DOI 10.1002/mnfr.201100694 *Mol. Nutr. Food Res.* 2012, *56*, 1122–1130

RESEARCH ARTICLE

Nutritional B vitamin deficiency disrupts lipid metabolism causing accumulation of proatherogenic lipoproteins in the aorta adventitia of ApoE null mice

Christopher J. McNeil[1], John H. Beattie[2], Margaret-Jane Gordon[2], Lynn P. Pirie[1] and Susan J. Duthie[1]

[1] Natural Products Group, Division of Lifelong Health, Rowett Institute of Nutrition and Health, University of Aberdeen, Aberdeen, United Kingdom
[2] Micronutrients Group, Division of Lifelong Health, Rowett Institute of Nutrition and Health, University of Aberdeen, United Kingdom

Received: October 17, 2011
Revised: March 2, 2012
Accepted: March 5, 2012

Scope: Cardiovascular disease is the major cause of death in the world. Low dietary folate, elevated homocysteine, and high circulating cholesterol are risk factors.
Methods and results: We investigated whether folate and/or B vitamin deficiency would change lipoprotein and fatty acid metabolism and lipid accumulation in the aorta adventitia of ApoE null mice. Mice ($n = 10$ per group) were fed a control (C; 4%) or high saturated fat (HF; 21%), and high cholesterol (0.15%) diet for 16 weeks. Folate (F-) or folate, B6 and B12 deficiency (F-B-) were imposed on these diets. Feeding a HF diet increased plasma and liver total cholesterol and HDL cholesterol (two- to threefold; $p < 0.05$). Total cholesterol increased (twofold; $p < 0.05$) in aorta adventitial lipid in response to HF. Feeding a diet depleted of folate and B vitamins (F-B-) significantly increased cholesterol accumulation in both liver and aorta adventitial lipid (approximately 50–70%; $p < 0.05$). Moreover, the proportions of fatty acids in hepatic and adventitial lipid was significantly changed by B vitamin depletion, measured as an increase in saturated fatty acids (approximately 15%) and a decrease (approximately 11%) in monounsaturated fatty acids ($p < 0.05$).
Conclusion: B vitamin deficiency perturbs lipid metabolism in ApoE null mice, causing accumulation of proatherogenic cholesterol and fatty acids in the aorta adventitia.

Keywords:
B vitamin deficiency in ApoE null mice / Hyperhomocysteinemia / Lipid metabolism / Vascular lipid deposition

1 Introduction

Cardiovascular disease (CVD) is the major cause of death and disability worldwide [1]. An estimated 17.3 million people died from CVD in 2008, representing 30% of all global deaths [1]. One in four men and one in six women will die from heart disease [1]. Atherosclerosis, a complex process of lipid accumulation, inflammation, and plaque formation leading to narrowing of the artery, is the primary underlying cause of CVD. While family history, male gender, and ageing are unavoidable risk factors [1], atherosclerosis and CVD are largely preventable. It has been estimated that 30–40% of deaths from CVD could be avoided by improved diet and lifestyle [1]. Elevated circulating total and LDL cholesterol, cigarette smoking, physical inactivity, obesity, hypertension, hyperhomocysteinemia, and diabetes are major independent risk factors for vascular disease [2]. Alcohol abuse, high salt and low wholegrain, fish, fruit, and vegetable intake are similarly associated with CVD incidence [1, 2].

Elevated blood concentrations of the modified amino acid homocysteine are associated with an increased risk of CVD in the general population [3–5]. Individuals with severe hyperhomocysteinemia (plasma Hcy in excess of 100 μmol/L) due to inborn errors in the cystathionine beta synthase (CBS) gene have a significantly increased risk of suffering a vascular event before the age of 30 years if untreated [6]. Low dietary

Correspondence: Dr. Susan J. Duthie, Natural Products Group, Division of Lifelong Health, Rowett Institute of Nutrition and Health, University of Aberdeen, Aberdeen AB21 9SB, United Kingdom
E-mail: s.duthie@abdn.ac.uk
Fax: +44-1224-716629

Abbreviations: **CVD**, cardiovascular disease; **HF**, high fat; **TG**, triglycerides; **SAH**, S-adenosylhomocysteine; **SAM**, S-adenosylmethionine

intake of the B vitamins folate, B6, and B12 similarly induce moderate plasma hyperhomocysteinemia by inhibiting intracellular methionine remethylation. Hyperhomocysteinemia adversely affects vascular function in animal models via several distinct mechanisms, including homocysteine-mediated induction of endoplasmic reticular (ER) stress, aorta smooth muscle cell (SMC) growth, plasma lipid oxidation and inflammation, and increased coagulation [7]. Nonetheless, there remains considerable debate whether hyperhomocysteinemia is causal for CVD [7]. A small number of studies indicate an independent role for B vitamins in vascular health. High blood folate has been shown in prospective human studies to be independently associated with reduced risk of coronary events [8, 9] and carotid intima-media thickness [10] while supplementation with folic acid positively influences vascular function both in hypercholesterolemic patients with CVD and in diabetics [11]. Similarly, supplementing at-risk patients either with folic acid alone or together with vitamins B6 and B12 reduces blood pressure [12] and carotid intima-media thickening [13], independently of circulating homocysteine concentrations.

A strong relationship exists between dietary saturated fat, elevated serum cholesterol, and CHD worldwide [1]. Moreover, increased fat intake in rodents alters lipoprotein metabolism and adipocyte function within the perivascular fat surrounding the aorta tunica adventitia [14]. Previously believed to have only a structural role, aorta adventitial fat has now been shown to actively regulate vascular responsiveness and function [14,15]. While changes in lipid metabolism, including hyperlipidemia, are associated with hyperhomocysteinemia in humans and in homocysteine-induced vascular pathology in rodents [16–19], the effect of B vitamin depletion and/or hyperhomocysteinemia on lipid metabolism directly in the aorta adventitia remains to be established.

