

DUTHIE, S.J., BEATTIE, J.H., GORDON, M.-J., PIRIE, L.P., NICOL, F., REID, M.D., DUNCAN, G.J., CANTLAY, L., HORGAN, G. and MCNEIL, C.J. 2015. 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. *Genes and nutrition* [online], 10(1), article 446. Available from: <https://doi.org/10.1007/s12263-014-0446-y>

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2015



# 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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Received: 3 June 2014 / Accepted: 21 November 2014 / Published online: 2 December 2014  
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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

## 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<sub>12</sub> and B<sub>6</sub>, 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

**Electronic supplementary material** The online version of this article (doi:10.1007/s12263-014-0446-y) contains supplementary material, which is available to authorized users.

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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 rodent 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<sub>6</sub> and B<sub>12</sub> 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  $-80^{\circ}\text{C}$  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 cyler (Stretton Scientific, UK). The homogenate was centrifuged at 14,000 rpm for 5 min at  $4^{\circ}\text{C}$  and the supernatant frozen at  $-80^{\circ}\text{C}$ . Protein was measured using the BioRad RC DC protein assay.

### 2D gel electrophoresis of aorta tissues

One 2D gel was run per aorta (100  $\mu\text{g}$ ) 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  $\times$  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<sub>12</sub> 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.

