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Plasma homocysteine, dietary B vitamins, betaine, and choline and risk of peripheral artery disease

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Abstract

Objective—Few studies have examined the roles of homocysteine and related nutrients in the development of peripheral artery disease (PAD). We examined the associations between plasma homocysteine, dietary B vitamins, betaine, choline, and supplemental folic acid use and incidence of PAD.

Methods—We used two cohort studies of 72,348 women in the Nurses' Health Study (NHS, 1990-2010) and 44,504 men in the Health Professionals Follow-up Study (HPFS, 1986-2010). We measured plasma homocysteine in nested matched case-control studies of clinically recognized PAD within both cohorts, including 143 PAD cases and 424 controls within the NHS (1990-2010) and 143 PAD cases and 428 controls within the HPFS (1994-2008). We examined the association between diet and risk of incident PAD in the cohorts using a food frequency questionnaire and 790 cases of PAD over 3.1 million person-years of follow-up.

Results—Higher homocysteine levels were positively associated with risk of PAD in men (adjusted IRR 2.17; 95% CI, 1.08-4.38 for tertile 3 vs. 1). There was no evidence of an association in women (adjusted IRR 1.14; 95% CI, 0.61-2.12). Similarly, higher folate intake, including supplements, was inversely associated with risk of PAD in men (adjusted HR 0.90; 95% CI, 0.82-0.98 for each 250 μg increase) but not women (HR 1.01, 95% CI, 0.88-1.15). Intakes of the

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other B vitamins, betaine, and choline were not consistently associated with risk of PAD in men or women.

Conclusion—Homocysteine levels were positively associated and dietary folate intake was inversely associated with risk of PAD in men but not in women.

Keywords

peripheral artery disease; homocysteine; folate; vitamin B12; vitamin B6; riboflavin; betaine; choline

Introduction

Elevated levels of the amino acid homocysteine are positively associated with endothelial dysfunction, oxidation of low-density lipoprotein, and monocyte adhesion. [1] Despite the widely-speculated influence of endothelial dysfunction and oxidative stress in peripheral artery disease (PAD), [2] the relationships between homocysteine, B vitamins and PAD have not been well studied. Furthermore, despite the well-known metabolic pathways that govern homocysteine, no prior studies have examined PAD risk in relation to the combination of plasma homocysteine and its dietary determinants using validated measurements, including dietary intakes of B vitamins, related betaine and choline, and supplements.

B vitamins lower homocysteine levels by promoting homocysteine metabolism. Homocysteine can be removed from circulation by catabolism to cysteine through a pyridoxal phosphate (vitamin B_6) dependent pathway or remethylation to methionine. Betaine or folate (vitamin B_9) can donate the methyl group, the latter of which requires cobalamin (vitamin B_{12}) and riboflavin (vitamin B_2). [3] Choline plays a peripheral role as betaine can be endogenously synthesized from choline.

The observed association between homocysteine levels and risk of CVD [4] led to a series of randomized controlled trials of B vitamin supplementation. Although these clinical trials consistently lowered homocysteine levels using supplemental folate, vitamin B_6, and vitamin B_{12}, meta-analyses show no impact on risk of cardiovascular events including myocardial infarction (MI) and, and death; [5] however, findings are mixed for stroke.[5,6] Furthermore these studies found no change in underlying atherosclerosis. [7] In contrast, prospective studies and clinical trials of homocysteine and PAD have so far presented inconclusive findings.

One previous prospective cohort study reported a positive association between homocysteine and PAD [8] but two reported no association. [9,10] Of three clinical trials, two found no effect of B vitamin supplementation on atherosclerotic progression, [11] arterial stiffening, [11] ankle-brachial index (ABI), [12] or carotid and femoral ultrasonography [12] however a third found small improvements in pulse wave velocity and ABI. [13] To address this question more fully, we examined the associations between plasma homocysteine, dietary B vitamins, betaine, choline, and supplemental folic acid use and risk of PAD in two prospective cohort studies including sizable numbers of both men and women. We

hypothesized that homocysteine levels would be positively associated and B vitamins, betaine, and choline inversely associated with risk of PAD.

Materials and Methods

Study Population

Cohort studies—The Nurses' Health Study (NHS) is a prospective cohort of 121,700 female nurses. [14] All women were age 30 to 55 years at baseline (1976) and continue to be followed. PAD cases in the NHS were confirmed beginning in 1990 until 2010; therefore, our analyses are restricted to 1990-2010. Women were excluded from our analyses if they had confirmed CVD (myocardial infarction, stroke, PAD, or revascularization of the coronary, carotid, or peripheral beds) at baseline. We additionally excluded women who reported implausible dietary energy intake (<600 or >3500 kcal/day) at baseline or during follow-up.

The Health Professionals Follow-up Study (HPFS) is a parallel prospective cohort of 51,529 male health professionals age 40-75 years at baseline (1986). [15] PAD cases were confirmed in the HPFS through 2010; our analyses include follow-up time between 1986 and 2010. We used the same exclusion criteria for men, with the exception of a higher cutoff for implausible energy intake, <800 or >4200 kcal/day.