Rodent models of atherosclerosis provide a good representation of human CVD and allow comprehensive analysis of the effect of diet on disease progression. The most commonly used genetic mouse model of atherosclerosis is deficient in ApoE, which acts as a ligand for receptors that remove lipoprotein particles [20]. ApoE null mice spontaneously develop atherosclerotic plaques that are morphologically comparable with human lesions [20]. We have recently reported that atherosclerotic plaque formation in the aorta of ApoE null mice is accelerated following a moderate nutritional folate and/or B vitamin deficiency [21]. Here, we report that B vitamin depletion causally alters lipoprotein and fatty acid profiles both in the liver and in the vascular adventitial tissue, resulting in a proatherogenic environment directly within the aorta.

2 Materials and methods

2.1 Animals and diets

The design of this animal study has been described in detail previously [21]. Briefly, ApoE null mice (males aged 4 weeks) were fed standard laboratory chow ad libitum for 1-week acclimatization before allocation by equal weight per group to experimental treatment ($n = 20$ animals per group). Six diets with different levels of fat/cholesterol and B vitamins were prepared. Two basal diets were designated either as control (C; 4% w/w lard) or high fat (HF; 21% w/w lard and cholesterol (0.15% w/w)). Different B vitamin compositions were applied to each of these diets; [1] folic acid and B vitamin complete, [21], folic acid deficient (F-), or [3] folic acid, B6 and B12 deficient (F-B-). Dietary composition has been described in detail previously [21]. Mice were housed individually on grid floors to prevent coprophagy. All procedures were carried out in accordance with the requirements of UK Animals (Scientific Procedures) Act 1986.

2.2 Blood B vitamin, homocysteine, lipid, and inflammation analysis

After 16 weeks treatment, mice were fasted (4–6 h) and killed by exsanguination under terminal anesthesia. Blood was collected from the vena cava for analysis of B vitamin status and for a range of lipid and inflammatory markers implicated in vascular disease ($n = 10$–20 per group depending on the assay). Whole blood and plasma folate and B12 were measured by radioassay [21]. Plasma homocysteine was measured by gas chromatography [22]. Total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides (TG) were measured using a Konelab 20 Clinical Chemistry Analyser (Thermo Scientific, Passau, Germany). Plasma markers of vascular disease and inflammation (TNF-α, MCP-1 KC, IL-1, IL-5, soluble ICAM-1, soluble VCAM-1, and soluble E-selectin) were measured using mouse multiplex panels of antibody-coated beads (Millipore, Watford, UK) and Luminex analysis (Luminex B.V., Oosterhouit NB, Netherlands).

2.3 Tissue collection, SAM:SAH, and lipid analysis

Post-exsanguination, any remaining blood was flushed from the heart by injection of DMEM into the left ventricle. The complete aorta was flushed with DMEM and microdissected of all tunica adventitia [23]. The aortas of all mice ($n = 20$ per group) were processed for plaque abundance [23] and the adventitial tissue of a subsample ($n = 10$ per group) snap frozen and stored at –80°C for lipid analysis. The liver was collected and immediately snap frozen for lipid analysis or for S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) measurement [24]. Total lipid was extracted from thawed liver or aortic adventitial tissue (approximately 200 mg) after the method of Folch et al. [25] and total cholesterol, LDL cholesterol, HDL cholesterol, and TG fractions measured by Kone analysis as described above. Individual fatty acids from aorta adventitial and hepatic lipid were transesterified to their respective fatty acid methyl esters (FAMEs) in methanolic HCL, separated by liquid–liquid solvent

Table 1. B vitamin, SAM, SAH, and homocysteine status in blood and atherosclerotic plaque formation in aorta from ApoE mice fed a control (C) or high fat (HF) diet depleted of folic acid (F-) or folic acid and vitamins B6 and B12 (F-B-) for 16 weeks. Values are means ± SEM for $n = 10$ mice sampled per group. Statistical differences between groups, detected by *t*-test, are shown by superscript letters; mean values with different superscript letters are significantly different, $p < 0.05$. These data are reproduced with permission of the journal, Clinical Epigenetics

Biomarkers	Control fat			High fat		
	C	C-F	C-F-B	HF	HF-F	HF-F-B
Plasma folate (ng/mL)	87.8 ± 4.8^{a}	16.2 ± 1.5^{b}	14.9 ± 0.9^{b}	104.9 ± 5.5^{c}	15.5 ± 1	15.0 ± 1.4^{b}
Whole blood folate (ng/mL)	498.9 ± 42.9^{a}	163.5 ± 8.9^{b}	184.7 ± 6.8^{b}	507.9 ± 26.0^{a}	149.6 ± 9.7^{b}	193.3 ± 18.7^{b}
Plasma B12 (ng/mL)	24.8 ± 2.3^{a}	15.3 ± 1.0^{b}	6.8 ± 0.1^{c}	25.6 ± 3.0^{a}	12.5 ± 0.5^{d}	6.1 ± 0.5^{c}
Whole blood B12 (ng/mL)	22.5 ± 1.0^{a,c}	22.0 ± 0.6^{a,c}	7.2 ± 0.7^{b}	24.9 ± 1.6^{a}	20.5 ± 1.1^{c}	6.9 ± 1.0^{b}
Plasma homocysteine (μM)	7.8 ± 0.4^{a}	18.1 ± 1.9^{b}	28.4 ± 3.6^{c}	6.7 ± 0.3^{a}	14.2 ± 1.0^{b}	45.5 ± 4.3^{d}
Liver SAM (nmol/g tissue)	100.2 ± 9.2^{a}	50.7 ± 4.0^{b}	43.7 ± 2.6^{c}	84.1 ± 4.7^{d}	47.6 ± 6.7^{b}	36.1 ± 1.8^{c}
Liver SAH (nmol/g tissue)	50.4 ± 2.3^{a}	71.6 ± 3.1^{b}	86.1 ± 4.3^{c}	47.6 ± 3.1^{a}	68.1 ± 3.8^{b}	89.7 ± 4.4^{c}

extraction and quantified by gas-liquid chromatography [GLC; 26]. All tissue and lipid samples were spiked with C17 and C19 standard respectively to assess extraction efficiency prior to analysis.

