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RESEARCH PAPER

Nutritional B vitamin deficiency alters the expression of key proteins associated with vascular smooth muscle cell proliferation and migration in the aorta of atherosclerotic apolipoprotein E null mice

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Abstract Low B vitamin status is linked with human vascular disease. We employed a proteomic and biochemical approach to determine whether nutritional folate deficiency and/or hyperhomocysteinemia altered metabolic processes linked with atherosclerosis in ApoE null mice. Animals were fed either a control fat (C; 4 % w/w lard) or a high-fat [HF; 21 % w/w lard and cholesterol (0/15 % w/w)] diet with different B vitamin compositions for 16 weeks. Aorta tissue was prepared and global protein expression, B vitamin, homocysteine and lipoprotein status measured. Changes in the expression of aorta proteins were detected in response to multiple B vitamin deficiency combined with a high-fat diet ($P < 0.05$) and were strongly linked with lipoprotein concentrations measured directly in the aorta adventitia ($P < 0.001$). Pathway analysis revealed treatment effects in the aorta-related primarily to cytoskeletal organisation, smooth muscle cell adhesion and invasiveness (e.g., fibrinogen, moesin, transgelin, vimentin). Combined B vitamin deficiency induced striking quantitative changes in the expression of aorta proteins in atherosclerotic ApoE null mice. Deregulated expression of these proteins is associated with human atherosclerosis. Cellular pathways altered by B vitamin status included cytoskeletal organisation, cell differentiation and migration, oxidative stress and chronic inflammation. These findings provide new insight into the molecular mechanisms through which B vitamin deficiency may accelerate atherosclerosis.

Keywords Aorta proteome · ApoE null mice · Atherosclerosis · B vitamins · Hyperhomocysteinemia

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Introduction

Low dietary folate is strongly associated with an increased risk of human vascular diseases including atherosclerosis and stroke (Voutilainen et al. 2000, 2004; Durga et al. 2005). This has been attributed primarily to suboptimal intracellular folate, together with low levels of vitamins B_{12} and B_6, inducing hyperhomocysteinemia (Splaver et al. 2004). However, there is evidence for folate alone influencing human vascular disease. High circulating folate is associated with reduced risk of primary coronary events and carotid intima-media thickness independently of total plasma homocysteine in several prospective human studies (Voutilainen et al. 2000, 2004; Durga et al. 2005). Moreover, supplemental folic acid positively influences vascular function in patients with, or at risk of, vascular disease (Mangoni et al. 2005; Tawakol et al. 2005; Till et al. 2005). These effects appear largely independent of homocysteine.

Determining whether low folate or hyperhomocysteinemia is causal for vascular disease in vivo is difficult due to the intimate metabolic relationship linking folate and

homocysteine. Transgenic mouse strains provide a representative model of human atherosclerosis and allow complete investigation of the effect of diet on vascular disease progression. The ApoE knockout mouse spontaneously develops atherosclerotic plaques that are morphologically analogous to lesions found in human tissue (Zhang et al. 2002). We have developed distinct nutritional deficiencies in transgenic ApoE null mice so that they exhibit either mild or moderate hyperhomocysteinemia (McNeil et al. 2011). We have shown that B vitamin deficiency increases atherosclerotic plaque formation in transgenic ApoE knockout mice and that this is strongly linked with accumulation of cholesterol and pro-atherogenic lipoproteins in the adventitial lipid surrounding the aorta (McNeil et al. 2011, 2012).

Proteomic technologies have been widely used to identify quantitative changes in protein expression as a consequence of several human vascular pathologies including atherosclerosis, aorta medial degeneration and myocardial revascularisation (Wu et al. 2007; Delbosc et al. 2008; Farina et al. 2010). Moreover, they have proven to be powerful tools in rodents studies for providing mechanistic insight into how vascular disease progresses and is influenced by environmental factors such as diet (Mayr et al. 2005; Almofti et al. 2006).

Here, we employed a combined biochemical and quantitative global proteomics approach to determine how prolonged nutritional folate deficiency and/or moderate hyperhomocysteinemia differentially modulates global protein expression directly in the aorta of ApoE null mice and to further identify whether these changes are associated with proteins and processes involved in the progression of human atherosclerosis.

Materials and methods

Animal study and diets

The design of this animal study and the composition of the experimental diets employed have been described in detail elsewhere (McNeil et al. 2011, 2012). All procedures were carried out in accordance with the requirements of the UK Animals (Scientific Procedures) Act 1986. ApoE null mice (males, 5 weeks old) were allocated (by weight) to one of six treatment groups ($n = 10$ animals per group). Diets were based on either a control [C; 4 % w/w lard] or high-fat [HF; 21 % w/w lard and cholesterol (0.15 % w/w)] background with different B vitamin compositions applied to each (1) folic acid and B vitamin replete, (2) folic acid depleted (F–), or (3) folic acid, B_6 and B_{12} depleted (F–B–). Mice were fed these diets for 16 weeks.

Blood and aorta collection

Mice were fasted up to 6 h and killed by exsanguination under terminal anaesthesia. Blood was collected from the vena cava for analysis of B vitamins, homocysteine and lipoproteins. Residual blood was flushed from the heart by injection of Dulbecco's modified Eagles medium (DMEM) into the left ventricle. The complete aorta was microdissected of tunica adventitia. The thoracic and abdominal aorta and the dissected adventitia were snap-frozen and stored at −80 °C for proteomic and lipid analysis. The aortic arch was fixed in 10 % formalin and atherosclerotic plaque volume quantified by staining plaques in situ with the neutral lipid-targeting lysochrome Oil Red O (ORO, Sigma, Poole, UK), which targets fatty acid deposits in the arterial plaques and solubilising and measuring spectrophotometrically the dye retained in the aorta. Solubilised ORO as a biomarker of atherosclerotic plaque volume was corrected for aorta tissue weight (Beattie et al. 2009; McNeil et al. 2011, 2012).