Of the 121,700 women participating in the NHS, 42,816 were missing dietary data at in 1990 (after 14 years of follow-up), 594 cases of clinically significant PAD were reported before 1990, 1,652 MI, 454 revascularization, 3,038 angina, and 485 stroke. After additionally excluding women with missing covariate data on age (n=23), smoking (n=207), and BMI (n=83), 72,348 women remained in our analyses. Of the 51,529 men participating in the HPFS, 1,595 were missing dietary data at baseline, 5 died before all baseline data was collected, 2,219 reported a history of MI before baseline, 967 revascularization, 732 angina, and 254 stroke. After additionally excluding men with missing data on age (n=36), BMI (n=1,027) and physical activity (n=190), 44,504 men remained in our analyses.

Nested case-control studies—In 1990 in NHS and 1994 in HPFS, surviving participants received blood collection kits. Participants collected fasting blood samples (heparin in women and EDTA in men) and shipped them on ice overnight to a central laboratory. Upon arrival, bloods were centrifuged under refrigeration and the blood components were aliquotted and stored in liquid nitrogen at -130 to -196°C. Among the subcohorts who provided blood specimens and were free of CVD at the time of blood collection, homocysteine was measured in nested 1:3 matched case-control studies within both cohorts, including 143 PAD cases and 424 controls within the NHS (1990-2010) and 143 PAD cases and 428 controls within the HPFS (1994-2008). Cases and controls were matched using risk set sampling on age, smoking, race, month of blood draw, and fasting status. Men and women who provided blood samples were younger on average, but otherwise similar to those who did not provide blood samples. [16,17]

Exposures

Plasma homocysteine—Plasma homocysteine was measured in all case-control samples (men and women) by the same laboratory. The lab used an enzymatic assay to measure homocysteine on the Roche P Modular system (Roche Diagnostics - Indianapolis, IN), with reagents and calibrators from Catch Inc. (Seattle, WA). In this assay, reduced homocysteine with serine was catalyzed by cystathionine b-synthase (CBS) to form L-cystathionine, which in turn was broken down by cystathionine b-lyase (CBL) to form homocysteine, pyruvate and ammonia. The pyruvate was then reduced by lactate dehydrogenase, with NADH forming NAD. The concentration of homocysteine in the sample was directly proportional to the amount of NADH converted to NAD. The change in absorbance was measured spectrophotometrically at 340 nm. Coefficients of variation for split homocysteine samples were 3.8% for women and 7.1% for men.

Dietary intakes of B vitamins, betaine, and choline—Food frequency questionnaires (FFQs) collected every four years from 1990 to 2006 were used to measure the intake of four B vitamins (folate, vitamin B_6, vitamin B_{12}, and riboflavin) and related compounds betaine, and choline in the NHS. The same FFQ was collected every four years from 1986 to 2006 in the HPFS. The semiquantitative FFQ [18] asked participants to report servings of specified portions of foods over the previous year in 9 categories ranging from “never or <1/mo” to “≥6/d.” The Harvard University food composition database, derived from the US Department of Agriculture data and other outside published sources, was used to calculate the amount of nutrients consumed from food items. The FFQ additionally asked about B vitamin supplement use, including folic acid, B_6, and B_{12}. Energy-adjusted Pearson correlations between the FFQ and multiple 1-week diet records were 0.71 for folate, 0.82 for vitamin B_6, 0.50 and for vitamin B_{12}.[18] This FFQ predicted plasma levels of folate, vitamin B_6, vitamin B_{12}, and homocysteine in previous analyses. [19-21]

Ascertainment of PAD

Participants reported PAD on questionnaires biennially. Permission to review medical records was requested for participants reporting PAD and trained adjudicators blinded to risk factor status confirmed self-reported PAD diagnoses and dates. Clinically recognized PAD required at least one of the following: (1) confirmed report of amputation, bypass, or other revascularization procedure (ex: angioplasty) for occlusive arterial disease, (2) angiogram or Doppler ultrasound report confirming at least 50% stenosis of at least one artery with congruent symptoms in the ipsilateral limb, (3) ABI < 0.9, or (4) documented physician's diagnosis.

Assessment of Covariates

Men and women in both cohorts completed biennial mailed questionnaires that asked about medical history and lifestyle habits, including medication use, smoking, weight, parental history of MI, physical activity, alcohol, diet, and postmenopausal hormone use. Weekly energy expenditure was calculated based on answers to questions about the average amount of time a participant spent per week on various activities like walking, jogging, running, bicycling, and tennis. Body mass index (BMI) was calculated by dividing weight in kg by

squared height in meters. These self-reported physical activity and BMI measures are highly valid. [22-24]

A laboratory certified by the National Heart, Lung and Blood Institute/Centers for Disease Control and Prevention Lipid standardization Program analyzed all other biochemical markers by means of commercially available analytic systems. High-density lipoprotein cholesterol (HDL-C) and triglycerides were measured enzymatically and low-density lipoprotein cholesterol (LDL-C) by a homogenous direct method from Roche Diagnostics (Indianapolis, IN). An immunoturbidimetric assay on the Roche P Modular system from Roche Diagnostics (Indianapolis, IN) quantified the concentration of high-sensitivity C-reactive protein (hsCRP), using reagents and calibrators from DiaSorin (Stillwater, MN). The Roche P Modular system uses turbidimetric immunoinhibition and a hemolyzed whole blood or packed red cells to determine hemoglobin A_{1c} (HbA_{1c}) (Roche Diagnostics, Indianapolis, IN).

