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RESEARCH PAPER

Nutrigenomic analysis of the protective effects of bilberry anthocyanin-rich extract in apo E-deficient mice

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Abstract Intake of anthocyanin-rich foods has been associated with a reduced risk of cardiovascular diseases. Supplementation with anthocyanin-rich extracts from black rice or purple sweet potato was reported to attenuate atherosclerotic lesion development in apolipoprotein E-deficient (apo $E^{-/-}$) mice. However, the mechanism(s) of their preventive action are not completely understood. Previous studies revealed that anthocyanins altered mRNA levels of genes related to atherosclerosis in cultured macrophages and endothelial cells, but in vivo studies remain scarce. The aim of the study was to investigate the impact of bilberry anthocyanin-rich extract (BE) supplementation on gene expression in the liver of apo $E^{-/-}$ mice, the widely used model of atherosclerosis. The liver was chosen because it is the main site of lipid metabolism. Apo $E^{-/-}$ mice received for 2 weeks a standard diet supplemented with a nutritional dose of BE (0.02%). This study focused on the early stage of atherosclerosis development for better assessment of anthocyanin action on initiation mechanisms of this pathology. The results showed that a 2-week supplementation significantly reduced plasmatic total cholesterol and hepatic triglyceride levels, whereas the plasmatic antioxidant status remained unchanged. Transcriptional analysis, using microarrays, revealed that the expression of 2,289 genes was significantly altered. BE over-expressed genes involved in bile acid synthesis and cholesterol uptake into the liver and down-regulated the expression of pro-inflammatory genes. These results suggest an anti-atherogenic effect of BE through the regulation of cholesterol metabolism and liver inflammation and provide a global integrated view of the mechanisms involved in the preventive action of this extract.

Keywords Anthocyanins · Apo E-deficient mice · Atherosclerosis · Microarray · Liver

Introduction

Anthocyanins are water soluble plant pigments that belong to the large group of polyphenols and more specifically to the subclass of flavonoids. They are abundant in the human diet due to their wide occurrence in fruits, such as berries, and fruit-based beverages [1]. Bilberry (*Vaccinium myrtillus* L.), commonly referred to as the European blueberry, is one of the richest sources of anthocyanins, with an anthocyanin glycoside content of 300–600 mg/100 g of fresh weight [2–4]. Once ingested, anthocyanin glycosides are rapidly absorbed in both the stomach and small intestine and appear in blood and urine as intact, methylated, glucurono- and/or sulfoconjugated forms [5]. Dietary intake of anthocyanin-rich foods has been associated with a reduced risk of coronary heart disease in the Iowa Women's Health Study, a prospective study of postmenopausal women [6]. Reduction

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of atherosclerotic lesions has been reported after supplementation of apolipoprotein E-deficient (apo $E^{-/-}$) mice, which spontaneously develop human-like atherosclerosis, with anthocyanin-rich extracts from black rice [7] and purple sweet potato [8]. Increasing evidence supports an effect of berry anthocyanins in vascular protection through reduced lipid peroxidation, anti-inflammatory properties and inhibition of platelet aggregation [9]; however, little is known about the molecular mechanisms involved. In vitro experiments showed that anthocyanins and their aglycones affect the expression of genes (assessed at the mRNA level) related to atherosclerosis, such as those encoding the cholesterol transporter ABCA-1, the pro-inflammatory enzyme COX-2, the scavenger receptor CD36 in mouse macrophages [10–12] and the vasoconstrictor ET-1 in human endothelial cells [13]. Global genomic approach, using microarray technology, allows studying the effects of foods or food-derived bioactive components at a genome-wide level. Up to now, only two studies using microarrays have reported effects of anthocyanins or anthocyanin-rich extracts on global gene expression [14, 15]. A bilberry extract has been found to attenuate the expression of inflammation and cell defence–related genes in cultured mouse macrophages [14]. Moreover, Lefevre et al. reported that a 6-week grape anthocyanin supplementation in C57BL/6 J mice modulated the expression of genes involved in the inflammatory response in both liver and skeletal muscle [15]. No similar genomic study has so far been carried out on the anti-atherosclerotic properties of anthocyanins or anthocyanin-rich extract.

The present study describes the effects of the dietary supplementation of a bilberry anthocyanin-rich extract (BE) on the global gene expression in the livers of apo $E^{-/-}$ mice, using microarrays. The liver is the main organ regulating plasma lipids levels and lipoprotein metabolism. It plays a central role in atherosclerosis. Reverse cholesterol transport and cholesterol efflux from peripheral tissues to liver have been seen to prevent atherogenesis [16]. High serum lipid levels, especially high low-density lipoprotein (LDL)-cholesterol levels, have been shown to be strongly related to the development of atherosclerosis [17]. Our data show that a 2-week supplementation of mice with BE affected the expression of multiple genes involved in processes relevant to atherogenesis in the liver. In particular, BE modulated the expression of genes involved in cholesterol metabolism and inflammatory response.

