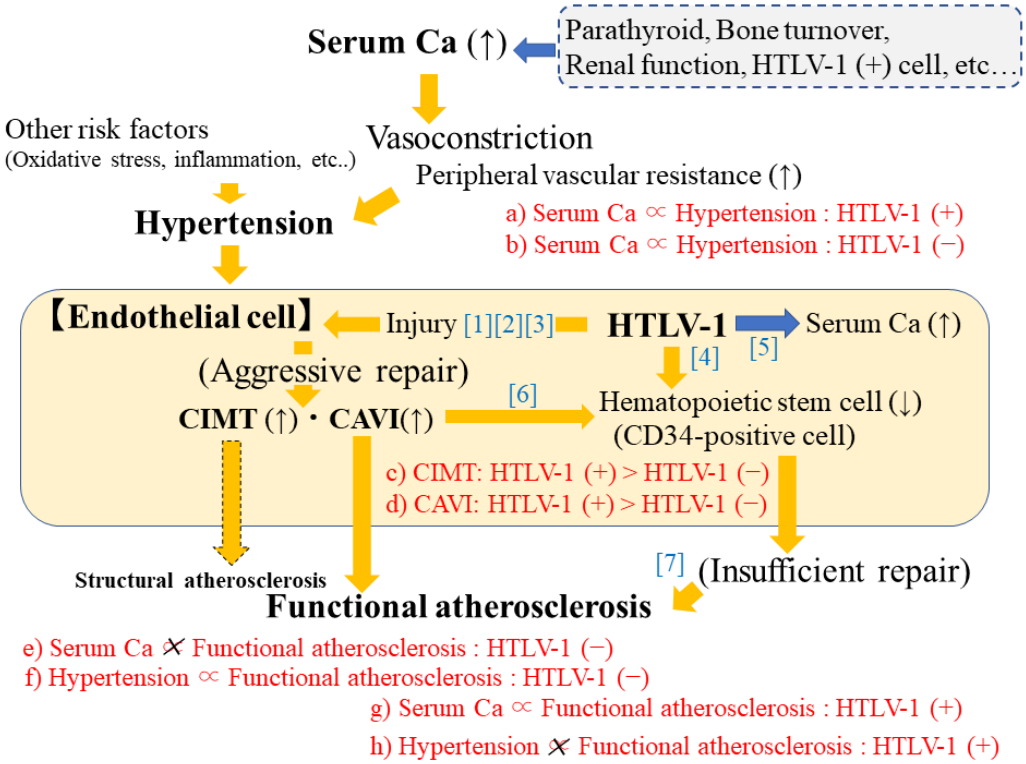

Figure 1. Possible mechanism underlying the association between serum calcium (Ca) level and functional atherosclerosis in relation to HTLV-1 carrier status. Associations in red (a–h) were observed in this study. Numbers in blue ([1]–[7]) were previously reported.

Endothelial repair leads to the progression of structural arterial stiffness. Upon vascular injury, production of CD34-positive hematopoietic stem cells is increased [39,40], leading to their differentiation into mature CD34-negative cells, such as endothelial cells [41], macrophages, and foam cells [40]. Since macrophages [42] and foam cells [43] contribute to the development of structural atherosclerosis, CD34-positive cells are necessary for the onset of this condition. Structural atherosclerosis simultaneously leads to functional atherosclerosis. Therefore, aggressive endothelial repair increases both structural arterial stiffness (CIMT) and functional arterial stiffness (CAVI). A previous report showed that HTLV-1 carriers had a higher risk of aggressive endothelial repair than HTLV-1 non-carriers [28,37]. In the present study, HTLV-1 carriers exhibited higher CIMT and CAVI values than HTLV-1 non-carriers (Table 1) (c, d in Figure 1). Since HTLV-1 enhances inflammation [12,13], possibly by activating the NF-κB pathway [14], its biochemical properties that progress structural atherosclerosis [15] could also worsen functional atherosclerosis ([1] in Figure 1).