Statistical Analyses

Nested case-control study analyses—To account for clustering by matching, we compared baseline characteristics between cases and controls using generalized linear mixed models for continuous variables and Cochran-Mantel-Haenszel tests for categorical variables. We used logistic regression, conditioning on matching factors, to estimate odds ratios for PAD according to tertiles of homocysteine as well as log-transformed homocysteine, based on model fit of serial models with and without quadratic terms, in units of one standard deviation (0.25 μmol/L among men and women). Risk set sampling was used to match controls to cases; therefore, these odds ratios are unbiased estimates of the incidence rate ratio (IRR). [25]

Covariates were included in multivariable models as linear variables or as categorical variables if discrete or their association with PAD was non-linear. We included the following risk factors for PAD in our multivariable models: matching factors [age, race (women only), month of blood draw, fasting status, and smoking], triglycerides, HDL-C, LDL-C, hsCRP, HbA1c, cystatin C, pack-years of smoking, hypertension, diabetes, family history of myocardial infarction, BMI, alcohol, and postmenopausal hormone use (women only). We additionally present models further adjusted for dietary intakes of total fiber and B vitamins.

We included an interaction term in our final model to test for potential effect modification by the following factors: fasting status, time (before and after 1998 when folic acid fortification of grains became mandatory in the US), age, alcohol, dietary intakes of folate, vitamin B_6, and vitamin B_{12}, cystatin C, diabetes, smoking, BMI, and postmenopausal hormone use. Finally, we included homocysteine and dietary B vitamins in a model together to examine whether their effects were independent.

Cohort study analyses—We used Cox proportional hazards models to estimate hazard ratios for PAD according to dietary intakes of B vitamins, categorized into quintiles and as continuous variables. Person-time (in months) was calculated from the return of the 1990 questionnaire in women (cases prior to 1990 were not confirmed) or the 1986 (baseline)

questionnaire in men to PAD, death, or the end of follow-up (2010). If dietary data were missing from one FFQ, we used data from the previous FFQ. We adjusted B vitamins, choline, and betaine intake for total energy using the residual method. [26]

We categorized exposure to B vitamins using the cumulative average[26] to best characterize long-term exposure, weighting the average of all previous reported intakes and current reported intake equally. We present results using total folate (supplemental and dietary combined) but tested dietary folate separately in a sensitivity analysis. We stopped updating diet if a participant developed an intermediate endpoint (cardiovascular disease, high cholesterol, high blood pressure, diabetes, or cancer) because of dietary changes in response to these diagnoses. Due to collinearity, we only present results for folate, vitamin B_6, and vitamin B_{12} modeled separately but tested them together in secondary analyses.

We updated covariate data every two years in our models. Participants with missing exposure or covariate data at baseline were excluded from our analyses. We checked the proportional hazards assumption and examined potential effect modification for the same set of variables mentioned above for the case-control analyses with the exception of fasting status and cystatin C.

We checked for heterogeneity between men and women using the Q statistic and continuous versions of homocysteine (per SD of log-transformed homocysteine) and folate (per SD). All tests were two-sided and used $\alpha = 0.05$ and all analyses used SAS statistical software version 9.2 (Cary, North Carolina). The study protocol was approved by the Institutional Review Board of the Brigham and Women's Hospital and by the Harvard School of Public Health Human Subjects Committee Review Board and all participants provided voluntary responses to mailed questionnaires which served as the participants' informed consent and research aims and use of data were fully explained to each participant.

Results

Plasma homocysteine (nested case-control studies)—PAD cases had higher levels of traditional CVD risk factors compared to controls including triglycerides, HDL-C, LDL-C, CRP, HbA_{1c}, cystatin C, history of hypertension, diabetes, and high cholesterol, and family history of MI (Table 1). Although cases were matched to controls on smoking status (never, past, current), cases had higher pack-years of smoking compared to controls, and thus we adjusted for pack-years of smoking in all analyses. As expected, plasma homocysteine levels were slightly higher in men than in women (Table 1).

In both crude and fully-adjusted models, men in the highest tertile had approximately twice the risk of PAD compared to men in the lowest tertile (Table 2): adjusted IRR 2.17, 95% CI 1.08-4.38. In contrast, there was no association in crude or adjusted models among women, adjusted IRR 1.14, 95% CI 0.61-2.12 (p heterogeneity = 0.18). This result remained similar even with additional adjustment for dietary intakes of B vitamins. Associations were similar when we examined homocysteine as a continuous variable: adjusted IRRs and 95% CIs 1.25, 0.94–1.67 for each one standard deviation increase in log-transformed homocysteine in men and 0.89, 0.67–1.17 in women. Finally, we found no interactions between

homocysteine and fasting status, time (before and after 1998), age, alcohol, folate, vitamin B_6, vitamin B_{12}, cystatin C, diabetes, smoking, BMI, and HRT use (women only).

