Elevated plasma homocysteine levels are associated with impaired peripheral microvascular vasomotor response

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A R T I C L E   I N F O

A B S T R A C T

Background: Hyperhomocysteinemia (HHcy) has been proposed as an important cardiovascular risk factor (cRF). However, little is known about the association between plasma homocysteine levels and peripheral microvascular endothelial dysfunction (PMED), which is an integrated index of vascular health.

Methods: This cross-sectional and retrospective cohort study included patients who underwent noninvasive PMED assessment using reactive hyperemia peripheral arterial tonometry (RH-PAT). The association between HHcy and PMED, and its impact on MACE (all-cause mortality and atherosclerotic cardiovascular events) was investigated.

Results: A total of 257 patients were enrolled (HHcy > 10.0 μmol/L, N = 51; lower levels of homocysteine [LHcy] ≤ 10 μmol/L, N = 206). Patients with HHcy were older, predominantly males, and with more comorbidities than patients with LHcy ($p < 0.05$ for all). RH-PAT index was lower in patients with HHcy versus LHcy ($p = 0.01$). A significant association between HHcy and PMED was observed in older (≥60 years), obese (≥30 kg/m^2), present/past smokers and hypertensive patients. HHcy was significantly associated with PMED even after adjusting for other cRF and B-vitamins supplementation. HHcy was associated with an increased risk of MACE with a hazard ratio of 3.65 (95% CI 1.41–9.48, $p = 0.01$) and an adjusted hazard ratio of 2.44 (95% CI 0.91–6.51, $p = 0.08$) after adjustment for age (≥60 years).

Conclusion: HHcy was independently associated with PMED after adjusting for cRF and B-vitamins supplementation. Thus, the link between homocysteine and MACE could be mediated by endothelial dysfunction, and will require further clarification with future studies.

1. Introduction

Since the recognition of premature arteriosclerosis and thromboembolic events as the sequelae of homocystinuria caused by the inherited deficiency of cystathionine synthase [1,2], elevated plasma levels of homocysteine (classified as mild, intermediate, and severe according to the levels of homocysteine: 15–30 μmol/L, 31–100 μmol/L, and > 100 μmol/L, respectively) have been linked to an increased risk of cardiovascular diseases [3,4]. These include coronary artery disease [5], peripheral artery disease [6,7], stroke [8,9], venous thrombosis [10], and elevated blood pressure during pregnancy [11]. Furthermore, hyperhomocysteinemia was suggested to be an independent predictor of cardiac death in a study of patients with stable coronary artery disease even though more than 80% of the study population were on statins [12].

Treatments targeting hyperhomocysteinemia by supplementing vitamins B6, B12 (B-vitamins), and folate successfully led to the reduction of hyperhomocysteinemia-induced atherosclerotic plaque, and decreased levels of plasma homocysteine in apoE knockout mice [13]. Also, folate supplementation was shown to

improve flow-mediated dilatation of the brachial artery in human subjects with hyperhomocysteinemia [14].

In contrast, randomized controlled trials have failed to show a beneficial effect on cardiovascular outcomes with B-vitamins and folate supplementation despite reducing the levels of homocysteine in patients with non-disabling cerebral infarction, advanced chronic kidney disease, coronary artery disease, and women at high risk of cardiovascular diseases [15–18]. However, a sub-study of the Vitamin Intervention for Stroke Prevention (VISP) trial demonstrated an association between high dose B-vitamins/folate supplementation and a reduced risk of stroke, myocardial infarction, and death in patients older than 67 years [19]. This highlights the potential benefit of B-vitamins/folate supplementation in certain populations, though rigorous evidence is still lacking.

The vascular endothelium is a prime site for the effects of cardiovascular risk factors. Thus reflecting the summative contribution of these risk factors, endothelial function can be viewed as a sensitive marker of cardiovascular disease risk [20]. Measurement of peripheral vasomotor response as a measure for endothelial dysfunction has been linked to adverse cardiovascular outcomes [21]. Reactive hyperemia peripheral arterial tonometry (RH-PAT) is a non-invasive method to measure the vasomotor response using fingertip device. RH-PAT index correlated well with coronary microvascular endothelial function and can be used to non-invasively assess peripheral microvascular endothelial dysfunction (PMED), which is associated with an increased risk of late adverse cardiovascular events in individuals with minimal cardiovascular risk factors, providing prognostic information above and beyond that provided by conventional cardiovascular risk factors [22].

One speculation of the gap in clinical studies is that the B-vitamins/folate supplementation didn't improve endothelial function. Therefore, we aimed to elucidate the association between hyperhomocysteinemia and PMED. In addition, we further categorized our study population on the basis of cardiovascular risk factors and B-vitamins/folate supplementation to investigate the effects of these factors on the indicator of endothelial function.