2.4 Statistical analysis

Data are presented as mean ± SEM with number of animals in parenthesis. Data were analyzed by 2-way ANOVA for the effect of fat intake, vitamin composition, and interaction between these factors. Significant differences between all groups were detected using the Least Significant Difference (LSD) post-hoc method for $p < 0.05$. Analyses were carried out using SPSS (version 17). Data not sharing superscript letters differ significantly.

3 Results

3.1 B vitamin, SAM:SAH, and homocysteine status

The effect of treatment of blood markers of folate, vitamin B12, and homocysteine has been described in detail previously [21]. Briefly, whole blood and plasma folate declined more than 65 and 80%, respectively in mice fed the C-F and HF-F diets ($p < 0.05$; Table 1). Feeding a F-B- diet depleted plasma B12 73% in the C and 76% in the HF group ($p < 0.05$; Table 1). Moreover, folate deficiency alone caused a significant decrease in both plasma and whole blood B12 that was dependent on fat intake ($p < 0.05$; Table 1). Plasma total homocysteine was increased by folate deficiency alone [2.3-fold in the C-F and 2.1-fold in the HF-F diet] and to a significantly greater extent by feeding a combined folate and B vitamin-depleted diet [3.6- and 6.8-fold in the C and HF group, respectively]. B vitamin depletion similarly decreased liver SAM and increased SAH levels to a greater extent than folate deficiency alone. The differential effect of folate and B vitamin deficiency on both homocysteine and liver SAM/SAH was significantly dependent on fat intake ($p < 0.05$ for all data; Table 1).

3.2 Total lipid and fatty acid profiles in plasma, liver, and aorta adventitial tissue

3.2.1 Effect of a high fat diet

Feeding a HF diet significantly increased plasma total cholesterol, HDL cholesterol, and LDL cholesterol, while plasma TG levels were significantly decreased ($p < 0.001$, Table 2).

Total lipid increased in the liver (but not aorta adventitia) of mice fed a HF diet ($p < 0.015$; Table 3). Liver and aorta adventitial total cholesterol and HDL cholesterol were significantly increased in these animals ($p < 0.001$; Figs. 1 and 2). Hepatic TG was lower in mice fed a HF diet compared with control animals ($p < 0.001$; Fig. 3). Adventitial TG levels were similar in animals fed either a C or HF diet (Fig. 3). The effects of dietary fat on individual fatty acids extracted from the liver and the aorta adventia were measured. For clarity, the data are grouped into saturated (SATFAs), polyunsaturated (PUFAs), and monounsaturated (MUFAs) fatty acids (Table 3). Hepatic and adventitial MUFA levels were unchanged by HF. Conversely, mice fed a HF diet had significantly lower levels of SATFAs both in the liver and aorta adventitial lipid than animals fed a control diet ($p < 0.001$ and $p < 0.007$ for liver and adventitia, respectively). Both liver and aorta PUFAs concentrations were elevated in mice fed a HF diet ($p < 0.001$ for liver and $p < 0.002$ for aorta adventitia).

3.2.2 Effect of a B vitamin deficient diet

Total plasma cholesterol and LDL cholesterol were reduced (approximately 15%) in mice fed a folate deficient C diet, but not in animals fed a folate-depleted HF diet ($p < 0.05$, Table 2). Combined folate and B vitamin deficiency did not affect these parameters, irrespective of fat intake. TG concentrations were not generally influenced by folate or B vitamin status (Table 2).

Folate depletion alone did not affect hepatic or adventitial total lipid levels in either treatment group (Table 3).

Table 2. Indices of plasma lipids from ApoE mice fed a control (C) or high fat (HF) diet depleted of folic acid (F-) or folic acid and vitamins B6 and B12 (F-B-) for 16 weeks. Values are means ± SEM for $n = 9$ or 10 animals per group. Statistical differences between groups, detected by *t*-test, are shown by superscript letters; mean values with different superscript letters are significantly different, $p < 0.05$

	Control fat			High fat		
Biomarkers of lipid metabolism	C	C-F	C-F-B	HF	HF-F	HF-F-B
Total cholesterol (mmol/L)	12.7 ± 0.51^{a}	10.9 ± 0.61^{b}	12.9 ± 0.40^{a,b}	26.2 ± 1.92^{c}	26.5 ± 0.96^{c}	24.9 ± 1.34^{c}
HDL cholesterol (mmol/L)	5.22 ± 0.33^{a}	5.51 ± 0.84^{a}	5.71 ± 0.92^{a}	9.23 ± 0.40^{b}	10.42 ± 0.87^{b}	8.86 ± 0.59^{b}
LDL cholesterol (mmol/L)	9.25 ± 0.38^{a}	8.04 ± 0.42^{b}	8.90 ± 0.52^{a,b}	24.19 ± 1.85^{c}	24.79 ± 1.11^{c}	22.58 ± 1.06^{c}
Triglycerides (TG) (mmol/L)	1.15 ± 0.05^{a,c}	1.27 ± 0.08^{a}	2.20 ± 0.25^{b}	0.89 ± 0.14^{c,e}	1.01 ± 0.08^{c,d,e}	0.91 ± 0.07^{e}

However, both hepatic ($p < 0.0001$) and adventitial lipid ($p < 0.015$) levels were significantly decreased in animals fed a HF combined folate and vitamin B deficient diet relative to the HF control group (Table 3). Folate deficiency alone did not alter cholesterol accumulation either in liver or adventitia (Fig. 1). However, both liver and adventitia total cholesterol increased significantly in animals fed a HF and combined folate and B vitamin deficient diet ($p < 0.05$; Fig. 1). Similarly, hepatic HDL cholesterol increased significantly in mice fed a combined B vitamin deleted HF diet ($p < 0.05$; Fig. 2). A similar but nonsignificant rise in HDL was observed in the aorta adventitia (Fig. 2). Hepatic TG concentrations were reduced in response to a combined folate and B vitamin deficient diet ($p < 0.05$; Fig. 3). TG levels in adventitial lipid were not generally influenced by B vitamin status.

