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Evaluation of Asymmetric Dimethylarginine and Homocysteine in Microangiopathy-Related Cerebral Damage

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BACKGROUND

Microangiopathy-related cerebral damage (MARCD) is an entity of cerebrovascular disease based on arteriosclerosis in deep white matter, which includes lacunar infarction and white matter hyperintensity (WMH). As asymmetric dimethylarginine (ADMA), an endogenous inhibitor of the nitric oxide (NO) synthases, and homocysteine are both potential risk factors for arteriosclerosis, the plasma levels of these two substances were evaluated in individuals with MARCD.

METHODS

Consecutive participants of a health examination (401 males and 311 females) were recruited for this cross-sectional study. All participants received an magnetic resonance imaging examination, and those with either lacunar infarction or WMH (grade ≥2) were classified into MARCD (+) ($N = 146$). The plasma ADMA concentration was measured with high performance liquid chromatography. The total homocysteine (tHcy) concentration was measured using a commercial kit.

RESULTS

The ADMA level ($P < 0.001$), symmetric dimethylarginine (SDMA) level ($P < 0.05$) and L-arginine (Arg)/ADMA ratio ($P < 0.01$) differed significantly between MARCD (+) and (–) according to nonparametric Wilcoxon test, while the tHcy level did not ($P = 0.37$). Classic risk factors such as age, blood pressure, and the presence of hypertension differed significantly between the two groups as well. In the logistic analysis, the association of Arg/ADMA with MARCD remained significant (odds ratio and 95% confidence interval, 0.19 (0.05, 0.73), $P < 0.05$) even after adjusting for the effects of age and hypertension.

CONCLUSIONS

ADMA and tHcy levels were studied in 712 subjects with or without MARCD. The Arg/ADMA ratio was suggested to be an independent risk factor for MARCD. A large-scale prospective study is warranted to confirm the causal relationship between Arg/ADMA and MARCD.

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Lacunar infarction and white matter hyperintensity (WMH) are cerebrovascular disorders often found in the elderly.[1,2] They frequently occur concomitantly and are known to share common risk factors, i.e., aging and hypertension.[1–3] As the two entities might well share a common etiology, the combined entity of small-vessel disease or microangiopathy-related cerebral damage (MARCD) has been proposed to integrate them.[2,4]

Acute and chronic ischemia based on arteriosclerosis in deep white matter is believed to be responsible for MARCD.[1,5] Accordingly, potential risk factors for arteriosclerosis have been evaluated in patients with MARCD.[6–11] Among them, nitric oxide (NO) has attracted much attention because it was thought not only to regulate cerebral blood flow but also to prevent arteriosclerosis by inhibiting fibrosis and the proliferation of smooth muscle cells in the arterial wall.[4,12,13] In fact, factors potentially influencing the level of NO *in vivo*, such as NO synthase (NOS) inhibitors and functional single-nucleotide polymorphisms in the endothelial NOS (*eNOS*) gene, were shown to be associated with MARCD.[4,12,14]

Recently, much attention has been paid to asymmetric dimethylarginine (ADMA), a unique endogenous inhibitor of the NO synthases.[15,16] A higher level of ADMA was reported to be associated with coronary artery diseases, hypertension, atherosclerosis, and cerebrovascular diseases.[17–24] In addition, the physiological importance of ADMA in the cerebral vasculature was directly indicated in several *in vitro* studies.[25–27] Considering the functional significance of NO *in vivo*, we hypothesized that ADMA was a good candidate for a risk factor for MARCD.

