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Berry intake changes hepatic gene expression and DNA methylation patterns associated with high-fat diet

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Abstract

The liver is a critical organ for regulation of energy homeostasis and fatty liver disease is closely associated with obesity and insulin resistance. We have previously found that lingonberries, blackcurrants and bilberries prevent, whereas açai berries exacerbate, the development of hepatic steatosis and obesity in the high-fat (HF)-fed C57Bl/6j mouse model. In this follow-up study, we investigated the mechanisms behind these effects. Genome-wide hepatic gene expression profiling indicates that the protective effects of lingonberries and bilberries are accounted for by several-fold downregulation of genes involved in acute-phase and inflammatory pathways (e.g. Saa1, Cxcl1, Lcn2). In contrast, açai-fed mice exhibit marked upregulation of genes associated with steatosis (e.g. Cfd, Crat, Crea). In silico analysis together with immunoblot analysis identified NF-κB, STAT3 and mTOR as upstream regulators involved in mediating the observed transcriptional effects. To gain further insight into mechanisms involved in the gene expression changes, the HELP-tagging assay was used to identify differentially methylated CpG sites. Compared to the HF control group, lingonberries induced genome-wide hypermethylation and specific hypermethylation of Cpg sites. The liver is a critical organ for regulation of whole body energy homeostasis due to its central role in lipid and glucose metabolism, as well as its close connection via the portal vein to nutrient uptake in the intestine. The prevalence of NASH is increasing in parallel with the epidemic of obesity and insulin resistance that is coupled to the diet of the Western lifestyle [3,4]. High-fat (HF) diets have been shown to cause dysregulated hepatic gene expression with perturbations in lipid, cholesterol, inflammatory and oxidative pathways [5]. NASH is characterized by accumulation of lipids and lipids derivates sensitizing the liver for further damage by inflammation and fibrosis, potentially by a second hit involving oxidative stress [6,7]. The hepatic fat accumulation may derive from increased dietary lipid intake, increased lipid synthesis (de novo lipogenesis) and/or decreased oxidation. In addition, obesity and T2DM states may associate with increased uptake of free fatty acids from adipose tissues into nonadipose tissues such as the liver [7,8].

1. Introduction

Overweight and obesity are health issues that continue to increase and are now affecting almost 40% of the global adult population [1]. Obesity is associated with metabolic changes including insulin resistance, nonalcoholic fatty liver disease (NAFLD), low-grade inflammation and dyslipidemia, which may lead to development of type 2 diabetes (T2DM) [2,3]. The liver is a critical organ for the regulation of whole body energy homeostasis due to its central role in lipid and glucose metabolism, as well as its close connection via the portal vein to nutrient uptake in the intestine. The prevalence of NAFLD is increasing in parallel with the epidemic of obesity and insulin resistance that is coupled to the diet of the Western lifestyle [3,4]. High-fat (HF) diets have been shown to cause dysregulated hepatic gene expression with perturbations in lipid, cholesterol, inflammatory and oxidative pathways [5]. NASH is characterized by accumulation of lipids and lipids derivates sensitizing the liver for further damage by inflammation and fibrosis, potentially by a second hit involving oxidative stress [6,7]. The hepatic fat accumulation may derive from increased dietary lipid intake, increased lipid synthesis (de novo lipogenesis) and/or decreased oxidation. In addition, obesity and T2DM states may associate with increased uptake of free fatty acids from adipose tissues into nonadipose tissues such as the liver [7,8].

The types of foods we eat are important for maintenance of a healthy body weight. It is possible that increased consumption of vegetables and fruit protects against body weight gain [9], thereby
preventing obesity and NAFLD. In a previous study [10], we found that addition of different berries to HF diets can prevent development of obesity and fatty liver in C57BL/6 mice, a mouse model used to study diet-induced obesity and prediabetes [11]. The previous findings are schematically described in Fig. 1. In brief, mice were protected against HF-induced weight gain when the diet was supplemented with lingonberry (Li), blackcurrant (Bc), bilberry (Br) or açai (Ac) for 13 weeks [10]. One group received a low-fat (LF) diet. Arrows indicate the statistically significant effects (P<0.05 or less) compared to the control mice receiving HF diet. PAI-1 and ALT are markers for inflammation and liver dysfunction, respectively. Homeostatic model assessment (HOMA) index reflects the level of insulin resistance. Illustrations were obtained and modified from Servier Medical Art by Servier, http://www.servier.com/Powerpoint-image-bank, licensed under Creative Commons Attribution 3.0 Unported License, http://creativecommons.org/licenses/by/3.0/.

