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Effects of three-months folate supplementation on early vascular abnormalities in hyperhomocysteinemic patients with epilepsy

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ABSTRACT

Background: Epilepsy has been associated with an increased risk of cardiovascular events. Anti-seizure medication (ASM) may contribute to vascular risk by several mechanisms, including increased homocysteine levels. This study aims to assess the global vascular burden in hyperhomocysteinemic people with epilepsy (PWE) on long-term ASM before and after folic acid supplementation and in subgroups of PWE treated with single enzyme-inducing or single non-enzyme inducing ASM.

Methods: One hundred and seventy-four hyperhomocysteinemic (HHcy) PWE who met the inclusion criteria were enrolled. Carotid Doppler ultrasonography, FMD and ultrasound assessment of the brachial artery properties at the baseline and after 90 days of folic acid supplementation were performed. The vascular biomarkers MMP-9 and TIMP-1 were also detected.

Results: After folic acid supplementation, in HHcy patients homocysteine levels reduced from 26.8 ± 10.5 to 20.2 ± 5.3 μmol/L, carotid Intima-Media-Thickness reduced from 0.83+0.06 mm to 0.79±0.05 mm, and FMD, distensibility coefficient and β-stiffness improved ($p < 0.05$). Moreover, MMP-9 and TIMP-1 reduced after supplementation ($p < 0.05$). PWE treated with a single enzyme-inducing ASM showed an impairment of vascular parameters compared to patients treated with non-enzyme inducing ASM.

Conclusions: The results highlight the importance of assessing homocysteine levels and estimating the cardiovascular risk of PWE, preferring non-enzyme inducing ASM in high cardiovascular-risk patients. An adequate correction of homocysteine levels with folate supplementation should be considered to improve the cardiovascular profile.

1. Introduction

Epilepsy is one of the most common brain disorders and affects over 50 million people worldwide [1]. It has been associated with several cardiovascular risk factors including inflammation, oxidative stress, insulin resistance, and high homocysteine level, and with an increased risk of cardiovascular events [2–4].

Anti-seizure medications (ASM) may contribute to the vascular risk of people with epilepsy (PWE), and especially enzyme inducer medications seem to be linked to atherosclerotic progression. The role of ASM in the pathogenesis of atherosclerosis is not yet fully understood, but alterations in the lipid profile, increased values of C-reactive protein and higher homocysteine levels, due to folate and vitamin B6 deficiencies, may be involved [5].

Homocysteine is a non-proteinogenic amino acid derived from methionine metabolism, and elevated homocysteine levels are associated with coronary and peripheral atherothrombosis [6]. According to current knowledge, folic acid supplementation may reduce homocysteine levels and improve cardiovascular abnormalities [5,7–9].

Aim of the study was to investigate the effects of three-month folate

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supplementation on early vascular alterations in hyperhomocysteinemic PWE on long-term antiepileptic drugs without pre-existing cardiovascular diseases.

In addition, the study was aimed at investigating the eventual differences in vascular disorders between hyperhomocysteinemic PWE treated with a single enzyme-inducing ASM or a single non-enzyme inducing ASM, before and after acid folic supplementation.

2. Methods

A total of 1014 PWE referred to the Department of Neurology of "Federico II" University School of Medicine, Naples, Italy, were consecutively screened from January 2018 to November 2020. The inclusion criteria were the following: age between 18 and 50 years; diagnosis of epilepsy for at least 5 years; treatment with anti-seizure medications; high homocysteine levels (total homocysteine concentration $>$ 15 μmol/L). Exclusion criteria included: diabetes, dyslipidemia, carotid atherosclerosis or others known cardiovascular diseases; thyroid pathologies and/or other endocrinology disorders; serological creatinine values major than 1.5 mg/dl; chronic dermatological (e.g. psoriasis) and rheumatological diseases; autoimmune gastritis and/or inflammatory bowel disease and/or coeliac disease and deficiency of folate and/or other B-complex vitamins; degenerative neurological diseases and active neoplasms. Patients taking folic acid routinely before enrollment were excluded from the study. Accordingly, women who were pregnant or trying to become pregnant were also excluded.

A total of 174 PWE met the inclusion criteria and were enrolled. All hyperhomocysteinemic (HHcy) patients performed a comprehensive vascular assessment before and after 90 days of folic acid supplementation.

At the beginning of the study, all patients performed carotid Doppler ultrasonography and flow-mediated dilation and ultrasound assessment of the brachial artery elastic wall properties, as described below. The ultrasound scans were performed by expert physicians, skilled in cardiovascular diseases and vascular ultrasonography; they were blinded to patient's lab results and medications. Moreover, all PWE carried out a laboratory evaluation including the dosage of metalloproteases MMP-9 and specific tissue inhibitor TIMP-1 by ELISA. After the baseline evaluation, PWE received Folic Acid 5 mg daily for 90 days. At the end of the study, the ultrasound evaluations and the laboratory tests for MMP-9/TIMP-1 dosage were repeated.

3. ELISA assay of MMP-9 and TIMP-1

MMP-9 and TIMP-1 levels were detected by quantitative sandwich ELISA using commercial kits obtained from R&D Systems (Minneapolis, MN, USA). These assays are based on a two-site sandwich format using two antibodies directed against various epitopes of the molecule. All analyses were performed according to the manufacturer's instructions.