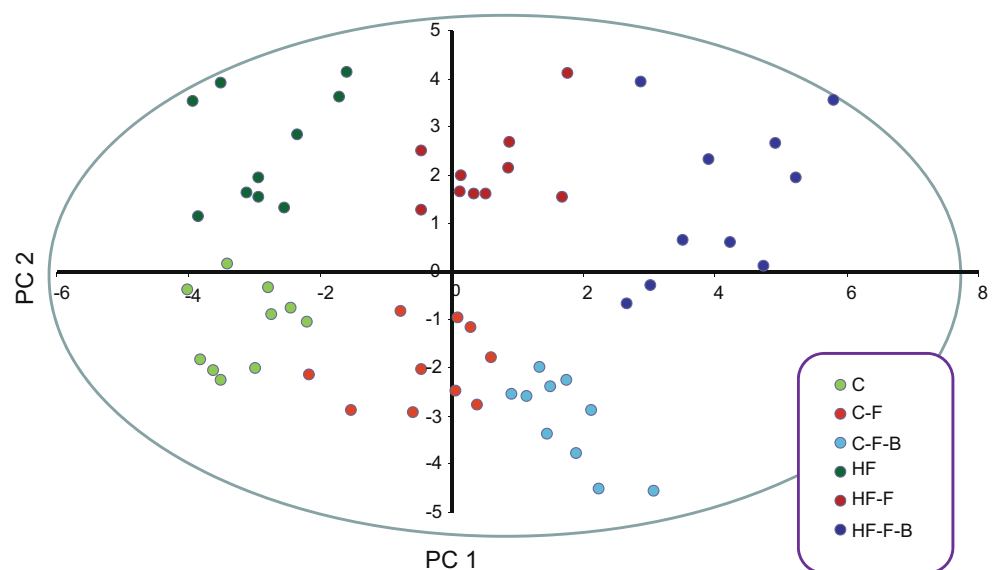
Biochemical data were analysed by two-way ANOVA for the effect of fat intake, vitamin composition and interaction between these factors. Significant differences between all groups were detected using the least significant difference (LSD) post hoc method. Analysis of associations among aorta tissue protein 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<sup>-</sup>) or folic acid and vitamins B<sub>6</sub> and B<sub>12</sub> (F<sup>-</sup>B<sup>-</sup>) 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
<i>B vitamin status</i>						
Plasma folate (ng/ml)	87.8 ± 4.8 <sup>a</sup>	16.2 ± 1.5 <sup>b</sup>	14.9 ± 0.9 <sup>b</sup>	104.9 ± 5.5 <sup>c</sup>	15.5 ± 1.7 <sup>b</sup>	15.0 ± 1.4 <sup>b</sup>
Whole blood folate (ng/ml)	498.9 ± 42.9 <sup>a</sup>	163.5 ± 8.9 <sup>b</sup>	184.7 ± 6.8 <sup>b</sup>	507.9 ± 26.0 <sup>a</sup>	149.6 ± 9.7 <sup>b</sup>	193.3 ± 18.7 <sup>b</sup>
Plasma B <sub>12</sub> (ng/ml)	24.8 ± 2.3 <sup>a</sup>	15.3 ± 1.0 <sup>b</sup>	6.8 ± 0.1 <sup>c</sup>	25.6 ± 3.0 <sup>a</sup>	12.5 ± 0.5 <sup>d</sup>	6.1 ± 0.5 <sup>c</sup>
Whole blood B <sub>12</sub> (ng/ml)	22.5 ± 1.0 <sup>a,c</sup>	22.0 ± 0.6 <sup>a,c</sup>	7.2 ± 0.7 <sup>b</sup>	24.9 ± 1.6 <sup>a</sup>	20.5 ± 1.1 <sup>c</sup>	6.9 ± 1.0 <sup>b</sup>
Plasma homocysteine (μM)	7.8 ± 0.4 <sup>a</sup>	18.1 ± 1.9 <sup>b</sup>	28.4 ± 3.6 <sup>c</sup>	6.7 ± 0.3 <sup>a</sup>	14.2 ± 1.0 <sup>b</sup>	45.5 ± 4.3 <sup>d</sup>
<i>Plasma lipids</i>						
Total cholesterol (mmol/L)	12.7 ± 0.51 <sup>a</sup>	10.9 ± 0.61 <sup>b</sup>	12.9 ± 0.40 <sup>a,b</sup>	26.2 ± 1.92 <sup>c</sup>	26.5 ± 0.96 <sup>c</sup>	24.9 ± 1.34 <sup>c</sup>
HDL cholesterol (mmol/L)	5.22 ± 0.33 <sup>a</sup>	5.51 ± 0.84 <sup>a</sup>	5.71 ± 0.92 <sup>a</sup>	9.23 ± 0.40 <sup>b</sup>	10.42 ± 0.87 <sup>b</sup>	8.86 ± 0.59 <sup>b</sup>
LDL cholesterol (mmol/L)	9.25 ± 0.38 <sup>a</sup>	8.04 ± 0.42 <sup>b</sup>	8.90 ± 0.52 <sup>a,b</sup>	24.19 ± 1.85 <sup>c</sup>	24.79 ± 1.11 <sup>c</sup>	22.58 ± 1.06 <sup>c</sup>
Triglycerides (TG) (mmol/L)	1.15 ± 0.05 <sup>a,c</sup>	1.27 ± 0.08 <sup>a</sup>	2.20 ± 0.25 <sup>b</sup>	0.89 ± 0.14 <sup>c,e</sup>	1.01 ± 0.08 <sup>c,d,e</sup>	0.91 ± 0.07 <sup>e</sup>
<i>Aorta adventitial lipids</i>						
Total cholesterol (mmol/g lipid)	35.1 ± 4.7 <sup>a,c</sup>	24.1 ± 2.1 <sup>b</sup>	33.0 ± 3.0 <sup>a</sup>	43.1 ± 4.6 <sup>a,c</sup>	53.4 ± 8.4 <sup>c,d</sup>	73.4 ± 9.1 <sup>d</sup>
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 <sup>a</sup>	5.8 ± 0.8 <sup>a</sup>	7.5 ± 1.3 <sup>a,b</sup>	7.5 ± 0.8 <sup>a,b</sup>	9.1 ± 1.4 <sup>a,b</sup>	10.2 ± 1.0 <sup>b</sup>
Triglycerides (TG) (μmol/g lipid)	919.0 ± 35.0 <sup>a,b</sup>	849.0 ± 58.7 <sup>a,c</sup>	880.6 ± 45.3 <sup>a,b,c</sup>	856.9 ± 61.6 <sup>a,b,c</sup>	1,032.7 ± 63.1 <sup>b</sup>	798.7 ± 23.1 <sup>c</sup>
SATFAs (% total fat)	31.14 ± 1.15 <sup>a,b</sup>	30.84 ± 0.69 <sup>a</sup>	33.38 ± 0.84 <sup>b</sup>	27.84 ± 0.59 <sup>c</sup>	28.81 ± 0.46 <sup>c</sup>	33.89 ± 0.49 <sup>b,d</sup>
MUFAs (% total fat)	47.38 ± 0.08 <sup>a,b</sup>	47.36 ± 0.44 <sup>a,b</sup>	46.11 ± 0.49 <sup>a</sup>	48.33 ± 0.67 <sup>b</sup>	48.60 ± 0.55 <sup>b,c</sup>	42.45 ± 0.69 <sup>d</sup>
PUFAs (% total fat)	20.76 ± 1.02 <sup>a,b</sup>	21.10 ± 0.70 <sup>a,b</sup>	19.56 ± 0.84 <sup>a</sup>	22.53 ± 0.54 <sup>b</sup>	21.77 ± 0.30 <sup>b,c</sup>	22.61 ± 0.64 <sup>b,d</sup>
Aorta plaque volume (nM/mg tissue)	0.274 ± 0.014 <sup>a</sup>	0.260 ± 0.018 <sup>a</sup>	0.261 ± 0.007 <sup>a</sup>	0.556 ± 0.024 <sup>b</sup>	0.637 ± 0.027 <sup>c</sup>	0.602 ± 0.030 <sup>b,c</sup>

