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# High dose folic acid does not improve endothelial function in hyperhomocysteinemic subjects without additional cardiovascular risk factors

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Short running title: Homocysteine, folic acid and endothelial function

## **Abstract**

### **Aims**

Folic acid supplementation lowers total plasma homocysteine (tHcy) and improves endothelial function in individuals with coronary artery disease (CAD) and in those with additional CAD risk factors. We assessed whether endothelial function is impaired in healthy subjects with hyperhomocysteinemia but without other CAD risk factors and whether folic acid supplementation improves endothelial function in these subjects.

### **Methods**

Flow-mediated dilatation (FMD) of the brachial artery was performed on 26 healthy subjects, age  $49 \pm 2$  yrs (mean  $\pm$  SEM), with high tHcy ( $15.6 \pm 1.5$   $\mu$ mol/l) and 16 healthy age-matched subjects with low tHcy ( $7.9 \pm 0.6$   $\mu$ mol/l) ( $p < 0.001$ ). High tHcy subjects were then randomised to receive 5mg/day of folic acid or placebo for 8 weeks in a double-blind cross-over trial with 4-week washout.

### **Results**

FMD was not associated with tHcy and was not different between high and low tHcy groups ( $7.0 \pm 0.6\%$  vs  $6.6 \pm 1.2\%$ ,  $p = 0.76$ ). Treatment with folic acid decreased tHcy by 34% in hyperhomocysteinemic subjects ( $p = 0.02$  vs placebo) but had no effect on FMD ( $+0.5 \pm 0.6\%$  vs  $-0.7 \pm 0.5\%$ ,  $p = 0.17$  vs placebo).

### **Conclusion**

In healthy subjects with hyperhomocysteinemia but without additional cardiovascular risk, endothelial function is unimpaired and folic acid supplementation has no additional effect.

### **Keywords:**

Endothelial function, Folic acid supplementation, Homocysteine, Atherosclerosis, Ultrasound

## INTRODUCTION

Hyperhomocysteinemia is now considered an independent and graded risk factor for atherosclerotic vascular disease (1), and for mortality in patients with coronary artery disease (CAD) (2). Mildly elevated levels of homocysteine occur commonly in the general population as a result of either genetic influences or sub-optimal nutrition (3;4). Since supplementation with folic acid lowers total plasma homocysteine (tHcy) in both healthy individuals and those with existing CAD (5), folic acid supplementation may be a simple and effective method for both primary and secondary prevention of CAD.

Experimental evidence suggests that hyperhomocysteinemia may cause vascular damage and dysfunction (4;6) and indeed, hyperhomocysteinemia has been associated with impaired endothelial function in healthy volunteers (7;8). Supplementation with folic acid lowers homocysteine and also improves endothelial function in subjects with CAD (9-11) or those with other risk factors for CAD (12). However the effects of homocysteine lowering on endothelial function in healthy subjects with mild to moderate hyperhomocysteinemia, but without other clearly defined risk factors is still uncertain. Whilst some studies have shown an improvement (13;14), others have found no change (15). A variable effect of folic acid supplementation may be a consequence of folic acid acting independently of reductions in homocysteine (16;17). In studies in which improved endothelial function has occurred, the changes have usually not been associated with the actual decreases in tHcy (9;10;14;16;18;19). In addition, folic acid or its active form, 5-methyltetrahydrofolate, restores endothelial function in familial hypercholesterolaemic subjects without hyperhomocysteinemia (12;18). In-vitro studies suggest that these improvements in endothelial function, rather than occurring directly via a reduction in homocysteine, may occur as either a direct or indirect reduction in oxidative stress (16;18).

This study assessed whether healthy subjects with basal tHcy levels in the upper quartile of a West Australian population but without other CAD risk factors had impaired endothelial function. We also determined whether the lowering of homocysteine with high dose (5mg/day) folic acid supplementation for 8 weeks, would improve endothelial function in these subjects.