3.1. Ultrasound assessment of carotid arteries

Carotid longitudinal ultrasound B-mode scans were performed with a 7.5 MHz linear-array transducer (Vivid E9 ultrasound system) according to current guidelines [10,11].

All measurements were performed on the R-wave of the electrocardiogram. Common carotid intima-media thickness (IMT) was measured as the distance of the intimal to the adventitial layer with semi-automated reading software that calculates the mean values obtained in the segment between 1 cm before and 1 cm after the carotid bifurcation [12]. IMT values were expressed as the mean of three measurements for the left and right carotid axes.

3.2. Flow mediated dilation

Flow-mediated dilation (FMD) evaluation was performed according to the guidelines [13,14] using a validated protocol [15,16]. All participants were studied in a quiet, temperature-controlled room (22 °C) after 12 h of fasting, including caffeine, tea, alcohol, and abstaining from cigarette smoking, physical exercise, and drugs for 24 h before the examination. Tests were performed at the same time of day (approximately 12:00) as endothelial function depends on circadian variation [17]. An ultrasound system adapted for Doppler analysis and equipped with a multi-frequency transducer with a minimum frequency of 7.5 MHz was used. The brachial artery was imaged approximately 5 cm proximal to the antecubital crease in the longitudinal axis, and were considered only FMD values obtained measuring brachial arteries with a baseline diameter comprised between 2.5 and 5 mm. Each measurement was performed in the dominant arm at the end-diastole cycle, on the electrocardiographic R-wave. To standardize and obtain reproducible evaluations of the blood flow velocity, an angle of 60° between the Doppler beam and the vessel axis was maintained during recording. A sphygmomanometer cuff was placed around the forearm 1 to 2 cm distal to the elbow crease. After the identification of the anatomical landmarks, we used a stereotactic adjustable probe holding device to increase the image quality and maintain the probe in the same position and relationship with the vessel throughout the study. After baseline measures were acquired, the cuff was inflated to 50 mmHg above systolic pressure to occlude arterial inflow for 5 min. After sudden cuff deflation, the peak velocity flow and BAD variation were automatically measured by dedicated hardware/software. Endothelial dependent vasodilatation was assessed by measuring the maximum increase of BAD during the reactive hyperemia according to the formula:

FMD (%): [(post hyperemia BAD –baseline BAD)/baseline BAD] x 100

We used FMD Studio (Institute of Clinic Physiology, National Research Council, Pisa, Italy), a system composed of a dedicated hardware/software device that was directly connected to the ultrasound equipment, allowing the FMD assessment to occur in real time. Peak shear rate (PSR), an estimate of wall shear stress, was computed as the ratio of 4 times the peak hyperemic blood velocity (cm/s) to BAD (cm). Data relative to the baseline BAD and PSR were evaluated to exclude that the differences found in FMD values could be related to different baseline BAD or PSR discrepancies.

3.3. Brachial artery elastic properties

Based on the technical approach used for FMD evaluation, at baseline, before inflating the cuff and placing stereotactic adjustable probe holding device, the arterial elastic properties were assessed, according to the validated protocol described elsewhere (17). Briefly, the following parameters were measured in M-mode: the lowest end-diastolic arterial diameter (Dd) on the electrocardiographic R-wave, the highest end-systolic arterial diameter (Ds) on the electrocardiographic T wave, and the diameter change during cardiac cycle (DD, defined as Ds-Dd). The mean of 3 consecutive measurements was used for the analyses. Brachial artery pulse pressure (DP), defined as systolic minus diastolic blood pressure in mmHg, was measured by a sphygmomanometer and then converted in kPa The properties of the vessel wall, such as distensibility and stiffness, were then derived using the following equations:

$\beta = \ln (SBP/DBP)/(\Delta D/Dd)$

$DC = (2\Delta D^{*}Dd + \Delta D2)/ \Delta P^{*}Dd2$ where ln is the natural logarithm and SBP and DBP are the brachial systolic and diastolic pressures, respectively [18–20]. The coefficient of variation for the arterial DC in our laboratory was 5%.

3.4. Statistical analysis

Data are presented as mean ± standard deviation. Shapiro-Wilk test was used as distribution test and distribution of data was non-normal. Statistical significance between groups was tested by the Mann-Whitney U test for independent samples, Wilcoxon test for paired samples and

chi-square test with Yate's correction for non-continuous variables. We performed the Holm-Bonferroni correction and assumed a p value less than 0.05 statistically significant. Multiple correlation among variables was performed by a stepwise regression analysis. A two-way ANOVA was conducted to perform a sub-analysis examining the additive effect of folate supplementation on vascular parameters in hyperhomocysteinemic PWE treated with a single enzyme-inducing ASM (HHcy+) and hyperhomocysteinemic PWE treated with a single non-enzyme inducing ASM (HHcy-). All calculations were performed with the SPSS software, version 26 (IBM, New York, NY, USA).

4. Results

All patients concluded the three-month follow up and no dropout was recorded.