Dietary B vitamins (cohort studies)—A total of 516 incident cases of PAD occurred over 26 years of follow-up in men and 274 cases over 20 years of follow-up among women. Plasma homocysteine levels were correlated with B vitamins similarly in both men and women (Table 3). Men and women with higher levels of folate, vitamin B_6, and vitamin B_{12} tended to be more active, drink less alcohol, report greater use of aspirin, and consume less saturated and *trans* fat (Supplemental Tables 1a and b). Men had slightly higher levels of folate, vitamin B_6, vitamin B_{12}, riboflavin, betaine, and choline compared to women, but these distributions overlapped substantially for all dietary nutrients (Tables 4 and 5).

Compared with the lowest quintile, the highest quintile of total folate, including diet and supplements, was inversely associated with risk of PAD in men but not women (Table 4, p heterogeneity 0.15). When we examined folate as a continuous variable, each 250 μg increase (approximately 1 SD) was associated with a 10% lower risk of PAD in men: adjusted HR 0.90, 95% CI 0.82-0.98 but was not associated with risk in women (adjusted HR 1.01, 95% CI 0.88 1.15).

Categorized into quintiles, intakes of vitamins B_6 and B_{12} were also generally inversely associated with risk of PAD in men, but these associations were not statistically significant. When we examined vitamin B_6 as a continuous variable, we found no increased risk of PAD per 25 mg higher intake (approximately 1 SD) in men (adjusted HR 0.97, 95% CI 0.88-1.07) or women (adjusted HR 1.03, 95% CI 0.91-1.16). Similarly, we found no significant associations of PAD with vitamin B_{12} as a continuous variable, as each 215 μg higher intake (approximately 1 SD) was associated with an adjusted HR of 0.90 (95% CI 0.79-1.02) in men and 1.03 (95% CI 0.97-1.09) in women.

In models that simultaneously adjusted for folate, vitamin B6, and vitamin B12, folate appeared to have the strongest inverse association with risk of PAD. The adjusted HRs and 95% CI in men were 0.91 (0.82-1.01) for each 250 μg higher intake of folate, 1.03 (0.92-1.15) for each 25 mg (approximately 1 SD) higher intake of vitamin B6, and 0.94 (0.82-1.08) for each 15 μg higher intake (approximately 1 SD) of vitamin B12. Associations were attenuated for dietary intake alone when we excluded participants who reported supplements (Supplemental Table 2). There were no associations between riboflavin, betaine, and choline intake and risk of PAD in men or women (Table 5).

In sensitivity analyses, we restricted our analyses to the subset of women with the lowest estrogen status (and hence most similar to men) by virtue of being postmenopausal and not using hormones to determine if this might explain the sex-specific associations observed earlier. Among these women, in whom 99 cases of PAD occurred, we observed an inverse association between total folate and vitamin B_6 and risk of PAD: HR (95% CI) across extreme quintiles 0.47 (0.24-0.93) for folate and 0.45 (0.22-0.89) for vitamin B_6 (p-trend 0.02 for both). There were too few cases to perform comparable analyses for homocysteine. Finally, we found no interactions of B vitamins with each other or other risk factors in both men and women.

Discussion

In two large cohorts of men and women, plasma homocysteine levels were positively associated and dietary folate inversely associated with risk of PAD in men, however the association with folate was not statistically significant. These associations were not present in women, and no significant associations with risk of PAD were observed for vitamin B_6, vitamin B_{12}, riboflavin, betaine, or choline in either men or women.

Although cross-sectional studies consistently show PAD to be positively associated with homocysteine and inversely associated with B vitamins, [27] previous cohort studies of incident PAD have yielded inconsistent findings. [8-10,28] Allison et al. [8] reported that men and women with higher homocysteine levels were more likely to progress to having an abnormal ABI (< 0.9). Pradhan et al., [10] on the other hand, reported no association between homocysteine and risk of PAD in the Women's Health Study, a similar cohort of female health professionals of the same age as women participating in the NHS. Ridker et al. [9] also reported no association between homocysteine and PAD within the Physicians' Health Study cohort of male physicians who were of similar age as the men included in the HPFS, but had lower, yet overlapping homocysteine levels. Two additional studies have examined homocysteine levels and PAD progression: neither found that higher homocysteine levels were positively associated with progression. [29,30]

The findings from clinical trials of B vitamin supplementation and PAD are likewise inconclusive. [11-13] Two trials found no effect of B vitamin supplementation on atherosclerotic progression, [11] arterial stiffening, [11] ABI, [12] or carotid and femoral atherosclerosis ascertained by ultrasonography. [12] On the other hand, a third trial of 133 patients, of whom 90 were men, found small improvements in pulse wave velocity and ABI. [13]

Our finding that homocysteine and B vitamins were associated with risk of PAD in men only is difficult to explain but not implausible. Homocysteine levels are higher in men compared to women, [31] indicating that there may possibly be gender differences in homocysteine metabolism. [32] One hypothesis is that these differences may be due to estradiol which lowered homocysteine levels in postmenopausal women participating in a small randomized clinical trial.[31,33] Alternately, these differences could be due to the presence of estrogen which may affect endothelial function through counteracting, positive pathways including reduced E-selectin levels and enhanced flow-mediated dilatation.[34-36] When we restricted our analysis to postmenopausal women who were not using hormones, we observed an inverse association between folate and vitamin B_6 intake and risk of PAD. Nonetheless, most, [37-44] but not all [45] longitudinal studies that stratify by gender find consistent associations (or lack thereof) across gender for homocysteine/B vitamins and CVD.