Materials and methods

Bilberry extract

Bilberry (*Vaccinium myrtillus* L.) anthocyanin-rich extract (BE), Antho 50®, was supplied by FERLUX S.A (Cournon d'Auvergne, France). It contains 52% of pure anthocyanins expressed as cyanidin 3-glucoside equivalent using HPLC analysis as previously described [18]. The total polyphenol content is 62 g gallic acid equivalent/100 g of BE, as determined by the Folin-Ciocalteu assay [19]. The detailed composition of BE is given in Supplemental figure S1.

Animals and diets

Pairs of homozygous apo E-deficient mice were purchased from Jackson Laboratories (Charles River Laboratories, L'Arbresle, France) and interbred to obtain the males used for the present study. Mice were individually housed in wire-bottomed cages in a temperature-controlled room (22 ± 0.8°C) with a 12-h light–dark cycle and a relative humidity of 55 ± 10% and had free access to food and water. All animals were maintained and handled according to the recommendations of the Institutional Ethics Committee of the INRA, in accordance with decree No 87-848. They were all fed with a standard breeding diet A03 (Safe, Epinay-sur-Orge, France) before the beginning of the experiment. Eight-week-old-male mice ($n = 40$) were then randomly divided into two groups and fed with ad libitum for 2 weeks either a semi-purified control diet (UPAE, INRA Jouy-en-Josas, France) or the same control diet supplemented with 0.02% bilberry (*Vaccinium myrtillus* L.) anthocyanin-rich extract (BE). These experimental diets were isoenergetic, and their detailed composition is given in Table 1. At the end of the experimental period, mice were anaesthetised using sodium pentobarbital (40 mg/kg body weight). Blood was collected from the abdominal cava vein into heparinised tubes. Plasma was prepared by centrifugation at 12,000*g* for 2 min, and samples were stored at −20°C. The organs were washed with physiological saline solution maintained at 37°C by direct injection in the heart's left ventricle. Livers were collected, immediately frozen in liquid nitrogen and stored at −80°C until the time of analysis.

Determination of cholesterol and triglyceride levels in plasma and liver

Plasma total cholesterol and triacylglycerol (TAG) concentrations were determined as previously described [20]. Liver samples were homogenised in NaCl (9 g l^{-1}) with a Polytron homogeniser PT-MR2100 (Kinematica AG, Littau/Luzern, Switzerland) and lipids were extracted by chloroform–methanol (2:1, v/v) under overnight agitation. The chloroform phase was recovered after centrifugation and evaporated under dry air. TAG from the lipid residue was saponified with 0.5 M KOH–ethanol at 70°C for 30 min followed by the addition of 0.15 M $MgSO_4$ to

Table 1 Composition of the experimental diets

Components (g/kg diet)	Group	
	Control diet	BE diet
Wheat starch	629.36	629.16
Casein	200	200
Corn oil	70	70
Alphacell	50	50
Mineral mix AIN-93G	35	35
Vitamin mix AIN-93G	10	10
L-cystein	3	3
Choline bitartrate	2.50	2.50
tert-Butyl-hydroquinone	0.14	0.14
Anthocyanin extract	0	0.2

Apo E-deficient mice received each diet as solid pellets

neutralise the mixture. After centrifugation (2,000*g*; 5 min), glycerol from TAG in the supernatant was estimated by an enzymatic assay (TG PAP 150 kits, BioMerieux®, Marcy-l'Etoile, France). Cholesterol in the lipid residue was dissolved with isopropanol and measured with an enzymatic assay (Cholesterol RTU™, BioMerieux®, Marcy-l'Etoile, France). HDL-cholesterol concentrations were measured by precipitation with phosphotungstic acid and $MgCl_2$ using a commercial kit from BioMerieux®. LDL-cholesterol was obtained by calculating the Friedewald's formula.

Determination of plasma antioxidant capacity

The plasma antioxidant capacity was determined using the Oxygen radical absorption capacity (ORAC) assay [21], which measures the ability of antioxidant compounds in a sample to scavenge peroxyl radicals generated from AAPH (2,2′-azobis (2-amidino-propane) dihydrochloride) at 37°C using fluorescein. The assay was performed in black-walled 96-well plates, and plasma samples were diluted 600-fold in phosphate buffer (75 mM, pH = 7.4). Trolox, a water-soluble analogue of vitamin E, was used as a control standard. Twenty-five micro litres of sample, standard or blank (phosphate buffer) were mixed with 25 µl of AAPH and immediately placed in an EL_X 808 ultra microplate reader (Bio-Tek Instruments, Winooski, USA) for a 1-h incubation at 37°C. Fluorescein (150 µl) was then automatically added and fluorescence was measured for 1 h using an excitation $\lambda = 485 \pm 20$ nm and an emission $\lambda = 530 \pm 20$ nm. The final results were calculated using the difference of areas under the fluorescein decay curve between the blank and each sample and were expressed as micromole Trolox equivalents (TE) per litre (µmol TE l^{-1}).