Table 1 shows the clinical features and the laboratory data at baseline and after 90-day folic acid supplementation in HHcy PWE. MMP-9 and TIMP-1 significantly reduced after folic acid supplementation ($p < 0.05$). Table 1 also summarizes the vascular findings of the study group at the beginning and the end of the study. After folic acid supplementation, homocysteine levels reduced from 26.8 ± 10.5 to 20.2 ± 5.3 μmol/L, carotid Intima-Media-Thickness reduced from 0.83+0.06 mm to 0.79 ± 0.05 mm, and FMD, distensibility coefficient (DC) and Beta stiffness improved ($p < 0.05$).

Percentage and mean dose for each ASM that patients assumed at the enrollment are shown in Table 2. A significant difference between the frequency of PWE on polypharmacy compared to PWE on monotherapy was observed.

We also analyzed results by stepwise multiple regression to determine which factor was predictive of vascular improvement. The analysis considered FMD as the dependent variable whereas glycaemia, SBP, DBP, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, Fibrinogen, C protein, S protein, Homocysteine, Folic Acid, Vitamin B, MMP-9 and TIMP were used as independent variables. The result showed a significant predictive value for the Homocysteine level (R: −0.66, β: −0.12; 95% CI, −0.17 to −0.04, $p = 0.032$).

Of the study group, 33 hyperhomocysteinemic PWE were treated with a single enzyme-inducing ASM (HHcy+) and 29 hyperhomocysteinemic PWE were treated with a single non-enzyme inducing ASM (HHcy-). Their baseline clinical features, laboratory data and vascular findings at the beginning are shown in Table 3. PWE treated with a single enzyme-inducing ASM showed worse vascular parameters than patients treated with non-enzyme inducing ASM ($p < 0.05$) at baseline. Table 4 shows response of vascular parameters to 90 days of folic acid supplementation in 33 hyperhomocysteinemic PWE treated with single enzyme-inducing ASM (HHcy+) and 29 hyperhomocysteinemic PWE treated with single non-enzyme inducing ASM (HHcy-). Two-way ANOVA for repeated measures was used to test if folate supplementation had a different effect between HHcy+ and HHcy- patients. The analysis showed a significative major improvement in vascular ultrasound parameters (carotid Intima-Media Thickness, FMD, DC and Beta Stiffness) in patients treated with a single enzyme-inducing ASM ($p < 0.05$).

Table 1
Clinical characteristics, laboratory data and vascular parameters of 174 hyperhomocysteinemic patients (HHcy) at baseline and after 90 days of folic acid supplementation.

	HHcy (n:174) T0	HHcy (n:174) T1	p
Age (years)	35.95 ± 13.21		
Sex, female (n,%)	91 (52%)		
Smokers (n,%)	80 (46%)		
SBP (mmHg)	124.45 ± 13.04	122.12 ± 0.92	ns
DBP (mmHg)	75.25 ± 13.06	71.71 ± 10.11	0.032
Total Cholesterol (mg/dl)	167.35 ± 21.02	165.5 ± 18.51	ns
HDL-C (mg/dl)	40.55 ± 8.88	43.3 ± 10.35	ns
LDL-C (mg/dl)	115.65 ± 19.15	118.4 ± 1242	ns
Triglycerides (mg/dl)	130.71 ± 68.65	131 ± 48.34	ns
Glucose (mg/dl)	97.74 ± 10.99	94.11 ± 10.12	ns
HbA1c (%)	5.23 ± 0.32	5.15 ± 0.14	ns
MMP-9 (ng/ml)	401.21 ± 208.22	355.98 ± 101.79	0.033
TIMP-1 (ng/ml)	217.44 ± 124.33	201.18 ± 105.98	0.033
Fibrinogen (mg/dl)	336.22 ± 58.75	326.4 ± 36.31	0.040
Protein C (%)	121.58 ± 13.30	124.3 ± 12.5	0.046
Protein S (%)	101.76 ± 9.46	103.9 ± 7.3	0.040
Homocysteine (μmol//L)	26.8 ± 10.5	20.2 ± 5.3	0.041
Folate (ng/ml)	4.34 ± 1.2	6.21 ± 1.84	0.036
CIMT (mm)	0.83 ± 0.06	0.79 ± 0.05	0.031
FMD (%)	11.79 ± 4.75	13.49 ± 4.72	0.032
DC (10^{-3}/kPa)	4.19 ± 2.32	4.98 ± 1.94	0.045
B	4.29 ± 2.05	3.91 ± 1.73	0.033

β: Beta stiffness; CIMT: Carotid Intima-Media Thickness; DC: Distensibility Coefficient; DBP: Diastolic Blood Pressure; FMD: Flow Mediated Dilation; HDL-C: High Density Lipoprotein Cholesterol; LDL-C: Low Density Lipoprotein Cholesterol; MMP-9: Matrix Metallopeptidase 9; SBP: Systolic Blood Pressure; TIMP-1: Metallopeptidase Inhibitor 1.
Data expressed as mean ± standard deviation or percentage.
p: Wilcoxon test. Comparison between HHcy at baseline and after 90 days Folic Acid supplementation.