2. Methods and materials

2.1. Animals and study design

The liver tissue used for gene expression analysis was taken from male C57BL/J6 BomTac mice receiving HF diets (45 kcal% fat) supplemented with 20% (w/w) of different freeze-dried berries for 13 weeks [10]. The general composition (kcal%): of the diets was as follows: for the HF diets, 45% fat, 35% carbohydrate and 20% protein; for the low-fat (LF) diet, 10% fat, 70% carbohydrates and 20% protein. In addition, the diets were formulated to have the same glucose, fructose and sucrose content. The study was approved by the Animal Ethics Committee in Lund, Sweden, ( Permit Number: M185-11) and was in accordance with the Council of Europe Convention (ETS 123). Dietary composition as well as phenotypical and metabolic characteristics of the mouse study is described in Ref [10]. In the current study, liver tissue and plasma (n=6–12) were used from 4-h-fasted mice receiving the following diets: HF diet (control group), LF diet (10 kcal% fat) or HF diet supplemented with lingonberries, blackcurrants, bilberries or açai.

2.2. DNA and RNA isolation from liver

DNA and RNA were extracted with the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) and purity and concentration were determined using spectrophotometry (NanoDrop). RNA integrity was evaluated by an Agilent 2100 Bioanalyzer (Agilent Technologies, Loveland, CO, USA).

2.3. Microarray processing

Global gene expression profiles in livers were determined by the Swegene Center for Integrative Biology Genomics DNA Microarray Resource Center (SCIBLU, Lund, Sweden) using MouseWG-6 v2.0 Whole-Genome Expression Beadchips (Illumina, San Diego, CA, USA). Expression analysis was conducted on 6 randomly selected mice (from a total of 12) per diet group, giving 36 microarrays in total. Images and raw signal intensities were acquired using the Illumina BeadArray Reader scanner. Data preprocessing and quantile normalization were performed using Illumina GenomeStudio software. The data have been deposited in NCBI’s Gene Expression Omnibus (GEO) database (accession number GSE66711).
2.4. Microarray data analysis

Data analysis was done using the R software environment (version 3.0) and the ‘limma’ package [18]. Raw data together with negative control probes were imported and quantile normalized. All samples were thoroughly quality controlled by assessing signal-to-noise ratios and manual inspection of MA and NUSE plots and deemed to pass. Probes with a detection P value < 0.05 in at least three samples were kept for further analysis. Differentially expressed probes were identified by fitting a linear regression model comparing each berry diet to the HF control diet. To illustrate group similarities, Venn diagrams were calculated and illustrated using the limma package. To interpret functional changes in the datasets, differentially expressed genes (compared to HF control) from each group were annotated with Gene Ontology (GO) biological process and analyzed with Kyoto encyclopedia of genes and genomes (KEGG) pathway using the Database for Annotation, Visualization and Integrated Discovery Bioinformatics Resources 6.7 (DAVID, http://david.abcc.ncifcrf.gov). Significantly upregulated or downregulated probes (FDR<0.05) from each diet group were separately uploaded to DAVID. The following annotation categories were used: GO Biological Process_ALL (GOTERM_BP_ALL includes all go-terms) and GOTERM_BP_FAT (mildly filtered list) and KEGG_PATHWAY to create functional annotation chart reports. Pathways with an EASE score (a modified Fisher’s Exact P value) less than 0.05 were selected for further analysis. To illustrate trends in gene expression, genes were put into context with other genes involved in similar pathways based on the information from the GO processes. KEGG pathways as well as scientific literature. Descriptions of the encoded proteins were derived using the UniProt database and NCBI/PubMed literature search.

The ‘upstream regulator’ analysis in Ingenuity Pathway Analysis (IPA, www.ingenuity.com, Redwood City, CA, USA) was used to determine likely upstream regulators of the genes compared to dataset genes through a set of direct or indirect relationships. Lists with Illumina identifiers and associated log ratios of differentially regulated genes (P<0.05) were used for IPA analysis. Due to the large metabolic effects observed by ligninon supplementation, the analysis was focused on differences between the ligninoberry and the HF control group. The computed z-score, a measure of significance as well as a predictor for the fraction of each site activated, was used to identify activated (z-score of more than 2) and inactivated (z-score of less than −2) transcription regulators and kinases predicted to regulate gene expression. The detected regulators were ranked on predicted activation based on the z-score, number of target genes in the dataset and P-value and were validated on protein expression level (Section 2.5).