Preparation of aorta tissue for proteomic analyses

Weighed frozen aorta (approx. 10 mg) was added to extraction buffer containing 7 M urea, 2 M thiourea, 4 % CHAPS and 2 % BioRad Biolite Ampholyte pH 3–10 and homogenised using a Precellys 24 lysis and homogenisation cycler (Stretton Scientific, UK). The homogenate was centrifuged at 14,000 rpm for 5 min at 4 °C and the supernatant frozen at −80 °C. Protein was measured using the BioRad RC DC protein assay.

2D gel electrophoresis of aorta tissues

One 2D gel was run per aorta (100 μg) sample ($n = 10$ mice per treatment group; 60 gels in total). Proteins were separated by isoelectric focusing in the first dimension [BioRad immobilised pH gradient (IPG) strips (pi range 3–10)] and SDS-PAGE in the second dimension on 8–16 acrylamide gels (18 × 18 cm) as described previously (Duthie et al. 2008). Gels were stained with Coomassie blue and imaged on a BioRad GS710 flat bed imager followed by image analysis using PD Quest version 8.0.1. Spots were excised from the gels using a robotic BioRad spot cutter, trypsinised in a MassPrep Station (Waters, MicroMass, Manchester, UK) and analysed by LC MS/MS using an Ultimate nano LC capillary chromatography system (LC Packings, Camberly, Surrey, UK), combined with an Applied Biosystems 2000 Q-Trap (Warrington, UK). Peak list were generated using Analyst 1.4.2 software set at default. Peptide fragment mass spectra yielded a sequence of separated peptides that were pasted into the fingerprinting web resource programme Mascot dll version 1.6b9 (Matrix Science Ltd, Boston, MA)

for protein identification. Protein identification was made using SWISS-PROT (version 2012.09) specifically for *Mus musculus* (mouse), with expectation values, cut-off scores and threshold values for individual proteins considered. Protein confidence parameters including Mowse scores, expected and actual masses, PI, number of unique peptides matched, percentage sequence coverage and gene and protein accession numbers, together with protein function, are shown for each identified peptide in the experimental results (Supplementary Table 1).

B vitamin and lipoprotein analyses

Blood and tissue folate and B_{12} were measured by radioassay and plasma total homocysteine by gas chromatography (McNeil et al. 2011, 2012). Lipid was extracted from adventitial tissue (approx. 200 mg) (Folch et al. 1957) and total cholesterol, LDL cholesterol, HDL cholesterol, TG and NEFA fractions measured in plasma and aorta adventitial lipid using a Konelab 20 Clinical Chemistry Analyser (Thermo Scientific, Passau, Germany). Fatty acids were trans-esterified to methyl esters (FAMEs) in methanolic HCL, separated by liquid–liquid solvent extraction and measured by gas–liquid chromatography (GLC; Burdge et al. 2000).

Statistical analysis

For proteomic analysis, 2D gel images were studied using PDQuest software (BioRad). After normalisation and matching, spot densities were exported for data analysis (multivariate) using Excel and "R" (R Foundation for Statistical Computing, Vienna, Austria; version 2.10). Log transformed spot densities from aorta tissue were analysed by two-way ANOVA for the effect of fat intake, vitamin composition and interaction between these factors ($P < 0.05$). These analyses provide quantitative protein expression data, as densitometry of the Coomassie-stained spots is carried out for each animal in each treatment group individually prior to statistical analysis.

Biochemical data were analysed by two-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. Analysis of associations among aorta tissue protein expression and blood B vitamin status markers, blood and vascular lipids, and atherosclerotic plaque volume was carried out for all mice in all treatment groups using Pearson's correlation coefficients. Spots with densities that differed significantly either due to treatment ($P < 0.05$) or were highly correlated with B vitamin and lipid status at the end of the study ($P < 0.001$) were excised from the gel and identified by LC MS/MS. Association networks between biochemical biomarkers and protein expression were displayed using Cytoscape software (version 2.8.3; http://www.cytoscape.org). Principal component analysis (PCA) of the physiological biomarkers measured from each mouse in the study was carried out and the component scores calculated.

Results

B vitamin, homocysteine, lipid status and aorta plaque volume

Principal component analysis (PCA) of biochemical data for all animals revealed that the treatment phenotypes

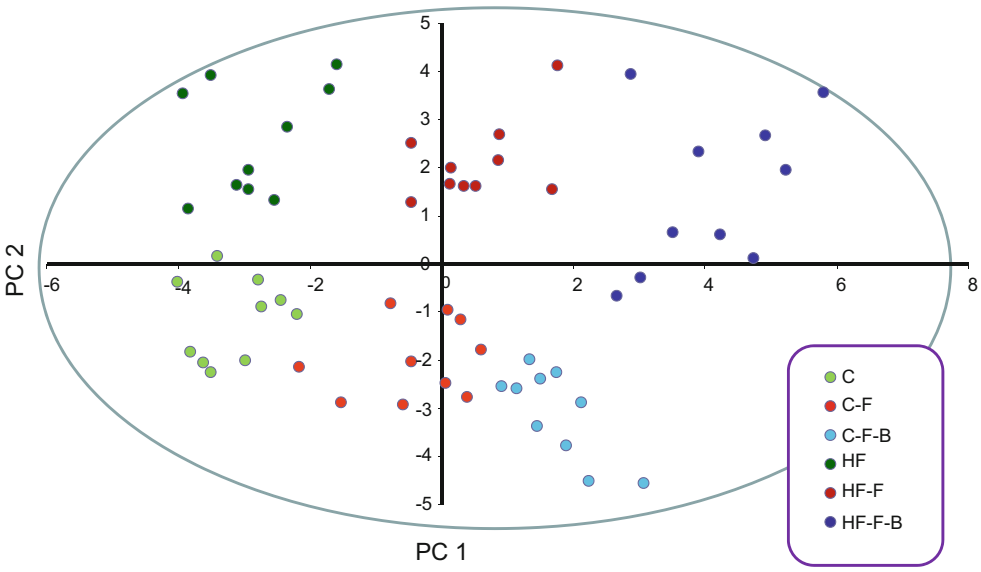

Fig. 1 Primary component analysis (PCA) score plot of blood and tissue biomarkers significantly altered by folic acid, B vitamins and fat intake in ApoE null mice. Mice were fed a control (*C*) or high-fat (*HF*) diet depleted of folic acid (F–) or folic acid and vitamins B_6 and B_{12} (F–B–) for 16 weeks. *Symbols* represent individual replicate animals within every treatment group