5. Discussion

To the best of our knowledge, this is the first prospective study aimed to assess the global vascular burden of hyperhomocysteinemic PWE before and after folic acid supplementation. HHcy PWE showed ultrasonographic features of vascular dysfunction, as highlighted by the measurements of carotid intima-media thickness and the assessment of flow-mediated dilation and elastic properties of the brachial artery, suggesting an early vascular injury in this clinical setting. Accordingly, the metalloproteases pathways, an early marker of subclinical atherosclerosis that promotes endothelial dysfunction, inflammation, and, finally, vascular remodeling [21,22], were significantly impaired in HHcy PWE.

After three months of folic acid supplementation, an improvement in all the above-mentioned vascular parameters was obtained. These findings seem to confirm previous data published by Talari et al. who showed that 12 weeks of folate supplementation resulted in a significant reduction in serum C-reactive protein and a significant increase in plasma nitric oxide (NO) levels in children with epilepsy receiving carbamazepine [23]. Homocysteine and 5-methyltetrahydrofolate (5-MTHF) levels modulate circulating NO levels through the inhibition of endothelial NO synthase, and can induce endothelial dysfunction through complex mechanisms, including the modulation of oxidative stress, the nuclear factor-kb (NF-kb) activation, and the trigger of the inflammatory response [24]. High levels of homocysteine induce oxidative stress, and act as a molecular signal leading to activation of MMPs' pathway, that is involved in pathological vascular remodeling [25]. The depression of endothelial function induces homocysteine atherogenic effects that could be reversed by folic acid supplementation [26], which indeed has been associated with a reduction in the progression of subclinical carotid atherosclerosis [27].

Peripheral endothelial function as assessed by noninvasive function tests (i.e. FMD) is a significant predictor of future cardiovascular events and all-cause mortality across different population subgroups [28,29]. A recent metanalysis showed that the extent of intervention effects on cIMT progression predicts the degree of cardiovascular risk reduction [30]. Similarly, among metalloproteinases, increased MMP-2 activity has been correlated with coronary, aortic and carotid atherosclerosis and elevated circulating levels of MMP-9 are associated with cardiovascular disease, including acute coronary syndrome [31] and documented coronary artery atherosclerosis [32]. Although folate supplementation improves endothelial function, c-IMT progression and serum matrix metalloproteinases' levels, large randomized clinical trials have provided inconclusive results on the "hard" cardiovascular

Table 2
Percentage and mean doses of anti-seizure medications (ASM) in people with epilepsy enrolled in the study.

ASM	PWE Mono-therapy (62)	PWE Poly-pharmacy (112)	ASM	PWE Monotherapy mean dose	PWE Polypharmacy mean dose
Valproic Acid (VPA) n (%)	19 (30.65)	52 (46.43)	**Valproic Acid (VPA)** (mg)	652.38±223.87	982.35 ± 398.1
Lamotrigine (LTG) n (%)	2 (3.23)	37 (33.03)	**Lamotrigine (LTG)** (mg)	333.33±115.47	256.94 ± 128.26
Oxacarbazepine (OXC) n (%)	6 (9.68)	34 (30.35)	**Oxacarbazepine (OXC)** (mg)	1221.43±491.75	1877.78±604.84
Levetiracetam (LEV) n (%)	1 (1.61)	27 (24.11)	**Levetiracetam (LEV)** (mg)	2000±707.17	2500±772.11
Topiramate (TPM) n (%)	0 (0)	15 (13.39)	**Topiramate (TPM)** (mg)	0	268.75±109.35
Vigabatrin (VGB) n (%)	0 (0)	3 (2.68)	**Vigabatrin (VGB)** (mg)	0	1833.33± 577.35
Gabapentin (GPT) n (%)	0 (0)	1 (0.89)	**Gabapentin (GPT)** (mg)	0	1600±0
Pregabalin (PGB) n (%)	1 (1.61)	1(0.89)	**Pregabalin (PGB)** (mg)	400±0	375±0
Clobazam (CLB) n (%)	0 (0)	8 (7.14)	**Clobazam (CLB)** (mg)	0	16.25±7.44
Ethosuximide (ESM) n (%)	0 (0)	3 (2.68)	**Ethosuximide (ESM)** (mg)	0	258.33±237.61
Felbamate (FLB) n (%)	0 (0)	1(089)	**Felbamate (FLB)** (mg)	0	3600±0
Clonazepam (CLN) n (%)	0 (0)	4 (3.57)	**Clonazepam (CLN)** (mg)	0	9 ± 7.57
Carbamazepine (CBZ) n (%)	21 (33.87)	31(27.68)	**Carbamazepine (CBZ)** (mg)	675±463.44	911.76±323.81
Phenobarbital (PB) n (%)	9 (14.51)	14 (12.5)	**Phenobarbital (PB)** (mg)	91.67±94.64	114.44±59.07
Phenytoin (PHT) n (%)	3 (4.84)	6 (5.36)	**Phenytoin (PHT)** (mg)	170.12±22.1	187.5 ± 25

Table 3
Baseline characteristics and vascular parameters of 33 hyperhomocysteinemic PWE treated with single enzyme-inducing ASM (HHcy+) and 29 hyperhomocysteinemic PWE treated with single non-enzyme inducing ASM (HHcy-).