Hyperhomocysteinemia has been recognized as a risk factor for atherosclerotic diseases.[28] In the study of cerebrovascular diseases, a high total homocysteine (tHcy) level has been reported to be a risk factor not only for atherothrombotic

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infarction but also for MARCD.[22,24,29–32] Concerning the pathophysiology of hyperhomocysteinemia in MARCD, recent studies pointed out the possibility that the deleterious effects of homocysteine on the cardiovascular system were mediated through ADMA.[33–35]

Based on the observations above, we performed a cross-sectional study to examine a hypothesis that ADMA and tHcy levels were associated with MARCD. In the assessment of the effects of ADMA, we used the L-arginine (Arg)/ADMA ratio. As ADMA competitively inhibited binding of Arg, the physiological substrate, to the NO synthases, the Arg/ADMA ratio was thought more relevant to *in vivo* effects of ADMA.[36,37]

In this communication, we described that the plasma Arg/ADMA ratio was associated with MARCD, while the plasma tHcy level was not. We additionally indicate that the plasma tHcy level was correlated not with the ADMA level but with the symmetric dimethylarginine (SDMA) and estimated glomerular filtration rate (eGFR). This is one of the largest studies to evaluate ADMA in cerebrovascular diseases.

METHODS

Subjects. A total of 712 consecutive participants (401 males and 311 females) who voluntarily visited the Shimane Institute of Health Science for a health screening examination between 2000 and 2003 were recruited into the study. Histories of smoking, hypertension, diabetes mellitus, and hypercholesterolemia were obtained through an interview. Participants were considered to have these diseases when they had already been diagnosed by medical doctors. Blood pressure or fasting blood glucose measured on site was not included in the criteria for the diagnosis. Smoking status was categorized based on the smoking index (number of cigarettes per day × years): non- or mild smokers with a smoking index <200 and heavy smokers with a smoking index ≥200 (ref. 10). Participants with a past history of cerebrovascular diseases were excluded from the study.

Blood pressure was measured three times after at least 15 min of rest. The mean of the three measurements was taken as representative of blood pressure. Venous blood was collected after overnight fasting. Plasma was separated within 30 min of the blood being drawn and kept frozen at −80 °C until the measurement of Arg, SDMA, ADMA, and tHcy. Total cholesterol, high-density lipoprotein cholesterol, and triglyceride levels were measured in the serum.

Estimated GFR was calculated using the following formula recently revised by a working group of Japanese Chronic Kidney Disease Initiative.[38]

$$\text{eGFR (ml/min/1.73m}^2) = 194 \times (\text{serum creatinine, mg/dl})^{-1.094} \times \text{age}^{-0.287} \times (0.739, \text{if female})$$

All participants gave informed consent. The study protocol was approved by the local ethics committee.

ADMA and tHcy measurements. Plasma was mixed with an equal volume of 3% sulfosalicylic acid for deproteinization. After centrifugation at 4 °C, 100 µl of the supernatant was injected into an high performance liquid chromatography system (TOSOH, Tokyo, Japan). The analysis was performed as described by Dobashi *et al.* using an ion-exchange column (TSKgel SP-2SW, TOSOH) and a reverse-phase column (TSKgel ODS-100V, TOSOH) with a solvent composed of 70 mmol/l phosphate buffer (pH6.75) and 3 mmol/l octanoate.[39] O-Phthaldialdehyde labeling was done automatically after the chromatographic separation of Arg and the dimethylarginines. The detection limit for ADMA and SDMA was 0.03 µmol/l and the relative standard deviation was <4.7%. This method allowed accurate and simultaneous evaluation of ADMA, SDMA, and Arg in a large number of samples. Accordingly, we were able to evaluate the Arg/ADMA ratio in MARCD, which had not been done in the previous study.[24]

The tHcy concentration in the plasma was measured with an enzymatic assay using a commercial kit (MBL, Nagoya, Japan).