Table 1 The effect of folic acid, B vitamins and dietary fat intake on B vitamin, homocysteine and plasma and aorta adventitial lipid status in ApoE null mice

Biomarkers	Control fat			High fat		
	C	C–F	C–F–B	HF	HF–F	HF–F–B
B vitamin status						
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 ± 1.7^{b}	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 B_{12} (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 B_{12} (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}
Plasma lipids						
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}
Aorta adventitial lipids						
Total cholesterol (mmol/g lipid)	35.1 ± 4.7^{a,c}	24.1 ± 2.1^{b}	33.0 ± 3.0^{a}	43.1 ± 4.6^{a,c}	53.4 ± 8.4^{c,d}	73.4 ± 9.1^{d}
HDL cholesterol (mmol/g lipid)	ND	ND	ND	1.0 ± 1.0	1.6 ± 1.4	6.1 ± 4.1
NEFA (mmol/g lipid)	6.9 ± 0.9^{a}	5.8 ± 0.8^{a}	7.5 ± 1.3^{a,b}	7.5 ± 0.8^{a,b}	9.1 ± 1.4^{a,b}	10.2 ± 1.0^{b}
Triglycerides (TG) (μmol/g lipid)	919.0 ± 35.0^{a,b}	849.0 ± 58.7^{a,c}	880.6 ± 45.3^{a,b,c}	856.9 ± 61.6^{a,b,c}	1,032.7 ± 63.1^{b}	798.7 ± 23.1^{c}
SATFAs (% total fat)	31.14 ± 1.15^{a,b}	30.84 ± 0.69^{a}	33.38 ± 0.84^{b}	27.84 ± 0.59^{c}	28.81 ± 0.46^{c}	33.89 ± 0.49^{b,d}
MUFAs (% total fat)	47.38 ± 0.08^{a,b}	47.36 ± 0.44^{a,b}	46.11 ± 0.49^{a}	48.33 ± 0.67^{b}	48.60 ± 0.55^{b,c}	42.45 ± 0.69^{d}
PUFAs (% total fat)	20.76 ± 1.02^{a,b}	21.10 ± 0.70^{a,b}	19.56 ± 0.84^{a}	22.53 ± 0.54^{b}	21.77 ± 0.30^{b,c}	22.61 ± 0.64^{b,d}
Aorta plaque volume (nM/mg tissue)	0.274 ± 0.014^{a}	0.260 ± 0.018^{a}	0.261 ± 0.007^{a}	0.556 ± 0.024^{b}	0.637 ± 0.027^{c}	0.602 ± 0.030^{b,c}

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Mice were fed a control (C) or high-fat (HF) diet depleted of folic acid (F–) or folic acid and vitamins B_6 and B_{12} (F–B–) for 16 weeks. Values are mean ± SEM for $n = 9$–10 mice sampled per group. Biochemical data were analysed by two-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. Data not sharing superscript letters differ by $P < 0.05$. *B_{12}* vitamin B_{12}, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *MUFAs* monounsaturated fatty acids, *PUFAs* polyunsaturated fatty acids, *NEFA* non-esterified fatty acids, *SATFAs* saturated fatty acids

separated differentially according to fat intake and folate and B vitamin treatment (Fig. 1). Aortic plaque volume, blood and lipid biomarkers are shown in Table 1. Folate was depleted more than 65 % in mice fed a folate-deficient diet. B_{12} decreased more than 70 % in animals fed the combined folate and B vitamin-depleted diet. Folate deficiency increased homocysteine twofold. Homocysteine was elevated further in mice fed a combined B vitamin-depleted diet (approx. fourfold). B_{12} depletion and hyperhomocysteinemia were exacerbated by high fat. Total cholesterol was increased in aorta adventitial lipid in response to a high-fat diet. Plasma cholesterol, HDL and LDL were elevated (approx. twofold). Folate depletion alone did not impact on circulating or adventitial lipids. However, feeding a combined folate and B vitamin-deficient diet exacerbated cholesterol accumulation and significantly reduced MUFA and increased pro-atherogenic SATFAs in the aorta adventitial lipid. Plaque volume was increased almost twofold ($P < 0.0001$) in mice fed a HF diet for 16 weeks when compared with animals on a control diet (Table 1). Plaque volume was significantly increased (17 %; $P < 0.05$) in mice depleted of folic acid. Imposing a combined B vitamin deficiency did not increase plaque development further (Table 1).

Atherosclerotic plaque abundance was correlated strongly with circulating and vascular lipid levels, and weakly with plasma vitamin B_{12} (Table 2). Plaque formation did not correlate directly either with blood folate or homocysteine. However, whole blood and plasma B_{12} did strongly influence both circulating and adventitial lipid concentrations, with low B_{12} associated with high levels of plasma TG, adventitial total cholesterol and SATFAs and,

conversely, a lower proportion of MUFAs. Low folate was similarly associated with accumulation of SATFAs in the aorta adventitia. Hyperhomocysteinemia was positively associated with elevated SATFA and reduced MUFA concentrations in the adventitia (Table 2).