2.5. Validation of microarray data by real-time qPCR

Differentially expressed genes of interest from the microarray analysis were selected for validation by real-time quantitative PCR (qPCR) (n=6). Total RNA (1 μg) was treated with DNaseI amplification grade (Invitrogen, Carlsbad, CA, USA) and reversely transcribed using random hexamers (Amer sham Bioscience, Piscataway, NJ, USA) and SuperScript II RNaseH reverse transcriptase (Invitrogen) according to the manufacturer’s recommendations. The cDNA was used in duplicates for qPCR using TaqMan chemistry (arrays on demand; Applied Biosystems, Foster City, CA, USA) or SYBR green chemistry, with an ABI 7900 system (Applied Biosystems). Primers were used to quantify mRNA expression of Saa1, Lcn2, Acacb, Pparg2, Cyp7a1, Hmgcr, Nco2 and Il16 (see Supplementary Table S1 for primer IDs and sequences). The relative quantification of mRNA was calculated using the ΔΔCT method with normalization by geometric average of the genes cyclophilin A (Ppia) and ribosomal protein S29 (Rp29) [19]. Ppis and Rp29 were chosen as they were well-accepted reference genes and the microarray data indicated that these genes were highly expressed (top 5%) with a low variation between different animals (~1% coefficient of variation).

2.6. HELP tagging to test epigenome-wide DNA methylation

Hpall tiny fragment enriched by ligation-mediated PCR (HELP) tagging was used to identify differentially methylated CpG sites (DMRs) in livers from the ligninoberry group compared to the HF control group (n=4 per group) as described by Suzuki et al. [2021]. HELP tagging is a technique based on restriction enzyme digestion using HpaII (methylation sensitive) together with its isoschizomer MspI (methylation insensitive). HELP tagging is a technique based on restriction enzyme digestion using HpaII. Briefly, high molecular-weight genomic DNA was extracted from livers using diethylamino ethanol precipitation. Five micrograms of DNA was digested with Hpall and ligated to two custom adapters containing Illumina adapter sequences, an EcoP151 recognition site and the T7 promoter sequence. The libraries were sequenced using Illumina HiSeq 2500 sequencer following the manufacturer’s instructions. Hpall profiles were obtained for each sample and the methylation scores were calculated using a previously generated MspI mouse reference library. The data analysis was performed as described by Delahaye et al. [22]. Briefly, DNA methylation scores from 0 (fully methylated) to 100 (unmethylated) were filtered based on confidence scores. Confidence scores were determined for each sample by determining the total number of Hpall-generated reads as a function of the total number of MspI-generated reads, excluding loci for which the confidence score was lower than the expected mean by locus. After confidence score filtering, the number of testable loci decreased from >1.5 million to 788,076. The locus-specific angle was compared between the ligninoberry and HF control groups and DMRs defined as the ones having an absolute difference in methylation angle value of >5 (which corresponds to ~5% difference in methylation [20]) and a P value of <0.05. We obtained 24,394 DMRs. The DMRs were defined in terms of genomic content such as promoter (~2 kb to ~2 kb from the transcription start site), gene body and intergenic regions using an arbitrary cutoff. DNAVid software was used to identify pathways enriched in genes containing DMRs in the promoter region. All HELP-tagging data were uploaded to the GEO database (GSE87277).

2.7. Bisulfite conversion, pyrosequencing and MassArray

All samples used in the genome-wide DNA methylation study (n=4 per group) were subjected to bisulfite conversion, PCR and pyrosequencing (Epitect Bisulfite Kit, PyroMark PCR Kit and PyroMark QR8 96 ID, Qiagen) according to the manufacturer’s instructions. The same primers were used at Beijing Genomics [24] (Shenzhen, China (pyrosequencing service provider), PCR and sequencing primers were designed using PyroMark Assay Design 2.0 and synthesized by Beijing Genomics Institute to assess percent methylation at 14 loci (Supplementary Table S1). To further validate HELP-tagging data at loci Nco2 and Il16, bisulfite MassArray was performed on n=6 samples per group [23]. Bisulfite conversion was performed using EZ DNA Methylation Gold kit (Zymo Research, California, USA) according to the manufacturer’s instruction. Primers are listed in Supplementary Table S1. The Albert Einstein College of Medicine (AECOM) Genomics Shared Facility performed Sequenom MassArray assays according to the company’s standard protocol and matched peak data were exported using Epityper software and data were analyzed as described by Thompson et al. [25] (same methods used to assess methylation of Nco2 and Il16 (n=6 samples per group) in livers from mice in a separate study receiving the same diets for 11 weeks (biological replicate).

2.8. Analysis of plasma samples

Plasma previously collected from each mouse (following fasting for 4 h, n=12 receiving the different diets [10] was used for determining concentrations of proteins secreted by liver to validate findings from the microarray gene expression data. Serum amyloid A (SAA) and CXCL1 were measured using enzyme-linked immunosorbent assay kits (Phase Marine Serum Amyloid A Assay, Triplet Development, Kildare, Ireland; Mouse CXCL1 ELSi Kit, Nordic Biosite, Täby, Sweden).