	HHcy+ n:33 T0	HHcy- n:29 T0	p
Age (years)	30.75±9.11	29.88±8.91	ns
Sex, female (n,%)	18 (54)	17 (59)	ns(**)
Smokers (n,%)	12 (36)	11 (38)	ns(**)
SBP (mmHg)	127.82±4.08	125.03±4.19	ns
DBP (mmHg)	81.46±4.17	76.19±6.73	0.038
Epilepsy duration (years)	7.11±1.95	7.36±2.02	ns
Total Cholesterol (mg/dl)	160.15±11.22	165.45±14.12	ns
HDL-C (mg/dl)	39.55±7.42	43.55±6.64	ns
LDL-C (mg/dl)	118.47±18.85	115.12±17.35	ns
Triglycerides (mg/dl)	131.85±65.63	134.51±61.85	ns
Glucose (mg/dl)	96.81±10.11	95.34±9.79	ns
HbA1c (%)	5.19±0.36	5.13±0.30	ns
MMP-9 (ng/ml)	400.31±207.87	395.01±201.52	0.031
TIMP-1 (ng/ml)	221.40±125.13	210.14±113.39	0.041
Fibrinogen (mg/dl)	333.81±48.77	316.88±38.55	0.038
Protein C (%)	120.48±13.91	124.18±11.21	0.041
Protein S (%)	102.66±9.16	104.55±8.65	0.041
CIMT(mm)	0.87±0.03	0.81±0.06	0.041
FMD (%)	10.92±3.97	12.12±3.81	0.031
DC (10^{-3}/kPa)	4.03±1.84	5.01±1.72	0.032
B	4.49±1.71	4.06±1.63	0.035

ASM: anti-seizure medication; β: Beta stiffness; CIMT: Carotid Intima-Media Thickness; DBP: Diastolic Blood Pressure; DC: Distensibility Coefficient; FMD: Flow Mediated Dilation; HDL-C: High Density Lipoprotein Cholesterol; LDL-C: Low Density Lipoprotein Cholesterol; MMP-9: Matrix Metallopeptidase 9; SBP: Systolic Blood Pressure; TIMP-1: Metallopeptidase Inhibitor 1.
Data expressed as mean ± standard deviation or percentage. p: Mann-Whitney U test.
(**): Chi Square test.

Table 4
Response of vascular parameters to 90 days of folic acid supplementation in 33 hyperhomocysteinemic PWE treated with single enzyme-inducing ASM (HHcy+) and 29 hyperhomocysteinemic PWE treated with single non-enzyme inducing ASM (HHcy-).

	HHcy+ n:33		HHcy- n:29		TWO-WAY ANOVA INTERACTION
	T0	T1	T0	T1	p
CIMT (mm)	0.87 ± 0.03	0.83 ±0.05	0.81 ±0.06	0.78 ±0.09	**0.043**
FMD (%)	10.92 ±3.97	12.23 ±2.57	12.12 ±3.81	14.22 ±2.63	**0.041**
DC (10^{-3}/kPa)	4.03 ±1.84	4.46 ±1.61	5.01 ±1.72	5.47 ±1.52	**0.033**
B	4.49 ±1.71	4.17 ±1.63	4.06 ±1.63	3.73 ±1.58	**0.048**
MMP-9 (ng/ml)	400.31 ±207.87	366.88 ±198.83	395.01 ±201.52	345.12 ±201.52	**ns**
TIMP-1 (ng/ml)	221.40 ±125.13	220.04 ±117.76	210.14 ±113.39	203.22 ±110.92	**ns**

ASM: anti-seizure medication; β: Beta stiffness; CIMT: Carotid Intima-Media Thickness; DC: Distensibility Coefficient; FMD: Flow Mediated Dilation; MMP-9: Matrix Metallopeptidase 9; TIMP-1: Metallopeptidase Inhibitor 1.

endpoints [33,34]. Despite such controversial data, the results of the present study strongly suggest the importance of measuring the homocysteine levels in PWE and assessing the related cardiovascular risk. An adequate correction of homocysteine level through folate supplementation could improve cardiovascular burden, particularly in PWE treated with single enzyme-inducing ASM. Some antiepileptic agents can cause high homocysteine levels more frequently as they induce cytochromes