Diagnosis of MARCD. WMH and lacunar infarction were diagnosed and rated on T2-weighted MR images (0.2T, Siemens, Munich, Germany).[10] The criteria for the diagnosis of lacunar infarction were described previously.[10] All participants were

Table 1 | Demographic data for the studied population

	With MARCD ($N = 146$)	Without MARCD ($N = 566$)	P[a]
Male/female (male %)	89/57 (61.0)	312/254 (55.1)	0.20
Age	62.7 (61.5, 63.8)	57.6 (57.0, 58.2)	<0.001
BMI, kg/m^2	23.7 (23.2, 24.2)	23.3 (23.0, 23.5)	0.10
SBP, mm Hg	132 (130, 135)	124 (123, 126)	<0.001
DBP, mm Hg	75 (73, 77)	71 (70, 72)	<0.001
MBP, mm Hg	94 (92, 96)	89 (88, 90)	<0.001
Creatinine, mg/dl	0.75 (0.72, 0.78)	0.73 (0.71, 0.74)	0.097
eGFR, ml/min/1.73 m^2	75.6 (73.4, 78.2)	78.3 (77.3, 79.4)	<0.05
T-C, mg/dl	212 (207, 218)	212 (209, 214)	0.76
HDL-C, mg/dl	63.5 (61.1, 65.9)	62.1 (60.8, 63.3)	0.31
TG, mg/dl	102 (94, 111)	110 (105, 115)	0.097
FBG, mg/dl	111 (105, 117)	107 (105, 109)	0.081
Heavy smokers, %	34.9	37.6	0.55
Hypertensives, %	54.5	25.8	<0.001
Antihypertensive Tx, %[b]	15.2	21.2	0.26
DM, %	12.5	10.8	0.57
tHcy, µmol/l	10.2 (9.8, 10.7)	10.0 (9.9, 10.5)	0.46
Arg, µmol/l	88.6 (84.8, 92.5)	90.6 (88.6, 92.6)	0.37
ADMA, µmol/l	0.60 (0.58, 0.62)	0.56 (0.55, 0.57)	<0.001
SDMA, µmol/l	0.53 (0.51, 0.54)	0.49 (0.48, 0.50)	<0.01
Arg/ADMA	144 (135, 151)	158 (155, 162)	<0.01

TG, FBG, tHcy, and Arg/ADMA were analyzed after log-transformation. eGFR was calculated with the formula indicated in Methods. The 95% confidence interval is shown in parentheses.
ADMA, asymmetric dimethylarginine; Arg, L-arginine; BMI, body mass index; DBP, diastolic blood pressure; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; FBG, fasting blood glucose; HDL-C, high-density lipoprotein cholesterol; MBP, mean blood pressure; SBP, systolic blood pressure; SDMA, symmetric dimethylarginine; T-C, total cholesterol; TG, triglyceride; tHcy, total homocysteine; Tx, treatment.
[a]Either by Student's *t*-test or χ^2-test. [b]Percentage of hypertensives.

physically examined by neurologists in the health examination, and were confirmed to have neither signs nor symptoms of cerebral stroke. Accordingly, those with lacunes on magnetic resonance imaging examination were all categorized into the "silent" lacunar infarction (SLI). WMH was graded according to Fazekas *et al.*[40] Following Schmidt *et al.*, subjects with MARCD were defined as those with SLI and/or WMH grade ≥2 (ref. 2). As the prevalence of other demyelinating diseases such as multiple sclerosis are considerably lower in Asians than in Caucasians,[41] it was expected that contamination of such demyelinating diseases in WMH was quite rare when nonsymptomatic participants were employed in Japan.

Statistical analysis. Continuous parameters were represented by the mean and the 95% confidence interval of the mean. Since several parameters were not in the normal distribution, differences between MARCD (+) and (−) were tested by nonparametric Wilcoxon test. Categorical parameters were analyzed by χ^2-test. The logistic regression analysis was employed to identify factors independently associated with MARCD. Because of co-linearity expected between the diagnosed hypertension and measured blood pressure levels, and between ADMA and Arg/ADMA, three different models were tested (see Results). Sex and eGFR were included in all the three models.

The statistical analysis was performed with JMP v.6 (SAS Institute, Cary, NC). Because of highly skewed distribution, triglyceride, fasting blood glucose, tHcy, and Arg/ADMA were analyzed after log-transformation.