2.9. Immunoblot analysis

The following groups with the strongest phenotypes were subjected to protein expression analysis: ligninoberry, acai, HF and LF diet (n=6). In addition, nuclear content of proteins NF-κB subunit p65 and SREBP1c was measured in all groups (n=12). Total liver lysates were prepared by homogenization in lysis buffer [50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% (w/v) Nonidet P-40, 50 mM Na2HPO4, 5 mM Na2HPO4, 0.25 M sucrose, 1 mM dithiothreitol and complete miniprotease inhibitor [1 tablet/10 ml)] followed by centrifugation at 13,000 × g for 10 min at 4°C. Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific, Rockford, IL, USA) according to the manufacturer’s instructions. Lysates and nuclear extracts were subjected to polyacrylamide gel electrophoresis on precast NuPAGE gels 4–12% (Invitrogen) and immunoblot analysis. Primary antibodies against the following proteins were used: signal transducer and activator of transcription 3 (STAT3) and phosphorylated STAT3 Tyr705 (pSTAT3), regulatory-associated protein of mTOR (Raptor), acetyl-CoA carboxylase (ACC), p65 subunit of NF-κB, AMPK (AMPK) subunit α1, AMPK α2, AMPK α1 Thr172, AMPK α2, AMPK γ2 and AMPK γ1 (Cell Signaling Technology, Beverly, MA, USA); sterol regulatory element–binding protein 1c (SREBP1c) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) p65 (Abcam, Cambridge, MA, USA). Antibodies against cyclin B1 (CypB) and TATA box–binding protein (TBP), used as loading control for total lysates and nuclear extracts, respectively, were from Abcam. Detection was performed with horseradish-peroxidase-conjugated secondary antibodies (antirabbit; Pierce, Rockford, IL, USA; antimouse: GE Healthcare, Buckinghamshire, UK) and chemiluminescent substrate (SuperSignal West Pico or Femto Maximum Sensitivity Substrate, Thermo Scientific). Images were acquired with a Bio-Rad Chemidox XRS–system and the band intensities were quantified using Image Lab 4.0 (Hercules, CA, USA).

2.10. Statistical analysis

Data are displayed as boxplots showing the median, quartiles and minimum and maximum values. Unless stated otherwise, results were analyzed by one-way ANOVA in conjunction with Dunnett’s multiple comparisons test. In cases where Gaussian distribution could not be assumed, groups were compared using Kruskal–Wallis postest. The ROUT test was performed to statistically reject outliers with 99% confidence level. The nonparametric Mann–Whitney U test (two-tailed) was used for comparisons involving only two groups. Unless stated otherwise, all results are compared to the HF control group. Differences with a P value <0.05 were considered significant: **P<0.01 and ***P<0.001. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Statistical analysis of microarray and HELP assay and DNA methylation data have been indicated in Sections 2.4 and 2.6 mentioned above.
3. Results

3.1. The effect of berries on hepatic gene expression

Gene transcription profiles in livers from mice receiving different berries were characterized by microarray. Of 46,255 tested probe sets in the microarray, 20,861 had a detection $P$ value $<.05$ in at least three animals and were carried forward for further analysis. When the expression of each gene probe was compared to the HF control group, 2777 unique probes were changed significantly by at least one of the diets (FDR corrected $P$ value $<.05$), and 1975 probes were changed by supplementation with berries (gene expression trends compared to HF control are illustrated in Fig. 2A and B). The microarray results revealed that 10 genes (Fig. 2A and C) were regulated by all diets compared to the HF control. Out of these, the genes Cidea, Anxa2 (markers for steatosis and oxidative stress, respectively) and Tceal8 were downregulated by all diets except açai, where they were upregulated. The LF diet had the largest number of differentially expressed genes compared to the HF diet group. The number of changed genes was also large in the mice receiving HF diet supplemented with lingonberries, indicating a switch from HF-induced changes in gene expression. The mice in the açai group, which had an obese phenotype similar to the HF control group [10], had the smallest number of differentially expressed genes and thus the most similar expression profile to the HF control group.

3.2. General analysis of affected GO processes and pathways

Pathways involving inflammatory response, lipid and cholesterol biosynthesis, glutathione/drug/xenobiotic metabolism and oxidation/
reduction were affected by several of the berry diets (Table 1) and hence became focus for further analysis (complete dataset in Supplementary Table S2). In general, the lingonberry- and bilberry-fed mice were protected against a HF-induced proinflammatory gene expression, as seen by downregulation of Saa1 and Lcn2 (Fig. 3A–C).