Microarray analysis

RNA extraction and fluorescent labelling

Livers stored at −80°C were ground in liquid nitrogen and the resulting powder (60 mg) was homogenised in buffer for total RNA extraction using the SV Total RNA Isolation System (Promega, Madison, WI, USA) as recommended by the manufacturer. In all, total RNAs were extracted from eight livers: four from mice that received the control diet and four from mice that received the diet supplemented with BE. The quality of total RNA was monitored by 1% agarose gel electrophoresis. With the ChipShot™ Direct Labelling System kit (Promega), cDNAs were obtained from 5 µg of total RNA with 1 µl of random primer and 1 µl of oligo(dT), and labelling was performed with Cy™3- or Cy™5-dCTP (GE Healthcare). The labelled cDNA was purified by application to an equilibrated filter cartridge using the ChipShot™ Membrane Clean-Up System (Promega). Quantities and labelling efficiencies of labelled cDNAs were determined by measuring the absorbencies at 260, 550 and 650 nm using an ND-1000 spectrophotometer (Nanodrop).

Hybridisation

Hybridisation was carried out on the Operon mouse microarray (OpArrays™). Array-Ready OligoSet Mouse Genome version 4.0 contains 35,852 longmer probes representing approximately 24,000 genes. In all, 8 microarrays were used for a total of four independent comparisons. Hybridisation was carried out in a Ventana hybridisation system (Ventana Medical Systems, S.A, Illkirch, France) at 42°C for 8 h. Slides were subsequently washed twice in 2× saline sodium citrate (SSC) and 0.1× SSC at room temperature. The buffer remaining on the slide was removed by rapid centrifugation (4,000*g*, 15 s). The fluorescence intensity was scanned using the Agilent Micro Array Scanner G2505B (Agilent Technologies, Inc., Santa Clara, CA, USA).

Image and data analysis

The signal and background intensity values for each spot in both channels were obtained using ImaGene 6.0 software (Biodiscovery, Inc, Proteigene, Saint Marcel, France). Data were filtered using the ImaGene "empty spot" option, which automatically flags low-expressed and missing spots to remove them from the analyses. After base-2 logarithm transformation, data were corrected for systemic dye bias by Lowess normalisation using GeneSight 4.1 software (BioDiscovery, Inc, Proteigene). Ratios were then filtered in accordance with their variability

among the four comparisons, and genes with high variability were removed from the analysis. Statistical analyses were performed using the free R 2.1 software (http://www.r-project.org). The log ratio between anthocyanin-supplemented and control samples was analysed with Student's t test to detect differentially expressed genes in the two nutritional conditions, and probability values were adjusted using the Bonferroni correction for multiple testing at 1% to eliminate false positives. Genes selected by these criteria are referred to as "differentially expressed genes". Differentially expressed genes were classified according to their role(s) in cellular or metabolic pathways using the online Pathway Miner analysis software (BioRag: Bio Resource for Array Genes; http://www.biorag.org), which combines the pathway analysis capabilities of three different tools (Babelomics, GenMAPP and Biocarta) through a Fisher Exact test [22]. Ingenuity pathway analysis (IPA; http://analysis.ingenuity.com) was used to confirm the identified pathways. Finally, Gene Ontology (GO) analysis (http://www.geneontology.org/) was also performed to describe the associated biological processes of the differentially expressed genes overall.

Quantitative real-time PCR

The expression level of differentially expressed genes in the liver of anthocyanin-fed and control-fed apo $E^{-/-}$ mice (*CYP7A1*, *HMGCR*, *LCAT*, *LPL*) was measured using the reverse transcription-polymerase chain reaction (RT-PCR). High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, California, USA) was used to reverse transcribe 2 μg of total RNA to cDNA. The following primers, GAPDH, CYP7A1, HMGCR, LCAT and LPL, were identified using Primer Express software (Applied Biosystem, CA, USA) and used for RT-PCR amplification (Supplemental table S2). The quantitative real-time PCR was carried out with 100-fold diluted cDNA on the Mastercycler epgradient S Realplex (Eppendorf, Hambourg, Germany) using the Power SYBR®Green PCR Master Mix kit (Applied Biosystems, Warrington, UK) as described previously [23]. The expression levels were calculated using the ΔΔCT method [24].

Statistical analyses

All values are given as means ± SEM. A one-way ANOVA coupled with the Student–Newman–Keuls multiple comparison test was carried out to compare the effect of the control diet versus the bilberry anthocyanin-rich diet. Values of $P < 0.05$ were considered significant.