Feeding a combined folate and B vitamin deficient diet significantly increased accumulation of SATFAs in the liver and aorta adventitia from mice fed a HF diet ($p < 0.05$ for both tissues; Table 3). In direct contrast, liver and adventitia MUFA levels were significantly lower in animals from this group ($p < 0.05$ for both tissue; Table 3). PUFA concentrations were unaffected by vitamin status in either tissue (Table 3). The effects of treatment on individual fatty acids extracted from the liver and the aorta adventia are shown in Supporting Information Tables S1 and S2.

3.3 Plasma markers of inflammation

Feeding a HF diet increased the levels of several plasma biomarkers associated with inflammation or atherosclerosis. The adhesion molecules, soluble E selectin, V-CAM, and I-CAM were increased (approximately 60, 15, and 60%, respectively) in plasma from the HF mice. Circulating levels of the cytokine IL-5, an immune cell stimulator, increased approximately twofold in response to a HF diet ($p < 0.05$; Table 4). None of these biomarkers were influenced by either folate deficiency alone or combined folate and B vitamin depletion.

4 Discussion

It has been suggested that hyperhomocysteinemia, which is associated with an increased risk of human CVD, may merely reflect low intracellular B vitamin status [8–13]. Proatherogenic changes in lipid levels are associated with low

Table 3. The effect of folic acid, B vitamins, and dietary fat intake on total lipid (% tissue) and saturated, monounsaturated, and polyunsaturated fatty acid levels (% lipid) in hepatic and aorta adventitial lipid from ApoE mice fed a control (C) or high fat (HF) diet depleted of folic acid (F-) or folic acid and vitamins B6 and B12 (F-B-) for 16 weeks. Values are means ± SEM for $n = 10$ animals per group. Statistical differences between groups, detected by *t*-test, are shown by superscript letters; mean values with different superscript letters are significantly different, $p < 0.05$. Data for individual-free fatty acids are presented in full in Supporting Information Tables S1 and S2

		Control fat			High fat		
Tissue		C	C-F	C-F-B	HF	HF-F	HF-F-B
Liver	Total lipid	13.25 ± 1.22^{a,c}	12.34 ± 0.66^{a}	10.22 ± 0.65^{b}	15.98 ± 0.83^{c}	15.71 ± 1.34^{c,d}	9.81 ± 0.53^{b}
	Saturated FAs	25.80 ± 0.25^{a}	25.89 ± 0.44^{a}	26.47 ± 0.53^{a}	20.31 ± 0.32^{b}	20.52 ± 0.31^{b}	23.21 ± 1.88^{c}
	Monounsaturated FAs	49.39 ± 1.49^{a}	47.52 ± 1.04^{a}	45.92 ± 1.49^{a,b}	48.95 ± 0.87^{a}	48.36 ± 1.07^{a}	43.70 ± 0.94^{b}
	Polyunsaturated FAs	22.94 ± 1.52^{a}	24.73 ± 1.28^{a,b}	25.89 ± 1.22^{a,b,d}	27.67 ± 0.69^{b,d}	27.63 ± 0.73^{b,c,d}	29.34 ± 0.49^{d}
Adventitia	Total lipid	9.05 ± 1.03^{a}	9.95 ± 0.69^{a}	8.28 ± 0.89^{a}	8.76 ± 1.32^{a}	9.53 ± 0.87^{a}	5.65 ± 0.76^{b}
	Saturated FAs	31.47 ± 1.08^{a,b}	30.87 ± 0.68^{a}	33.39 ± 0.84^{b}	27.85 ± 0.59^{c}	28.82 ± 0.46^{c}	33.92 ± 0.49^{b,d}
	Monounsaturated FAs	46.76 ± 0.92^{a,b}	47.44 ± 0.44^{a,b}	46.19 ± 0.49^{a}	48.38 ± 0.67^{b}	48.62 ± 0.55^{b,c}	42.53 ± 0.71^{d}
	Polyunsaturated FAs	20.55 ± 0.94^{a,b}	21.09 ± 0.70^{a,b}	19.56 ± 0.84^{a}	22.53 ± 0.54^{b}	21.77 ± 0.30^{b,c}	22.62 ± 0.64^{b,d}

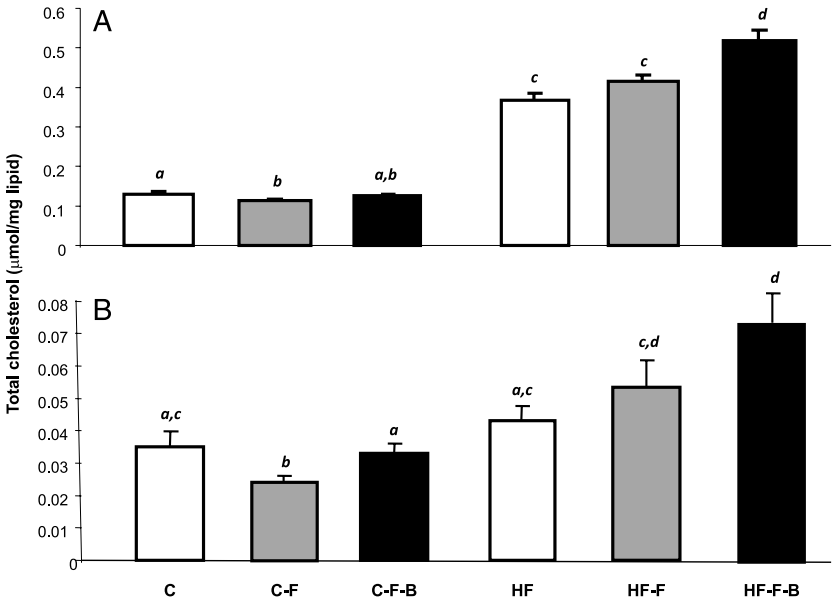

Figure 1. The effect of folic acid, B vitamins and dietary fat intake on total cholesterol in liver (A) and aorta tunica adventitia (B) Results are mean ± SEM for $n = 10$ animals per group. Statistical differences between groups, detected by *t*-test, are shown by superscript letters; mean values with different superscript letters are significantly different, $p < 0.05$.