The relative distributions of men and women above and below the current RDA for folate was similar by gender, and therefore differences in the percentage of each cohort with adequate folate levels is unlikely to explain the gender difference we found. Although the storage of plasma in heparin tubes in women versus EDTA tubes in men may have created

differential measurement of homocysteine by gender, correlations between plasma homocysteine levels and dietary intakes of B vitamins and coefficients of variation in measurement were comparable and, if anything, measurement variation was lower for women (Table 3). It is also unlikely that differential confounding explains the gender differences for homocysteine because even unadjusted models in women demonstrated no association with risk of PAD.

The positive association between homocysteine and PAD in men could relate to homocysteine-induced endothelial dysfunction. [1] The finding that individuals with a C677T mutation of the methylenetetrahydrofolate reductase gene have an elevated risk of PAD lends support to this hypothesis. [46] Folate intake itself may also drive the association between homocysteine and PAD, possibly by reducing oxidative stress [47] and improving endothelial dysfunction independent of homocysteine,[48] or by reducing oxidation of LDL.[49] In contrast, folate supplementation may not reduce oxidative stress in individuals whose homocysteine levels are not lowered.[50] Neither hypothesized mechanism is consistent with a gender-specific effect.

Because B vitamin intakes are so highly correlated, it is difficult to say with certainty that folate is most relevant in men; folate may be a marker of other B vitamin intake, the effect of which is obscured due to a greater degree of measurement error. Due to their high correlation, collinearity arose when we included all B vitamins in our models together. Nonetheless, when we included all B vitamins in a model together, the association between folate and PAD appeared the most robust.

The lack of association with riboflavin may not be surprising given evidence that supplementation only lowers homocysteine levels among individuals homozygous for the T allele of the C677T polymorphism of the methylenetetrahydrofolate reductase (*MTHFR*) gene [5153] (about 15% of the general population [54]). There are currently no clear recommendations for choline or betaine intake, and choline and/or betaine supplementation only lowers homocysteine levels in specific populations, such as those with pyridoxine-resistant homocystinuria and hyperhomocysteinemia due to deficient cystathionine β-synthase activity [55] or after a post-methionine load rise in homocysteine. [56,57] This may explain the lack of associations between choline and betaine in our two cohorts.

Our study is not without limitation. The correlation among B vitamins and between B vitamins and homocysteine makes it difficult to tease apart their independent associations with risk of PAD. The lack of ethnic diversity in either cohort means that these results are not necessarily generalizable to men and women of non-White ethnicity; African-Americans are at particularly high risk for PAD. We had only a single measure of homocysteine and did not have genotype information available to determine if these findings might differ according to *MTHFR* status. Furthermore, supplementation of the food supply with folic acid beginning in 1996[58] could have changed levels of plasma homocysteine during follow-up. Finally, as with all other prospective studies, there remains the possibility of residual confounding due to unmeasured or poorly measured confounders.

Strengths of our study include a relatively large number of events due to the size of the two cohorts, confirmed clinically significant PAD, and a comprehensive list of nutrients and covariates measured repeatedly. Finally, this is the first study to our knowledge that measured both plasma homocysteine and supplemental and dietary intakes of B vitamins including riboflavin, betaine, and choline using validated measurements in relation to risk of PAD in both men and women.

In conclusion, homocysteine levels were positively associated and dietary folate intake was inversely associated with risk of PAD in men but not in women. The basis for this sex-specificity is uncertain but may bear on hormonal differences or the role of homocysteine in the progression of atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations list

PAD	peripheral artery disease
CVD	cardiovascular disease
MI	myocardial infarction
ABI	ankle-brachial index
NHS	Nurses' Health Study
HPFS	Health Professionals Follow-up Study
FFQ	food frequency questionnaire
IRR	incidence rate ratio
SD	standard deviation
CI	confidence interval

HR	hazard ratio
EDTA	ethylenediaminetetraacetic acid
HDL-C	high-density lipoprotein cholesterol
LDL-C	low-density lipoprotein cholesterol
CRP	c-reactive protein
HbA_{1c}	hemoglobin A_{1c}
BMI	body mass index

Highlights

- Few studies have examined homocysteine or B in peripheral artery disease (PAD).
- We examine plasma homocysteine and dietary B vitamin intake among 116,852 adults.
- Higher plasma homocysteine levels were associated with a higher risk of PAD in men.
- Higher dietary folate was associated with a lower risk of PAD in men.
- There was no association between homocysteine or B vitamins and PAD among women.

Table 1

Baseline characteristics of cases and matched controls.