Aorta tissue proteomics

Comparative quantitative proteomics revealed approximately 800 spots in the aorta samples that matched across all 60 treatment gels. The primary proteins (approx. 35 % of total identified proteins) influenced by treatment in the aorta were involved in regulating cytoskeletal organisation, cell adhesion and cell motility (Fig. 2). Mean (and group SEM) spot densities for aorta proteins that differed between treatment groups ($P < 0.05$) are shown in Table 3. The independent effects of fat or vitamin treatment on protein expression are described (P values) together with the synergistic effect of a HF and combined vitamin-depleted diet. The effect of B_6 and B_{12} depletion, above folate deficiency alone is also shown. Vitamin depletion alone altered expression of only six aorta proteins [fibrinogen, gelsolin, glutamate dehydrogenase 1, lactate dehydrogenase and talin-1 (all upregulated) and peptidyl-prolyl *cis–trans* isomerise A (downregulated)]. Feeding a combined vitamin-deficient and high-fat diet had a strong synergistic effect on 25 aorta proteins (including dermatopontin, fibrinogen, glutathione *S*-transferase Mu 1, peroxiredoxin-2, serotransferrin, transgelin and vimentin; Table 3).

Correlation analysis revealed strong ($P < 0.001$; mean $q = 0.03$) associations between lipid and vitamin status, and aorta protein expression (Table 4). Protein expression in the aorta was exclusively correlated with aorta adventitial lipid levels (total fat, cholesterol, HDL, NEFA) and with the proportion of saturated and monounsaturated fat. Figure 3 constructed from biochemical and aorta protein correlations demonstrates the impact that B vitamins, homocysteine and methionine cycle intermediates have on lipoprotein accumulation directly in aorta adventitial lipid (e.g., increased SATFAs, decreased MUFAs, increased total cholesterol) and the influence that aorta lipid levels have on the expression of more than 80 aorta proteins (identified by spot number in Supplementary Table 1).

Table 2 Correlation analyses of B vitamin, lipid status and atherosclerotic plaque formation in ApoE null mice

Biomarker		Correlation	*P* value
Plaque associations			
Plaque abundance	Plasma HDL	0.763	8E−12
Plaque abundance	Plasma LDL	0.736	5E−10
Plaque abundance	Plasma total cholesterol	0.708	1.3E−08
Plaque abundance	Adventitia total cholesterol	0.586	2.1E−06
Plaque abundance	Adventitia HDL	0.446	0.00055
Plaque abundance	Plasma TG	−0.427	0.00106
Plaque abundance	Adventitia SATFA	0.374	0.00526
Plaque abundance	Plasma B_{12}	−0.271	0.04303
B vitamin status and lipid metabolism			
Adventitia total cholesterol	Plasma B_{12}	−0.339	0.00802
Adventitia SATFA	Whole blood B_{12}	−0.632	1.3E−07
Adventitia SATFA	Plasma homocysteine	0.534	1.9E−05
Adventitia SATFA	Plasma B_{12}	−0.502	6.8E−05
Adventitia SATFA	Plasma folate	−0.396	0.00231
Adventitia MUFA	Plasma homocysteine	−0.564	4.8E−06
Adventitia MUFA	Plasma B_{12}	0.456	0.00037
Adventitia MUFA	Whole blood B_{12}	0.445	0.00052

Analysis of associations between blood B vitamin status markers and blood, vascular lipids and plaque formation were carried out for all mice in all treatment groups using Pearson's correlation coefficients. Biomarker and strength of associations (Pearson's correlation coefficient; positive and negative) are shown together with the corresponding P value. *B_{12}* vitamin B_{12}, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *MUFAs* monounsaturated fatty acids, *PUFAs* polyunsaturated fatty acids, *NEFA* non-esterified fatty acids, *SATFAs* saturated fatty acids

Discussion

The primary aim of this study was to examine and quantify the differential effects of folate and/or B vitamin depletion on global protein expression in the aorta of ApoE mice and to consider the role of these proteins on cellular processes implicated in human vascular disease. Feeding a folate and/or B vitamin depleted diet against a control fat background altered expression of only very few aorta protein [fibrinogen, gelsolin, lactate dehydrogenase, talin (upregulated) and peptidyl-prolyl *cis–trans* isomerise (decreased)]. However, when combined with a major dietary challenge (a high-fat diet), B vitamin depletion strongly perturbed many more processes [including oxidative stress, inflammation and vascular smooth muscle cell (VSMC) activation and migration] causally associated both with rodent and human atherosclerosis (see Fig. 2). Suboptimum folate status and hyperhomocysteinemia are linked with hyperlipidemia and oxidative stress, major risk factors for vascular disease (Kris-Etherton et al. 2002; Lohm et al. 2002; Mikael et al. 2006; Devlin et al. 2007). Here, B vitamin depletion was associated with significant changes in vascular adventitial lipid metabolism and protein markers of