B vitamin concentrations and hyperhomocysteinemia in humans, and in homocysteine-induced vascular pathology in rodents [16–19]. We have shown previously that feeding a high fat diet containing cholesterol significantly exacerbates hyperhomocysteinemia and aortic plaque formation in a mouse model of nutritional folate and/or B vitamin deficiency [21].

We report here how combined B vitamin depletion, rather than folate deficiency alone, may accelerate atherosclerosis in response to a high fat intake by causing proatherogenic lipoproteins, including cholesterol, to accumulate directly in the aorta tunica adventitia.

Previously believed to have a passive structural role, the perivascular adventitial fat within the tunica adventitia is now known to regulate vascular responsiveness and remodeling via secretion of vasoactive substances [14, 15]. Adipocyte-derived relaxing factor (ADRF) secreted by rat vascular fat cells induces vasodilation in mesenteric arteries by inhibiting the action of vasoactive modulators [14]. Critically, these actions are dependent on the quantity of lipid in the tissue [14]. Moreover, the growth of human vascular SMCs in culture is stimulated in response to conditioned medium from rat perivascular adipose tissue isolated from animals fed a HF

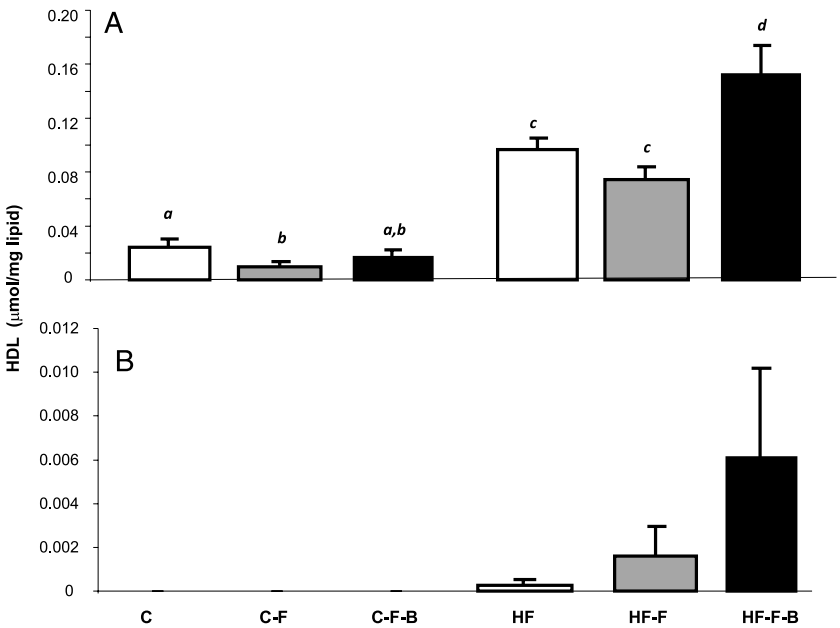

Figure 2. The effect of folic acid, B vitamins and dietary fat intake on HDL in liver (A) and aorta tunica adventitia (B) Results are mean ± SEM for $n = 10$ animals per group. Statistical differences between groups, detected by *t*-test, are shown by superscript letters; mean values with different superscript letters are significantly different, $p < 0.05$.

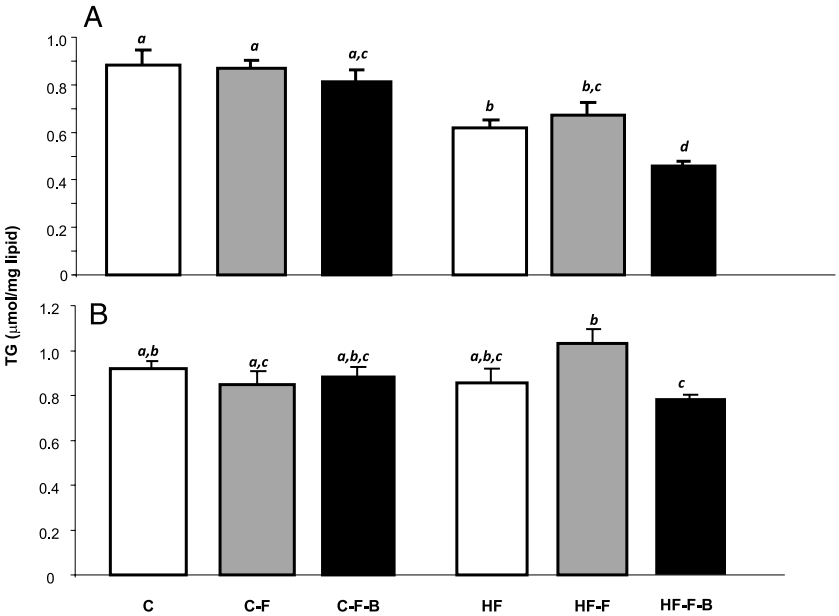

Figure 3. The effect of folic acid, B vitamins and dietary fat intake on triglyceride levels in liver (A) and aorta tunica adventitia (B) Results are mean ± SEM for $n = 10$ animals per group. Statistical differences between groups, detected by *t*-test, are shown by superscript letters; mean values with different superscript letters are significantly different, $p < 0.05$.

diet for 3 months [15]. Dysregulation of adipocytes within the adventitia is implicated in the pathogenesis of CVD, obesity, and diabetes [14]. The ability of dietary factors to influence perivascular adipose lipid deposition and tissue function is currently under investigated.