Several acute-phase proteins were expressed at even lower levels than in the LF diet group (Table 1 and Fig. 3). Lipid-related gene expression was affected by the berry diets (Fig. 4); for example, lingonberries and blackcurrants tended to downregulate genes encoding enzymes involved in lipid synthesis compared to the HF control. Mice receiving acai, bilberries and LF diet had a different profile with several lipid- and cholesterol-synthesizing genes being upregulated (Figs. 4 and 5).

3.3. Effect of berry supplementation on inflammation

Several inflammatory factors were among the top hits in the microarray data (Fig. 3A). The mRNA of Saa isoforms, Lcn2 and Cxcl1...
were downregulated several-fold in livers from mice receiving lingonberries, bilberries and the LF diet in comparison to HF control mice. Specifically, in the lingonberry group, Saa1, Saa2, Saa4, Cxcl1, Cfd and Lcn2 were downregulated 6, 3, 8, 5, 3 and 11 times, respectively, compared to the HF control. The decrease in expression of Saa1 and Lcn2 was validated by qPCR (Fig. 3B and C). The acute-phase SAA proteins are induced and secreted by the liver in response to inflammatory stimuli (reviewed in Ref. [25]). In line with the mRNA

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**Gene Description**

**Acute phase proteins**

Saa1;2;4 Acute-phase serum amyloid A (SAA) 1, 2 and 4; Acute-phase proteins and constitutive SAA protein.

Orm1;2 Alpha-1-acid glycoprotein 1 and 2; Acute-phase proteins.

**Macrophage-derived cytokines and markers**

Aoah Acyloxyacyl hydrolase; Lipase inactivating bacterial lipopolysaccharide.

Ccl4 C-C motif chemokine 4; Chemokine produced by macrophages in response to infection/inflammation.

Cxcl1 Chemokine (C-X-C motif) ligand 1; Cytokine expressed by macrophages.

Emr1 EGF-like module-containing mucin-like hormone receptor-like 1; Macrophage marker F4/80.

Cfd Complement factor D (adipsin); Alternative complement pathway component.

Lcn2 Neutrophil gelatinase-associated lipocalin (NGAL).
compared to HF control (Fig. 3D) and were elevated in the açai group. In plasma from the groups receiving lingonberry, bilberry and LF diet in fold change compared to the control. (B and C) qPCR results of gene expression of expression data, the plasma levels of SAA were significantly downregulated in neutrophils and its expression was significantly downregulated in the açai group. The chemokine CXCL1 plays a role in inflammation by recruiting neutrophils and its expression was significantly downregulated in is depicted *P<0.05 and ***P<0.001. processes, fatty acid and triglyceride metabolic processes, PPAR signaling pathways and glycerolipid metabolic processes (identified using DAVID). The fold change is displayed for genes that were significantly changed compared to the HF control. Shades of red and blue indicate the degree of upregulation or downregulation, respectively. White indicates zero difference.
control (Fig. 3A). In addition, the plasma levels of CXCL1 protein were significantly decreased in all groups except the açai group (Fig. 3E) compared to the HF control. Genes for other macrophage-derived cytokines tended to be downregulated by lingonberries and upregulated by açai (Fig. 3A). Acyloxyacyl hydrolase, produced by Kupffer cells and involved in detoxification of lipopolysaccharide (LPS) [26], was upregulated 1.2-fold by açai supplementation.

3.4. Effects of berries on genes involved in lipid metabolism

Many genes involved in lipid synthesis, transport and regulation were differentially expressed compared to the HF control (Fig. 4). The general trends are that LF diet-fed mice show the most significantly changed gene expression profile, and the açai group displays upregulated expression of genes involved in lipid synthesis compared to the HF control group. Cidea and Cidec, involved in the regulation of lipid droplets, were both found to be downregulated in all berry groups except the açai group, where they were upregulated compared to HF control. In the LF group, several genes involved in acetyl and acyl-CoA metabolism were significantly regulated, such as the Acot and Elovl gene families. The mRNA levels for the gene transcribing the fatty acid-binding protein FABP5 were elevated 8.4 times in livers from the LF control group. FABPs are implicated in fatty acid uptake and metabolism, and polymorphisms in human Fabp5 are associated with T2DM [27]. Except for açai, the mRNA levels for apolipoproteins ApoA5 and ApoM were induced by berry supplementation and LF diet, whereas ApoA4 was downregulated (Fig. 4A). The Acacb gene, encoding ACCβ, was significantly downregulated in the lingonberry and blackcurrant groups and was upregulated in the açai and LF diet group compared to the HF control. The qPCR validation of Acacb and Pparg2 shows the same trends in mRNA expression as in the array (Fig. 4B and C).