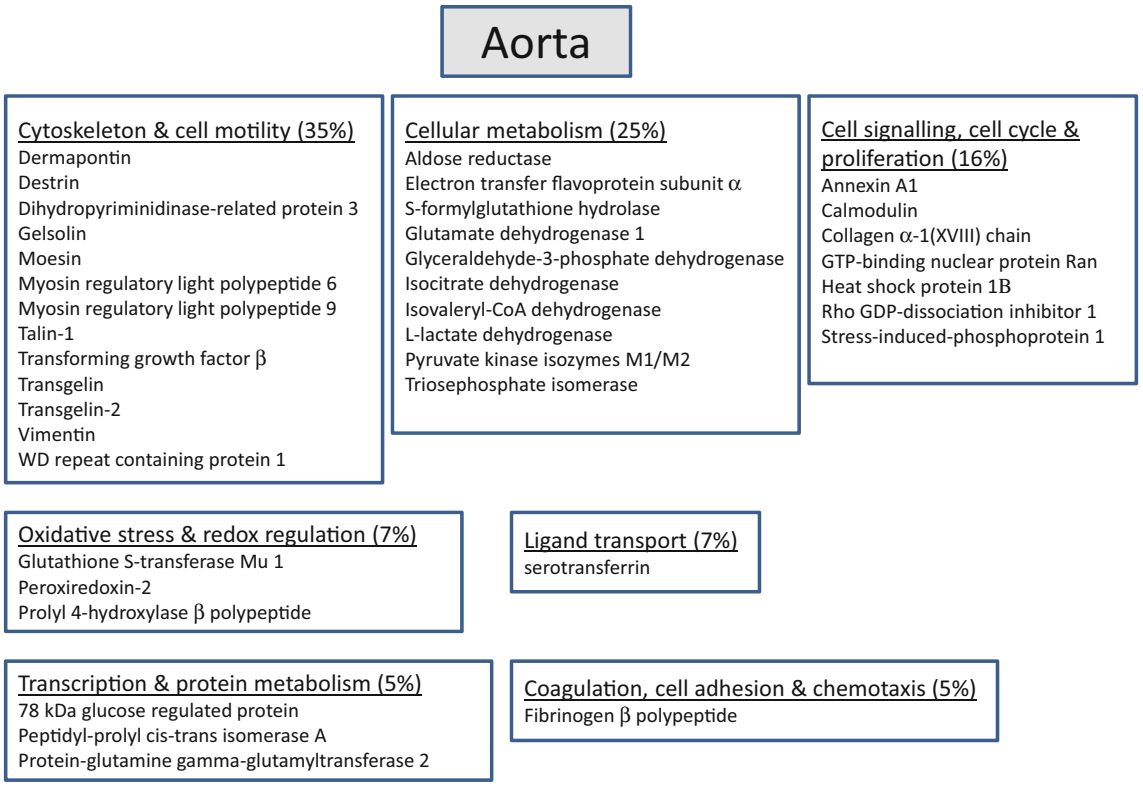

Fig. 2 Proteins and major cellular pathways altered by folic acid, B vitamins and fat intake in aorta from ApoE null mice. All proteins (influenced by treatment and associated with B vitamin and lipid status at the end of intervention), together with primary cellular function are presented as percentage of total identified proteins

cellular stress known to be altered in atherosclerosis (Brownlee 2001; Vikramadithyan et al. 2005) only when combined with this additional oxidative insult. Glutathione *S*-transferase Mu was elevated, as was aldose reductase, a protein that induces atherosclerosis in mice (Vikramadithyan et al. 2005) and is upregulated in human foam cells and plaque macrophages (Gleissner et al. 2008). Peroxiredoxin expression was decreased in this study. Low peroxiredoxin expression is associated with atherosclerosis in mice and humans (Gleissner et al. 2008). Elevated serotransferrin is linked with oxidative stress and chronic disease risk. Multiple aorta spots were identified as serotransferrin in this study and feeding a high-fat/B vitamin-depleted diet increased expression of each of these ($P < 0.05$). Moreover, serotransferrin expression was positively associated with total cholesterol and HDL accumulation in the adventitia and negatively linked with monounsaturated fatty acid concentration. Collective changes in these proteins indicate an upregulated metabolic response to hyperlipidemia in the aorta and an increased requirement to resist vascular ROS generation.

Inflammation and accelerated growth and migration of VSMC are critical in the progression of atherosclerosis. Here, combined B vitamin depletion altered expression of multiple protein molecular markers indicative both of a pro-inflammatory environment and a pro-proliferative VSMC phenotype. Annexin A1 (lipocortin), a potent anti-inflammatory agent (Perretti and Dalli 2009) is present in human atherosclerotic plaques, with low protein levels indicative of advanced disease (Cheuk and Cheng 2011). Lipocortin was negatively associated with adventitial cholesterol accumulation. Atherosclerosis-driven inflammation increases expression of acute phase proteins including fibrinogen, C-reactive protein and serotransferrin. Hyperfibrinogenemia is a marker for advanced human atherosclerosis and a predictor for cardiovascular events (Kofoed et al. 2003; Sabeti et al. 2005). Protein-glutamine gamma-glutamyltransferase 2 (fibrinogen-stabilising factor) promotes fibrin deposition within the arterial lumen and vascular endothelial cell dysfunction, VSMC proliferation and atherosclerosis (Smith 1995). Here, fibrinogen and fibrinogen-stabilising factor protein expression increased and was positively linked with total and HDL cholesterol and with reduced MUFA concentrations. Specific heat shock proteins (including Hsp60 and Hsp70) are elevated in early human cardiovascular disease and are predictive for atherosclerotic progression (Xu 2002; Pockley et al. 2003). Hsp70 (identified here as 78 kDa

Table 3 Aorta proteins significantly altered in response to folic acid, B vitamins and fat intake