	WOMEN			MEN		
	Cases (143)	**Controls (424)**	**p-value[1]**	**Cases (143)**	**Controls (428)**	**p-value[1]**
Age (y)	59.9 (5.2)	60.0 (5.2)	Matched	65.4 (8.1)	65.3 (8.1)	Matched
Plasma homocysteine (μmol/L)	13.6 (3.1)	13.9 (3.8)	0.32	16.3 (6.0)	15.0 (4.4)	0.01
Dietary folate (μg)	339 (264,548)	374 (270,607)	0.16	420 (298,750)	451 (322,698)	0.72
Dietary vitamin B6 (mg)	2.3 (1.7,3.8)	2.4 (1.7,4.0)	0.41	3.1 (2.2,4.9)	3.2 (2.2,4.9)	0.83
Dietary vitamin B12 (μg)	7 (5,11)	7 (5,12)	0.05	10 (6,16)	10 (6,14)	0.78
Dietary riboflavin (mg)	2.0 (1.5,3.6)	2.1 (1.5,3.5)	0.49	2.8 (1.8,4.3)	2.9 (2.0,4.2)	0.95
Dietary betaine (mg)	107 (40)	110 (45)	0.42	123 (44)	134 (50)	0.02
Dietary choline (mg)	321 (61)	316 (57)	0.35	373 (66)	369 (67)	0.58
Total calories (kcal)	1786 (469)	1711 (491)	0.04	1977 (580)	2075 (596)	0.08
Lipids						
Triglycerides (mg/dL)	110 (85,161)	106 (73,144)	0.29	143 (105,195)	115 (80,165)	0.001
HDL-C (mg/dL)	60.5 (19.9)	62.1 (17.1)	0.41	41.7 (11)	48.5 (14)	<0.001
LDL-C (mg/dL)	148 (44)	143 (38)	0.17	139 (35)	131 (33)	0.02
High-sensitivity CRP (mg/L)	2.56 (1.25,4.70)	1.63 (0.73,3.33)	0.07	2.24 (1.2,3.5)	1.18 (0.5,2.3)	0.005
HbA_{1c} (%)	5.68 (0.87)	5.40 (0.50)	<0.001	5.96 (1.15)	5.56 (0.84)	<0.001
Cystatin C (mg/L)	0.96 (0.16)	0.95 (0.17)	0.49	1.08 (0.24)	0.99 (0.22)	<0.001
Smoking status						
Never	30 (21%)	90 (21%)	Matched	23 (17%)	82 (20%)	Matched

	WOMEN			MEN		
	Cases (143)	**Controls (424)**	**p-value[1]**	**Cases (143)**	**Controls (428)**	**p-value[1]**
Past	56 (39%)	168 (39%)	Matched	78 (59%)	242 (58%)	Matched
Current	58 (40%)	172 (40%)	Matched	32 (24%)	90 (22%)	Matched
Pack-years (y)	32.3 (25.6)	22.0 (21.4)	<0.001	28.7 (24)	22.5 (22)	<0.001
Physical activity (MET hr/wk)	12.8 (4.7,24.9)	13.3 (4.9,27.4)	0.79	22.7 (8.0,43.8)	27.4 (10.3,52.8)	0.003
History of hypertension	68 (47%)	137 (32%)	<0.001	70 (49%)	130 (30%)	<0.001
History of diabetes	19 (13%)	12 (3%)	<0.001	28 (20%)	16 (4%)	<0.001
History of hypercholesterolemia	84 (58%)	200 (47%)	0.01	82 (57%)	187 (44%)	0.005
Alcohol (g/day)	1.9 (0,11)	2.1 (0,9.9)	0.85	7.6 (1,18)	9.8 (2,20)	0.48
Parental history of MI < age 60 y	31 (22%)	61 (14%)	0.03	22 (15%)	44 (10%)	0.09
BMI (kg/m^2)	25.3 (4.5)	24.8 (4.0)	0.20	25.8 (3.3)	25.6 (4.4)	0.41
Aspirin use	29 (20%)	89 (21%)	0.87	80 (56%)	184 (43%)	0.007
Postmenopausal	131 (95%)	388 (95%)	0.72			
Ever used postmenopausal hormones[2]	96 (72%)	255 (62%)	0.04			
Currently using postmenopausal hormones[2]	57 (43%)	181 (44%)	0.89			

[1] Generalized linear mixed models for continuous variables and Cochran-Mantel-Haenszel test for categorical variables (to account for matching/correlation between controls), matching criteria were age, race (women only), month of blood draw, fasting status, and smoking status.
Note: data are expressed as mean (SD), median (interquartile range), or n (%).

[2] Among postmenopausal women

Table 2

IRRs and 95% CIs for peripheral artery disease according to level of plasma homocysteine.