Meanwhile, aggressive endothelial repair reduces the number of circulating CD34-positive cells by causing many of these cells to differentiate into CD34-negative cells ([6] in Figure 1) [1,16]. Under conditions of aggressive endothelial repair with reduced numbers of circulating CD34-positive cells, functional atherosclerosis develops, but structural atherosclerosis does not ([7] in Figure 1) [1,16]. We hypothesized that the serum calcium level might act as an indicator of HTLV-1 activity. This study confirmed that the serum calcium level was positively associated with functional atherosclerosis, but only among HTLV-1 carriers and not HTLV-1 non-carriers, and this association was independent of structural arterial stiffness (CIMT) (Table 4) (e, g in Figure 1).

Endothelial cells participate in heterocellular communication with adult T-cell leukemia cells [17] ([2] in Figure 1). Since HTLV-1 infects cells mainly through cell-to-cell contact [11], many endothelial cells can be infected by HTLV-1 [18,19] and subsequently cause en-

dothelial dysfunction. Further studies should investigate the association between HTLV-1 infection and maintenance of the endothelium.

Although HTLV-1 infection of CD4-positive lymphocytes induces cellular replication and transformation, infection of CD34-positive cells strikingly results in G(0)/G(1) cell cycle arrest [20] ([4] in Figure 1). Hence, endothelial function is likely to be impaired by HTLV-1 infection.

Serum calcium is regulated by the parathyroid glands; parathyroid hormone (PTH) secretion is increased by hypocalcemia and decreased by hypercalcemia [44]. Parathyroid activity is also known to be associated with the serum phosphate levels [45]. Therefore, the association among PTH, serum calcium levels, and serum phosphate levels is complex [46]. Renal function [47], bone resorption [48], and age [49] also play roles in this mechanism. This is presumably why the serum calcium level in the present study was significantly positively associated with TRACP-5b and serum phosphate level but inversely associated with age and renal function in HTLV-1 non-carriers (Table 2). In contrast, there was no significant association among the serum calcium level and TRACP-5b, serum phosphate level, or renal function in HTLV-1 carriers (Table 2). These results suggest the existence of another mechanism that regulates the serum calcium levels in HTLV-1 carriers.

In HTLV-1-infected cells, the transcription of PTHrP was induced by tax, a protein induced by HTLV-1, through activation of NF-κB [23] ([5] in Figure 1). Since PTHrP is known to increase the serum calcium levels, adult T-cell leukemia patients often experience hypercalcemia [24]. As a result, even in asymptomatic HTLV-1 carriers, the serum calcium levels might indicate HTLV-1 activity. In the present study, HTLV-1 carriers had a significantly lower serum calcium level than HTLV-1 non-carriers (Table 1). Unlike PTH, PTHrP does not provide normal negative feedback [24]. Further investigation of data related to PTHrP, PTH, and albumin levels is necessary to clarify the mechanisms that regulate the serum calcium levels in asymptomatic HTLV-1 carriers.

Furthermore, HTLV-1-infected cells create a conjugate formation in a calcium-dependent manner [50]. This presumably includes HTLV-1-infected endothelial cells [18,19] ([3] in Figure 1), which could impair normal endothelial function.

Independent of CIMT, the serum calcium level was positively associated with functional atherosclerosis in HTLV-1 carriers but not HTLV-1 non-carriers (Table 4) (e, g in Figure 1). A significantly positive association between hypertension and functional atherosclerosis was observed in HTLV-1 non-carriers but not in HTLV-1 carriers (Table 6) (f, h in Figure 1), presumably because both aggressive and deficient endothelial repair occur simultaneously in HTLV-1 carriers. In HTLV-1 non-carriers, factors that induce hypertension might contribute to the progression of functional atherosclerosis, independent of the serum calcium levels.

In the present study, HTVL-1 carriers had a significantly lower serum calcium level than HTLV-1 non-carriers. The mechanism underlying this relationship is unclear since HTLV-1 infection might itself increase the serum calcium level by activating the NF-κB pathway [23,24].