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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<sub>6</sub> and B<sub>12</sub> (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<sub>12</sub> vitamin B<sub>12</sub>, 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<sub>12</sub> 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<sub>12</sub> 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<sub>12</sub> (Table 2). Plaque formation did not correlate directly either with blood folate or homocysteine. However, whole blood and plasma B<sub>12</sub> did strongly influence both circulating and adventitial lipid concentrations, with low B<sub>12</sub> 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<sub>6</sub> and B<sub>12</sub> 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).

#### Discussion

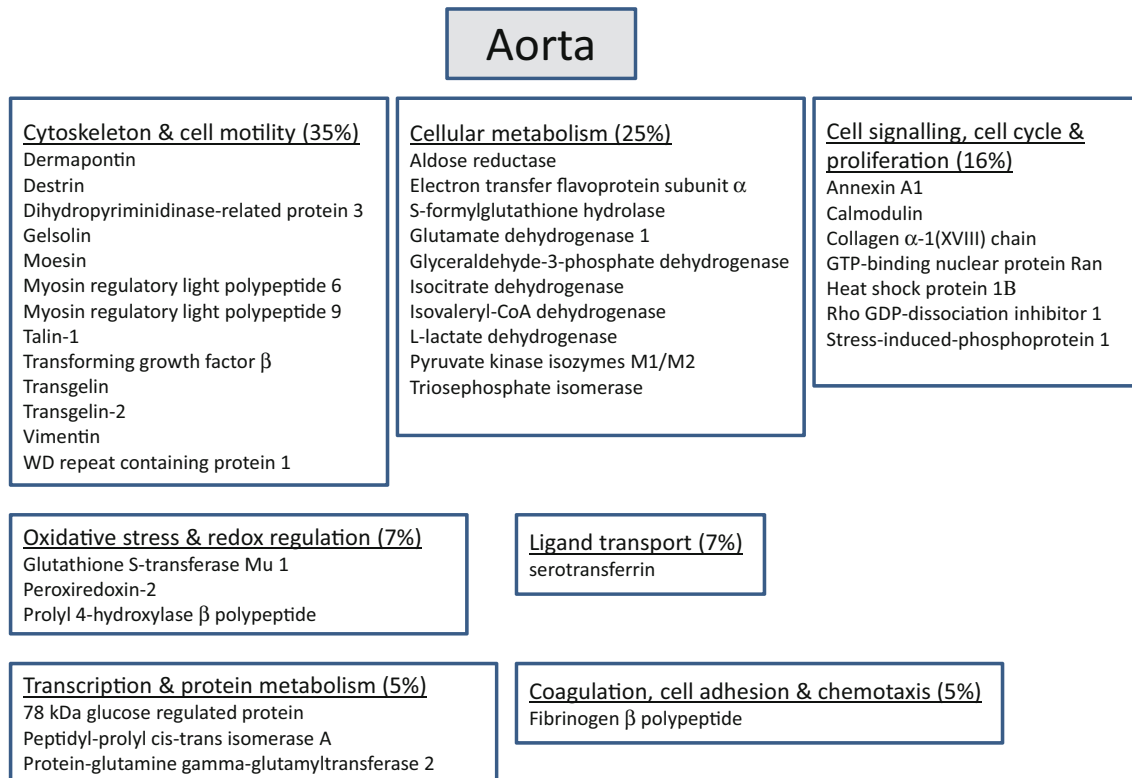
The primary aim of this study was to examine and quantify the differential effects of folate and/or B vitamin depletion on global protein expression in the aorta of ApoE mice and to consider the role of these proteins on cellular processes implicated in human vascular disease. Feeding a folate and/

