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The association of homocysteine and related factors to brachial artery diameter and flow-mediated dilation

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Abstract

Brachial artery flow-mediated dilation (BAFMD) has been proposed as a measurement of the degree and severity of cardiovascular disease. The purpose of this study was to (1) evaluate the associations between BAFMD and homocysteine, folate, vitamin B_{12}, vitamin B_6; (2) examine the influence of 5,10-methylenetetrahydrofolate reductase (MTHFR) genotypes on homocysteine levels and BAFMD; and (3) evaluate the effect of homocysteine on the baseline diameter of the vessel vs BAFMD. A total of 174 healthy research subjects were examined for BAFMD, homocysteine, folate, vitamin B_{12}, vitamin B_6, and MTHFR genotype, nucleotide 677 C→T. The data indicated a significant inverse correlation between homocysteine and BAFMD ($r = -0.1763$, $P = .02$). There was a significant difference in BAFMD between MTHFR genotype groups ($P = .01$) (T/T vs C/C, $P = .042$; C/C vs C/T, $P = .13$; T/T vs C/T, $P = .003$). Homocysteine was significantly associated with the baseline brachial artery diameter ($r = 0.1878$, $P = .013$). The data confirmed a significant inverse correlation between baseline diameter and BAFMD ($r = -0.3321$, $P = .0001$). Regression analysis indicated that the MTHFR genotype, homocysteine, and age were significant predictors of BAFMD ($P = .0001$, $r^2 = 0.118$). When the baseline brachial diameter was incorporated into the model, the effect of homocysteine on BAFMD disappeared. The present data indicate an association between homocysteine and BAFMD and reduced BAFMD in individuals with the MTHFR nucleotide 677 T/T genotype, despite similar blood values for folate and homocysteine. Finally, the data suggest that the effect of homocysteine on vascular reactivity is in part a consequence of its influence on baseline brachial artery diameter.

1. Introduction

Plasma levels of homocysteine, an amino acid produced from the metabolism of methionine, are independently associated with increased risk for vascular disease [1-5] and have been shown to augment the effects of other traditional risk factors [6]. Because of their intimate involvement in homocysteine metabolism, dietary intakes of folic acid, vitamin B_{12}, and vitamin B_6 are associated with homocysteine levels and, therefore, possibly the development of cardiovascular disease. However, this relationship is complicated by the presence of genetic variation in a key enzyme involved in homocysteine metabolism, 5,10-methylenetetrahydrofolate reductase (MTHFR).

MTHFR is a critical enzyme in homocysteine metabolism that regenerates the active form of folate, 5-methyltetrahydrofolate, from 5,10-methyltetrahydrofolate. Individuals with a genetic alteration in the MTHFR enzyme (nucleotide [nt] 677 C→T), which substitutes a valine for an alanine, have elevated plasma homocysteine concentrations that contribute to an increased risk for cardiovascular disease [7]. Furthermore, individuals with coronary artery disease have an increased prevalence of the MTHFR variant compared with control subjects [8].

Brachial artery flow-mediated dilation (BAFMD) has been proposed as a measurement of the degree and severity of cardiovascular disease and is used as a research tool in the

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study of cardiovascular disease [9]. Assessment of BAFMD is related to structural changes of the carotid arteries [10], coronary artery reactivity [11,12], coronary stenosis [13], and the extent and severity of coronary artery disease [14]. In addition, an impaired BAFMD has been shown to independently predict long-term cardiovascular events in patients with peripheral artery disease [15]. Likewise, when evaluating patients with coronary artery disease, BAFMD has been shown to be an independent prognostic measurement [16-22] and correlated to coronary events [23].

Because of the prognostic ability of BAFMD, studies have examined the associations between BAFMD and cardiovascular disease risk factors, including homocysteine. However, in this regard, the data are conflicting. An association between BAFMD and fasting plasma homocysteine concentrations was found in subjects with hyperhomocysteinemia [24,25] and between BAFMD and postprandial plasma homocysteine concentrations after methionine loading [26]. Other studies with healthy individuals, however, showed no correlation between homocysteine levels and BAFMD [27,28]. The inconsistent findings in the literature concerning the relationship between homocysteine and BAFMD may in part be related to failure to account for a genetic variation in MTHFR.