SSP	C	C F-	CF-B-	HF	HF F-	HF F-B-	SEM (group)	Protein	Treatment Effect (P value)				
									1	2	3	4	5
409	169199	131778	127946	129378	185040	285012	29955	Serotransferrin	0.025		0.008		0.0113
410	91893	71044	68660	58533	84969	117914	14399	Serotransferrin			0.022		
411	129200	119989	77969	78473	99832	190848	26757	Glyceraldehyde-3-phosphate dehydrogenase			0.009		0.0102
438	58942	109474	80501	72767	64377	70374	10918	Moesin			0.033		
479	492999	342549	398663	304003	376391	467241	51955	WD repeat-containing protein 1			0.035		
503	153085	110359	165007	106566	146999	155681	16490	Stress-induced-phosphoprotein 1			0.051	0.0449	
524	498232	464730	538282	530668	587944	614039	33763	Pyruvate kinase isozymes M1/M2	0.008				
539	656381	727487	702774	856387	950910	901150	91547	Prolyl 4-hydroxylase β polypeptide	0.008				
557	328576	294149	308150	296549	359980	516807	29077	Fibrinogen β polypeptide	0.001	0.002	0.001		0.0001
575	22136	12396	34560	11979	11998	22247	5854	Talin-1		0.024			
604	548680	476027	574233	551877	583150	666979	32718	Glutamate dehydrogenase 1	0.015	0.020			0.0384
657	229799	178195	253643	207606	207407	253802	20119	Gelsolin		0.015			
682	245727	194803	197442	200100	203449	227977	14725	Serotransferrin			0.037		
711	108825	77684	79255	65819	83371	96248	8896	Isovaleryl-CoA dehydrogenase			0.004		
752	2242222	2015031	2467761	2401648	2355783	1722231	208096	Vimentin			0.027		
772	928923	790189	805569	735218	903424	904891	63386	Aldose reductase			0.031	0.0335	
812	252143	233199	229379	235829	257636	298407	16480	Electron transfer flavoprotein subunit α			0.044		0.0435
813	290271	211098	234300	210192	246316	272842	21596	S-formylglutathione hydrolase			0.012		
875	77106	94554	107753	88791	85480	73829	6916	Rho GDP-dissociation inhibitor 1			0.008		
887	18953	27727	23519	23528	18234	25198	2521	Glutathione S-transferase Mu 1			0.019		0.0285
890	131019	178025	164288	144794	105335	135759	14158	Collagen α-1(XVIII) chain	0.015		0.014	0.0275	
892	334883	303318	273171	338496	322572	375678	25266	Triosephosphate isomerase	0.049				
914	387578	703301	716437	634069	556591	473778	100735	Vimentin			0.046		
924	120507	111070	129078	108301	126114	91251	8964	Peroxiredoxin-2			0.046		0.0013
933	169080	115685	60049	58594	58341	132509	36678	Transgelin-2			0.046		
938	720961	1034511	1301660	1226349	940180	864543	152056	Vimentin			0.012		
1060	267500	403249	582440	409082	483310	235974	74044	Transgelin			0.003		
1085	3094843	3518178	4007414	3367393	2758845	2390348	308204	Myosin regulatory light polypeptide 9	0.008		0.014		
1107	777311	673830	770344	569560	576034	599060	65528	Destrin	0.005				
1114	869818	728904	790691	939706	784068	750674	64394	Peptidyl-prolyl cis-trans isomerase A		0.049		0.0472	
1123	1644039	1653905	2488633	3304958	2542275	2236073	417555	Calmodulin	0.030				
1125	3441316	4658617	3970620	4648551	3186374	2841366	537017	Transgelin			0.033	0.0303	
1126	3441316	4658617	3970620	4648551	3186374	2841366	537017	Myosin light polypeptide 6			0.033		
1169	1765177	1996358	2467280	2705766	2535734	1795372	301493	Dermatopontin			0.028		0.0447
1186	178428	126071	178951	184640	165308	239859	23082	L-lactate dehydrogenase		0.029			0.0136

Spot number, protein ID, mean spot density ± SEM (group mean) and level of significance (*P*) for each protein affected by treatment is shown for $n = 10$ mice per group. Log transformed spot densities from aorta tissue were analysed by two-way ANOVA for the effect of fat intake, vitamin composition and interaction between these factors ($P < 0.05$). ANOVA with post hoc testing was carried out to establish the differential effects of folic acid, B vitamin and fat intake on protein expression. Green represents upregulated proteins and red represents downregulated proteins, respectively. 1; fat status, 2; vitamin status, 3; fat and vitamin status, 4; HF versus HF–F, 5; HF–F versus HF–F–B, where C; control, HF; high fat, F–; diet depleted of folic acid, F–B–; diet depleted of folic acid and vitamins B_6 and B_{12}

Table 4 Associations (correlation analyses) between aorta lipid and aorta protein expression

Biomarker	Spot	Protein	Correlation	*P* value
Adventitia total cholesterol	829	Annexin A1	−0.500	4.69E−05
Adventitia total cholesterol	582	Protein-glutamine gamma-glutamyltransferase 2	0.413	0.00102
Adventitia total cholesterol	912	Isocitrate dehydrogenase	0.418	0.00088
Adventitia total cholesterol	524	Pyruvate kinase isozymes M1/M2	0.421	0.00079
Adventitia total cholesterol	468	78 kDa glucose-regulated protein	0.424	0.00072
Adventitia total cholesterol	473	Heat shock protein 1B	0.450	0.00030
Adventitia total cholesterol	604	Glutamate dehydrogenase 1	0.472	0.00013
Adventitia total cholesterol	557	Fibrinogen b polypeptide	0.484	9.09E−05
Adventitia total cholesterol	812	Electron transfer flavoprotein subunit α	0.495	5.85E−05
Adventitia total cholesterol	409	Serotransferrin	0.545	6.68E−06
Adventitia HDL	557	Fibrinogen b polypeptide	0.461	0.00020
Adventitia HDL	468	78 kDa glucose-regulated protein	0.556	4.02−06
Adventitia HDL	409	Serotransferrin	0.676	3.14E−09
Adventitia HDL	473	Heat shock protein 1B	0.687	1.30E−09
Adventitia NEFA	914	Vimentin	−0.451	0.00030
Adventitia NEFA	772	Aldose reductase	0.414	0.00101
Adventitia NEFA	812	Electron transfer flavoprotein subunit α	0.443	0.00038
Adventitia NEFA	468	78 kDa glucose-regulated protein	0.471	0.00014
Adventitia NEFA	524	Pyruvate kinase isozymes M1/M2	0.472	0.00014
Adventitia NEFA	604	Glutamate dehydrogenase 1	0.487	8.32E−05
Adventitia NEFA	912	Isocitrate dehydrogenase	0.497	5.45E−05
Adventitia NEFA	909	GTP-binding nuclear protein Ran	0.573	1.71E−06
Adventitia MUFA	557	Fibrinogen β polypeptide	−0.467	0.00024
Adventitia MUFA	410	Serotransferrin	−0.457	0.00035
Adventitia MUFA	503	Stress-induced-phosphoprotein 1	−0.429	0.00085
Adventitia SATFA	1,125	Transgelin	−0.512	4.72E−05