Here, we measured the abundance of key lipoproteins known to influence atherosclerosis (total cholesterol, LDL cholesterol, HDL cholesterol) in lipid extracted directly from the aorta periadventitial tissue of ApoE null mice fed either a folate deficient or a combined folate, B6- and B12-depleted diet. The most significant finding from this study is that combined B vitamin deficiency, together with a high fat diet, produces a proatherogenic lipid environment directly within the aorta adventitial lipid. Feeding a hyperlipidemic and B vitamin-depleted diet increased total cholesterol and HDL cholesterol accumulation both in the liver and in adventitial lipid. B vitamin deficiency was also associated with an increase in the proportion of saturated and a decrease in monounsaturated fatty acids in both tissues. Folate deficiency alone did not substantially alter lipid metabolism.

Table 4. Plasma markers of inflammation and atherosclerosis in ApoE mice fed a control (C) or high fat (HF) diet depleted of folic acid (F-) or folic acid and vitamins B6 and B12 (F-B-) for 16 weeks. Values are means ± SEM with no. of mice sampled per group in parenthesis. Statistical differences between groups, detected by *t*-test, are shown by superscript letters; mean values with different superscript letters are significantly different, $p < 0.05$

Biomarkers	Control fat			High fat		
	C	C-F	C-F-B	HF	HF-F	HF-F-B
sE selectin (ng/mL)	32.5 ± 3.5^{a} (13)	$37.7 \pm 3.2^{a,b,d}$ (12)	$34.6 \pm 3.5^{a,b}$ (12)	$50.5 \pm 7.5^{b,c}$ (13)	53.3 ± 5.9^{c} (13)	$55.5 \pm 8.1^{c,d}$ (13)
sV CAM 1 (ng/mL)	1275 ± 121^{a} (13)	1335 ± 83^{a} (12)	1248 ± 101^{a} (12)	1422 ± 85^{b} (8)	1448 ± 129^{b} (13)	1571 ± 142^{b} (13)
sI CAM 1 (ng/mL)	23.2 ± 1.5^{a} (13)	23.8 ± 1.0^{a} (12)	20.5 ± 1.6^{a} (12)	35.6 ± 2.2^{b} (13)	38.7 ± 1.5^{b} (13)	34.9 ± 2.0^{b} (13)
IL-1α (ng/mL)	147.1 ± 78.6 (11)	48.7 ± 14.4 (13)	45.2 ± 15.9 (9)	66.8 ± 21.8 (9)	44.0 ± 11.8 (12)	39.1 ± 16.6 (13)
IL-5 (ng/mL)	$23.3 \pm 8.9^{a,b}$ (13)	13.4 ± 2.3^{a} (13)	15.5 ± 2.4^{a} (12)	$35.5 \pm 13.5^{a,b,c}$ (11)	$53.8 \pm 16.9^{b,c}$ (12)	55.5 ± 9.9^{c} (13)
KC (ng/mL)	55.3 ± 23.1 (12)	52.6 ± 16.6 (11)	39.5 ± 9.8 (12)	69.5 ± 20.6 (13)	85.6 ± 26.6 (13)	78.3 ± 33.5 (12)
MCP-1 (ng/mL)	77.3 ± 28.7 (12)	28.2 ± 12.2 (12)	29.2 ± 17.9 (10)	64.9 ± 19.7 (11)	53.8 ± 21.1 (13)	28.5 ± 9.7 (13)
TNF-α (ng/mL)	39.7 ± 12.5 (9)	12.1 ± 5.0 (11)	16.2 ± 6.6 (9)	25.3 ± 11.2 (11)	26.5 ± 9.4 (11)	12.8 ± 4.5 (11)

Surprisingly, combined B vitamin deficiency was associated with lower total lipid levels both in aorta and liver of ApoE mice fed a high fat diet. Hyperhomocysteinemia, while strongly linked with an increased risk of CVD, is not associated with lipid accumulation in the vasculature. Patients with severe hyperhomocysteinemia due to a mutation in the CBS gene typically do not present with conventional lipid-dense atherosclerotic plaques [27]. Similarly, hyperhomocysteinemia is associated with lipid-poor fibrous aortic plaques in patients with severe CVD postmortem [28].

The findings from this study suggest that atherogenesis is dependent on the type of lipid that accumulates rather than on total lipid levels.

Feeding methionine in excess induces hyperhomocysteinemia in rodents and does appear to increase hepatic lipogenesis and, in some cases, plaque formation. However, the data are highly conflicting. Total cholesterol increases twofold after feeding ApoE null mice a Westernized diet [29]. Plasma homocysteine increased two- to threefold after methionine was added to the diet, but circulating lipid levels and plaque formation were unaltered [29]. Feeding methionine concurrently with an atherogenic diet containing high fat, cholesterol, and cholic acid (that increases circulating LDL and VLDL cholesterol and depresses TG) accelerates atherosclerotic plaque formation in C57Bl/6J mice but without further altering the lipid profile [30]. Surprisingly, feeding ApoE null mice folic acid (75 μg/kg/day for 10 weeks) on a normal chow diet background increased total plasma cholesterol (as VLDL and LDL cholesterol), yet reduced atherosclerotic plaque formation [31]. Homocysteine levels were unchanged in this study.

Few studies have examined mechanistically B vitamin and/or homocysteine-mediate changes in lipid metabolism and atherosclerosis. ER stress/protein unfolding or altered methylation capacity may partly explain how B vitamin deficiency and/or hyperhomocysteinemia cause lipid metabolism dysregulation. Exposing human aorta smooth muscle and liver cells in vitro to high homocysteine (1–5 mM for 48 h) increases expression of SREBP-1, a transcription factor that activates enzymes in the cholesterol/TG biosynthetic and uptake pathways [32]. Hyperhomocysteinemia in this system caused cholesterol accumulation intracellularly [32]. Similar findings have been reported in mice fed a hyperhomocysteinemic diet, with increased hepatic lipogenesis (rather than decreased lipoprotein export) causing hepatic cholesterol [32]. Critically, cholesterol is elevated and atherosclerosis accelerated in the aorta of hyperhomocysteinemic CBS and ApoE double knockout mice without dietary manipulation [16].