[35]. In our study, a subgroup analysis was performed to assess the effects of folate replacement in hyperhomocysteinemic PWE monotherapy-treated with a single enzyme-inducing ASM (HHcy+) or with a single non-enzyme inducing ASM (HHcy-). Baseline vascular parameters resulted worse in HHcy+ than in HHcy-. A significative major improvement in vascular parameters (carotid Intima-Media Thickness, FMD, distensibility coefficient and Beta Stiffness) was observed in patients treated with a single enzyme-inducing ASM after folic acid supplementation. The data of the present study are in line with the results of previous investigation by Mintzer et al., who demonstrated a significant amelioration of cholesterol, C-reactive protein, and other markers of cardiovascular risk switching PWE from enzyme-inducing ASM to non-enzyme inducing ASM [6]. Similar results were observed when enzyme-inducing ASM was replaced by low-dose topiramate [36] or carbamazepine was switched to oxcarbazepine [37]. Non-enzyme inducing ASM treatment is associated with lower homocysteine levels and with a successful response to folic acid supplementation. Accordingly, Bhosale et al. observed that folic acid supplementation resulted more effective on homocysteine levels in PWE taking non-enzyme inducing ASM [38]. Furthermore, it is remarkable that enzyme-inducing ASM, in addition to being associated with the presence of early vascular abnormalities may contribute to the development of other pathologies, including osteoporosis and sexual dysfunction [39, 40]. Thus, enzyme induction should be considered a predominant mechanism for the increase in homocysteine in PWE patients on ASM, but not the only one, as demonstrated by data obtained in patients treated with not-enzyme inducer ASM. These findings are consistent with data reported in the literature: the meta-analysis by Ni et al. showed that plasma homocysteine levels were significantly higher in PWE treated with valproate than in controls [41]. Interference with the absorption and movement of folate in the gastrointestinal tract appears to be the leading mechanism [41,42].

For these reasons, the findings of our study also imply the relevance of considering the cardiovascular profile and the overall vascular burden of patients candidates for anti-epileptic therapy, preferring non-enzyme inducing ASM treatment in PWE with pre-existing vascular abnormalities. Besides, the approach to PWE should be multidisciplinary and integrated, and it should involve both Neurologists and Cardiologists, for tailoring therapy and during the follow-up of all the clinical features.

Our study presents several limitations. Firstly, a clear limitation is represented by the small number of patients, who were recruited from and studied in a single center. Secondly, data derived from a follow-up of a control group of untreated PWE are not available. Finally, the small sample of patients treated with a single enzyme-inducing ASM prevents drawing firm conclusions concerning the vascular effects of this subgroup of antiepileptic drugs. Thus, the results of this trial should be taken as preliminary evidence regarding the potential beneficial vascular effects of folic acid therapy in hyperhomocysteinemic PWE and stimulate robust clinical trial to test the efficacy of folic acid supplementation on hard clinical endpoints.

6. Conclusions

The results of our study strongly suggest assessing the homocysteine levels of PWE and estimating their cardiovascular risk by performing appropriate vascular tests, possibly preferring non-enzyme inducing ASM in patients at higher cardiovascular risk. An adequate correction of homocystein levels with folate supplementation should be considered to improve the cardiovascular disease burden in this setting of patients.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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CRediT authorship contribution statement

Mariarosaria De Luca: Conceptualization, Writing – original draft, Data curation, Formal analysis, Visualization. **Antonio Valvano:** Conceptualization, Writing – original draft, Data curation, Formal analysis, Visualization. **Pasquale Striano:** Data curation, Formal analysis, Visualization. **Giorgio Bosso:** Data curation, Formal analysis, Visualization. **Daniela Pirone:** Data curation, Formal analysis, Visualization. **Assunta Trinchillo:** Data curation, Formal analysis, Visualization. **Leonilda Bilo:** Data curation, Formal analysis, Visualization. **Ugo Oliviero:** Conceptualization, Writing – original draft, Data curation, Formal analysis, Visualization.

Declaration of Competing Interest

The authors declare that they have no conflict of interest/competing interests.