2. Methods

2.1. Study population

In this cross-sectional and retrospective cohort study, we enrolled 687 patients who visited Mayo Clinic between January 2006 and February 2014 for the assessment of chest pain and/or cardiovascular risk and underwent endothelial function testing using the EndoPAT 2000 device (Itamar Medical Inc., Caesarea, Israel). The decision to assess endothelial function was at the clinical discretion of the evaluating physician. Two hundred fifty-seven patients with available baseline serum homocysteine levels at the time of the endothelial function assessment were included in the analysis. The study was conducted in accordance with the guidelines of the Declaration of Helsinki. Mayo Clinic Institutional Review Board approved the study protocol. All patients provided written informed consent for participation in the current study.

2.2. Assessment of peripheral microvascular endothelial function

RH-PAT was used to evaluate peripheral microvascular endothelial function, as previously described [22–24]. Briefly, the study protocol included a 5-minute baseline measurement, followed by 5-minutes of inflation of a blood pressure cuff around the study participant's test arm with a pressure of 60 mmHg above baseline systolic blood pressure or 200 mmHg, followed by a 6-minute period of PAT measurement after deflation of the cuff. RH-PAT ratio was determined as the average pulse wave amplitude

for a 1-minute-period beginning 1 min after pressure cuff deflation divided by the average pulse wave amplitude during the 5-minute baseline period before pressure cuff inflation. The RH-PAT index was computed by indexing the RH-PAT ratio on the test arm to that of the contralateral arm. Per clinical protocol, patients were instructed to stop all vasoactive medications, including calcium channel blockers, β blockers, and long-acting nitrates, for at least 24 h before endothelial function testing. Patients fasted for 4 h before the study and abstained from coffee and tobacco use on the day of the RH-PAT testing. A calculated RH-PAT index ≤ 1.7 was used as a cut-off value for the diagnosis of PMED in this study [25,26].

2.3. Clinical assessment

Clinical history, laboratory data, and current medications were collected from a detailed chart review by an investigator blinded to RH-PAT data. Data were collected on the following parameters: 1) sex, age, body mass index (BMI), and traditional cardiovascular disease risk factors (smoking status and obesity [BMI > 30 kg/m^2]), 2) dyslipidemia, defined by a documented history of hyperlipidemia, treatment with lipid-lowering therapy, a low-density lipoprotein cholesterol level above the target (<130 mg/dL for low risk patients, < 100 mg/dL for moderate-high risk patients, < 70 mg/dL for very high risk, and < 55 mg/dL for extreme high risk patients based on 10-year atherosclerotic cardiovascular disease risk) [27], high-density lipoprotein cholesterol < 40 mg/dL in men or < 50 mg/dL in women, or triglycerides > 150 mg/dL, 3) type 2 diabetes mellitus, defined as a documented history of or treatment for type 2 diabetes, 4) hypertension, defined as a documented history of or treatment for hypertension, 5) coronary artery disease, defined as documented history of revascularization or more than 50% luminal stenosis in any coronary artery diagnosed by coronary angiography or computed tomography coronary angiography, and 6) the occurrence of major adverse cardiovascular events (MACE: all-cause death, myocardial infarction, clinically-driven coronary revascularization, cerebrovascular disease such as transient ischemic attack, ischemic stroke, and hemorrhagic stroke, and peripheral artery disease causing claudication) since the date of RH-PAT testing over follow-up.

2.4. Statistical analysis

Continuous variables distributed normally were expressed as the mean ± standard deviation, and those with a skewed distribution were expressed as the median with interquartile range. Categorical variables were expressed as frequency (percentage). Enrolled patients were divided into two groups—those with PMED (RH-PAT index ≤ 1.7) and those without PMED (RH-PAT index > 1.7). For between-group comparisons, unpaired *t*-test was used for normally distributed continuous variables, Mann-Whitney *U* test for non-normally distributed variables, and χ^2 test (and Fisher's exact test) for categorical variables. Univariate analyses were performed to show the association between PMED and serum homocysteine levels, with additional stratification by age, sex, and the presence of cardiovascular risk factors. Multivariate logistic regression analyses were performed to estimate the independent association between serum homocysteine levels and PMED. In the multivariate analyses, three covariate sets were investigated: (1) higher levels of homocysteine (>10.0 μmol/L), age, sex, chronic kidney disease, obesity, and B-vitamins/folate supplementation, (2) higher levels of homocysteine (>10.0 μmol/L), hypertension, diabetes mellitus, dyslipidemia, obesity, smoking history, and B-vitamins/folate supplementation, and (3) higher levels of homocysteine (>10.0 μmol/L), obesity, smoking history, coronary artery disease, and B-vitamins/folate supplementation.