## METHODS

### *Subjects*

Subjects were original participants in a random electoral survey of 2000 people from the metropolitan area of Perth, Western Australia (20). From these invited subjects, a total of 1111 (558 men, 553 women), age  $52 \pm 13$  yrs (mean  $\pm$  SD; range 27 to 77 yrs), were assessed between June 1995 and December 1996 for tHcy and standard cardiovascular risk factors (3), and then divided into sex-specific quartiles according to their tHcy. We randomly selected 26 healthy male and female subjects from those in the upper tHcy quartile (men  $> 14.4$ ; women  $> 12.8$   $\mu\text{mol/l}$ ) who were also aged between 35-60 years, who had never smoked, had a total cholesterol  $< 6.0$  mmol/l, were non-diabetic, normotensive (systolic blood pressure  $< 140$  mmHg), were not receiving medication for cholesterol, blood pressure, or asthma and who did not take vitamin supplements. Sixteen healthy age-matched subjects in the bottom tHcy quartile (men  $\leq 10.5$ ; women  $\leq 8.4$   $\mu\text{mol/l}$ ) were recruited as a control population and were also used to assess the reproducibility of the brachial artery ultrasound technique. However they were not used in the folic acid intervention phase of the study. The study was approved by the Institutional Ethics Committee of the University of Western Australia and conforms with the principles outlined in the Declaration of Helsinki (21).

### *Study design*

The intervention study was performed in the hyperhomocysteinemic subjects ( $n=26$ ) in a double-blinded cross-over fashion with subjects randomly allocated to either placebo or folic acid (5mg/day) for 8 weeks followed by a 4-week washout, then crossed over to either folic acid or placebo for the final 8 weeks. Subjects were tested at the beginning and end of each 8-week intervention period. On each occasion subjects attended after a 12-hour fast for blood tests, and a brachial artery ultrasound study to determine endothelium-dependent and endothelium-independent function. Subjects were instructed to avoid changing their normal diet for the duration of the study and on the morning of their visits to only drink water. The control participants with low tHcy ( $n=16$ ) had the same blood tests, and brachial ultrasound studies were performed on 2 visits one week apart without any treatment or change of diet between visits in order to assess the reproducibility of the technique.

### *Laboratory measurements*

Fasting venous blood samples were obtained at each visit for subjects in both the high and low homocysteine groups. Samples were collected into vacutainers containing EDTA or heparin for the measurement of red cell folate (RCF), vitamins B<sub>6</sub> and B<sub>12</sub> and total plasma homocysteine (tHcy). The samples were centrifuged shortly after venipuncture. For tHcy assays, the plasma was separated, transported on ice, and stored at  $-70^{\circ}$  until assay. tHcy was determined by reverse-phase high performance liquid chromatography after treatment with tributylphosphine, deproteinization, and fluorogenic derivatization by the method of Araki and Sako (22). The inter-assay coefficient of variation (CV) is 6% in our laboratory (23). RCF was measured by a procedure based upon ion capture technology (AxSYM Folate, Abbott Laboratories, Illinois). The inter-assay CV is 4.1 % and the mean (normal range) 331.4 ng/mL (180.4-617.5 ng/mL). Vitamin B<sub>6</sub> (pyridoxal -5'-phosphate) was measured by microbial assay (24). The inter-assay CV is 7.1 % and the normal range for healthy middle-aged subjects 21.6-66.0 nmol/L. Vitamin B<sub>12</sub> was determined from serum obtained at baseline using a micro particle enzyme immunoassay (AxSYM B12, Abbott Laboratories, Illinois). The inter-assay CV is 6.8 % and the mean (normal ranges) for healthy subjects 474 pg/mL (100 - 2437 pg/mL). At baseline, total cholesterol, HDL cholesterol and triglyceride levels were determined enzymatically with a Hitachi 747 autoanalyser. LDL cholesterol was calculated using the method by Friedwald et al. (25).