An additional factor may be a failure to consider the influence of homocysteine on the baseline diameter of the vessel. Several traditional cardiovascular disease risk factors including body mass index (BMI), body surface area, weight, diabetes, hypertension, dyslipidemia, and an increased atherosclerosis risk score, influence the baseline brachial artery diameter [29]. Given that the baseline diameter is inversely related to the BAFMD measure [30], it is important to understand that risk factors may exert a direct or indirect effect on the vessel's reactivity.

Therefore, the purpose of this study was (1) to evaluate the associations between serum homocysteine, folate, vitamin B_{12}, vitamin B_6, and BAFMD; (2) to examine the possible influence of MTHFR genotype on homocysteine levels and BAFMD; and (3) to evaluate the effect of homocysteine on the baseline diameter of the vessel vs BAFMD. We hypothesized that plasma homocysteine would be inversely related to BAFMD and that individuals

Table 1
Participant characteristics, biochemical measurements, and brachial artery measurements

	Sex	n	Mean	SD	Range	Minimum	Maximum
Age (y)	Female	127	56	4.74	21	45	66
	Male	47	54*	6.08	19	46	65
Height (cm)	Female	127	163	5.36	31	150	181
	Male	47	177*	6.44	31	158	189
Weight (kg)	Female	127	71.67	11.97	51.20	47.20	105.60
	Male	47	88.10*	12.77	54.80	61.40	116.20
BMI (kg/m^2)	Female	127	26.94	4.19	19.87	18.57	38.44
	Male	47	28.09†	3.47	15.21	21.46	36.67
SBP (mm Hg)	Female	127	123	13.61	71	67	168
	Male	47	121	13.15	57	85	152
DBP (mm Hg)	Female	127	75	6.94	41	60	96
	Male	47	76	8.32	34	60	94
Resting heart rate (beats/min)	Female	127	66	8.92	48	45	93
	Male	47	62*	9.49	40	43	83
Baseline diameter (mm)	Female	127	2.97	.41	2.03	2.16	4.19
	Male	47	3.81*	.44	2.09	2.94	5.03
BAFMD (%)	Female	127	5.25	3.89	14.23	−0.97	13.26
	Male	47	4.99	2.93	11.28	−0.32	10.96
HDL (mmol/L)	Female	127	63	13.68	78	33	111
	Male	47	47*	7.49	36	34	70
LDL (mmol/L)	Female	127	131	25.20	124	80	204
	Male	47	138+	20.94	98	93	191
Triglycerides (mmol/L)	Female	127	125	56.50	370	33	403
	Male	47	139	84.10	566	25	591
Cholesterol (mmol/L)	Female	127	219	27.12	127	164	291
	Male	47	213	27.10	126	159	285
Folate (nmol/L)	Female	127	13.60	2.23	14.80	4.80	19.60
	Male	47	13.35	2.00	10.40	6.40	16.80
Vitamin B_{12} (pmol/L)	Female	127	529.99	287.63	1789	166	1955
	Male	47	463.48†	155.68	722	187	909
Vitamin B_6 (nmol/L)	Female	127	83.89	97.97	515	3.90	519.00
	Male	47	82.21	96.42	593	15.50	608.50
Homocysteine (μmol/L)	Female	127	7.20	1.85	9.80	3.50	13.30
	Male	47	8.83*	1.88	10.50	5.30	15.80

SBP indicates systolic blood pressure; DBP, diastolic blood pressure.

* $P = .05$.

† $P = .01$ (females vs males).

with the T/T genotype would have decreased BAFMD. In addition, we hypothesize that at least part of the effect of homocysteine on vasoreactivity is secondary to its effect on the baseline brachial artery diameter.