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

Biomarker		Correlation	$P$ value
<i>Plaque associations</i>			
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 <sub>12</sub>	-0.271	0.04303
<i>B vitamin status and lipid metabolism</i>			
Adventitia total cholesterol	Plasma B <sub>12</sub>	-0.339	0.00802
Adventitia SATFA	Whole blood B <sub>12</sub>	-0.632	1.3E-07
Adventitia SATFA	Plasma homocysteine	0.534	1.9E-05
Adventitia SATFA	Plasma B <sub>12</sub>	-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 <sub>12</sub>	0.456	0.00037
Adventitia MUFA	Whole blood B <sub>12</sub>	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<sub>12</sub> vitamin B<sub>12</sub>, 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

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-	C F-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	129700	119989	77969	78473	99832	190848	26757	Glyceraldehyde-3-phosphate dehydrogenase			0.009		0.0102
438	58942	109474	80501	72767	64377	70374	10918	Moessin			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 $\beta$ polypeptide	0.008				
557	328576	294149	308150	296549	359980	516807	29077	Fibrinogen $\beta$ 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	224222	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 $\alpha$			0.044		0.0435
819	290371	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			0.014		0.0285
890	131019	178025	164288	144794	105335	135759	14158	Collagen $\alpha$ -1(XVIII) chain	0.015				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	720963	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				
1107	777311	673830	770344	569560	576034	59060	65528	Dextrin	0.005	0.049			0.0472
1114	869818	728904	790691	939706	784068	750674	64394	Peptidyl-prolyl dis-trans isomerase A					
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  $\pm$  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 and vitamins B<sub>6</sub> and B<sub>12</sub>



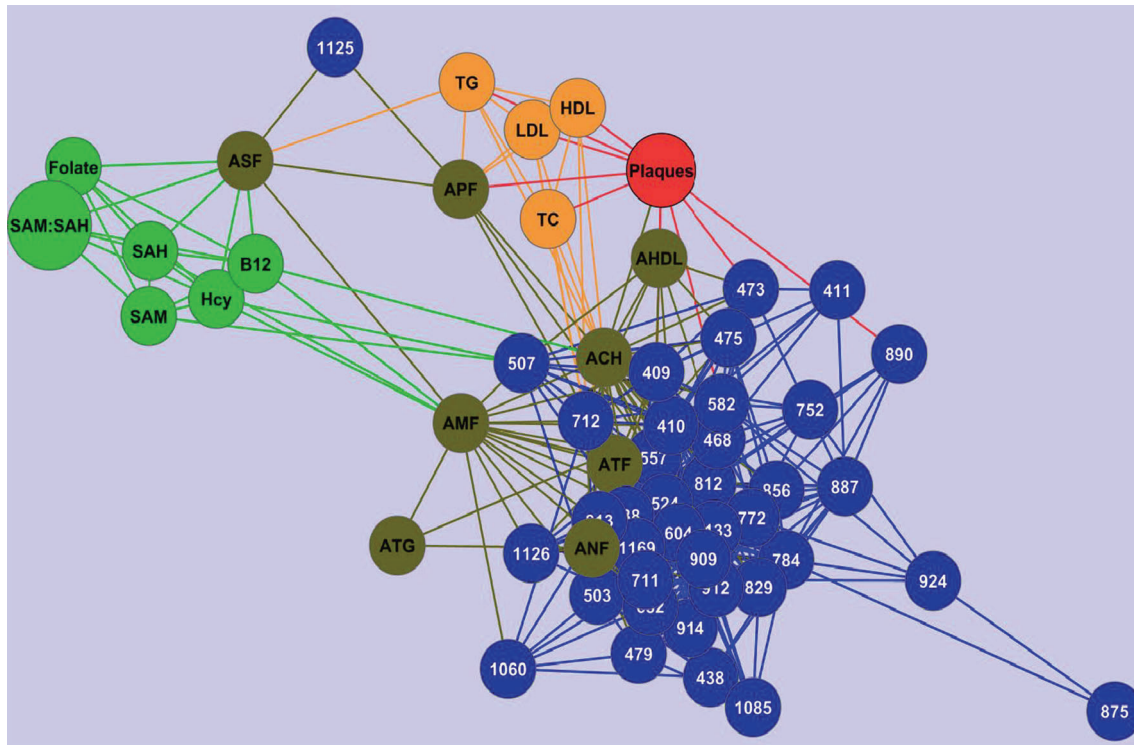
**Table 4** Associations (correlation analyses) between aorta lipid and aorta protein expression