### *Brachial artery ultrasound*

Flow-mediated dilatation (FMD) and glyceryl trinitrate-mediated dilatation (GTNMD) were used to assess endothelium-dependent and endothelium-independent function respectively. The technical aspects of the ultrasound procedure used for the assessment of FMD and GTNMD have been described previously by Celermajer et al (26). Briefly, 2-dimensional B-mode ultrasound images of the lumen/arterial wall interface of the left-hand brachial artery were obtained in the distal third of the upper arm using a high resolution 12 MHz linear array transducer (Sequoia, Accuson instruments, Colorado, USA). Baseline scans assessing vessel diameter were recorded over one minute. Arterial flow velocity was measured using a pulsed Doppler signal at  $70^{\circ}$  to the vessel with the range gate (1.5mm) in the centre of the artery. A rapid

inflation/deflation pneumatic cuff, previously placed around the forearm immediately distal to the humeral epicondyle, was inflated to 250 mm Hg for 4.5 min to induce FMD. Recordings commenced 30 sec prior to cuff deflation and continued for 90 seconds after cuff deflation. After 10 mins, a second baseline scan was recorded. This was followed by sublingual administration of glyceryl trinitrate (400  $\mu$ g) and recording of images for a further 5 minutes. All images were recorded on super-VHS videotape (Sony MQSE 180), for retrospective analysis. Baseline volume flow was calculated by multiplying the mean velocity time integral of the Doppler flow signal for 2 consecutive pulse waves, by the heart rate and vessel cross-sectional area. Peak hyperaemic response was calculated using 2 consecutive pulse waves within the first 15 seconds following cuff deflation, and dividing by baseline flow. FMD and GTNMD measurements were performed using traditional manual analysis techniques with ultrasonic callipers (26). The mean of 2 observers measurements were used for all scans. Observers were blinded to patient group and treatment order.

#### *Reproducibility of brachial artery ultrasound*

Ultrasound scans were repeated one week later on subjects with low tHcy (n=16) under the same conditions as their first scan. Values for each visit were calculated as the mean measurement of 2 observers and within-subject variability of FMD and GTNMD was calculated as the between-visits CV.

#### *Statistical analysis*

Data were analysed using SPSS (SPSS Inc, Chicago). Differences in baseline characteristics between subjects with high and low tHcy, and between the 2 groups of subjects with high tHcy were compared with unpaired t-tests. Effects of treatment and placebo were assessed using a paired t-test on the changes during each period. Treatment period interactions were assessed for RCF, tHcy, FMD and GTNMD by comparing changes in the 2 groups during the two treatment periods with an unpaired t-test. Linear regression analysis was conducted to examine the relationships between RCF, tHcy, and FMD. Differences were considered significant at a p value <0.05. All variables were tested for normality using histograms and Kolmogorov-Smirnov statistics. Values are reported as mean  $\pm$  SE except for changes due to treatment, which are reported as mean (95% confidence interval). FMD and GTNMD responses

were calculated as the percent increases from baseline diameter. CV's for FMD and GTNMD were calculated as the pooled SD for replicates  $\div$  mean. The statistical power for a cross-over study using the ultrasound technique was determined using an MS-DOS based statistical power calculation program (POWER) (27). Power was calculated for detecting an absolute 2% change in FMD.

## RESULTS

### *Within-subject reproducibility of brachial artery ultrasound*

In the control population with low tHcy (n=16), the mean ( $\pm$  SD) %FMD for visits 1 and 2 were  $6.6 \pm 4.8$  % and  $6.4 \pm 3.0$  %, respectively. The combined mean was  $6.5 \pm 3.8$  % and the mean absolute difference between visits was  $1.6 \pm 1.9$  %. The between visit CV for %FMD was 24.6 % and the Pearson correlation coefficient  $r=0.91$ . The power to detect an absolute 2% change in FMD in the cross-over study was 83% at an alpha of 0.05.