Analysis of associations between aorta tissue protein expression and vascular lipids was carried out for all mice in all treatment groups using Pearson's correlation coefficients. Lipid biomarker, spot number, protein name and strength of association (positive and negative) are shown together with the corresponding *P* value. *B_{12}* vitamin B_{12}, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *MUFAs* monounsaturated fatty acids, *PUFAs* polyunsaturated fatty acids, *NEFA* non-esterified fatty acids, *SATFAs* saturated fatty acids

glucose-related proteins and Hsp 1B) and stress-induced phosphoprotein 1 (a modulator of Hsp70 activity) were positively associated with total and HDL adventitial cholesterol and negatively linked with MUFA concentration. Rho GDP dissociation inhibitor 1 negatively regulates VSMC adhesion and enhances migration (Qi et al. 2008). Here, feeding a high-fat combined B vitamin-depleted diet significantly downregulated Rho GDP dissociation inhibitor 1 expression in mouse aorta. Conversely, GTP-binding nuclear protein Ran expression is positively associated with VSMC migration (Fuastino et al. 2010). GTP-binding nuclear protein Ran expression was upregulated in this experiment. Actin assembly and disassembly drives vascular cytoskeletal organisation and regulates VSMC phenotype. Many aorta proteins (including transgelin, vimentin, dermatopontin, collagen, moesin, myosin regulatory light polypeptide 6 and 9, and talin) that influence actin filament regulation, cell differentiation, cell adhesion and cell migration were altered by treatment in this study. Reduced expression of transgelin-1 (sm22α) is related to VSMC proliferation and atherosclerotic plaque formation (Feil et al. 2004; Shen et al. 2010). Transgelin expression was strongly downregulated in the aorta of mice fed a high-fat and combined B vitamin-depleted diet. Moreover, a strong negative relationship between transgelin and saturated fatty acid accumulation in adventitial lipid was observed. Overexpression of gelsolin increases vascular endothelial cell motility (Silacci et al. 2003). Gelsolin was