2. Materials and methods

2.1. Participants

A total of 174 research subjects were available for study from the baseline period of the Reversal of Early Atherosclerotic Changes (REACH) by Diet study at the Pennington Biomedical Research Center, Baton Rouge, LA. The subjects consisted of 47 men and 127 postmenopausal women. These volunteers were recruited during a screening period based on an age range of 45 to 66 years, an age- and gender-adjusted mean low-density lipoprotein (LDL) cholesterol between the 15th and 85th percentile, and high-density lipoprotein (HDL) cholesterol and triglyceride levels between the 5th and 95th percentile as defined by National Health and Nutrition Examination Survey (NHANES) II data [31]. Exclusions included a history of coronary artery disease (angina, myocardial infarction, or abnormal electrocardiogram), diabetes mellitus, systolic pressure of 160 mm Hg or higher, or diastolic pressure 95 mm Hg or higher, use of medications for treatment of elevated lipids or hypertension, or a history of drug or alcohol abuse. After an explanation of the study and its benefits and risks, volunteers signed an informed consent approved by the Pennington Biomedical Research Center Institutional Review Board.

2.2. Brachial artery assessments

Brachial artery assessments were obtained by high-resolution ultrasound (Toshiba Powervision SSA-380A with a 7.5-MHz linear array transducer, Toshiba America, New York, NY) before, during, and after 5 minutes of forearm occlusion. Individuals were instructed to fast and refrain from exercise for 12 hours and alcohol for 48 hours before the measurements. Ultrasound images were obtained after 15 minutes of supine rest. Images were obtained in longitudinal view, approximately 4 cm proximal to the olecranon process, in the anterior/medial plane. Image depth was initially set at 4 cm, and gain settings were adjusted to provide an optimal view of the anterior and posterior walls of the artery. Once settings were optimized they were kept constant. Imaging was performed on the nondominant arm with the forearm extended and slightly supinated. Forearm occlusion consisted of inflation of a blood pressure cuff to 240 mm Hg for 5 minutes. Images were recorded on super VHS videotape for 30 seconds at baseline and continuously from the final 30 seconds of occlusion until 5 minutes after release. Digital still images captured during diastole, as defined by the onset of the QRS complex, were analyzed using specialized imaging software (Media Cybernetics, Image-Pro Plus, Silver Spring, MD). Arterial diameters (millimeters) were calculated as the mean distance between the anterior and posterior wall at the vessel-blood interface. Reproducibility of the technique, in our hands, has yielded average mean differences in vessel diameter change for days and testers of 1.91% and 1.40% with intraclass correlation coefficients of 0.92 and 0.94, respectively [32]. In that same study, the mean absolute difference in BAFMD between readers was 0.21 ± 0.014 mm, with an intraclass correlation coefficient of 0.90 [32]. Baseline diameter was taken as the brachial diameter during the resting phase. BAFMD is defined as follows: peak = (peak diameter after hyperemia minus the baseline diameter) × 100%/(baseline diameter).

2.3. Laboratory analyses

Fasting blood draws were obtained after the imaging procedure or on a subsequent day. After aliquoting, samples were frozen at −80°C until analysis. Plasma homocysteine, vitamin B_{12}, and folate analyses were performed on the Abbott IMx (Abbott Park, IL) using Abbott IMx total homocysteine, vitamin B_{12}, and folate reagents, controls, and calibrators, respectively. Plasma vitamin B_6 levels were determined with ALPCO Diagnostics (Windham, NH) vitamin B_6 (pyridoxal-5′-phosphate) ^{3}H radioenzymatic assay according to standard protocol. Total cholesterol and triglycerides were determined as part of the lipid profile. These were analyzed on the Beckman Synchron CX7 (Brea, CA) using Beckman reagents and calibrators. Sigma Diagnostics (St Louis, MO) EZ HDL Cholesterol reagent was used on the Beckman Synchron CX7 for HDL cholesterol analysis. LDL cholesterol was calculated by using the Friedewald equation. Genomic DNA was isolated from buffy coats by the method of John et al [33] and the prevalence of the nt 677 CT mutation was determined by polymerase chain reaction and *Hin*fI restriction enzyme digestion as described by Frosst et al [34].