The mean ( $\pm$  SD) %GTN for visits 1 and 2 were  $18.4 \pm 6.6$  % and  $19.4 \pm 8.5$  %, respectively. The combined mean was  $19.6 \pm 8.0$  % and the mean absolute difference between visits was  $1.5 \pm 1.0$  %. The between visit CV for %GTN was 7.7% and the Pearson correlation coefficient  $r=0.96$ . The power to detect an absolute 2% change in GTN in the cross-over study was 99.4% at an alpha of 0.05.

### *Subject characteristics*

Table 1 shows the characteristics of all subjects at baseline. Subjects with high tHcy had higher levels of tHcy ( $15.6 \pm 1.5$   $\mu\text{mol/l}$  vs  $7.9 \pm 0.6$   $\mu\text{mol/l}$ ,  $p<0.001$ ), and plasma creatinine ( $91 \pm 3$  vs  $74 \pm 3$ ,  $p<0.001$ ), but lower levels of RCF ( $332 \pm 21$  ng/ml vs  $425 \pm 41$  ng/ml,  $p=0.03$ ) than those with low tHcy. However, there were no significant differences in other baseline clinical variables. At baseline, tHcy was inversely associated with RCF amongst all subjects ( $r=-0.4$ ,  $p<0.01$ ) and also amongst the subjects with high tHcy alone ( $r=-0.38$ ,  $p=0.05$ ). However, there were no associations between FMD and tHcy for all subjects ( $r=0.23$ ,  $p=0.14$ ) or for those with high tHcy alone ( $r=0.16$ ,  $p=0.45$ ). FMD, GTNMD, and hyperaemic responses were no different between the high and low tHcy subjects (Table 1).



Amongst subjects with high tHcy, there were no significant differences between the initial folic acid and placebo groups in their baseline characteristics including tHcy, RCF, FMD, GTNMD, and reactive hyperaemia (data not shown).

#### *Effect of treatment*

Table 2 shows the effect of treatment in each group between visits 1 and 2, and between visits 3 and 4. When the results of both groups were combined (n=26), treatment with folic acid increased RCF by an average of 89% ( $+300 \pm 26$  ng/mL) compared to a decrease of 8% ( $-33 \pm 26$  ng/mL) during treatment with placebo (treatment minus placebo effect = 333 ng/mL (254, 411),  $p < 0.001$ ). Similarly, tHcy decreased by an average of 34% ( $-5.0 \pm 1.6$   $\mu$ mol/l) with folic acid compared with a mean increase of 2% ( $+0.2 \pm 0.6$   $\mu$ mol/l) with placebo (treatment minus placebo effect =  $-5.2$   $\mu$ mol/l (-9.5, -1.0),  $p = 0.02$ ). However, folic acid had no effect on either FMD (treatment minus placebo effect = 1.2% (-0.5, 2.8),  $p = 0.17$ ) or GTNMD (treatment minus placebo effect = -0.4% (-3.4, 2.6),  $p = 0.77$ ) compared to placebo (Figure 1) and changes in tHcy during treatment were not significantly associated with changes in FMD ( $r = 0.02$ ,  $p = 0.94$ ). Following the 4-week washout period, RCF was significantly higher ( $499 \pm 23$  ng/ml vs  $352 \pm 34$  ng/ml,  $p = 0.002$ ), and tHcy significantly lower ( $10.3 \pm 0.5$   $\mu$ mol/l vs  $13.6 \pm 0.8$   $\mu$ mol/l,  $p = 0.001$ ) in the subjects who received treatment first compared to those who received placebo first (Table 2). However, no significant treatment-period interactions between the 2 groups occurred in any of the variables (Table 2).

#### **Discussion**

This study has demonstrated that endothelial function is not significantly impaired in healthy subjects with mild to moderate basal hyperhomocysteinemia but without other risk factors for CAD. In addition, 8-weeks of high-dose folic acid supplementation did not have any additional effect on endothelial function in this population despite substantial reductions in tHcy, increases in RCF, and sufficient statistical power to detect a small (2%) absolute change in FMD.