These covariate sets were chosen for clinical relevance. Kaplan-Meier method was used to estimate MACE-free survival rates. The difference among groups was analysed using the log-rank test. Univariate and multivariate Cox proportional hazard analysis was performed to esitimate the hazard ratio of MACE. For all tests, a p value < 0.05 was considered statistically significant. All statistical analyses were performed using JMP Pro software (SAS Institute, Inc., Cary, NC, USA).

3. Results

3.1. Baseline characteristics

The baseline patients' characteristics at the time of RH-PAT testing categorized by normal versus abnormal peripheral microvascular endothelial function are summarized in Table 1. The median (interquartile range) of the RH-PAT index was 2.09 (1.68–2.44). Of 257 patients, 70 patients (27.2%) had PMED (RH-PAT index $\leq$ 1.7), and 187 (72.8%) had a normal peripheral microvascular endothelial function (RH-PAT index > 1.7) at baseline. Patients with PMED were more likely to have cardiovascular risk factors such as obesity, dyslipidemia, diabetes mellitus, and smoking history. Homocysteine levels tended to be higher in patients with PMED as compared to those with normal microvascular endothelial function (8.0 [6.8–11.0] vs. 8.0 [6.0–10.0], p = 0.09) (Table 1). Further categorization based on BMI (BMI $\geq$ 30 kg/m^2 vs. < 30 kg/m^2) showed that homocysteine levels were significantly higher in patients with PMED than those with normal microvascular endothelial function in obese patients (10.0 [8.0–13.0] vs. 8.0 [6.8–10.0], p = 0.02), but not in non-obese patients (p = 0.99).

The distribution of homocysteine levels in this study population is shown in Fig. 1. Only 8 patients had homocysteine levels > 15 µmol/L (mild hyperhomocysteinemia). Therefore, we divided patients by the quartiles of homocysteine levels. The asso-
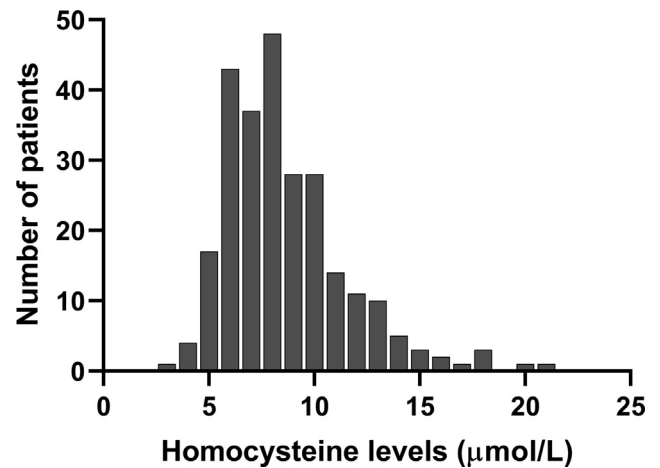

Fig. 1. The distribution of homocysteine levels. Homocysteine levels showed skewed distribution with a median value of 8.0 (interquartile range 6.0–10.0, range 3.0–21.0) µmol/L.

ciation between quartiles of homocysteine levels and the RH-PAT index is shown in Table 2. Only patients with the highest quartile of homocysteine (>10.0 µmol/L) had a significantly lower RH-PAT index than patients in the first quartile of homocysteine levels (p = 0.03) (Table 2). Therefore, we divided patients into two groups separated by the cut-off of plasma homocysteine levels of 10 µmol/L, which was the 75th percentile in this study population.

Table 3 compares baseline characteristics between patients with higher (>10.0 µmol/L, N = 51 [19.8%]) versus lower ($\leq$10.0 µmol/L, N = 206 [80.2%]) levels of homocysteine. Patients with higher levels of homocysteine were significantly older, more likely to be male and obese, more likely to have diabetes mellitus,

Table 1
Baseline characteristics of patients with normal versus abnormal peripheral microvascular endothelial function.