2.4. Statistical analysis

Statistical analyses were performed by using SPSS for Windows (version 11.0, SPSS, Chicago, IL). Data are presented as means and SDs. To examine the associations between homocysteine, vitamin B_{12}, vitamin B_6, folate, and

Table 2
Correlations of homocysteine and related factors to brachial artery measurements (N = 174)

	Vitamin B_{12} (pmol/L)	Folate (nmol/L)	Homocysteine (μmol/L)	Baseline diameter (mm)	BAFMD (%)
Vitamin B_6 (nmol/L)	0.31, P = .0001	0.26, P = .001	−0.17, P = .025	0.09, P = .23	0.06, P = .45
Vitamin B_{12} (pmol/L)		0.28, P = .0001	−0.40, P = .0001	−0.06, P = .41	−0.08, P = .31
Folate (nmol/L)			−0.38, P = .0001	0.06, P = .42	−0.09, P = .24
Homocysteine (μmol/L)				.19, P = .013	−0.18, P = .02
Baseline diameter (mm)					−0.33, P = .0001

BAFMD, a partial correlation coefficient with height as a covariate was used. To examine the influence of the MTHFR genotype on BAFMD, a multivariate linear model was used. Finally, to determine the possible direct and/or indirect effect of homocysteine on BAFMD, multiple general linear models were compared with and without the baseline diameter in the model. Significance was tested at the 95% confidence level ($P \leq .05$).

3. Results

3.1. Participant characteristics

Participant characteristics, biochemical measurements, and brachial artery measurements for all subjects are shown in Table 1. Participants consisted of 47 men and 127 postmenopausal females within an age range of 45 to 66 years. In addition, most of all participants' biochemical analytes were within reference ranges, including a participant homocysteine range of 3.5 to 15.8 μmol/L (reference range, 5-15 μmol/L).

3.2. Association of homocysteine, folate, vitamin B12, and vitamin B6 levels and BAFMD

The data indicated strong inverse correlations between serum homocysteine and serum folate, vitamin B_{12}, and vitamin B_6 ($r = -0.38$, $P = .0001$; $r = -0.39$, $P = .001$; $r = -0.17$, $P = .025$, respectively) (Table 2). In addition, the data showed a significant inverse correlation between homocysteine and BAFMD ($r = -0.18$, $P = .020$) (Fig. 1). In contrast, the data indicated no significant associations between serum folate, vitamin B_{12}, or vitamin B_6 and BAFMD.

3.3. Influence of MTHFR genotypes on homocysteine levels and BAFMD

Of the 174 men and women who were analyzed for the MTHFR C677T genotype, 83 (48%) were homozygous for the common allele, C/C; 20 (11%) were homozygous for the variant allele T/T; and 71 (41%) were heterozygous, C/T. No significant group differences for age, BMI, lipids, blood pressure, heart rate, or baseline diameter were noted between genotypes. The average folate, vitamin B_{12}, vitamin B_6, and homocysteine concentrations were not significantly different between the 3 MTHFR groups. In contrast, regression analysis incorporating MTHFR genotype and gender, and covarying for age, homocysteine, folate, and baseline diameter, revealed significant differences in BAFMD between MTHFR genotype groups ($P = .01$) (post hoc comparison: T/T vs C/C, $P = .042$; C/C vs C/T, $P = .13$; T/T vs C/T, $P = .003$) (Fig. 2). The correlation of homocysteine and folate by MTHFR genotype is shown