Other studies have not clearly established whether basal elevation of tHcy is associated with impaired FMD in otherwise healthy subjects. Pullin et al (15) found no difference in FMD in healthy individuals with elevated tHcy who were

homozygous (TT) for the C677T mutation in the methylenetetrahydrofolate reductase gene compared to wild-type (CC) individuals with normal tHcy (15). By comparison, Woo et al (7) and Tawakol et al (8) reported that hyperhomocysteinemia was associated with impaired FMD in “healthy” subjects. Differences in clinical risk characteristics of the subjects such as smoking history (7), older-age (8) and higher tHcy levels (7) may explain the discordance in these data with our own findings and those of Pullin et al (15).

The absence of endothelial dysfunction in our subjects despite hyperhomocysteinemia is also plausible in light of numerous reports showing the lack of an association between improvements in endothelial function and reductions in tHcy subsequent to folic acid supplementation (9;10;14;16;18;19). Similarly, improvements in FMD were not associated with reductions in either tHcy or free Hcy following either an acute dose (2 hours or 4 hours) or long-term administration (6 weeks) of 5mg/day of folic acid in CAD patients (9). However, free plasma Hcy has been shown to be a significant independent predictor of FMD in another group of CAD patients (10),

Our finding of no effect on FMD with high-dose folic acid supplementation in a healthy population is supported by Pullin et al. (15) using lower doses of folic acid (400µg/day) in healthy subjects with hyperhomocysteinemia in the presence of genetic mutations of methylenetetrahydrofolate reductase. Thus, no improvement in FMD occurred despite increases in plasma folate and reductions in tHcy. Whilst their subjects only received 400µg of folic acid/day compared to our higher dose of 5mg/day, this should have been sufficient to reduce tHcy levels maximally in most subjects (5).

The disparate findings concerning the effects of folic acid on endothelial function in subjects with hyperhomocysteinemia may be a result of the presence or absence of CAD or other cardiovascular risk factors. Atherosclerotic vascular disease or added risk may be necessary before endothelial function is impaired as a consequence of hyperhomocysteinemia (28). For example, the combination of smoking and hyperhomocysteinemia appears to greatly increase cardiovascular risk (29), possibly via a synergistic effect on antioxidant defences (30). Accordingly, folic acid has been demonstrated to cause improvement of endothelial function in hyperhomocysteinemic patients with CAD (10;11).

Whilst small increases in FMD have been observed in studies of healthy subjects with hyperhomocysteinemia following high-dose folic acid supplementation, uncertainty exists regarding the subjects' risk-profile (13,14). Thus, in the study by Bellamy et al (13) using blood donors, the age of the "healthy" volunteers were not specified and they also allowed higher inclusion levels of blood pressure and serum cholesterol than in our study. In the other study by Woo et al (14) using Chinese subjects, their smoking status was not stated and serum cholesterol levels were not measured. In addition, neither of these two studies included control subjects without hyperhomocysteinemia to determine if endothelial function was impaired at baseline.

Possibility exists for the acute effects of folic acid, rather than its homocysteine-lowering effects to be responsible for improvements in endothelial function. FMD was increased only 2 hours after consuming 5mg of folic acid, despite no change in tHcy (9). Likewise, folic acid or its active form, 5-methyltetrahydrofolate, restores endothelial function in familial hypercholesterolaemic subjects without hyperhomocysteinemia (12;18). These effects may be a consequence of folic acid reducing LDL-induced eNOS increases in the generation of superoxide production (18) and/or enhancing NO synthesis by increasing the effectiveness of tetrahydrobiopterin (a co-factor for NO synthesis) on NO synthase uncoupling (17). The acute effects of folic acid, may thus explain the improvements in endothelial function observed in healthy subjects with hyperhomocysteinemia who were instructed to take their folic acid supplement before attending for tests of vascular function (14). In our own study, subjects were requested to drink water only on the morning of their visits, thereby ensuring that chronic rather than acute effects of folic acid supplementation were assessed.