Results

Effects of anthocyanin-rich bilberry extract on weight and lipid parameters

No significant difference in weight gain was observed between BE-fed and control mice during the 2-week supplementation (data not shown). As shown in Table 2, plasma total cholesterol concentrations were significantly reduced (−20%; $P < 0.05$) after ingestion of the BE diet for 2 weeks, whereas plasma triglyceride concentrations did not differ from the controls. In the liver, a significant decrease of triglyceride concentration (−30%, $P < 0.01$) was observed with the BE diet, whereas total cholesterol level remained unchanged. No significant difference was observed in plasma HDL/LDL ratio and antioxidant capacity (ORAC) between the BE and the control groups (Table 2).

Effects of anthocyanin-rich bilberry extract on gene expression in the liver

Microarray analyses performed on the livers of apo $E^{-/-}$ mice revealed that the BE-supplemented diet affected the expression of 2,289 genes (fold change (FC) higher than 1.2, $|FC| > 1.2$), with 1,331 genes up-regulated and 958 down-regulated. Mean fold changes were 1.32. Among these genes, Pathway Miner analyses identified genes belonging to cholesterol metabolism and its regulation as well as to inflammatory pathways and cell migration/communication processes (Table 3). Ingenuity Pathway Analysis (IPA) showed that anthocyanin supplementation modulated the expression of genes associated with cardiovascular disease and affected cholesterol biosynthesis-related genes in agreement with previous results (Table 4).

Table 2 Plasma and hepatic concentrations of lipids and antioxidant capacity of apo $E^{-/-}$ mice fed with the control diet or bilberry anthocyanin-supplemented (BE) diet for 2 weeks

Analysis	Group	
	Control	BE
Plasma ($n = 20$)		
Triacylglycerol (mmol L^{-1})	1.02 ± 0.11	0.96 ± 0.11
Total-cholesterol (mmol L^{-1})	7.0 ± 0.5	5.6 ± 0.4*
HDL/LDL	0.038 ± 0.006	0.030 ± 0.005
ORAC (μmol TE L^{-1})[b]	5,519 ± 272	4,925 ± 210
Liver ($n = 16$)		
Triacylglycerol (mg g^{-1})	43.9 ± 3.0	30.9 ± 3.2**
Total-cholesterol (mg g^{-1})	3.0 ± 0.1	2.8 ± 0.1

Values are means ± SEM; * $P < 0.05$, ** $P < 0.01$

[a] Data expressed as micromoles of Trolox equivalents (TE)/litre

Table 3 Metabolic and cellular processes affected by bilberry anthocyanin-rich extract in the liver of apo $E^{-/-}$ mice using Pathway Miner analyses

Pathways	Total (*n*)	Up-regulated genes (*n*)	Down-regulated genes (*n*)
Babelomics			
Metabolic process			
Energy and lipid metabolism			
Oxidative phosphorylation	9	6	3
Arachidonic acid metabolism	11	5	6
Fatty acid metabolism	11	6	5
Glycerophospholipid metabolism	10	5	5
Glycerolipid metabolism	6	4	2
Bile acid biosynthesis	3	2	1
Biosynthesis of steroids	3	3	0
Fatty acid biosynthesis	2	2	0
Cellular process			
Cell migration and communication			
Regulation of actin cytoskeleton	31	19	12
Cell Communication	21	14	7
Focal adhesion	27	17	10
Leukocyte transendothelial migration	18	9	9
Cell adhesion molecules (CAMs)	17	7	10
Inflammation			
Natural killer cell mediated cytotoxicity	13	7	6
T cell receptor signalling pathway	12	6	6
Complement and coagulation cascades	11	6	5
Signalling			
Neuroactive ligand-receptor interaction	46	25	21
MAPK signalling pathway	36	16	20
Cytokine-cytokine receptor interaction	23	14	9
Jak-STAT signalling pathway	15	10	5
GenMAPP			
Metabolic process			
Statin pathway	5	4	1
Electron Transport Chain	5	3	2
Steroid Biosynthesis	3	1	2
Mitochondrial fatty acid betaoxidation	3	3	0
Cellular process			
Regulation of actin cytoskeleton	24	14	10
Integrin-mediated cell adhesion	17	6	11
TGF Beta Signalling Pathway	10	6	4
Nuclear receptors	6	3	3
Inflammatory Response Pathway	3	2	1
Complement Activation Classical	2	0	2
Biocarta			
Metabolic process			
SREBP control of lipid synthesis	3	2	1
FXR and LXR regulation of cholesterol metabolism	2	2	0
Cellular process			
Nuclear receptors in lipid metabolism and toxicity	11	8	3
PDGF signalling pathway	8	5	3
TNFR1 signalling pathway	6	4	2
CXCR4 signalling pathway	6	4	2
IL-10 anti-inflammatory signalling pathway	3	2	1