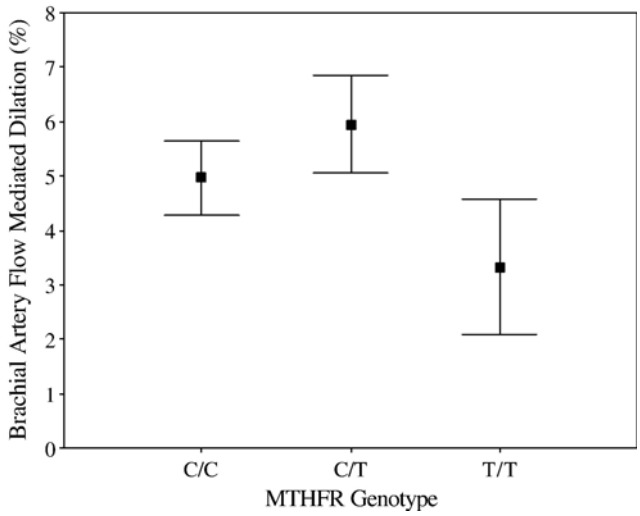

Fig. 2. The relationship between MTHFR genotype and BAFMD. Data show the mean ± SE for each genotype (C/C genotype, 5.04 ± 0.37; C/T genotype, 5.9 ± 0.40; T/T genotype, 3.3 ± 0.75) (T/T vs C/C, $P = .042$; C/C vs C/T, $P = .13$; T/T vs C/T, $P = .003$).

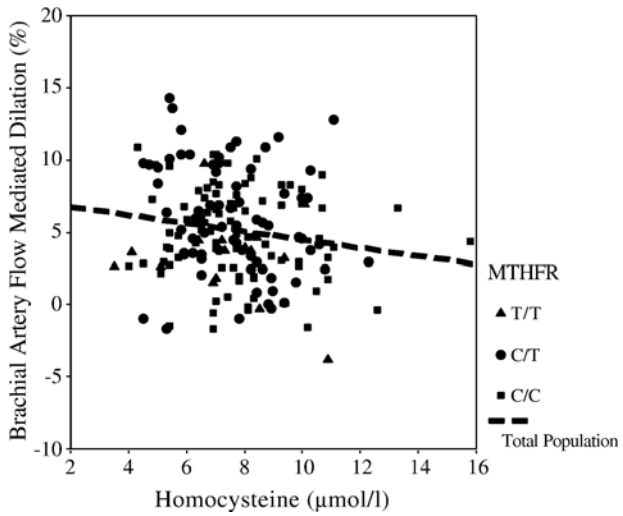

Fig. 1. Correlation between brachial artery flow-mediated dilation and homocysteine ($y = -0.282x + 7.29$; $r = 0.18$, $P = .020$).

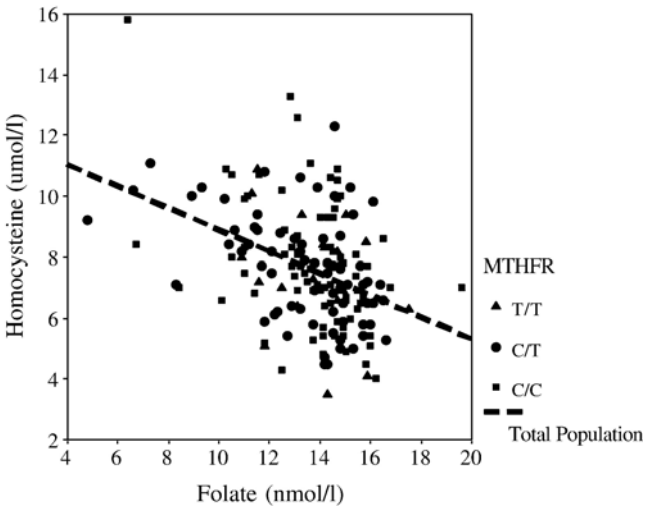

Fig. 3. Correlation of homocysteine and folate ($y = -0.4543x + 16.98$; $r = -0.394$, $P = .001$).