	All Patients N = 257	RH-PAT index		p value
		$\leq$1.7 N = 70	>1.7 N = 187	
Clinical parameters				
Age	49.8 ± 13.5	52.2 ± 14.1	49.0 ± 13.2	0.10
Male, n (%)	110 (42.8)	32 (45.7)	78 (41.7)	0.56
White race	250 (97.3)	69 (98.6)	181 (96.8)	0.44
Obesity (BMI $\geq$ 30 kg/m^2)	81 (31.5)	31 (44.3)	50 (26.7)	0.01
Body mass index (kg/m^2)	27.7 ± 5.4	29.3 ± 5.8	27.1 ± 5.1	0.01
Hypertension, n (%)	107 (41.6)	30 (42.9)	77 (41.2)	0.81
Systolic blood pressure (mmHg)	121.5 ± 16.8	123.1 ± 17.6	121.0 ± 16.5	0.36
Diastolic blood pressure (mmHg)	75.6 ± 12.0	75.6 ± 11.7	75.6 ± 12.2	0.99
Dyslipidemia, n (%)	194 (75.5)	59 (84.3)	135 (72.2)	0.05
LDL (mg/dL)	106.9 ± 42.1	112.9 ± 39.3	104.7 ± 43.1	0.15
HDL (mg/dL)	54.5 ± 16.8	52.6 ± 20.1	55.3 ± 15.4	0.32
Diabetes mellitus, n (%)	24 (9.3)	12 (17.1)	12 (6.4)	0.01
Glucose (mg/dL)	99.5 ± 21.3	100.6 ± 12.7	99.0 ± 23.9	0.51
HbA1c (%)	5.5 ± 0.7	5.6 ± 0.6	5.5 ± 0.8	0.8
Creatinine (mg/dL)	0.95 ± 0.24	0.99 ± 0.29	0.93 ± 0.21	0.15
eGFR (mL/min/1.73 m^2)	76.2 ± 19.2	76.3 ± 22.9	76.2 ± 17.7	0.99
Smoking history, n (%)	95 (37.0)	35 (50.0)	60 (32.1)	0.01
Coronary artery disease, n (%)	69 (27.0)	24 (34.3)	45 (24.2)	0.10
RH-PAT index	2.06 (1.68–2.44)	1.47 (1.38–1.61)	2.24 (1.98–2.54)	<0.0001
Homocysteine (µmol/L)	8.0 (6.0–10.0)	8.0 (6.8–11.0)	8.0 (6.0–10.0)	0.09
Medications				
Aspirin, n (%)	132 (51.3)	36 (51.4)	96 (51.3)	0.99
Statin, n (%)	121 (47.1)	31 (44.3)	90 (48.1)	0.58
Anti-hypertensive, n (%)	126 (49.0)	40 (57.1)	86 (46.0)	0.11
Anti-diabetic, n (%)	14 (5.5)	7 (10.1)	7 (3.7)	0.05
Diuretics, n (%)	40 (15.6)	13 (18.6)	27 (14.5)	0.43
Vitamin B6, B12/folate, n (%)	101 (39.3)	28 (40.0)	73 (39.0)	0.89

eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; RH-PAT, reactive hyperemia peripheral arterial tonometry.

Table 2
Association between Homocysteine levels and RH-PAT index.

Homocysteine level Quartile	Homocysteine level range (µmol/L)	RH-PAT index median	RH-PAT index 95% CI	p value vs. Quartile 1
1	≤6.0	2.08	1.70–2.54	
2	>6.0, ≤8.0	2.06	1.75–2.47	0.68
3	>8.0, ≤10.0	2.11	1.80–2.35	0.77
4	>10.0	1.74	1.55–2.28	0.03

CI, confidence interval; RH-PAT, reactive hyperemia peripheral arterial tonometry.

Table 3
Baseline characteristics comparing patients with higher (>10.0 µmol/L) versus lower (≤10.0 µmol/L) levels of homocysteine.

	Homocysteine (µmol/L)		p value
	≤10.0 N = 206	>10.0 N = 51	
Clinical parameters			
Age	47.6 ± 12.3	58.9 ± 14.4	<0.0001
Male, n (%)	76 (36.9)	34 (66.7)	0.0001
White race	202 (98.1)	48 (94.1)	0.12
Obesity (BMI > 30 kg/m^2)	59 (28.6)	22 (43.1)	0.05
Body mass index (kg/m^2)	27.3 ± 5.4	29.2 ± 5.3	0.03
Hypertension, n (%)	82 (39.8)	25 (49.0)	0.23
Systolic blood pressure (mmHg)	120.1 ± 15.9	127.1 ± 19.2	0.02
Diastolic blood pressure (mmHg)	75.3 ± 12.3	77.1 ± 10.8	0.29
Dyslipidemia, n (%)	156 (75.7)	38 (74.5)	0.86
LDL (mg/dL)	107.9 ± 42.8	103.2 ± 39.4	0.47
HDL (mg/dL)	54.5 ± 15.6	54.6 ± 21.1	0.99
Diabetes mellitus, n (%)	15 (7.3)	9 (17.7)	0.02
Glucose (mg/dL)	98.7 ± 22.9	102.7 ± 11.7	0.11
HbA1c (%)	5.5 ± 0.8	5.7 ± 0.5	0.26
Creatinine (mg/dL)	0.91 ± 0.21	1.08 ± 0.30	0.001
eGFR (mL/min/1.73 m^2)	78.3 ± 19.4	67.2 ± 15.8	0.0002
Smoking history, n (%)	72 (35.0)	23 (45.1)	0.18
Coronary artery disease, n (%)	49 (23.9)	20 (39.2)	0.03
RH-PAT index	2.08 (1.74–2.46)	1.74 (1.55–2.28)	0.01
Homocysteine (µmol/L)	8.0 (6.0–9.0)	13.0 (11.0–14.0)	<0.0001
Medications			
Aspirin, n (%)	102 (49.5)	30 (58.8)	0.23
Statin, n (%)	95 (46.1)	26 (51.0)	0.53
Anti-hypertensive, n (%)	95 (46.1)	31 (60.8)	0.06
Anti-diabetic, n (%)	8 (3.9)	6 (11.8)	0.03
Diuretics, n (%)	28 (13.7)	12 (23.5)	0.08
Vitamin B6, B12/folate, n (%)	90 (43.7)	11 (21.6)	0.004

eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; RH-PAT, reactive hyperemia peripheral arterial tonometry.

worse renal function, and coronary artery disease, and more likely to be treated with anti-diabetic agents and supplementation of B-vitamins/folate.

3.2. Impact of higher homocysteine levels on microvascular endothelial function

RH-PAT index was significantly lower in patients with higher levels of homocysteine compared to that in patients with lower levels of homocysteine (1.74 [1.55–2.28] vs. 2.08 [1.74–2.46], p = 0.01). The association between higher homocysteine levels and PMED was significant in all individuals, and after stratification, in both sexes, patients with and without chronic kidney disease, and patients with and without diabetes mellitus. Further, the association between higher homocysteine levels > 10.0 µmol/L and PMED persisted in patients ≥ 60 years, obese patients, those with hypertension, those with a smoking history, and those treated with supplementation of B-vitamins/folate (Table 4). Baseline characteristics were similar between patients who were versus those who

were not taking supplementation of B-vitamins/folate. However, homocysteine levels were significantly lower in patients taking supplementation of B-vitamins/folate (8.0 [7.0–11.0] vs. 8.0 [6.0–9.0], p = 0.04) (Supplemental Table 1).

Univariate logistic regression analysis demonstrated that higher levels of homocysteine, as well as diabetes mellitus, dyslipidemia, obesity, and a smoking history were associated with PMED with an odds ratio (OR) 2.78 (95% confidence interval [CI] 1.46–5.27, p = 0.002), 3.01 (95% CI 1.29–7.08, p = 0.011), 2.07 (95% CI 1.01–4.24, p = 0.048), 2.18 (95% CI 1.23–3.86, p = 0.008), and 2.12 (95% CI 1.21–3.71, p = 0.009), respectively (Table 5).

In multivariate analyses, higher levels of homocysteine were an independent predictor of PMED after adjustment for (1) age, sex, chronic kidney disease, obesity, and B-vitamins/folate supplementation, (2) hypertension, diabetes mellitus, dyslipidemia, obesity, smoking history, and B-vitamins/folate supplementation, and (3) obesity, smoking history, coronary artery disease, and B-vitamins/folate supplementation (multivariate 1: adjusted OR 3.30, 95% CI 1.50–7.28, p = 0.003; multivariate 2: adjusted OR 2.70, 95% CI 1.35–5.41, p = 0.001; multivariate model 3: adjusted OR 2.51, 95% CI 1.27–4.96, p = 0.01) (Table 5).

3.3. Impact of higher levels of homocysteine on MACE

Follow-up data were available in 194 patients (75.5%). Baseline characteristics of patients with available follow-up are summarized in Supplemental Table 2. A total of 18 patients developed MACE over median follow-up of 4.9 years (10/157 [6.4%, 11 events] patients with lower levels of homocysteine vs. 8/37 [21.6%, 8 events] patients with higher levels of homocysteine, p = 0.004). Individual MACE events are summarized in Supplemental Table 3. Higher levels of homocysteine at baseline was significantly associated with decreased MACE-free survival (p = 0.02) (Fig. 2). Univariate COX proportional hazard ratio analysis demonstrated that higher levels of homocysteine was significantly associated with an increased risk of MACE with a hazard ratio of 3.65 (95% CI 1.41–9.48, p = 0.01); however, multivariate COX proportional hazard ratio analysis showed only a trend between higher levels of homocysteine and an increased risk of MACE after adjustment for age (≥60 years) (adjusted hazard ratio 2.44, 95% confidence interval 0.91–6.51, p = 0.08).