WOMEN (1990 - 2010)				
	Tertile of plasma homocysteine (μmol/L)			
	1	**2**	**3**	**p-trend**
Median	10.7	13.2	16.7	
Cases/Controls	46/142	50/141	47/141	
Model 1	1.0 (ref)	1.12 (0.70-1.78)	1.05 (0.65-1.71)	0.86
Model 2	1.0 (ref)	1.03 (0.58-1.82)	1.14 (0.61-2.12)	0.68
Model 3	1.0 (ref)	1.03 (0.58-1.85)	1.01 (0.53-1.93)	0.98

MEN (1994 - 2008)				
	Tertile of plasma homocysteine (μmol/L)			
	1	**2**	**3**	**p-trend**
Median	11.7	14.3	18.6	
Cases/Controls	32/156	51/143	60/129	
Model 1	1.0 (ref)	1.72 (1.05-2.81)	2.40 (1.45-3.98)	<0.001
Model 2	1.0 (ref)	1.44 (0.77-2.68)	2.17 (1.08-4.38)	0.03
Model 3	1.0 (ref)	1.46 (0.78-2.75)	2.37 (1.16-4.82)	0.02

Model 1: adjusted for matching factors [age, race (women in the NHS only), month of blood draw, fasting status, and smoking].

Model 2: model 1+ triglycerides, HDL-C, LDL-C, hsCRP, HbA_{1C}, cystatin C, pack-years of smoking, hypertension, diabetes, family history of myocardial infarction, BMI, alcohol, and postmenopausal hormone use (women only).

Model 3: model 2 + dietary intakes of total fiber and B vitamins.

Table 3

Correlations between plasma homocysteine, dietary B vitamins, betaine, and choline, adjusted for age and total energy at baseline (1990 women, 1994 men).

	Plasma homocysteine	Folate (B_9)	Vitamin B_6	Vitamin B_{12}	Riboflavin (B_2)	Betaine	Choline
Plasma homocysteine		**-0.31**	-0.32	**-0.21**	**-0.31**	**-0.18**	**-0.09**
Folate (B_9)	**-0.25**		**0.77**	**0.64**	**0.74**	**0.19**	**0.23**
Vitamin B_6	**-0.23**	**0.78**		**0.62**	**0.85**	**0.13**	**0.28**
Vitamin B_{12}	**-0.19**	**0.60**	**0.60**		**0.73**	0.03	**0.35**
Riboflavin (B_2)	**-0.25**	**0.73**	**0.88**	**0.68**		0.07	**0.33**
Betaine	**-0.15**	**0.20**	**0.14**	0.01	**0.11**		0.02
Choline	-0.04	0.07	**0.10**	**0.33**	**0.17**	-0.06	

Bold coefficients are significant ($p < 0.05$).

Unshaded represent women in the NHS (n=567) and shaded correlations represent men in the HPFS (n = 571).

Table 4

HRs and 95% CIs for peripheral artery disease according to level of dietary B vitamins (including supplements).

WOMEN						
	Quintile of folate (μg)					
	1	**2**	**3**	**4**	**5**	**p-trend**
Median	226	299	383	555	770	
# Cases	61	64	49	37	63	
P-years	403,645	414,499	416,742	429,524	453,426	
Model 1	1.0 (ref)	0.96 (0.67-1.36)	0.71 (0.48-1.03)	0.51 (0.34-0.77)	0.73 (0.51-1.03)	0.02
Model 2	1.0 (ref)	1.07 (0.75-1.53)	0.83 (0.56-1.22)	0.66 (0.43-0.99)	1.01 (0.70-1.45)	0.60
	Quintile of vitamin B_6 (mg)					
	1	**2**	**3**	**4**	**5**	**p-trend**
Median	1.5	1.9	2.4	3.7	8.3	
# Cases	50	57	58	52	57	
P-years	346,520	419,100	479,383	423,682	449,151	
Model 1	1.0 (ref)	0.95 (0.65-1.39)	0.80 (0.55-1.17)	0.75 (0.51-1.11)	0.78 (0.53-1.14)	0.28
Model 2	1.0 (ref)	1.02 (0.69-1.50)	0.93 (0.63-1.36)	0.90 (0.61-1.34)	1.00 (0.68-1.49)	0.92
	Quintile of vitamin B_{12} (μg)					
	1	**2**	**3**	**4**	**5**	**p-trend**
Median	4.0	5.5	7.5	11.0	20.0	
# Cases	48	69	41	55	61	
P-years	374,595	451,482	328,336	493,924	469,499	
Model 1	1.0 (ref)	1.22 (0.84-1.76)	0.96 (0.63-1.46)	0.82 (0.56-1.21)	0.87 (0.59-1.27)	0.13
Model 2	1.0 (ref)	1.19 (0.82-1.74)	1.01 (0.66-1.55)	0.84 (0.57-1.25)	1.05 (0.71-1.55)	0.75
MEN						
	Quintile of folate (μg)					
	1	**2**	**3**	**4**	**5**	**p-trend**