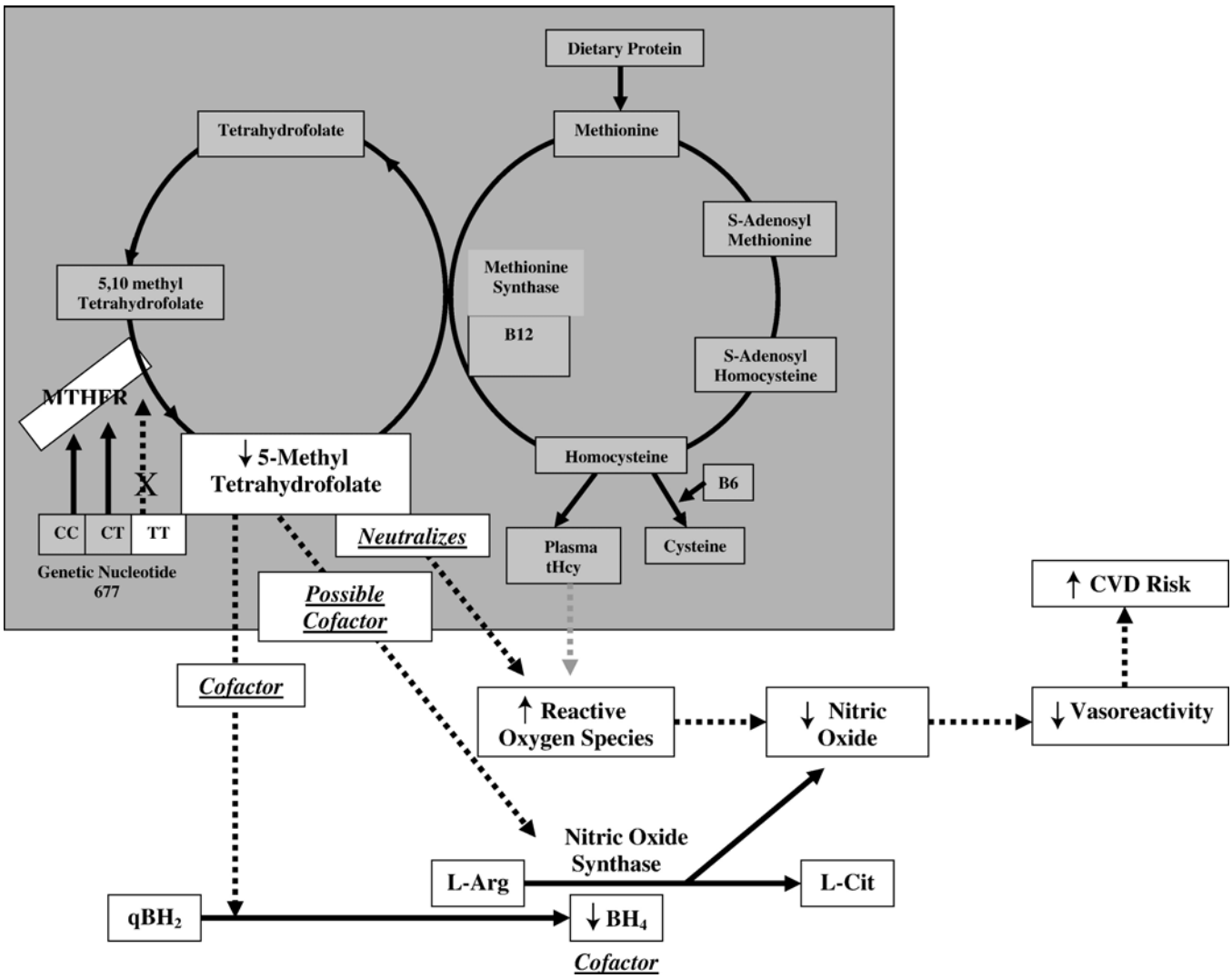

Fig. 4. Hypothetical pathway by which an MTHFR variant increases cardiovascular risk. L-Arg indicates L-arginine; L-Cit, L-citruline; qBH_2, quinoid 7,8-dihydrobiopterin; BH_4, 5,6,7,8-tetrahydrobiopterin.

in Fig. 3. The correlation between homocysteine and folate was not affected by the MTHFR genotype.