4. Discussion

In this study, we demonstrated that the highest quartile of homocysteine levels (10.0–21.0 µmol/L), which were within high-normal to mildly elevated range, were independently associated with PMED even after adjustment for other cardiovascular risk factors and B-vitamins and/or folate supplementation. In subgroup analysis, higher levels of homocysteine (>10.0 µmol/L) were significantly associated with PMED in older (age ≥ 60 years), obese (≥30 kg/m^2), and hypertensive patients. Interestingly, the association between higher levels of homocysteine and PMED seemed to be particularly prominent in patients with supplementation of B-vitamins/folate. Thus the association between homocysteine and

Table 4
Association between higher levels of homocysteine and peripheral microvascular endothelial dysfunction.

Stratified by	No. with higher homocysteine/all (%)	No. with PMED/all (%)	Odds Ratio	95% CI	*p* value
All individuals	51/257 (19.8)	70/257 (27.2)	2.78	[1.46–5.27]	0.002
Age (years)					
<60	14/147 (9.5)	36/147 (24.5)	1.26	[0.37–4.30]	0.71
≥60	37/110 (33.6)	34/110 (30.9)	4.08	[1.73–9.64]	0.001
Sex					
Male	34/110 (30.9)	32/110 (29.1)	2.74	[1.15–6.51]	0.02
Female	17/147 (11.6)	38/147 (25.9)	2.96	[1.05–8.35]	0.04
eGFR (mL/min/1.73 m^2)					
<60	12/35 (34.3)	12/35 (34.3)	5.04	[1.11–22.96]	0.03
≥60	31/196 (15.8)	53/196 (27.0)	3.13	[1.42–6.92]	0.01
BMI (kg/m^2)					
<30	29/176 (16.5)	39 (22.2)	1.76	[0.73–4.24]	0.21
≥30	22/81 (27.2)	31/81 (38.3)	4.32	[1.54–12.18]	0.01
Hypertension					
(−)	26/150 (17.3)	40/150 (26.7)	1.96	[0.80–4.77]	0.14
(+)	25/107 (23.4)	30/107 (28.0)	4.14	[1.60–10.70]	0.003
Diabetes mellitus					
(−)	42/233 (18.0)	58/233 (24.9)	2.18	[1.07–4.44]	0.03
(+)	9/24 (37.5)	12/24 (50.0)	7.00	[1.04–46.95]	0.05
Smoking history					
(−)	28/162 (35.7)	35/162 (35.7)	2.42	[0.99–5.88]	0.05
(+)	23/95 (24.2)	35/95 (36.8)	2.95	[1.13–7.75]	0.03
Vitamin B6, B12/folate					
(−)	40/156 (25.6)	42/156 (26.9)	1.69	[0.78–3.68]	0.18
(+)	11/101 (10.9)	28/101 (27.7)	16.82	[3.35–84.43]	0.001

BMI, body mass index; CI, confidence interval; eGFR, estimated glomerular filtration rate; PMED, peripheral microvascular endothelial dysfunction.

Table 5
Univariate and multivariate logistic regression analysis for peripheral microvascular endothelial dysfunction.

	Univariate			Multivariate 1			Multivariate 2			Multivariate 3		
	OR	95% CI	*p* value	OR	95% CI	*p* value	OR	95% CI	*p* value	OR	95% CI	*p* value
Homocysteine > 10.0 μmol/L	2.78	[1.46–5.27]	0.002	3.30	[1.50–7.28]	0.003	2.70	[1.35–5.41]	0.001	2.51	[1.27–4.96]	0.01
Age, 10-year increment	1.20	[0.97–1.48]	0.09	0.99	[0.78–1.27]	0.97						
Male sex	1.18	[0.68–2.05]	0.56	1.13	[0.61–2.10]	0.69						
Hypertension	1.07	[0.61–1.87]	0.81				0.59	[0.30–5.97]	0.11			
Diabetes mellitus	3.01	[1.29–7.08]	0.01				2.07	[0.80–5.34]	0.13			
Dyslipidemia	2.07	[1.01–4.24]	0.05				2.04	[0.94–4.43]	0.07			
eGFR < 60 mL/min/1.73 m^2	1.41	[0.65–3.03]	0.38	1.07	[0.45–2.52]	0.87						
BMI ≥ 30 kg/m^2	2.18	[1.23–3.86]	0.01	2.03	[1.08–3.83]	0.02	1.75	[0.92–3.34]	0.09	1.82	[0.99–3.35]	0.05
Smoking history	2.12	[1.21–3.71]	0.01				2.02	[1.09–2.41]	0.03	1.86	[1.03–3.34]	0.04
Coronary artery disease	1.63	[0.90–2.97]	0.11							1.16	[0.61–2.21]	0.65
Vitamin B6, B12/folate	1.04	[0.59–1.82]	0.89	1.17	[0.62–2.20]	0.62	1.31	[0.71–2.41]	0.39	1.20	[0.66–2.19]	0.56

BMI, body mass index; CI, confidence interval; eGFR, estimated glomerular filtration rate; OR, odds ratio.
Multivariate 1: adjusted for homocysteine, age, sex, eGFR, BMI, B-vitamins.
Multivariate 2: adjusted for homocysteine, hypertension, diabetes mellitus, dyslipidemia, BMI, smoking history, B-vitamins.
Multivariate 3: adjusted for homocysteine, BMI, smoking history, coronary artery disease, B-vitamins.