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 $\alpha$	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	4.02E-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 $\alpha$	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 $\beta$ 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<sub>12</sub>* vitamin B<sub>12</sub>, *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 (sm22 $\alpha$ ) 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 mono-unsaturated 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<sub>12</sub>* vitamin B<sub>12</sub>, *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 1 $\beta$ ; 479, WD repeat-containing protein 1; 503, stress-induced-phosphoprotein 1; 507, transforming growth factor  $\beta$ -induced; 524, pyruvate kinase isozymes M1/M2; 557, fibrinogen B  $\beta$  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  $\alpha$ ; 813, *S*-formylglutathione hydrolase; 829, annexin A1; 875, Rho GDP dissociation inhibitor 1; 887, glutathione *S*-transferase Mu 1; 890, collagen  $\alpha$ -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. Dermatoptin, 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 *in 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  $\alpha$ -polypeptide, 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.

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

**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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SSP	Mr [exp] (kDa)	Mr [theor] (kDa)	pI	Mowse score-10Log P	No. significant peptides matched	Sequence coverage (%)	Protein name	Accession No	Gene name	Function
409	82.172	78.814	7.12	120	8	11	Serotransferrin	Q92111	Tf	Ligand transport
410	82.110	78.814	7.23	223	8	14	Serotransferrin	Q92111	Tf	Ligand transport
411	81.927	36.093	7.35	254	7	32	Glyceraldehyde-3-phosphate dehydrogenase	P16858	Gapdh	Intermediate metabolism
438	74.343	67.708	7.38	73	6	12	Moesin	P26041	Msn	Structural, cytoskeleton & cell motility
468	71.320	72.491	5.90	830	23	42	78 kDa glucose regulated protein	P20029	Hspa5	Transcription, protein modelling & metabolism
473	70.585	70.321	5.99	420	15	31	Heat shock protein 1 $\beta$	Q3TU85	Hspa1a	Cell signalling, cell cycle, cell proliferation
479	68.584	66.918	6.70	295	13	28	WD repeat-containing protein 1	O88342	Wdr1	Structural, cytoskeleton and cell motility
503	64.714	62.970	6.95	90	3	6	Stress-induced-phosphoprotein 1	Q60864	Stip1	Cell signalling, cell cycle, cell proliferation
507	63.649	59.029	6.60	96	5	10	Transforming growth factor $\beta$ -induced	Q3TDZ5	Tgfb1	Structural, cytoskeleton & cell motility
511	62.856	62.140	6.68	160	8	20	Dihydropyrimidinase-related protein 3	Q3TT92	Dpysl3	Structural, cytoskeleton & cell motility
524	61.506	58.32	8.08	436	9	19	Pyruvate kinase isozymes M1/M2	P52480	Pkm2	Intermediate metabolism
539	39.682	57.422	4.75	86	3	5	Prolyl 4-hydroxylase $\beta$ polypeptide	Q922C8	P4hb	Xenobiotic metabolism, oxidative stress & redox regulation
557	57.425	55.402	6.45	263	9	27	Fibrinogen $\beta$ polypeptide isoform	Q3TGR2	Fgb	Coagulation, cell adhesion and chemotaxis
575	53.977	271.832	8.59	183	9	5	Talin-1	P26039	Tln1	Structural, cytoskeleton & cell motility
582	54.337	78.167	5.29	283	9	18	Protein-glutamine gamma-glutamyltransferase 2	P21981	Tgm2	Transcription, protein modelling & metabolism
604	51.976	61.640	7.40	213	13	22	Glutamate dehydrogenase 1	P26443	Glud1	Intermediate metabolism