Our previous study of 2989 individuals aged 60 to 99 years reported that asymptomatic HTLV-1 carrier status was inversely associated with hypertension, possibly due to the promotion of endothelial maintenance, which can contribute to structural atherosclerosis progression [37]. Angiogenesis is necessary for the development of structural atherosclerosis [51]; therefore, its development might have a beneficial association with hypertension [52]. Serum calcium regulates smooth muscle tone [38], and it is reported to be positively associated with hypertension [33]. Further investigation related to NF-κB is necessary since a lower risk of hypertension might be associated with a lower serum calcium level in HTLV-1 carriers than in HTLV-1 non-carriers. In the present study, the prevalence of hypertension was slightly lower in HTLV-1 carriers (61.5%) than in HTLV-1 non-carriers (63.8%), even though the difference was not significant.

Furthermore, genetic characteristics related to lower angiogenic activity might be associated with a reduced chance of HTLV-1 infection [53]. Genetically, therefore, HTLV-1 carriers might have higher angiogenic activity than HTLV-1 non-carriers. In addition,

because angiogenesis reduces peripheral blood resistance, HTLV-1 carriers might have a lower risk of hypertension than HTLV-1 non-carriers.

Anti-hypertensive medications might also influence the serum calcium level. However, even though HTLV-1 infection might impact the association between the serum calcium level and functional atherosclerosis, a positive association between the serum calcium level and anti-hypertensive medication use was observed in both HTLV-1 carriers and non-carriers. Therefore, anti-hypertensive medication use might not explain the present results.

The clinical implication of the present findings is that the serum calcium level may act as a marker of endothelial maintenance activity related to HTLV-1 infection. Since the majority of HTLV-1 carriers remain asymptomatic throughout their lives [2–5], our results may help estimate their risk of functional atherosclerosis. Our multifaceted analysis revealed many associations that indicate potential mechanisms underlying the present results.

5. Limitations

However, this study has some limitations that warrant consideration. First, because we had no data on serum albumin, we could not derive the corrected calcium levels. Second, we had no data on CD34-positive cells. Our hypothesis is that CD34-positive cells may play an important role in the association between the serum calcium levels and functional atherosclerosis in HTVL-1 carriers. Further research that includes information on CD34-positive cells is necessary. Third, we had no data on related disorders (e.g., osteoporosis, bone tumor, and hyperparathyroidism) or medications (e.g., bisphosphonate, calcitonin, and corticosteroids) that could influence the serum calcium levels. However, we did adjust for factors related to bone turnover and renal function. Fourth, the causal relationship between the serum calcium level and functional atherosclerosis could not be established because this was a cross-sectional study. However, we performed a multifaceted analysis that enabled us to explore potential mechanisms to explain the present results.

6. Conclusions

In conclusion, the serum calcium levels were positively associated with functional atherosclerosis, but only in HTLV-1 carriers and not in HTLV-1 non-carriers. This finding may make it possible to estimate the risk of functional atherosclerosis in asymptomatic HTLV-1 carriers.

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Informed Consent Statement: Written consent forms were used to ensure that participants understood the objectives of the study when obtaining informed consent.

Data Availability Statement: According to ethical guidelines in Japan, we cannot provide individual data due to participant privacy considerations. In addition, the informed consent obtained did not include a provision for publicly sharing data. Qualified researchers may apply to access a minimal dataset by contacting Prof. Takahiro Maeda, Principal Investigator, Department of General Medicine,

Nagasaki University, Nagasaki, Japan, at tamaeda@nagasaki-u.ac.jp or the Office of Data Management at ritouken@vc.fctv-net.jp. Information about data requests is also available online at https://www.mh.nagasaki-u.ac.jp/soshin/ (accessed on 7 July 2022) and http://www.med.nagasaki-u.ac.jp/cm/ (accessed on 7 July 2022).

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