3.5. Effects of berries on the expression of cholesterol metabolism genes

In general, genes involved in cholesterol biosynthesis (such as Cyp51, Fdps, Hmgcr, Lss, Nsdhl, Pmvk, Tm7sf2) tended to be upregulated by all diets compared to HF control, but it was most pronounced in the bilberry, açai and LF diet groups (Fig. 5). Notably, the genes encoding
Gene Description

Glutathione conjugating and synthetizing enzymes
Gsta1; a2; a4; k1; m4; t; t3
Glutathione S-transferase alpha 1; alpha 2; alpha 4; kappa 1; mu 2; mu 4; theta 1; theta 2 and 3; Conjugation of glutathione to exogenous and endogenous hydrophobic electrophiles.

Gss Glutathione synthetase; Catalyzes second step of glutathione synthesis.

Response to oxidative stress and xenobiotic metabolism
Aldh1a1; 1a7; 1b7; 3a2; 3b1
Aldehyde dehydrogenase family 1 member A1, A7 and B1;family 3 member A2 and B1; Oxidation and detoxification of various substrates, including reactive lipid-derived aldehydes.

Cyp2a5 CYP, family 2, subfamily A, polypeptide 5 (CYP2A5); Phase I xenobiotic metabolism

Nnmt Nicotinamide N-methyltransferase; Catalyses N-methylation which is important for biotransformation of drugs and xenobiotic compounds. Proposed regulator of adiposity and energy expenditure.

Nqo1 Nad(p)h:quinone oxidoreductase 1; Phase II xenobiotic metabolism

Txnip Thioredoxin interacting protein; Possible oxidative stress mediator.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Gsta1</td>
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</tr>
<tr>
<td>Gsta2</td>
<td>-1.3</td>
</tr>
<tr>
<td>Gsta3</td>
<td>-1.3</td>
</tr>
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<td>Gsta4</td>
<td>1.8</td>
</tr>
<tr>
<td>Gstk1</td>
<td>-1.3 -1.3 -1.4</td>
</tr>
<tr>
<td>Gstm2</td>
<td>-1.6 -1.6</td>
</tr>
<tr>
<td>Gstm4</td>
<td>1.7 1.4</td>
</tr>
<tr>
<td>Gstt1</td>
<td>1.7</td>
</tr>
<tr>
<td>Gstt2</td>
<td>1.7</td>
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<tr>
<td>Gstt3</td>
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<tr>
<td>Gss</td>
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<tr>
<td>Aldh1a1</td>
<td>-1.2 -2.2</td>
</tr>
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<td>Nnmt</td>
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<td>Nqo1</td>
<td>-1.5 -1.5</td>
</tr>
<tr>
<td>Txnip</td>
<td>1.7 1.4 1.5 1.3 1.4</td>
</tr>
</tbody>
</table>

Fig. 6. Effect on the expression of genes involved in redox processes and glutathione metabolism. Genes changed by at least one diet involved in pathways related to glutathione, xenobiotic and drug metabolism and redox activities (identified using DAVID). The fold change is displayed for genes that were significantly changed compared to the HF control. Shades of red and blue indicate the degree of upregulation or downregulation, respectively. White indicates zero difference in fold change compared to the control.

<table>
<thead>
<tr>
<th>Top upstream regulators affected by lingonberries</th>
<th>Effect of lingonberry supplementation</th>
<th>Z-score</th>
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</thead>
<tbody>
<tr>
<td>STAT3 Transcription regulator</td>
<td>Inhibited</td>
<td>-2.1 0.03 -2.5 -</td>
</tr>
<tr>
<td>mTOR Upstream kinase</td>
<td>Inhibited</td>
<td>-2.5 -2.7 -2.2 0.4 -</td>
</tr>
<tr>
<td>SOX11 Transcription regulator</td>
<td>Activated</td>
<td>2.8 - 2.1 -</td>
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</table>

Fig. 7. Upstream regulators predicted in silico to affect gene expression and validation by immunoblotting. (A) The transcription regulators and kinase predicted to be most activated or inhibited by lingonberries (no upstream kinase was predicted to be activated). The prediction z-scores are depicted for all berry groups and represents inhibition (less than −2) or activation (more than 2) by the experimental diet compared to the HF control. Hepatic protein expression of the regulators was measured by immunoblotting in the lingonberry, açai and HF and LF control groups (n=6). Representative blots and quantification of the protein levels of (B) the phosphorylated signal transducer and activator of transcription pSTAT Tyr705 and total STAT3 and (C) the phosphorylated regulatory-associated protein of mTOR pRaptor are shown. pRaptor was normalized against CypB. Values significantly different from the HF control (ANOVA, Dunnett’s posttest) are denoted *P<0.05 and **P<0.01 (n=6).
the rate-limiting steps of cholesterol \((Hmgcr)\) and bile acid \((Cyp7a1)\) synthesis were upregulated 1.8-fold and 3-fold, respectively, in the bilberry group. \(Hmgcr\) was also upregulated in the \(\text{açai}\) group (1.8-fold). The expression of \(Cyp7a1\) and \(Hmgcr\) was quantified by qPCR and found to be significantly upregulated by bilberries compared to HF control (Fig. 5B and C). The gene \(Abcg8\), encoding a cholesterol efflux transporter, tended to be downregulated by all berry groups, although significantly only in the group receiving \(\text{açai}\) (−1.4-fold).