The absence of any effects on endothelial function in this study are unlikely to have been due to the slight carry-over effects following folic acid washout since there was no significant treatment-period interaction. Although the washout period was not sufficient to normalise tHcy, it was sufficient for the studies of vascular function. In our study we assessed RCF, an indicator of long-term folate status, as opposed to plasma folate, which varies rapidly after folic acid intake. Whilst there was an association between RCF and tHcy at baseline, there were no associations between either RCF or tHcy and endothelial function. This supports our main findings that endothelial function is not necessarily impaired in healthy subjects with basal

hyperhomocysteinemia, and that in this case, reducing homocysteine does not have any additional effect on endothelial function.

In conclusion, our results suggest that mild to moderate hyperhomocysteinemia alone does not cause endothelial dysfunction in healthy subjects in the absence of other cardiovascular risk factors. Folic acid supplementation may therefore not be beneficial in reducing cardiovascular risk in this section of the general population.

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**TABLE 1:** Baseline characteristics of subjects

	<b>High homocysteine (n=26)</b>	<b>Low homocysteine (n=16)</b>	<b>P-value between groups<sup>1</sup></b>
Age (years)	49 ± 2	50 ± 2	NS
Male/Female	18/8	11/5	NS
Homocysteine (µmol/l)	15.6 ± 1.5	7.9 ± 0.6	< 0.001
BMI (kg/m <sup>2</sup> )	28.1 ± 1.0	25.2 ± 2.1	NS
Waist/hip ratio	0.90 ± 0.06	0.87 ± 0.02	NS
SBP (mm/Hg)	125 ± 2	129 ± 4	NS
DBP (mm/Hg)	81 ± 2	87 ± 2	NS
Total cholesterol (mmol/l)	5.3 ± 0.1	5.4 ± 0.2	NS
LDL cholesterol (mmol/l)	3.2 ± 0.2	3.4 ± 0.2	NS
HDL cholesterol (mmol/l)	1.39 ± 0.07	1.36 ± 0.06	NS
Triglycerides (mmol/l)	1.27 ± 0.17	1.29 ± 0.16	NS
Creatinine (µmol/l)	91 ± 3	74 ± 3	< 0.001
Red cell folate (ng/mL)	332 ± 21	425 ± 41	0.03
Vitamin B6 (nmol/L)	39 ± 5	44 ± 5	NS
Vitamin B12 (pg/mL)	360 ± 21	386 ± 31	NS
Flow-mediated dilatation (%)	7.0 ± 0.6	6.6 ± 1.2	NS
GTN-mediated dilatation (%)	20.4 ± 0.9	18.4 ± 1.7	NS
Basal blood flow (ml <sup>3</sup> /min)	29.3 ± 5.0	23.1 ± 3.1	NS
Reactive hyperaemia (%)	786 ± 118	650 ± 104	NS