3.4. Relationship between homocysteine, baseline diameter, and BAFMD

As previously stated, the association between homocysteine and BAFMD was weakly significant. However, analysis revealed that homocysteine was significantly associated with the baseline brachial artery diameter (r = 0.19, P = .013). Moreover, the data confirmed a significant inverse correlation between baseline diameter and BAFMD (r = −0.33, P = .0001).

Regression analysis using a multivariable general linear model indicated that the MTHFR genotype, homocysteine, and age were significant predictors of BAFMD (P = .0001, r^2 = 0.118) (homocysteine, t = −1.97, P = .050; age, t = −2.49, P = .014; and MTHFR genotype, P = .008). When the baseline brachial diameter was incorporated into the model, the effect of homocysteine on BAFMD was no longer significant.

4. Discussion

The present data confirm an association between homocysteine and BAFMD [24,25]. Uniquely, the present study indicates reduced brachial artery reactivity in individuals with the MTHFR nt 677 T/T genotype, despite similar blood values for folate and homocysteine. Finally, the present study suggests that the effect of homocysteine on vascular reactivity may in part be secondary to its influence on the vessel's baseline diameter.

Elevated homocysteine levels have been shown to increase cardiovascular disease risk proportionally [6]. Even within the reference range of homocysteine levels of 10 and 15 μmol/L there is a continuum of increasing cardiovascular disease [35]. Unlike the findings of some studies evaluating

healthy subjects [27,28,36], our data show that homocysteine levels are associated with a decrease in reactivity of the vessel. Although the relationship between homocysteine and reactivity was weak, these findings are intriguing given that the homocysteine values were all in the reference range. In fact, the homocysteine values in our participants ranged from 3.5 to 15.8 μmol/L, with only one participant having a value greater than the reference range of 5 to 15 μmol/L. Likewise, all folate levels were within or above the reference range, with only 4% and 16% of the vitamin B_{12} and vitamin B_6 levels, respectively, below the reference ranges. Even within these ranges there are strong inverse correlations between homocysteine and the nutritional analytes, folate, vitamin B_{12}, and vitamin B_6. This information, coupled with evidence that individuals with hyperhomocysteinemia have a marked reduction in vessel reactivity [24,25] and that there is a graded risk for homocysteine levels within the reference range [6], further emphasizes the importance of minimizing homocysteine levels. The possible mechanism that explains the apparent association between homocysteine and BAFMD is not entirely understood but is thought to involve the influence of homocysteine on the bioavailability of nitric oxide, a mediator of vasoreactivity [24] (Fig. 4).

Regarding the second objective of this study, the present data indicate reduced BAFMD in individuals with the MTHFR nt 677 T/T genotype. The prevalence rate for the MTHFR nt 677 T/T genotype in this study was 11%, which is similar to the reports of Frosst et al [34], who found an incidence of 14% in a French Canadian population. Previous research has indicated a greater incidence of cardiovascular disease in patients with the MTHFR T/T genotype [37]. In fact, de Franchis et al [38] reported a relationship between MTHFR genotype and homocysteine levels and cardiovascular disease risk [38]. In contrast, de Bree et al [39] reported no differences in homocysteine concentrations among MTHFR genotypes at high folate intake. The biological mechanism for the reduced vasoreactivity in individuals with the MTHFR T/T genotype in the present study cannot be explained by differences in plasma levels of homocysteine or folate, as these were similar in all 3 genotypes. Recognizing that flow-mediated dilation is in part modulated by endothelial derived nitric oxide [40], our present findings may indicate an alteration in nitric oxide bioavailability in individuals with the MTHFR T/T genotype.

Bioavailability of nitric oxide is affected by both nitric oxide production and degradation. Nitric oxide is synthesized by endothelial nitric oxide synthase, which converts L-arginine to L-citrulline. This reaction requires the presence of the essential cofactor tetrahydrobiopterin (BH_4) [41,42]. Tetrahydrobiopterin ensures that endothelial nitric oxide synthase produces nitric oxide instead of superoxide [43-45]. Tetrahydrobiopterin is produced from the inactive quinoid form (qBH_2) in the presence of 5-methyltetrahydrofolate [46]. In addition, 5-methyltetrahydrofolate may directly interact with the active site of endothelial nitric oxide synthase [47] resulting in nitric oxide production. Finally, evidence suggests that 5-methyltetrahydrofolate is involved in decreasing the degradation of nitric oxide, in part because of a reduction of reactive oxygen species [43-45,48]. Thus, decreased MTHFR activity may result in a decrease in nitric oxide availability due to a reduction in 5-methyltetrahydrofolate and/or tetrahydrobiopterin.