References

[1] World Health Organization. Epilepsy: a public health imperative. Summary. Geneva: World Health Organization; 2019. WHO/MSD/MER/19.2.
[2] Elliott JO, Jacobson MP, Haneef Z. Cardiovascular risk factors and homocysteine in epilepsy. Epilepsy Res 2007;76:113–23.
[3] Hamed SA, Hamed EA, Hamdy R, Nabeshima T. Vascular risk factors and oxidative stress as independent predictors of asymptomatic atherosclerosis in adult patients with epilepsy. Epilepsy Res 2007;74:183–92.
[4] Cleary P, Shorvon S, Tallis R. Late-onset seizures as a predictor of subsequent stroke. Lancet 2004;363:1184–6.
[5] Mintzer S, Skidmore CT, Abidin CJ, Morales MC, Chervoneva I, Capuzzi DM, Sperling MR. Effects of antiepileptic drugs on lipids, homocysteine, and C-reactive protein. Ann Neurol 2009;65(4):448–56.
[6] Ganguly P, Alam SF. Role of homocysteine in the development of cardiovascular disease. Nutr J 2015;14:6.
[7] Till U, Röhl P, Jentsch A, Till H, Müller A, Bellstedt K, Plonné D, Fink HS, Vollandt R, Sliwka U, Herrmann FH, Petermann H, Riezler R. Decrease of carotid intima-media thickness in patients at risk to cerebral ischemia after supplementation with folic acid, vitamins B6 and B12. Atherosclerosis 2005;181 (1):131–5.
[8] Woo KS, Chook P, Lolin YI, Sanderson JE, Metreweli C, Celermajer DS. Folic acid improves arterial endothelial function in adults with hyperhomocystinemia. J Am Coll Cardiol 1999;34(7):2002–6.
[9] Chambers JC, Ueland PM, Obeid OA, Wrigley J, Refsum H, Kooner JS. Improved vascular endothelial function after oral B vitamins: an effect mediated through reduced concentrations of free plasma homocysteine. Circulation 2000;102(20): 2479–83.
[10] Title LM, Ehud U, Giddens K, McQueen MJ, Nassar BA. Folic acid improves endothelial dysfunction in type 2 diabetes – an effect independent of homocysteine-lowering. Vasc Med 2006;11(2):101.
[11] Mancia G, De Backer G, Dominiczak A, Cifkova R, Fagard R, Germano G, Grassi G, Heagerty AM, Kjeldsen SE, Laurent S, Narkiewicz K, Ruilope L, Rynkiewicz A, Schmieder RE, Boudier HA, Zanchetti A. 2007 ESH-ESC practice guidelines for the management of arterial hypertension: ESH-ESC task force on the management of arterial hypertension. J Hypertens 2007;25(9):1751–62.
[12] Stein JH, Korcarz CE, Hurst RT, Lonn E, Kendall CB, Mohler ER, Najjar SS, Rembold CM, Post WS, American Society of Echocardiography Carotid Intima-Media Thickness Task Force. Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: a consensus statement from the American Society of Echocardiography Carotid Intima-Media Thickness Task Force. Endorsed by the society for vascular medicine. J Am Soc Echocardiogr 2008;21(4):376.
[13] Vlachopoulos C, Xaplanteris P, Aboyans V, Brodmann M, Cífková R, Cosentino F, De Carlo M, Gallino A, Landmesser U, Laurent S, Lekakis J, Mikhailidis DP, Naka KK, Protogerou AD, Rizzoni D, Schmidt-Trucksäss A, Van Bortel L, Weber T, Yamashina A, Zimlichman R, Boutouyrie P, Cockcroft J, O'Rourke M, Park JB, Schillaci G, Sillesen H, Townsend RR. The role of vascular biomarkers for primary and secondary prevention. A position paper from the European Society of Cardiology Working Group on peripheral circulation: endorsed by the Association for Research into Arterial Structure and Physiology (ARTERY) society. Atherosclerosis 2015;241(2):507–32.
[14] VanderLaan PA, Reardon CA, Getz GS. Site specificity of atherosclerosis: site-selective responses to atherosclerotic modulators. Arterioscler Thromb Vasc Biol 2004;24(1):12–22.
[15] Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith SC, Spertus JA, Costa F, American Heart