RESULTS

Among the 712 participants, 146 were identified to have MARCD (68 with SLI, 54 with WMH grade ≥2, and 24 with SLI + WMH grade ≥2). Demographic data of the MARCD (+) and (−) populations are shown in **Table 1**. In addition to the classic risk factors of age and hypertension, fasting blood glucose, plasma ADMA and SDMA levels, and the Arg/ADMA ratio were significantly different between the two populations. A marginal difference was observed in triglyceride ($P = 0.080$), while no significant difference was observed in the tHcy level ($P = 0.37$).

Based on previous observations,[30–32] we examined the correlation between the tHcy level and the dimethylarginine levels in **Figure 1**. There was no significant correlation between tHcy (log-transformed) and ADMA, Arg, or Arg/ADMA (log-transformed) in the present study. By contrast, SDMA showed a modest but significant correlation with tHcy ($r = 0.15$, $P < 0.001$). Estimated GFR showed significant inverse correlations with tHcy ($r = -0.20$, $P < 0.001$) and with SDMA ($r = -0.50$, $P < 0.001$). The strong inverse correlation of SDMA with eGFR confirmed the previous result[42] and implied usefulness of

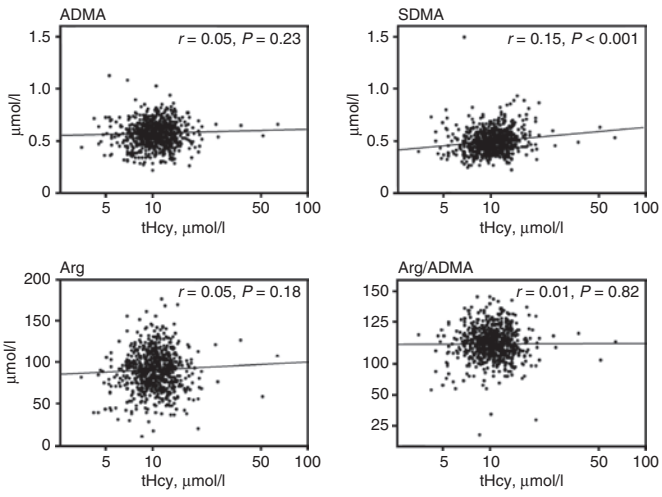

Figure 1 | Correlation between the concentration of total homocysteine (tHcy) and concentrations of dimethylarginines. Arg/ADMA and tHcy were plotted with a log-scale because of a skewed distribution. ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; Arg, arginine; tHcy, total homocysteine.

Table 2 | Association of the factors with MARCD examined by the logistic regression analysis

	β	s.e.	χ^2	P	OR (95% CI)
Model 1					
Age	0.082	0.015	29.5	<0.001	1.09 (1.05, 1.12)
Hypertension	0.50	0.10	23.4	<0.001	1.65 (1.34, 2.01)
log Arg/ADMA	−1.64	0.67	5.9	<0.05	0.19 (0.05, 0.73)
Sex (female vs. male)	0.14	0.11	1.75	0.19	1.15 (0.93, 1.43)
log TG	−1.30	0.51	6.55	<0.05	0.27 (0.10, 0.73)
log FBG	2.44	1.14	4.55	<0.05	11.5 (1.13, 103)
SDMA	0.18	1.00	0.03	0.86	1.20 (0.16, 8.76)
eGFR	−0.0017	0.009	0.04	0.84	1.00 (0.98, 1.02)
Model 2					
Age	0.091	0.015	38.0	<0.001	1.10 (1.07, 1.13)
MBP	0.030	0.008	12.6	<0.001	1.03 (1.01, 1.05)
log Arg/ADMA	−1.72	0.67	6.51	<0.05	0.18 (0.05, 0.68)
Sex	0.081	0.11	0.54	0.46	1.08 (0.88, 1.35)
log TG	−1.24	0.50	6.12	<0.05	0.29 (0.11, 0.76)
log FBG	2.14	1.12	3.67	0.0554	8.50 (0.90, 73.0)
SDMA	0.31	1.00	0.09	0.76	1.36 (0.19, 10.2)
eGFR	−0.0013	0.009	0.02	0.88	1.00 (0.98, 1.02)
Model 3					
Age	0.086	0.015	34.9	<0.001	1.09 (1.06, 1.13)
MBP	0.031	0.008	13.9	<0.001	1.03 (1.02, 1.05)
ADMA	1.75	0.96	3.31	0.069	5.75 (0.88, 38.1)
Sex	0.070	0.11	0.4	0.53	1.07 (0.87, 1.34)
log TG	−1.15	0.50	5.29	<0.05	0.32 (0.12, 0.83)
log FBG	1.75	1.11	2.51	0.11	5.75 (0.61, 48.4)
SDMA	−0.012	1.15	0.00	0.99	0.99 (0.10, 9.78)
eGFR	−0.0031	0.009	0.12	0.73	1.00 (0.98, 1.02)