Regarding the direct and/or indirect role of homocysteine on brachial artery reactivity, the present study indicates that homocysteine is correlated to both BAFMD and the baseline diameter of the brachial artery. In fact, multiple regression analysis strongly suggests that the effect of homocysteine on reactivity occurs predominantly through its influence on the baseline diameter with the diameter increasing with increasing levels of homocysteine. These findings contradict the findings of Wiltshire et al [49] who found that homocysteine was inversely related to the brachial artery resting diameter in diabetic children. However, other studies have found cardiovascular risk factors, such as BMI, glucose, blood pressure, and severity of coronary artery disease to be associated with an increase in the diameter of not only the brachial artery, but also the common carotid artery and the coronary arteries [29,50-54]. It is presently not known how homocysteine influences the baseline diameter. Recognizing that vascular tone is predominantly affected by the autonomic nervous system, it is possible that homocysteine effects autonomic balance. In fact, there is evidence that homocysteine levels are associated with autonomic neuropathy [55]. It is possible that early changes in autonomic balance may contribute to an increase in vascular resistance in smaller resistance vessels. As compensation for the changes in the resistance vessels, larger conduit vessels may initially increase diameter size in an attempt to normalize blood pressure. Other factors that may cause homocysteine to influence the vasculature include alterations in the coagulation system, increased platelet aggregation, increased hydrogen peroxide production causing endothelial damage, increased growth of smooth muscle cells, or a reduction in antioxidant levels [6]. Combined, these factors may contribute to structural and physiologic alterations in different segments of the vasculature.

Future studies are needed to evaluate whether the influence of individual cardiovascular risk factors on the reactivity of the vessel is direct (eg, endothelial mechanisms) or indirect (eg, autonomic balance). In doing so, it will be possible to determine modulators of each effect separately, perhaps leading to new methods of reducing cardiovascular disease risk.

The present findings were noted despite several limitations. Specifically, the ability to detect significant effects of the variables of interest may, in part, be limited due to the homogeneous nature of our study population. It certainly is important for future studies to widen the study population (in terms of age, gender, ethnicity, physical fitness, and type and severity of disease). In addition, it

should be noted that our study was conducted after folate fortification of grains and cereals [56], which may have accounted for the lack of differences in homocysteine levels among the MTHFR genotypes. Nevertheless, our data indicate promising new possibilities in the study of BAFMD and cardiovascular disease risk. It is well known that increasing folate levels will eventually compensate for the decreased enzyme in the MTHFR T/T genotype and result in normal homocysteine levels. In fact, Christensen et al [57] found that although 5-methyl tetrahydrofolate levels were lower in the T/T MTHFR genotype, total folate was not significantly lower than in the C/C variant. Finally, our folate immunoassay measured total folate rather than individual forms of folate. Perturbations in the specific forms of folate could occur although the total levels are normal [58]. It is conceivable that the levels for the specific forms of folate differ between the MTHFR genotypes. This could then affect nitric oxide levels by the mechanism we propose in Fig. 4.

In conclusion, the present data indicate that even within normal serum levels of vitamins, there are strong associations between homocysteine and folate, vitamin B_{12}, and vitamin B_6 levels. There is also a relatively weak association between homocysteine and BAFMD. The present data indicate reduced brachial artery reactivity in individuals with the MTHFR nt 677 T/T genotype, despite similar blood values for folate and homocysteine. Finally, the present study suggests that the effect of homocysteine on vascular reactivity is in part a consequence of its influence on baseline brachial artery diameter.

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