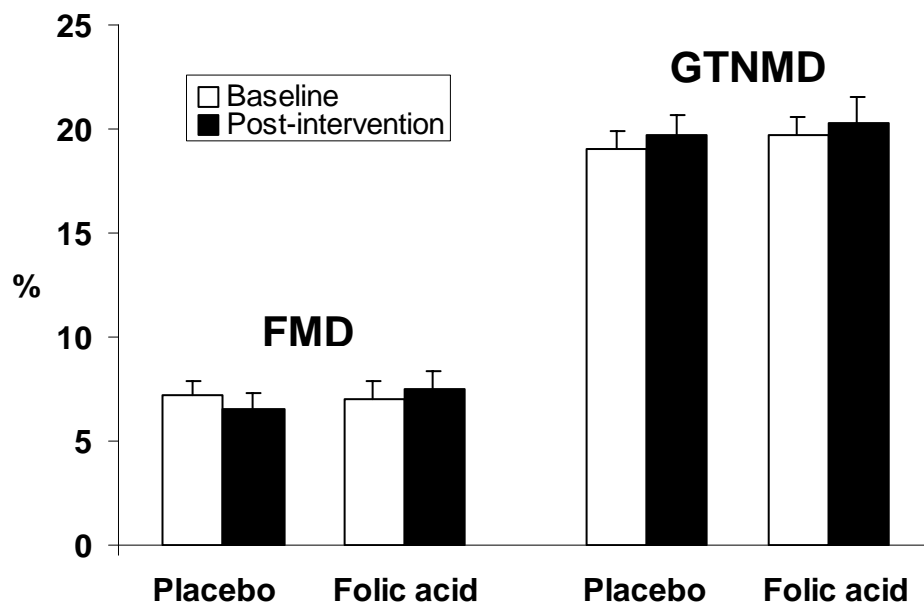
Values are Mean ± SEM. <sup>1</sup>Unpaired t-test

**TABLE 2**  
**Effects of treatment period on HCY, RCF, %FMD and %GTN**

		1 <sup>st</sup> Treatment period	Visit 1	Visit 2	$\Delta 1$ (Visit 2-1)	2 <sup>nd</sup> Treatment period	Visit 3	Visit 4	$\Delta 2$ (Visit 4-3)	Treatment- period interaction <sup>1</sup>
<b>HCY</b>	Group 1	<b>Placebo</b>	14.9 ± 1.0	13.2 ± 0.9	-1.7 ± 0.50	<b>Folic acid</b>	13.6 ± 0.8	10.0 ± 0.7	-3.5 ± 0.6	NS
	Group 2	<b>Folic acid</b>	16.4 ± 3.0	9.8 ± 0.7	-6.6 ± 3.1	<b>Placebo</b>	10.3 ± 0.5	12.4 ± 1.0	2.1 ± 0.9	
<b>RCF</b>	Group 1	<b>Placebo</b>	338 ± 23	320 ± 29	-18 ± 19	<b>Folic acid</b>	352 ± 34	683 ± 52	331 ± 39	NS
	Group 2	<b>Folic acid</b>	326 ± 35	595 ± 37	269 ± 40	<b>Placebo</b>	499 ± 23	451 ± 48	-47 ± 48	
<b>%FMD</b>	Group 1	<b>Placebo</b>	6.5 ± 0.7	5.3 ± 0.7	-1.2 ± 0.6	<b>Folic acid</b>	6.5 ± 0.7	6.3 ± 1.0	-0.2 ± 0.6	NS
	Group 2	<b>Folic acid</b>	7.5 ± 1.1	8.7 ± 1.3	1.2 ± 1.1	<b>Placebo</b>	8.0 ± 1.1	7.9 ± 1.2	-0.1 ± 0.8	
<b>%GTN</b>	Group 1	<b>Placebo</b>	19.3 ± 1.2	19.0 ± 1.1	-0.3 ± 1.4	<b>Folic acid</b>	17.5 ± 1.0	19.7 ± 1.2	2.2 ± 1.3	NS
	Group 2	<b>Folic acid</b>	21.6 ± 1.4	20.9 ± 2.0	-0.7 ± 1.7	<b>Placebo</b>	18.8 ± 1.4	20.5 ± 1.5	1.7 ± 1.5	

<sup>1</sup> Unpaired t-test,  $\Delta 1$  vs  $\Delta 2$  for placebo and  $\Delta 1$  vs  $\Delta 2$  for folic acid.

FIGURE 1



**Figure Legend:** Mean ( $\pm$  SEM) flow-mediated dilatation (FMD) and glyceryl-trinitrate mediated dilatation (GTNMD) at the end of the placebo and folic acid phases of supplementation.