Factors with $P < 0.1$ in the univariate analysis as well as sex and eGFR were included in the models. In model 2 and 3, MBP was substituted for the diagnosed hypertension. In model 3, ADMA was substituted for log Arg/ADMA.
ADMA, asymmetric dimethylarginine; Arg, L-arginine; CI, confidence interval; eGFR, estimated glomerular filtration rate; FBG, fasting blood glucose; MBP, mean blood pressure; SDMA, symmetric dimethylarginine; TG, triglyceride.

SDMA as a sensitive parameter for renal function and other cardiovascular disorders.[43] In this context, the significant difference in SDMA between MARCD (+) and (−) might reflect a small difference in renal function (see **Table 1**).

A logistic regression analysis was performed with the factors selected from the univariate analysis. The factors with $P < 0.1$ in the univariate analysis were included (see **Table 1**). When systolic blood pressure and diastolic blood pressure were included simultaneously in a model, neither of them showed significant association (systolic blood pressure; $P = 0.11$, diastolic blood pressure; $P = 0.33$) with MARCD probably because of a significant co-linearity between systolic blood pressure and diastolic blood pressure ($r = 0.74$, $P < 0.001$). Accordingly, diagnosed hypertension and mean blood pressure were included in models 1 and 2, respectively. In the same way, ADMA was substituted for Arg/ADMA (log-transformed) in model 3. Sex and eGFR were included in all the three models.

The results of the analysis are summarized in **Table 2**. The Arg/ADMA ratio was independently associated with MARCD both in model 1 and 2. When the ADMA level was included in model 3, the association was marginal ($P = 0.069$). The triglyceride level associated significantly with MARCD while the fasting blood glucose level showed a marginal association.

Treatment for hyperlipidemia as well as for hypertension may modulate the level of ADMA and tHcy. We therefore excluded 61 participants receiving medication for hyperlipidemia or hypertension from the analysis. A significant association of Arg/ADMA with MARCD was still observed both in model 1 ($\chi^2 = 5.25$, $P < 0.05$) and in model 2 (χ^2=6.71, $P < 0.01$).

DISCUSSION

In the present cross-sectional study, the Arg/ADMA ratio was shown to have an independent association with MARCD, which supported the pathological role of ADMA in this disease entity.

Although the inhibition of eNOS with ADMA was expected to induce hypertension, the logistic regression analysis indicated that the association of Arg/ADMA with MARCD was independent of hypertension (or mean blood pressure). This was consistent with the observation by Kielstein *et al.* that the infusion of ADMA induced a significant reduction in cerebral blood flow without affecting blood pressure in humans.[14] The cerebral arteries may be more sensitive to ADMA or other regulatory systems may counterbalance the effects of ADMA on systemic blood pressure. Recently, a case–control study by Khan *et al.* indicated that ADMA levels were greater in those with small-vessel disease.[24] The present observations basically confirmed their result. However, our study design was unique in its size and the method of subject recruitment; Khan *et al.* recruited stroke patients visiting a hospital as a case population ($n = 47$) and a small number of community-based subjects without magnetic resonance imaging findings as a control ($n = 38$). By contrast, we employed 712 consecutive participants of a health examination who did not have clinically overt symptoms and signs of cerebral stroke, and then sorted them into the "case" and "control" populations based on magnetic resonance imaging findings. This method was probably beneficial in avoiding unexpected selection biases.