Phosphatidylcholine (PC) synthesis takes place in the liver by two pathways that are both dependent on the methionine cycle. The first liver-specific pathway involves methylation of phosphatidylethanolamine (PE) to PC by phosphatidylethanolamine N-methyltransferase (PEMT) and is directly dependent on SAM availability. PC is synthesized in the second pathway from choline obtained from the diet or via the PEMT pathway [19, 32]. In our study, B vitamin deficiency significantly decreased hepatic SAM. This effect was aggravated by feeding a high fat diet. Reduced methylation capacity has been shown to impact on lipid metabolism in a small number of rodent studies. Liver SAM is depleted, PC is reduced and PEMT activity is inhibited in hyperhomocysteinemic CBS ± mice fed a low folate/high methionine diet [19]. Crucially, low folate, low SAM, and elevated homocysteine are associated with lipid deposition in the aorta of mice deficient in the folate metabolizing enzyme, MTHFR [33]. In addition to low intracellular SAM directly deregulating lipid metabolism, inadequate provision of SAM may also cause vascular dysfunction by causing aberrant DNA methylation and gene expression [34–36]. Low SAM is associated with methylation silencing and underexpression of Fads2 in CBS ± mice. The enzyme encoded by this gene catalyses metabolism of the long chain fatty acids, linoleic, and linolenic acid to arachidonic and decosahexanoic acid, respectively [19]. Hepatic fatty acid distribution was abnormal in these animals [19].

Finally, we investigated whether induction of a proatherogenic environment in the aorta of ApoE null mice would affect circulating markers of inflammation and vascular dysfunction. Vascular inflammation and atherosclerotic plaque formation are characterized by the release of inflammatory cytokines. However, B vitamin deficiency did not change the circulating levels of any of the inflammatory markers measured, irrespective of fat intake. It remains to be investigated whether B vitamin deficiency can modify an immune response locally, possibly by affecting recruitment and/or cytokine secretion by immunologically active cells directly in the aorta tissue.

In summary, low B vitamin status, hyperhomocysteinemia, and hyperlipidemia all contribute to human CVD risk. A high fat and low B vitamin containing diet, as typically consumed in developed countries, provides a potentially considerable proatherogenic dietary stress. Here, we report that nutritional B vitamin deficiency, together with a high fat diet, promotes atherosclerosis by perturbing lipid metabolism and causing accumulation of proatherogenic lipoproteins (such as cholesterol) in the aorta. B vitamin deficiency was also associated with an increase in the proportion of saturated-free fatty acids and a decrease in monounsaturated fatty acids in this tissue. While these data generated using a mouse model of atherosclerosis must be interpreted carefully the evidence that B vitamin deficiency significantly impacts on lipoprotein deposition directly in the aorta tunica adventitia is strong.

This work was funded by The Scottish Government Rural and Environment Science and Analytical Services Division (RESAS).

The authors have declared no conflict of interest.

5 References

[1] Mendis, S., Puska, P., Norrving, B., (Eds.), Global Atlas on Cardiovascular Disease Prevention and Control, World Health Organization, Geneva 2011.

[2] Kris-Etherton, P. M., Etherton, T. D., Carlson, J., Gardner, C., Recent discoveries in inclusive food-based approaches and dietary patterns for reduction in risk for cardiovascular disease. *Curr. Opin. Lipidol.* 2002, *13*, 397–407.

[3] Boushey, C. J., Beresford, S. A. A., Omenn, G. S., Mutulsky, A. G. et al., A quantitative assessment of plasma homocysteine as a risk factor for vascular disease: probable benefits of increasing folic acid intakes. *JAMA* 1995, *274*, 1049–1057.

[4] Verhaar, M. C., Stroes, E., Rabelink, T. J., Folates and cardiovascular disease. *Arterioscler. Thromb. Vasc. Biol.* 2002, *22*, 6–13.

[5] McNulty, H., Pentieva, K., Hoey, L. Ward, M., Homocysteine, B vitamins and CVD. *Proc. Nutr. Soc.* 2008, *67*, 232–237.

[6] Mudd, S. H., Skovby, F., Levy, H. L., Pettigrew, K. D., The natural history of homocystinuria due to cystathione-B-synthase deficiency. *Am. J. Hum. Genet.* 1985, *37*, 1–31.

[7] Splaver, A., Lamas, G. A., Hennekens, C. H., Homocysteine and cardiovascular disease: biological mechanisms, observational epidemiology and the need for randomised trials. *Am. Heart J.* 2004, *148*, 34–40.

[8] Voutilainen, S., Lakka, T. A., Porkkala-Sarataho, E., Rissanen, T. et al., Low serum folate concentrations are associated with an excess incidence of acute coronary events: the Kuopio Ischaemic Heart Disease Risk Factor Study. *Eur. J. Clin. Nutr.* 2000, *54*, 424–428.

[9] Voutilainen, S., Virtanen, J. K., Rissanen, T. H. Alfthan, G. et al., Serum folate and homocysteine and the incidence of acute coronary events: the Kuopio Ischaemic Heart Disease Risk Factor Study. *Am. J. Clin. Nutr.* 2004, *80*, 317–323.

[10] Durga, J., Bots, M. L., Schouten, E. G., Kok, F. J., Low concentrations of folate not hyperhomocysteinemia are associated with carotid intima-media thickness. *Atherosclerosis* 2005, *179*, 285–292.

[11] Mangoni, A. A., Sherwood, R. A., Asonganyi, B., Swift, C. G. Short-term folic oral acid supplementation enhances endothelial function in patients with type 2 diabetes. *Am. J. Hypertens.* 2005, *81*, 220–226.

[12] Tawakol, A., Migrino, R. Q., Aziz, K. S., Waitkowska, J. et al., High dose folic acid acutely improves coronary vasodilator functions in patients with coronary artery disease. *J. Am. Coll. Cardiol.* 2005, *45*, 1580–1584.

[13] Till, U., Rohl, P., Jentsch, A., Till, H., 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*, 131–135.