### 3.6. Effects of berries on the expression of genes related to oxidation/reduction processes, glutathione and xenobiotic metabolism

Overall, the gene expression profile of glutathione and redox enzymes in the blackcurrant group mimicked the downregulation observed in the LF diet group, whereas especially \(\text{açai}\), but also lingonberry- and bilberry-fed mice, had a more upregulated expression profile of these enzymes compared to the HF control (Fig. 6), exemplified by \(Gsta1\) that was upregulated by lingonberries and \(\text{açai}\) (2.3- and 2.3-fold) and downregulated by blackcurrant and LF diet (−2.4- and −4.1-fold). \(Cyp2a5\) and \(Nqo1\), involved in xenobiotic metabolism, were significantly downregulated in the blackcurrant and LF diet groups compared to the HF control group. Overall, several glutathione-conjugating and glutathione-synthesizing enzymes were downregulated by the LF diet. The pattern of gene expression of aldehyde dehydrogenases (ALDHs), involved in detoxification of lipid-derived aldehydes, tended to be downregulated in the lingonberry, blackcurrant and LF groups, whereas the opposite was observed in the \(\text{açai}\) group. Nicotinamide N-methyl transferase (\(Nnmt\)) was significantly downregulated in the lingonberry group (−3.2-fold) compared to the HF control.

### 3.7. Identification of upstream regulators driving gene expression changes induced by berry supplementation

Next, we sought to identify transcription factors responsible for the gene expression changes observed following supplementation with lingonberries, the berry with the most pronounced health-promoting effect according to our previous study [10] and with the strongest effects on gene expression (Fig. 2A). Based on the differentially expressed genes, an \textit{in silico} tool for prediction of upstream regulators revealed that the most inhibited transcription regulator was STAT3, the most inhibited kinase was mammalian target of rapamycin (mTOR) and the most activated transcription regulator was sex-determining region Y box (SOX11) (no kinase was predicted to be activated by lingonberries) (Fig. 7A; for total lists, see Supplementary Tables S3 and S4). These results were validated at the protein level by immunoblotting, and the phosphorylation of STAT3 protein was decreased in the lingonberry and the LF control group compared to the HF control (Fig. 7B). LF diet significantly decreased the protein expression of Raptor compared to HF control (Fig. 7C). Protein expression of AMPK and ACC, downstream targets of mTOR, was also measured as effects on AMPK signaling have been described to mediate metabolic effects of resveratrol and other polyphenols [28]. However, no significant differences in total AMPK (subunit \(\alpha_1/\alpha_2, \alpha_3, \beta_1, \beta_2 \text{ or } \gamma_1\)), phosphorylation of AMPK or phosphorylation of the target protein ACC were observed (Supplementary Fig. S1).

The \textit{in silico} upstream analysis predicted that NF-\(\kappa\)B and SREBP1c were regulated in several berry groups (Supplementary Table S4 and Fig. 8). As these transcription factors are master regulators of inflammation and fatty acid synthesis, respectively, we measured their concentration in nuclear extracts of all groups. Interestingly, the NF-\(\kappa\)B complex was predicted to be significantly inhibited by lingonberries (−2.3) and tended to be inhibited also by blackcurrants and bilberries, when compared to the HF control. Immunoblot analysis showed that NF-\(\kappa\)B protein was significantly reduced in nuclear fractions of livers from mice receiving lingonberries, blackcurrants and bilberries compared to the HF control (Fig. 8A). SREBF1 was predicted to be significantly activated by aci and bilberry (−2.8- and 2.9) and inhibited by blackcurrant