Table 4 Identification of significant biological processes affected by bilberry anthocyanin-rich extract in the liver of apo $E^{-/-}$ mice using ingenuity pathway analysis

Summary of analysis	*P* value
Biological functions	
Diseases and disorders	
Neurological disease	4.25E-06
Genetic disorder	5.40E-05
Cardiovascular disease	5.75E-05
Molecular and cellular functions	
Cellular development	7.66E-05
Cellular assembly and organisation	3.66E-04
Physiological system development and function	
Nervous system development and function	7.66E-05
Cardiovascular system development and function	2.04E-04
Canonical pathways	
Endothelin-1 Signalling	2.02E-04
Ephrin Receptor Signalling	8.57E-04
Tox Lists	
Cholesterol Biosynthesis	8.32E-02
LXR/RXR Activation	1.16E-01

As described in Fig. 1, classification of the differentially expressed genes according to gene ontology (GO) analysis revealed that lipid metabolism, immune system function and cell adhesion function were most affected by BE intake. Changes in expression of 4 genes revealed by microarray analysis (*CYP7A1*, *HMGCR*, *LPL* and *LCAT*) were verified by real-time RT-PCR. Profiles obtained using both techniques confirmed the down- or up-regulation of these genes. The mean values obtained by RT-PCR were 1.23 for CYP7A1; 1.14 for HMGCR; 1.33 for LPL and 0.82 for LCAT.

Discussion

In the present study, the intake of bilberry anthocyanin-rich extract during 2 weeks did not affect the plasma antioxidant capacity measured by ORAC assay. This result could be related to the low bioavailability of anthocyanins as described by Garcia-Alonso et al. The authors demonstrated low plasma concentrations of anthocyanins after consumption of 12 g anthocyanin extract (containing 183.8 mg anthocyanin monoglucosides) by healthy volunteers and no significant improvement of plasma antioxidant status (according to FRAP and TEAC assays) [25]. Additionally, it has been shown that pyranoanthocyanins naturally present in strawberries or grape pomace, did not scavenge hydroxyl radicals, suggesting that interaction of anthocyanins with pyruvic acid could decrease the antioxidant potential of anthocyanin adducts [26].

We show that the short-term supplementation of BE to the diet of apo $E^{-/-}$ mice reduced hypercholesterolemia and triglyceride accumulation in the liver. Similar results with another anthocyanin extract have been previously reported. The supplementation with an anthocyanin-rich extract from black rice, for 16–20 weeks improved serum lipid profile (total cholesterol, triglycerides) in apo $E^{-/-}$ mice [7, 27]. However, the supplementation with anthocyanins from sweet potato had no effect in cholesterol-fed apo $E^{-/-}$ mice after a 4-week period [8]. To our knowledge, no data are available about the effects of anthocyanins or anthocyanin-rich extracts on hepatic triglyceride levels in apo $E^{-/-}$ mice.

The analysis of global gene expression in the liver showed a modification by BE supplementation of the expression of genes involved in various molecular and cellular pathways, such as cholesterol metabolism, inflammatory processes and cell adhesion. From this

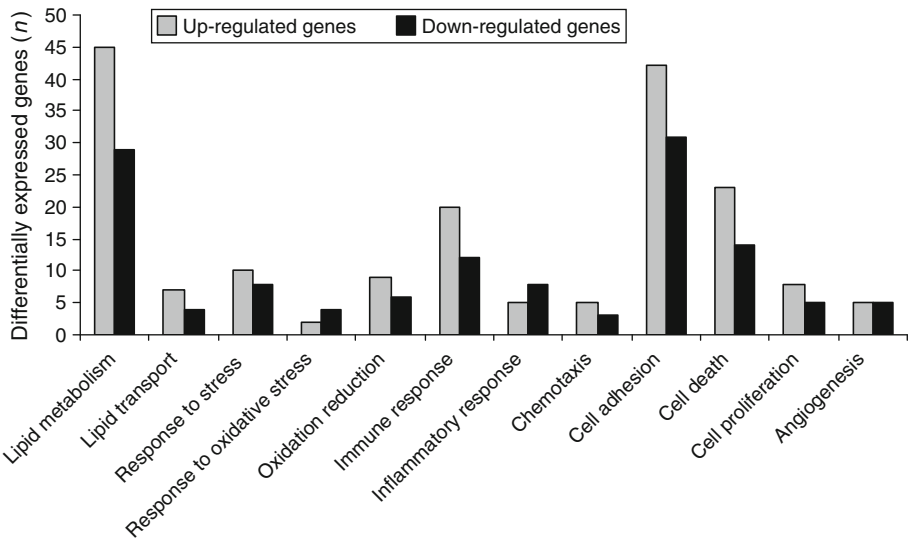

Fig. 1 Main gene ontology processes modulated by bilberry anthocyanin-rich extract in the liver of apo $E^{-/-}$ mice