[14] Lohn, M., Dubrovska, G., Lauterbach, B., Luft, F. C. et al., Periadventitial fat releases a vascular relaxing factor. *FASEB J.* 2002, *16*, 1057–1063.

[15] Barandier, C., Montani, J.-P., Yang, Z., Mature adiposites and perivasuclar adipose tissue stimulate vascular smooth muscle cell proliferation: effects of ageing and obesity. *Am. J. Physiol. Heart Circ. Physiol.* 2005, *289*, H1807–H1813.

[16] Wang, H., Jiang, X.-H., Yang, F., Gaubatx, J. W. et al., Hyperhomocysteinemia accelerates atherosclerosis and cystathionine b-synthase and apolipoprotein E double knockout mice with and without dietary perturbation. *Blood* 2003, *101*, 3901–3907.

[17] Namekata, K., Enokido, Y., Ishi, I., Nagai, Y. et al., Abnormal lipid metabolism in cystathionine B-synthase-deficient mice, and animal model for hyperhomocysteinemia. *J. Biol. Chem.* 2004, *279*, 52961–52969.

[18] Mikael, L. G., Genest, J., Rozen, R. Elevated homocysteine reduces apolipoprotein A-1 expression in hyperhomocysteinemic mice and in males with coronary artery disease. *Circ. Res.* 2006, *98*, 564–571.

[19] Devlin, A. M., Singh, R., Wade, R. E., Innis, S. M. et al., Hypermethylation of *Fads2* and altered hepatic fatty acid and phospholipid metabolism in mice with hyperhomocysteinemia. *J. Biol. Chem.* 2007, *282*, 37082–37090.

[20] Zhang, S. H., Reddick, R. L., Piedrahita, J. A., Maeda, N., Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science 258*, 468–471.

[21] McNeil, C. J., Beattie, J. H., Gordon, M.-J., Pirie, L. P. et al., Differential effects of nutritional folic acid deficiency and moderate hyperhomocysteinemia on aortic plaque formation and genome-wide DNA methylation in vascular tissue from ApoE -/- mice. *Clin. Epigenet.* 2011, *2*, 361–368.

[22] Duthie, S. J., Grant, G., Pirie, L. P., Watson, A. J. et al., Folate deficiency alters hepatic and colon MGMT and OGG-1 DNA repair protein expression in rats but has no effect on genome-wide DNA methylation. *Cancer Prev. Res.* 2010, *3*, 92–100.

[23] Beattie, J. H., Duthie, S. J., Kwunm, I.-S., Ha, T.-Y., Rapid quantification of aortic lesions in ApoE -/- mice. *J. Vasc. Res.* 2009, *46*, 347–352.

[24] Wang, W., Kramer, P. M., Yang, S., Pereira, M. A., et al., Reversed-phase high performance liquid chromatography procedure for the simultaneous determination of S-adenosyl-L-methionine and S-adenosyl-L-homocysteine in mouse liver and the effect of methionine on their concentrations. *J. Chromatogr. B* 2001, *762*, 59–65.

[25] Folsch, J., Less, M., Sloane Stanley, G. H., A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 1957, *226*, 497–509.

[26] Burdge, G. C., Wright, P., Jones, A. E., Wooton, S. A., A method for the separation of phosphatidylcholine and triacylglycerol, non-esterified fatty acids and cholesterol seters from plasma by solid-phase extraction. *B. J. Nutr.* 2000, *84*, 781–787.

[27] Wilcken, D. E. L., in: Scriver, C. R., Beaudet, A. L., Sly, W. S., Valle, D. et al. (Eds.), *The Metabolic and Molecular Basis of Inherited Disease*. 8th Edn., McGraw Hill, New York, pp. 3987–3933.

[28] Burke, A. P., Fonseca, V., Kolodgie, F., Zieske, A. et al., Increased serum homocysteine and sudden death resulting from coronary atherosclerosis with fibrous plaques. *Arterioscler. Thromb. Vasc. Biol.* 2002, *22*, 1936–1941.

[29] Wilson, K. M., McCaw, R. B., Leo, L., Arning, E. et al., Prothrombotic effects of hyperhomocysteinemia and

hypercholesterolemia in ApoE-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 2007, *27*, 233–240.

[30] Zhou, J., Werstruck, G. H., Lhotak, S., Shi, Y. Y. et al., Hyperhomocystenemia induced by methionine supplementation does not independently cause atherosclerosis in C57BL/6J mice. *FASEB J.* 2008, *22*, 2569–2578.

[31] Carnicer, R., Navarro, M. A., Arbones-Mainar, J. M., Acin, S. et al., Folic acid supplementation delays atherosclerotic lesion development in ApoE-deficient mice. *Life Sci.* 2007, *80*, 638–643.

[32] Werstruck, G. H., Lentz, R. S., Dayal, S., Hossain, G. S. et al., Homocysteine-induced endoplasmic reticulum stress causes dysregulation of the cholesterol and triglyceride biosynthetic pathways. *J. Clin. Invest.* 2001, *107*, 1263–1273.

[33] Chen, Z., Karaplis, A. C., Ackerman, S. L. Pogribny, I. P. et al., Mice deficient in methylenetetrahydrofolate reductase exhibit hyperhomocysteinemia and decreased methylation capacity with neuropathology and aortic lipid deposition. *Hum. Mol. Genet.* 2001, *10*, 433–443

[34] Dong, C., Yoon, W., Goldschmidt-Clermont, P. J., DNA methylation and atherosclerosis. *J. Nutr.* 2002, *132*, 2406S–2409S.

[35] Zaina, S., Lindholm, M. W., Lund, G., Nutrition and aberrant DNA methylation patterns in atherosclerosis: more than just hyperhomocystenemia? *J. Nutr.* 2005, *153*, 5–8.

[36] Turunen, M. P., Aavik, E., Yla-Herttula, S., Epigenetics and atherosclerosis. *Biochim. Biophys. Acta.* 2009, *1790*, 886–891.