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WOMEN						
	Quintile of folate (µg)					
	1	**2**	**3**	**4**	**5**	**p-trend**
Median	254	333	416	575	863	
# Cases	120	119	108	91	78	
P-years	189,097	191,702	192,522	193,271	193,297	
Model 1	1.0 (ref)	1.04 (0.80-1.35)	0.91 (0.69-1.18)	0.77 (0.58-1.01)	0.63 (0.47-0.85)	<0.0001
Model 2	1.0 (ref)	1.14 (0.88-1.49)	1.01 (0.77-1.33)	0.95 (0.71-1.26)	0.78 (0.58-1.05)	0.03
	Quintile of vitamin B_6 (mg)					
	1	**2**	**3**	**4**	**5**	**p-trend**
Median	1.7	2.2	2.8	4.3	12.0	
# Cases	115	98	108	96	99	
P-years	187,648	193,223	188,198	198,456	192,364	
Model 1	1.0 (ref)	0.78 (0.60-1.03)	0.89 (0.68-1.16)	0.69 (0.52-0.91)	0.76 (0.58-1.01)	0.21
Model 2	1.0 (ref)	0.87 (0.66-1.16)	1.01 (0.77-1.33)	0.78 (0.60-1.06)	0.87 (0.66-1.15)	0.43
	Quintile of vitamin B_{12} (µg)					
	1	**2**	**3**	**4**	**5**	**p-trend**
Median	5.0	7.0	10.0	13.7	23.0	
# Cases	69	148	95	112	92	
P-years	133,930	249,900	189,655	184,364	202,041	
Model 1	1.0 (ref)	1.09 (0.82-1.46)	0.83 (0.61-1.14)	0.94 (0.69-1.28)	0.70 (0.51-0.95)	0.002
Model 2	1.0 (ref)	1.10 (0.82-1.48)	0.86 (0.62-1.18)	0.99 (0.72-1.34)	0.77 (0.56-1.07)	0.03

Model 1: adjusted for age, total energy, race (women only), and smoking.

Model 2: model 1 + pack-years, hypertension, high cholesterol, diabetes, family history of myocardial infarction, BMI, alcohol, physical activity, aspirin, and postmenopausal hormone use (women only).

Table 5

HRs and 95% CIs for peripheral artery disease according to level of dietary intake of riboflavin, betaine, and choline.

WOMEN						
	Quintile of riboflavin (mg)					
	1	**2**	**3**	**4**	**5**	**p-trend**
Median	1.3	1.7	2.2	3.2	9.6	
Model 1	1.0 (ref)	0.91 (0.63-1.30)	0.74 (0.51-1.07)	0.65 (0.45-0.95)	0.76 (0.53-1.09)	0.33
Model 2	1.0 (ref)	0.99 (0.68-1.43)	0.87 (0.59-1.28)	0.83 (0.56-1.22)	0.95 (0.65-1.37)	0.94
	Quintile of betaine (mg)					
	1	**2**	**3**	**4**	**5**	**p-trend**
Median	67	85	101	120	159	
Model 1	1.0 (ref)	1.30 (0.91-1.86)	1.07 (0.73-1.56)	0.97 (0.66-1.42)	0.91 (0.61-1.35)	0.24
Model 2	1.0 (ref)	1.39 (0.97-2.01)	1.16 (0.79-1.71)	1.08 (0.73-1.59)	1.02 (0.69-1.52)	0.57
	Quintile of choline (mg)					
	1	**2**	**3**	**4**	**5**	**p-trend**
Median	246	282	307	334	377	
Model 1	1.0 (ref)	0.94 (0.62-1.43)	1.34 (0.91-1.97)	1.42 (0.97-2.06)	1.21 (0.82-1.79)	0.12
Model 2	1.0 (ref)	0.91 (0.59-1.38)	1.30 (0.88-1.91)	1.40 (0.95-2.05)	1.07 (0.72-1.60)	0.32
MEN						
	Quintile of riboflavin (mg)					
	1	**2**	**3**	**4**	**5**	**p-trend**
Median	1.5	1.9	2.5	3.8	12.9	
Model 1	1.0 (ref)	0.78 (0.59-1.02)	0.69 (0.52-0.91)	0.71 (0.54-0.92)	0.74 (0.56-0.96)	0.32
Model 2	1.0 (ref)	0.87 (0.66-1.15)	0.78 (0.59-1.03)	0.83 (0.63-1.08)	0.81 (0.61-1.06)	0.38
	Quintile of betaine (mg)					
	1	**2**	**3**	**4**	**5**	**p-trend**

WOMEN						
	Quintile of riboflavin (mg)					
	1	**2**	**3**	**4**	**5**	**p-trend**
Median	81	102	121.0	144	191	
Model 1	1.0 (ref)	1.12 (0.86-1.45)	1.00 (0.76-1.31)	0.80 (0.60-1.07)	0.95 (0.73-1.25)	0.27
Model 2	1.0 (ref)	1.19 (0.91-1.55)	1.10 (0.83-1.45)	0.85 (0.63-1.13)	1.02 (0.77-1.35)	0.48
	Quintile of choline (mg)					
	1	**2**	**3**	**4**	**5**	**p-trend**
Median	304	348	379	415	488	
Model 1	1.0 (ref)	1.15 (0.83-1.59)	1.37 (1.01-1.87)	1.51 (1.12-2.04)	1.36 (1.00-1.84)	0.03
Model 2	1.0 (ref)	1.14 (0.82-1.59)	1.33 (0.97-1.83)	1.46 (1.08-1.98)	1.24 (0.91-1.68)	0.16

Model 1: adjusted for age, total energy, race (women only), and smoking.

Model 2: model 1 + pack-years, hypertension, high cholesterol, diabetes, family history of myocardial infarction, BMI, alcohol, physical activity, aspirin, and postmenopausal hormone use (women only).