In the present study, the difference in ADMA between the case and control populations was found to be very small. Such a small difference in the level of ADMA between case and control populations is commonly observed in literature.[23,24] An argument was accordingly made that even such a small difference in the ADMA level could reflect the risks for various cardiovascular diseases.[44] In this regard, it is of interest that neuronal NOS was shown to be more sensitive to ADMA than was eNOS.[45] This suggests that small changes in ADMA may have more profound effects on neural tissue than on the vasculature, which may contribute to the pathogenesis of MARCD beyond cerebral perfusion.

All studies on ADMA in cerebrovascular diseases reported to date measured ADMA after the diagnosis and/or treatment for cerebral stroke had already been made.[22–24] This raised a possibility that the ADMA level was modified by the treatment of stroke, which may have obscured a causal relationship. As the subjects in the present study were not recruited in a hospital, we may be able to exclude this possibility. In spite of that, the cross-sectional study design itself does not allow us to infer a causal relationship between ADMA and MARCD. Prospective studies are essential to resolve this issue.

In contrast to ADMA, tHcy did not have a significant association with MARCD in the population we studied (see **Table 1**). Furthermore, there was no significant correlation between tHcy and ADMA (see **Figure 1**). This observation contrasted with previous reports of a positive correlation of tHcy with MARCD and with ADMA.[6–8,24,32] In addition, studies *in vivo* and *in vitro* indicated that the administration of homocysteine induced an increase in ADMA.[34,35] Contrary to these observations, Wilcken *et al.* indicated that in patients deficient in cystathionine β-synthase, who showed extreme variations in the level of tHcy (15–285 μmol/l), no significant correlation was observed between tHcy and ADMA.[46] Furthermore, they clearly indicated that treatment for hyperhomocysteinemia did not change the ADMA level while it dramatically decreased the tHcy level from 300 to 10 μmol/l.[46] Their results strongly suggested that hyperhomocysteinemia itself did not influence the ADMA level. Although the acute administration of homocysteine increases the level of ADMA, the chronic increase in tHcy *in vivo* may be compensated for by other physiological mechanisms to prevent an increase in ADMA.

Concerning the role of hyperhomocysteinemia in MARCD, Pezzini *et al.* discussed in their review that only a very high level of tHcy in those with the cystathionine β-synthase deficiency could induce arteriosclerosis and fibrinoid necrosis in the small arteries and arterioles, while a modest increase in the serum tHcy level found in the general population promoted not arteriosclerosis but atherosclerosis.[47] This implied that homocysteine *per se* did not have a major effect on arteriosclerosis, a pathological basis of MARCD, when the hyperhomocysteinemia was as modest as that found in most MARCD patients. In this context, it should be emphasized that some

atherothrombotic infarctions might be mixed with larger symptomatic lacunar infarctions.[10] This might partly explain the discrepancy found in the relation between hyperhomocysteinemia and MARCD.

Regarding the association between tHcy and MARCD, tHcy showed a significant correlation both with SDMA and with eGFR (see **Figure 1** and the Results). This suggested that tHcy was substantially influenced by renal function. Van Guldener indeed pointed out that the GFR was closely correlated with tHcy,[48] which was later confirmed in a large meta-analysis.[49] This observation reveals the need for careful evaluation of renal function and other confounding factors when examining the effects of tHcy in clinical and epidemiological studies. Even in the case of atherosclerotic diseases, it is argued that hyperhomocysteinemia may be an innocent surrogate marker for unknown causative factors.[47,48,50]

In summary, the findings of the present study indicate that Arg/ADMA, but not tHcy, is associated with MARCD in a Japanese population. Studies on the pathological role of ADMA in MARCD as well as large-scale prospective studies would be warranted.

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