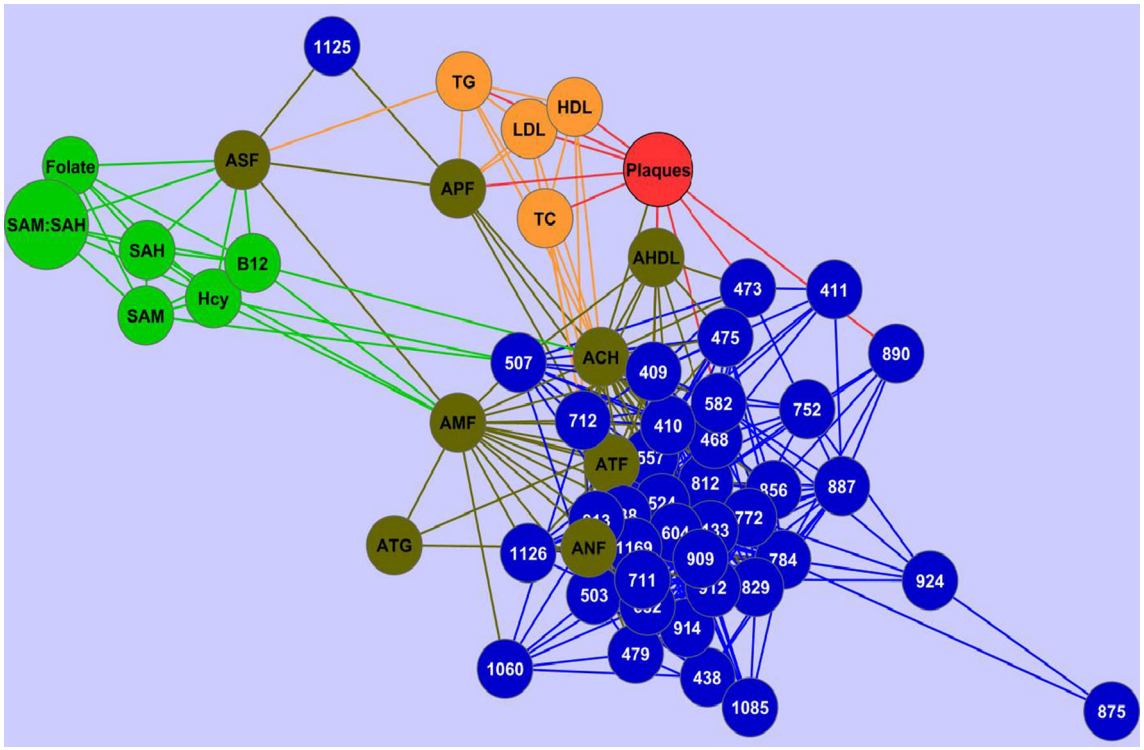

Fig. 3 Association network 1 (*ball* and *stick*) of aorta proteins influenced by B vitamin and fat intake in ApoE null mice. Association networks between biochemical biomarkers and protein expression were displayed using Cytoscape software (version 2.8.3; http://www.cytoscape.org). *Lines* connecting proteins and biomarkers indicate a correlation with $R > 0.3$ or <-0.3. *ACH* adventitial total cholesterol, *AHDL* adventitial high-density lipoprotein, *AMF* adventitial monounsaturated fatty acid, *ANF* adventitial non-esterified fatty acid, *APF* adventitial polyunsaturated fatty acid, *ASF* adventitial saturated fatty acid, *ATF* adventitial total fat, *ATG* adventitial triglyceride, B_{12} vitamin B_{12}, *HDL* high-density lipoprotein, *Hcy* homocysteine, *LDL* low-density lipoprotein, *SAH* *S*-adenosylhomocysteine, *SAM* *S*-adenosylmethionine, *SAM: SAH* *S*-adenosylmethionine: *S*-adenosylhomocysteine ratio, *TC* total cholesterol, *TG* triglyceride. Spot number and protein name: 409, serotransferrin; 410, serotransferrin; 411, glyceraldehyde-3-phosphate dehydrogenase; 438, moesin; 468, 78 kDa glucose-regulated protein; 473, heat shock protein 1β, 479, WD repeat-containing protein 1; 503, stress-induced-phosphoprotein 1; 507, transforming growth factor β-induced; 524, pyruvate kinase isozymes M1/M2; 557, fibrinogen B β polypeptide isoforms; 582, protein-glutamine gamma-glutamyltransferase 2; 604, glutamate dehydrogenase 1; 682, serotransferrin; 711, isovaleryl-CoA dehydrogenase; 752, vimentin; 772, aldose reductase; 812, electron transfer flavoprotein subunit α; 813, *S*-formylglutathione hydrolase; 829, annexin A1; 875, Rho GDP dissociation inhibitor 1; 887, glutathione *S*-transferase Mu 1; 890, collagen α-1(XVIII) chain; 909, GTP-binding nuclear protein Ran; 912, isocitrate dehydrogenase; 924, peroxiredoxin-2; 938, vimentin; 1,060, transgelin; 1,085, myosin regulatory light polypeptide 9; 1,125, transgelin; 1,126, myosin light polypeptide 6; 1,169, dermatopontin

elevated in the aorta of ApoE null mice in this study. While collagen deposition is associated both with plaque formation and stability, polymerised collagen type 1 maintains VSMC quiescence (Adiguzel et al. 2009). Here, expression of this anti-proliferative and anti-inflammatory protein was significantly decreased. Dermatopontin, an extracellular matrix protein, regulates collagen deposition and promotes cell adhesion (Okamoto et al. 2010), while moesin, a member of the ezrin/radixin/moesin (ERM) protein family (together with Rho GDP dissociation inhibitor 1), inhibits cell migration (Takahashi et al. 1997). Feeding a high-fat B vitamin-depleted diet decreased aorta moesin and dermatopontin levels and (as described previously) Rho GDP dissociation inhibitor 1 expression. Conversely, expression of talin, a cytoskeletal protein which promotes cell dispersal (Albiges-Rizo et al. 1995) was doubled.

These parallel quantitative changes in numerous proteins implicated in VSMC proliferation and motility provide strong but indirect evidence that feeding a combined B vitamin-depleted high-fat diet may promote an invasive VSMC phenotype in mice vivo. Whether B vitamin depletion impacts on human aorta protein expression and on SMC phenotype in the same way remains to be established. ApoE null mice are recognised as a good model for human vascular disease, spontaneously developing atherosclerotic plaques morphologically similar to lesions found in humans (Zhang et al. 2002). Additionally, here, we have employed a nutritionally relevant nutritional rodent model that better reflects a moderate B vitamin deficiency when compared with previous studies inducing supraphysiologically high levels of homocysteine (McNeil et al. 2011). Furthermore, our findings are consistent with

other studies using proteomic technologies to profile aorta or plasma protein expression patterns in several human vascular pathologies. Many proteins, acknowledged as characteristic of vascular disease progression, are identified in this animal study as strongly linked to nutritional B vitamin depletion (noted in parentheses). Medial degeneration in the human ascending aorta alters the expression of proteins critical in vascular remodelling, cytoskeleton organisation and muscle contraction (actin, collagen, profilin, myosin, vimentin), VSMC phenotype (transgelins), protein folding and ROS detoxification (Hsps, peroxiredoxin, protein disulphide isomerase), and iron transport and inflammation (serotransferrin) (Mayr et al. 2005). The HUPO Plasma Proteome Project identified more than 300 human plasma proteins as strong biomarkers of vascular function and/or disease. These include markers of inflammation (transferrin), coagulation (fibrinogen A α-polypetide, fibronectin) and cytoskeletal organisation (actin, myosin, talin and villin) (Sanzen et al. 2010). Protein profiling has also been employed to investigate mechanism of vascular dysfunction in animal models. Serotransferrin, 78 kDa glucose-regulated protein and pyruvate kinase isozyme M1/M2 are elevated in experimentally induced rat heart failure (Ingwall and Weiss 2004), while fibrinogen, collagen, peroxidase and transgelin are overexpressed in atherosclerotic mice (Mayr et al. 2005; Wu et al. 2007). Transgelin-1 is increased in the artery wall of hypertensive rats (Delbosc et al. 2008).

In summary, nutritional B vitamin depletion when combined with a high-fat diet in a mouse model of human vascular disease quantifiably altered the expression of many aorta proteins implicated in human atherosclerosis, primarily proteins associated with regulating the VSMC phenotype. It remains to be established whether these significant changes in vascular protein expression are driven by elevated homocysteine or are a consequence of other B vitamin-sensitive mechanisms.

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Conflict of interest All institutional and national guidelines for the care and use of laboratory animals were followed. Susan Duthie, John H Beattie, Margaret-J Gordon, Lynn P Pirie, Fergus Nicol, Martin D Reid, Gary J Duncan, Louise Cantlay, Graham Horgan and Christopher J McNeil declare that they have no conflict of interest.

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