PMED may, in part, explain the mechanism of increased cardiovascular events associated with hyperhomocysteinemia.

4.1. Effects of homocysteine on endothelial dysfunction

Homocysteine is a degradation product of methionine, which is an essential sulfur-containing amino acid and plays a critical role in numerous metabolic processes such as polyamine and nucleotide (purine and pyrimidine) synthesis. Homocysteine levels are maintained at low levels (5 to 15 μmol/L) both by the vitamin B12- and folate-dependent *trans*-methylation pathway to form methionine, and by the vitamin B6-dependent *trans*-sulfuration pathway to form cysteine [28]. Therefore, deficiencies of B-vitamins/folate as well as genetic variations affecting these enzymatic pathways lead to hyperhomocysteinemia. In this study, we demonstrated the association between high-normal to mildly elevated range of homocysteine levels and PMED, which is an early feature of vascular damage/dysfunction [22]. Similar results were shown in a previous case-control study demonstrating that a mod-

est increase of homocysteine > 12.1 μmol/L was associated with an increased risk of vascular diseases, including coronary, cerebrovascular, and peripheral artery diseases [29]. Interestingly, concurrent smoking increased the risk of vascular disease up to 12-fold in combination with mild hyperhomocysteinemia [29], which is consistent with our findings. The synergistic effect of smoking and hyperhomocysteinemia on vascular health may be related to the fact that smoking was shown to produce a localized B-vitamins/folate deficiency in peripheral tissues [30]. Also, obesity might be contributory to deficiency of fat-soluble vitamins such as folic acid and vitamin B12 [31], which supports our current observation that the association between homocysteine and PMED in obese patients. Our current study also suggests that high-normal to mildly elevated range of homocysteine levels is strongly associated with PMED in patients aged ≥ 60 years and hypertension, which is consistent with the previous observation showing that hyperhomocysteinemia is associated with cerebrovascular stiffness in elderly hypertensive patients [32]. This finding may offer a potential explanation for the reason as to why the beneficial effects
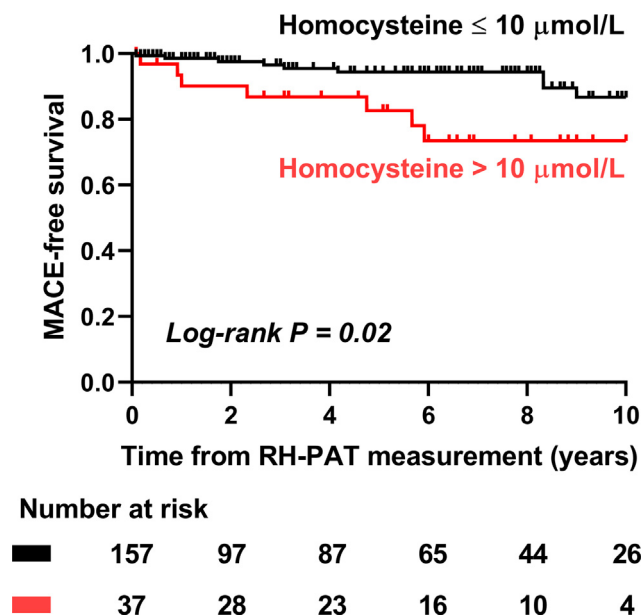
Fig. 2. Comparison of MACE-free survival between patients with higher versus lower levels of homocysteine. Follow-up data were available in 194 patients (75.5%). Patients with higher homocysteine levels > 10 μmol/L was significantly associated with decreased MACE-free survival as compared to patients with lower homocysteine levels (≤10 μmol/L) (log-rank p = 0.02).

of B-vitamins/folate therapy on vascular risk were observed only in patients ≥ 67 years of age in a post hoc analysis of the VISP trial [19]. Though we did not find an independent prognostic predictive value of higher levels of homocysteine for MACE after adjustment for age (≥60 years) in our small samples, our current data suggests that homocysteine even in high-normal to mildly elevated range can be associated with PMED in the presence of other risk factors which may aggravate or unveil the potential deleterious effects of homocysteine on endothelial function.