611	51.649	61.640	7.67	111	7	13	Glutamate dehydrogenase 1	P26443	Glud1	Intermediate metabolism
657	46.175	80.997	9.14	290	10	22	Gelsolin	Q6PAC1	Gsn	Structural, cytoskeleton & cell motility
682	45.519	78.814	7.32	93	6	19	Serotransferrin	Q92111	Tf	Ligand transport
711	43.060	46.596	6.82	233	11	39	Isovaleryl-CoA dehydrogenase	Q9JH15	lvd	Intermediate metabolism
752	39.137	53.581	4.83	294	11	25	Vimentin	P20152	Vim	Structural, cytoskeleton & cell motility
772	35.364	35.921	7.22	136	4	9	Aldose reductase	P45376	Akr1b1	Intermediate metabolism
812	31.725	35.358	7.27	151	4	15	Electron transfer flavoprotein subunit $\alpha$	Q99LC5	Etfa	Intermediate metabolism
813	31.779	31.870	7.09	106	5	30	S-formylglutathione hydrolase	Q9R0P3	Esd	Intermediate metabolism
829	30.863	38.864	8.18	87	4	13	Annexin A1	P10107	Anxa1	Cell signalling, cell cycle, cell proliferation
875	27.728	23.319	5.33	168	8	37	Rho GDP-dissociation inhibitor 1	Q99PT1	Arhgdia	Cell signalling, cell cycle, cell proliferation
887	27.003	25.936	8.24	130	3	12	Glutathione S-transferase Mu 1	P10649	Gstm1	Xenobiotic metabolism, oxidative stress & redox regulation
890	26.971	19.145	8.62	134	4	26	Collagen $\alpha$ -1(XVIII) chain	P39061	Col18a1	Cell signalling, cell cycle, cell proliferation
892	26.847	26.907	7.74	571	10	46	Triosephosphate isomerase	P17751	Tpi1	Intermediate metabolism
909	25.127	24.448	7.46	114	4	20	GTP-binding nuclear protein Ran	P62825	Ran	Cell signalling, cell cycle, cell proliferation
912	24.986	47.044	7.59	195	10	32	Isocitrate dehydrogenase	Q3TJ51	Idh1	Intermediate metabolism
924	22.538	21.805	5.07	100	3	22	Peroxiredoxin-2	Q61171	Prdx2	Xenobiotic metabolism, oxidative stress & redox regulation
933	21.049	23.679	7.13	431	8	38	Transgelin-2	Q9WVA4	Tagln2	Structural, cytoskeleton & cell motility
938	21.419	53.581	4.58	255	9	18	Vimentin	P20152	Vim	Structural, cytoskeleton & cell motility
1060	18.376	22.487	8.11	198	8	42	Transgelin	P37804	Tagln	Structural, cytoskeleton & cell motility
1085	18.422	19.767	4.74	244	6	40	Myosin regulatory light polypeptide 9	Q9CQ19	Myl9	Structural, cytoskeleton & cell motility
1107	17.087	18.721	6.79	247	8	58	Destrin	Q9R0P5	Dstn	Structural, cytoskeleton & cell motility
1114	16.503	18.131	7.62	109	4	26	Peptidyl-prolyl cis-trans isomerase A	P17742	Ppia	Transcription, protein modelling & metabolism
1123	16.400	16.696	3.50	211	7	44	Calmodulin	P62204	Calm1	Cell signalling, cell cycle, cell

										proliferation
1125	15.724	22.487	8.17	144	6	34	Transgelin	P37804	Tagln	Structural, cytoskeleton & cell motility
1126	16.012	17.121	4.21	316	8	47	Myosin light polypeptide 6	Q60605	Myl6	Structural, cytoskeleton & cell motility
1169	22.888	24.563	4.26	186	6	29	Dermatopontin	Q9QZZ6	Dpt	Structural, cytoskeleton & cell motility
1186	32.819	36.818	7.98	105	7	23	L-lactate dehydrogenase	Q3THB4	Ldha	Intermediate metabolism
1195	30.814	55.402	8.97	399	16	42	Fibrinogen $\beta$ polypeptide	Q3TGR2	Fgb	Coagulation, cell adhesion & chemotaxis

**Supplementary Table 1. Measures of confidence for protein identification in aorta proteins significantly altered in response to folic acid, B vitamins and fat intake.**

Spot number, calculated and theoretical molecular weight (kDa) and pI are presented. Probability-based Mowse Scores for MS/MS-based identifications [where ion scores greater than 35 indicate identity of significant homology ( $P < 0.05$ )], number of sequence peptides matched by “Mascot” searching (Matrix Science Ltd, Boston USA), percentage sequence coverage, accession number, gene and protein name are shown for all identified proteins.