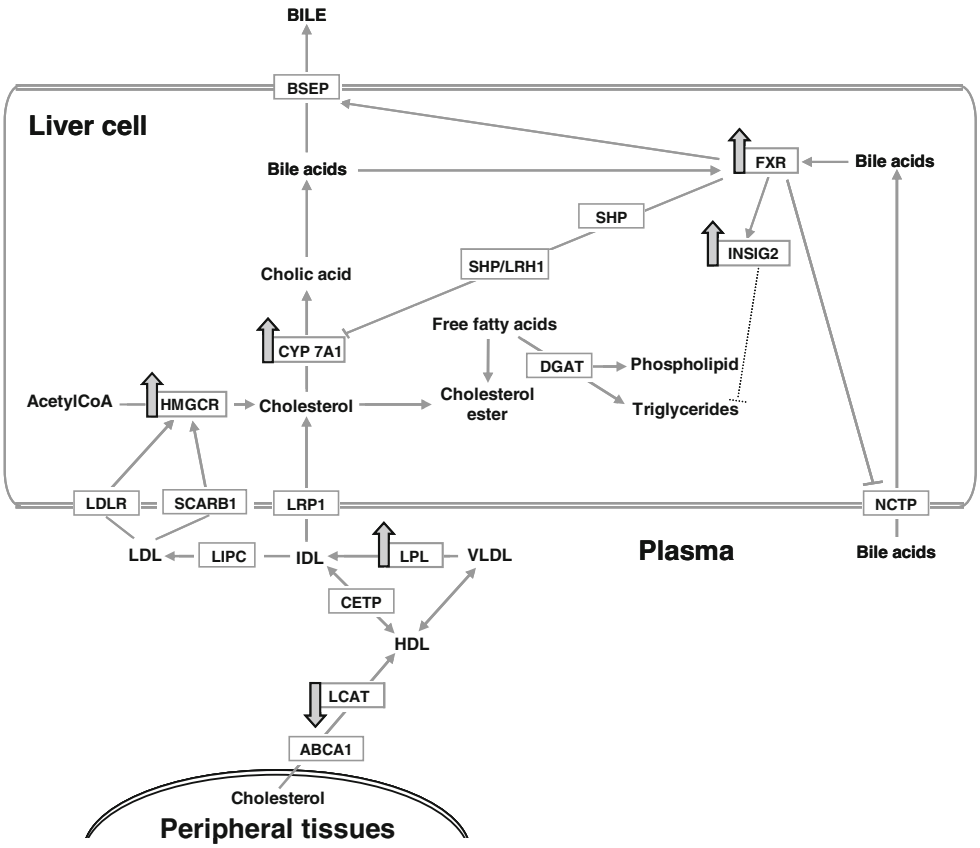

Fig. 2 Changes in the expression of genes involved in cholesterol metabolism by an anthocyanin-rich bilberry extract in apoE$^{-/-}$ mice (adapted from GenMAPP Statin pathway). *Large arrows* indicate up- or down-regulation of genes. The expression of genes *without an arrow* did not significantly change

transcriptional analysis, diverse hypothesis could be formulated in an attempt to explain atheroprotective effects of the bilberry anthocyanin-rich extract in association with observed modifications of lipid parameters.

Effects of the bilberry anthocyanin-rich extract on lipid metabolism

The liver is the main organ maintaining cholesterol homeostasis by balancing its de novo biosynthesis and elimination in the bile directly as free cholesterol or after conversion to bile acids [28, 29].

Cholesterol metabolism in the liver

The 2-week supplementation with the anthocyanin-rich BE extract induced a significant reduction of plasma total cholesterol, possibly explained by an increase in cholesterol elimination from plasma in the liver. In agreement with this finding, we observed an over-expression of *CYP7A1* in the liver (FC = 1.31), suggesting a higher cholesterol elimination via bile acids. Indeed, *CYP7A1* is the gene encoding the rate-limiting enzyme cholesterol-7-α-hydroxylase, which controls the synthesis of bile acids from cholesterol. Transgenic over-expression of *CYP7A1* in C57BL/6 J mice fed with an atherogenic diet has been found to reduce plasma lipoprotein-associated cholesterol and prevented atherosclerotic lesion formation [30]. Therefore, the observed over-expression of *CYP7A1* could explain the lower cholesterolemia (Fig. 2).

An up-regulation by BE of *HMGCR* gene (FC = 1.26), which encodes the microsomal 3-hydroxy-3-methylglutaryl CoA reductase, the rate-limiting enzyme in de novo cholesterol biosynthesis, was also observed. Despite this over-expression of *HMGCR*, the total hepatic cholesterol level was not significantly modified, suggesting that newly synthesised cholesterol in the liver is encouraged to participate in increased bile acid synthesis (Fig. 2). Similar results have been observed with grape seed procyanidins, which induced over-expression of *CYP7A1* and *HMGCR* in rat liver without increasing hepatic cholesterol levels [31].