4.2. Treatment of hyperhomocysteinemia

Growing evidence suggests that hyperhomocysteinemia is contributory to the progression of cardiovascular diseases [28]. Nevertheless, the role of homocysteine on the progression of vascular disease has aroused much controversy in light of the lack of treatment efficacy with B-vitamins/folate supplementation. Several explanations have been considered to explain the gap between epidemiological studies and interventional trials for B-vitamins/folate therapy. One hypothesis is that B-vitamins/folate treatment per se could exacerbate atherosclerosis, neutralizing the beneficial effects of reducing homocysteine levels. It should be noted that post hoc analysis of the Western Norway B vitamin Intervention Trial (WENBIT) study showed that B-vitamins/folate therapy was associated with more rapid progression of coronary diameter stenosis as evaluated by quantitative coronary angiography [33]. Our data also suggest that the association between higher levels of homocysteine and PMED was prominent in patients taking B-vitamins/folate supplementation. Though, the cross-sectional design of this study does not allow us to draw any conclusions regarding a causal relationship, supplementation with B-vitamins/folate should be undertaken cautiously, and may not be recommended for the general population with higher levels of homocysteine.

4.3. Limitations

This study has several limitations. First, the study's retrospective design makes it challenging to derive causal associations,

and the results should be considered as hypothesis-generating. The evaluation of RH-PAT index and homocysteine levels was performed at the discretion of the evaluating physician. Therefore, some selection bias cannot be excluded. Second, serum folate concentrations are directly influenced by the intake of folate fortified foods [34]. Further, alcohol consumption from red wine or spirits, but not from beer (a rich source of folate and vitamin B6) elevates homocysteine levels [35]. Alcohol consumption might also be associated with endothelial dysfunction [36]. Our lack of data regarding the intake of B-vitamins/folate fortified foods and alcohol makes it difficult to adjust for the potential effects of these substances. Also, the indication of taking B-vitamins/folate supplementation was not clear. Given that the patients were taking B-vitamins/folate to compensate for the lack of vitamins-rich food consumption, unhealthy lifestyle behaviors accumulated in such populations might have affected endothelial function [37]. Homocysteine levels before B-vitamins supplementation and duration of B-vitamins supplementation were not available; however, baseline characteristics between patients with and without vitamins supplementation were not different (Supplemental Table 1). Third, detrimental effects of homocysteine on endothelial function have been hypothetically linked to decreased levels of tetrahydrobiopterin (BH4), co-factor of nitric oxide synthase, and increased levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase [38–40]. Homocysteine reduces intracellular levels of BH4 by inhibiting its synthesis and activating its degradation, leading to uncoupling of endothelial nitric oxide synthase with resultant increase of reactive oxygen species and decrease of nitric oxide bioavailability [41]. Homocysteine in turn increases levels of ADMA by inhibiting dimethylaminohydrolase, the enzyme degrades ADMA, resulting in decrease in nitric oxide production [42]. It is beyond the scope of the current study to elucidate the association between levels of homocysteine and BH4/ADMA, and its association with PMED, though supplementation of BH4 might be a therapeutic option to attenuate homocysteine-induced endothelial dysfunction [43]. Finally, though we calculated the predictive value of the higher levels of homocysteine using a multivariate analysis, we could not adjust for all the variables due to small sample sizes. Nevertheless, higher levels of homocysteine > 10.0 μmol/L were independently associated with PMED after adjustment for variables shown to be relevant to PMED and homocysteine levels.

5. Conclusions

Our study demonstrates that homocysteine even in the high-normal to mildly elevated range is independently associated with PMED after adjustment for cardiovascular risk factors. Thus, the link between homocysteine and adverse cardiovascular events could be mediated by endothelial dysfunction, and will require further clarification with future studies. Also, treatment strategies targeting hyperhomocysteinemia require further investigation, though supplementation with B-vitamins/folate should potentially be avoided.

6. Grants

CRediT authorship contribution statement

Takumi Toya: Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Jaskanwal D. Sara:** Conceptualization, Methodology, Data

curation, Writing - review & editing. **Ben Lerman:** Data curation, Writing - original draft, Writing - review & editing. **Ali Ahmad:** Data curation, Writing - original draft, Writing - review & editing. **Riad Taher:** Conceptualization, Methodology, Formal analysis, Data curation. **Shigeo Godo:** Data curation, Writing - original draft, Writing - review & editing. **Michel T. Corban:** Data curation, Writing - original draft, Writing - review & editing. **Lilach O. Lerman:** Data curation, Writing - original draft, Writing - review & editing. **Amir Lerman:** Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

Amir Lerman declared consulting for Itamar Medical.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcha.2020.100515.

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