Association; National Heart, Lung, and Blood Institute. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. Circulation 2005;25(17):2735–52. 112.
[16] Valvano A, Bosso G, Apuzzi V, Riccone F, Saccà L, Oliviero U. Mesoglycan improves vascular reactivity and insulin sensitivity in patients with metabolic syndrome. Atherosclerosis 2015;243(2):407–13.
[17] Bosso G, Valvano A, Apuzzi V, Mercurio V, Di Simone V, Cittadini A, Napoli R, Oliviero U. Pheripheral vascular function in dilated cardiomyopathy of different etiology. Angiology 2020;71:726–33.
[18] Otto ME, Svatikova A, Barretto RB, Santos S, Hoffmann M, Khandheria B, Somers V. Early morning attenuation of endothelial function in healthy humans. Circulation 2004;109(21):2507–10.
[19] Van Bortel LM, Duprez D, Starmans-Kool MJ, Safar ME, Giannattasio C, Cockcroft J, Kaiser DR, Thuillez C. Clinical applications of arterial stiffness, task force III: recommendations for user procedures. Am J Hypertens 2002;15(5): 445–52.
[20] Messas E, Pernot M, Couade M. Arterial wall elasticity: state of the art and future prospects. Diagn Interv Imaging 2013;94(5):561–9.
[21] Jourdan C, Wühl E, Litwin M, Fahr K, Trelewicz J, Jobs K, Schenk JP, Grenda R, Mehls O, Tröger J, Schaefer F. Normative values for intima–media thickness and distensibility of large arteries in healthy adolescents. J Hypertens 2005;23(9): 1707–15.
[22] Tan J, Hua Q, Xing X, Wen J, Liu R, Yang Z. Impact of the metalloproteinase-9/ tissue inhibitor of metalloproteinase-1 system on large arterial stiffness in patients with essential hypertension. Hypertens Res 2007;30(10):959–63.
[23] Peeters SA, Engelen L, Buijs J, Chaturvedi N, Fuller JH, Jorsal A, Parving HH, Tarnow L, Theilade S, Rossing P, Schalkwijk CG, Stehouwer CDA. Circulating matrix metalloproteinases are associated with arterial stiffness in patients with type 1 diabetes: pooled analysis of three cohort studies. Cardiovasc Diabetol 2017; 16(1):139.
[24] Talari HR, Bahrami M, Ardestani AT, Bahmani F, Famili P, Asemi Z. Effects of folate supplementation on carotid intima-media thickness, biomarkers of inflammation, and oxidative stress in carbamazepine-treated epileptic children. Int J Prev Med 2019;10:4.
[25] Basu A, Jenkins AJ, Stoner JA, Thorpe SR, Klein RL, Lopes-Virella MF, Garvey WT, Lyons TJ, DCCT/EDIC Research Group. Plasma total homocysteine and carotid intima-media thickness in type 1 diabetes: a prospective study. Atherosclerosis 2014;236(1):188–95.
[26] Vacek TP, Rehman S, Neamtu D, Yu S, Givimani S, Tyagi SC. Matrix metalloproteinases in atherosclerosis: role of nitric oxide, hydrogen sulfide, homocysteine, and polymorphisms. Vasc Health Risk Manag 2015;11:173–83.
[27] Holven KB, Holm T, Aukrust P, Christensen B, Kjekshus J, Andreassen AK, Gullestad L, Hagve TA, Svilaas A, Ose L, Nenseter MS. Effect of folic acid treatment on endothelium-dependent vasodilation and nitric oxide-derived end products in hyperhomocysteinemic subjects. Am J Med 2001;110(7):536–42.
[28] Xu Y, Arora RC, Hiebert BM, Lerner B, Szwajcer A, McDonald K, Rigatto C, Komenda P, Sood MM, Tangri N. Non-invasive endothelial function testing and the risk of adverse outcomes: a systematic review and meta-analysis. Eur Heart J Cardiovasc Imaging 2014;15(7):736–46.
[29] Matsuzawa Y, Kwon TG, Lennon RJ, Lerman LO, Lerman A. Prognostic value of flow-mediated vasodilation in brachial artery and fingertip artery for cardiovascular events: a systematic review and meta-analysis. J Am Heart Assoc 2015;4(11):e002270.
[30] Willeit P, Tschiderer L, Allara E, Reuber K, Seekircher L, PROG-IMT and the Proof-ATHERO Study Groups. Carotid intima-media thickness progression as surrogate marker for cardiovascular risk: meta-analysis of 119 clinical trials involving 100 667 patients. Circulation 2020;142(7):621–42.
[31] Inokubo Y, Hanada H, Ishizaka H, Fukushi T, Kamada T, Okumura K. Plasma levels of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 are increased in the coronary circulation in patients with acute coronary syndrome. Am Heart J 2001;141(2):211–7.
[32] Blankenberg S, Rupprecht HJ, Poirier O, Bickel C, Smieja M, Hafner G, Meyer J, Cambien F, Tiret L, AtheroGene Investigators. Plasma concentrations and genetic variation of matrix metalloproteinase 9 and prognosis of patients with cardiovascular disease. Circulation 2003;107(12):1579–85.
[33] Vermeulen EG, Stehouwer CD, Twisk JW, van den Berg M, de Jong SC, Mackaay AJ, van Campen CM, Visser FC, Jakobs CA, Bulterjis EJ, Rauwerda JA. Effect of homocysteine-lowering treatment with folic acid plus vitamin B6 on progression of subclinical atherosclerosis: a randomised, placebo-controlled trial. Lancet 2000;355(9203):517–22.
[34] Saposnik G, Ray JG, Sheridan P, McQueen M, Lonn E. Heart outcomes prevention evaluation 2 investigators. Homocysteine-lowering therapy and stroke risk, severity, and disability: additional findings from the HOPE 2 trial. Stroke 2009;40 (4):1365–72.
[35] Ebbing M, Bleie Ø, Ueland PM, Nordrehaug JE, Nilsen DW, Vollset SE, Refsum H, Pedersen EK, Nygård O. Mortality and cardiovascular events in patients treated with homocysteine-lowering B vitamins after coronary angiography: a randomized controlled trial. JAMA 2008;300(7):795–804.
[36] Paknahad Z, Chitsaz A, Zadeh AH, Sheklabadi E. Effects of common anti-epileptic drugs on the serum levels of homocysteine and folic acid. Int J Prev Med 2012;3: S186–90. Suppl 1.
[37] Mintzer S, Skidmore CT, Rankin SJ, Chervoneva I, Pequinot E, Capuzzi DM, Sperling MR. Conversion from enzyme-inducing antiepileptic drugs to topiramate: effects on lipids and C-reactive protein. Epilepsy Res 2012;98(1):88–93.
[38] Isojärvi JI, Pakarinen AJ, Rautio A, Pelkonen O, Myllylä VV. Liver enzyme induction and serum lipid levels after replacement of carbamazepine with oxcarbazepine. Epilepsia 1994;35(6):1217–20.
[39] Bhosale UA, Yegnanarayan R, Agrawal A, Patil A. Efficacy study of folic acid supplementation on homocysteine levels in adolescent epileptics taking antiepileptic drugs: a single blind randomized controlled clinical trial. Ann Neurosci 2019;26(3–4):50–4.
[40] Brodie MJ, Mintzer S, Pack AM, Gidal BE, Vecht CJ, Schmidt D. Enzyme induction with antiepileptic drugs: cause for concern? Epilepsia 2013;54(1):11–27.
[41] Ni G, Qin J, Fang Z, Chen Y, Chen Z, Zhou J, et al. Increased homocysteine levels in valproate-treated patients with epilepsy: a meta-analysis. BMJ Open 2014;4(7): e004936.
[42] Sun M, Zhou R, Wang X, Cheng Y. Influence of different antiepileptic drugs on blood ammonia and homocysteine levels in children with epilepsy. Evid Based Complement Alternat Med 2021;2021:5698765.