VLDL removal from the plasma

Apo E deficiency in mice results in a severe hypercholesterolemia due to a defect in the clearance of remnant lipoproteins from the plasma [32]. In these mice, cholesterol is present mostly in the VLDL and chylomicron remnant (CMR) fractions [33, 34]. The anthocyanin-rich BE supplementation induced an over-expression in the

liver of *LPL* (lipoprotein lipase; FC = 1.26), the lipolytic enzyme involved in the clearance of VLDL and chylomicrons. Therefore, BE may increase the hydrolysis of cholesterol-enriched VLDL into IDL (intermediate-density lipoprotein), removed from the plasma by the liver via the receptor LRP1 (LDL-receptor-related protein) [35]. This would explain the reduction of cholesterol level in the plasma (Fig. 2). This hypothesis is in agreement with previous results showing that the adenovirus-mediated gene transfer of human LPL into apo $E^{-/-}$ mice was associated with a reduction of plasma total cholesterol, VLDL/CMR and triglyceride (TG) concentrations [36]. In this study, the plasma triglyceride concentrations remained unchanged after BE supplementation possibly because the VLDL present in the plasma of apo $E^{-/-}$ mice are already relatively poor in triglycerides.

Reverse cholesterol transport

In contrast to previous genes, BE supplementation induced a down-regulation of *LCAT* (FC = 0.71), which encodes the lecithin-cholesterol acyltransferase. LCAT is a plasmatic enzyme synthesised by the liver which is involved in high density lipoprotein (HDL) maturation and free cholesterol efflux from peripheral cells. This under-expression could reduce HDL levels without affecting the reverse cholesterol transport. Indeed, in a previous study, a lowered HDL concentration has been observed in LCAT$^{-/-}$ × apo $E^{-/-}$ mice [37]. LCAT deficiency was not associated with reduced plasma levels of pre-β HDL, which promote cholesterol efflux, signifying that reverse cholesterol transport was still efficient. Additionally, LCAT$^{-/-}$ × apo $E^{-/-}$ mice showed a decreased level of plasma total cholesterol and a significantly reduced aortic lesion area [37]. Therefore, by down-regulating *LCAT*, BE may reduce total cholesterol levels by modulating HDL maturation and maintaining reverse cholesterol transport (Fig. 2).

FXR activation and bile acid excretion

Using C57BL/6 J mice fed with an atherogenic diet, Lefevre et al. demonstrated that supplementation with an anthocyanin-rich extract from grapes significantly affected cholesterol biosynthesis and fatty acid β-oxidation, both pathways being regulated by nuclear receptors such as liver x-receptor (LXR) or peroxisome proliferator-activated receptors (PPARs) [15]. The authors suggested that grape anthocyanins could alter lipid metabolism in the liver through the modulation of the activities of these receptors. Bile acids are known activators of the nuclear receptors involved in the regulation of cholesterol and bile acid metabolism. An up-regulation of *NR1H4* (FC = 1.44), encoding farnesoid X receptor (FXR), a member of the nuclear receptor superfamily, was observed after BE intake. FXR stimulates hepatic BSEP expression (canalicular bile salt export pump) leading to bile acid excretion into the bile, and inversely inhibits the expression of NCTP (Na-taurocholate cotransport protein), which is responsible for plasmatic bile acid uptake into the liver [38]. The observed up-regulation of *NR1H4* in the liver may thus favour plasmatic cholesterol elimination through enhanced bile acid excretion consecutive to BE-induced increase of bile acid synthesis (Fig. 2).

FXR is also known to positively regulate *INSIG2* gene and protein levels, thus preventing the dissociation of the INSIG-2/SCAP/SREBP-1c complex, which is necessary for the activation of the SREBP-1c (sterol regulatory element-binding protein) transcription factor which itself up-regulates the expression of lipogenic genes [39]. In our study, *INSIG2* was up-regulated (FC = 1.32) by the BE supplementation, possibly as a consequence of the *FXR* up-regulation, and this may induce a reduction of hepatic lipogenesis and explain the decreased liver TG levels. In agreement with this hypothesis, elevated hepatic TG content has been shown in FXR$^{-/-}$ mice [40].

Overall, our results suggest that the anthocyanin-rich BE extract enhances cholesterol elimination through bile acid synthesis and their further excretion. This may lead to both a *de novo* cholesterol biosynthesis and an increase in cholesterol uptake by the liver, explaining the observed reduction of serum cholesterol (Fig. 2). The increase of bile acid synthesis and their further excretion could lead to a reduction of hepatic lipogenesis and explain the observed reduction of TG levels in the liver.

Other studies in rats or rabbits have shown some cholesterol-lowering effects of grapefruit polyphenols and black tea associated with increased bile acid excretion [41–43]. Apple polyphenols were also found to enhance in rats the cholesterol-7α-hydroxylase activity in the liver and to increase the excretion of faecal bile acids [44].

Effects of the bilberry anthocyanin-rich extract on liver inflammation

Hypercholesterolemia in atherosclerosis-prone apo $E^{-/-}$ mice elicits an early inflammatory response in the liver that is characterised by increased hepatic steatosis, necroinflammation and increased hepatic expression of pro-inflammatory factors when compared with wild type C57BL/6 J mice [45]. The liver may exert an initiating role in early atherogenesis through the production of the pro-inflammatory proteins such as CRP (C-reactive protein) and SAA (Serum amyloïd A), direct mediators of atherosclerosis [46]. This inflammation was shown to precede atherogenesis in the arterial wall [47, 48].

Down-regulation of hepatic and inflammatory cell activation. The anthocyanin-rich BE extract was found to down-regulate in the liver the expression of pro-inflammatory genes related to the activation of leukotrienes (*ALOX5AP*; FC = 0.80), chemokines (*CX3CL1*; FC = 0.78), cytokines (*TNFRSF14*; FC = 0.82) and complement components (*C3*; FC = 0.80) as well as cell adhesion molecules (*VCAM1*; FC = 0.66). A similar inhibition of the acute inflammatory response by an anthocyanin-rich grape extract has also been observed in the liver of C57BL/6 J mice fed with an atherogenic diet [15].

Different data emphasise the importance of the four genes related to inflammation in the atheroprotective action of the BE extract.

- The *ALOX5AP* gene encodes the helper protein, 5-lipoxygenase (5-LO)-activated protein (FLAP), which is implicated in the biosynthesis of pro-inflammatory leukotriene lipid mediators [49]. Leukotrienes produced in the liver, such as LTC4, are known to stimulate hepatic endothelial cells in a paracrine manner [50]. These pro-inflammatory leukotrienes induce the production in endothelial cells of cell adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) [51], thus promoting the recruitment of leukocytes into the liver. This process leads to the subsequent production of hepatic inflammatory molecules (serum amyloïd A, cytokines and complement factors) by leukocytes, which are able to enter the bloodstream and promote atherogenesis [48].
- The *CX3CL1* gene encodes for a cell-bound CXC chemokine involved in the activation and adhesion of leukocytes [52, 53]. Once activated, these leukocytes secrete inflammatory mediators [54]. The reduction of the expression of the *CX3CL1* gene by the BE supplementation may limit leukocyte adhesion and inflammation. A similar reduction by a *Vaccinium myrtillus* extract has been observed in an inflammatory model of macrophages [14].
- TNFRSF14, a member of the tumour necrosis factor receptor superfamily, is primarily expressed in T lymphocytes. Its activation has been found to induce the expression of pro-atherogenic cytokines in human monocyte leukaemia (THP-1) cells [55].
- C3 is a plasma-localised type I acute-phase protein, mainly produced in the hepatocyte. It is involved in the complement system activation, associated with atherosclerosis. Hypercholesterolemic patients with coronary artery disease (CAD) present increased plasmatic levels of C3 compared to healthy subjects or hypercholesterolemic patients without CAD [56]. Stimulation of human or rat hepatoma cells with the acute-phase inducer IL-6 increases the expression of the complement component C3 gene [57, 58]. Observed hepatic down-regulation of *C3* gene expression by BE could result in reduced levels of plasmatic C3 and further attenuate the development of atherosclerosis.

Our overall results suggest that BE may also prevent atherosclerosis by reducing the release of pro-inflammatory mediators, leading simultaneously to lessened activation of both hepatocytes and hepatic endothelial cells, inhibition of leukocyte activation and limited production of cytokines and complement components by the liver. However, these results are still hypothesis that need to be confirmed, since it was reported that anthocyanins and their corresponding vitisins A (pyranoanthocyanins) did not inhibit NO production and TNF-α secretion in activated macrophages [59].

In conclusion, the results presented here show that an anthocyanin-rich bilberry extract fed at a nutritional dose affects the expression of numerous hepatic genes encoding proteins that are involved in lipid metabolism and inflammation at an early-onset stage of atherosclerosis. The observed modulation of hepatic gene expression may explain the reduction of cholesterol level in the plasma possibly via an increased elimination as bile acid. They may also explain the reduction of TG level in the liver via a decreased hepatic lipogenesis. A down-regulation of the expression of pro-inflammatory genes in the liver may also participate in the protection against atherosclerosis. These results allow formulating new hypotheses on the mechanisms of action of anthocyanins in the prevention of atherosclerosis. Further work is required to evaluate whether the observed changes in mRNA levels are translated into biochemical and physiological processes relevant for the protection against atherosclerosis.

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Conflict of interest statement None.

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