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**Fig. 8. Immunoblot analysis of transcription factors NF-\(\kappa\)B and SREBF1c in nuclear extracts.** (A) NF-\(\kappa\)B was predicted by IPA to be significantly inhibited by lingonberries (z-score of less than −2), with a tendency to be inhibited also by blackcurrants and bilberries, when compared to the HF control. Nuclear extracts were subjected to immunoblotting and quantification showed that NF-\(\kappa\)B (p65) protein was significantly reduced in liver nuclear fractions from mice receiving lingonberries, blackcurrants and bilberries. Values significantly different from the HF control (ANOVA, Dunnett’s posttest) are denoted \(*p<0.05\) and \(**p<0.0001\) \((n=11−12)\). (B) Sreb1f1 encodes SREBP1c and was predicted to be highly regulated by different berries, e.g., significantly activated by açai and bilberry (z-score of more than 2) and inhibited by blackcurrant (z-score of less than −2). Compared to the HF control, there were no significant differences in nuclear SREBP1c protein concentration (ANOVA, Dunnett’s posttest). However, there were significant differences among the berry groups, as well as the LF control group (ANOVA, Tukey’s posttest), \(*p<0.05\) and \(**p<0.01\) \((n=11−12)\). The protein expression was normalized against the nuclear marker TBP.
Z-score of −2.1. Immunoblot analysis showed a tendency ($P$=.13) to increase nuclear SREBP1c protein in the açai group (Fig. 8B). Comparison of nuclear SREBP1c between all groups revealed that SREBP1c was increased in the açai and bilberry groups compared to the blackcurrant and LF groups. There was also a significant increase of nuclear SREBP1c in the açai group compared to the lingonberry group.

3.8. Epigenome-wide DNA methylation patterns in mice receiving HF diet with or without lingonberry supplementation

Since lingonberry supplementation had large effects on liver gene expression and phenotype, we decided to further investigate the mechanisms behind the transcription pattern in this group by assessing genome-wide DNA methylation at specific CpG sites. The DNA methylation profile in liver from mice receiving lingonberries was distinct from the profile in control mice receiving HF diet, as seen in Fig. 9A. A total of 24,304 loci were differentially methylated (methylation score difference: $N$=5, $P$<.05), 16,550 loci were hypermethylated and 7754 loci were hypomethylated in lingonberries compared to the HF control group. Out of these, 4413 were in promoter regions, 8253 were in the gene body and 11,638 loci were intergenic (Fig. 9B). Methylation in all samples at 14 CpG sites was assessed using both HELP tagging and pyrosequencing (negative correlation value between techniques: $−0.74$, $P$<.0001) (Supplementary Fig. S2A). Validation by MassArray of two loci of interest

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**Fig. 9. General trends in DNA methylation derived from HELP-tagging analysis of liver from mice receiving HF diet supplemented with lingonberries.** (A) Volcano plot comparing DNA methylation in liver of mice receiving HF diet supplemented with lingonberries versus control mice receiving only HF diet (plot of loci-specific methylation differences ($x$-axis) vs. $−\log P$ value ($y$-axis)). The black dots represent the loci with a significant methylation difference greater than 5, $P$<.05. (B) Visualization of significantly DMRs in promoter (Prom), gene body (GB) or intergenic regions (IG). Black bars represent DMRs with hypomethylation, and gray bars represent DMRs with hypermethylation in lingonberry-supplemented mice. (C) The analyzed CpG sites were mapped according to gene region, here defined as being in the promoter [−2 kb/+2 kb of the transcription start site (TSS)] or inside the gene body. All other loci were classified as intergenic. (D) Supplementation with 20% lingonberries to the HF control diet induced hypermethylation at sites associated with Ncor2 and Il16 (mean values and difference, $n$=6, MassArray). The mean fold changes (FC) in mRNA expression in the lingonberry group vs. HF control are reported; however, the changes were not significant ($P$>.05). (E) The top most significant pathways enriched in genes with differentially hypomethylated or hypermethylated loci ($N$=5, $P$<.05) in the promoter region are visualized. The number of DMR-associated genes in each pathway, percentage of mapped genes/total number of genes and $P$ values are displayed.

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<table>
<thead>
<tr>
<th>Location</th>
<th>DNA methylation (%)</th>
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<tr>
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**Table:**

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showed that a CpG site (loci chr5:125521063) in the gene body of nuclear receptor corepressor 2 (Ncor2) and a CpG site (loci chr7:90885449) in the promoter region of interleukin 16 (Il16) were significantly hypermethylated in the lingonberry group compared to HF control (+13.5 and +8.5, respectively, P<0.01) (Fig. 9D). The hypermethylation of Ncor2 was validated in a biological replicate cohort (+11.3, P<0.05); however, the smaller difference in methylation of Il16 (+1.8) was not significant (Supplementary Fig. S2B). Gene expression of Ncor2 tended to be downregulated in the lingonberry group (Fig. 9D); however, the effect was not significant (P=0.2). The 100 top most significant DMRs with a difference in methylation score of >5 are listed in Supplementary Table S5.

Several DMRs were in the promoter or gene body region of genes that were differentially expressed or characterized as important for the phenotype based on the microarray gene expression analysis, for example the inflammatory-related genes Cfd, Cxcl11 and Cdf5 and lipid/cholesterol genes Apoa5, Elov6 and Cyp7a1 (Supplementary Table S6). Overall, the pathways associated with genes containing differentially methylated loci in the promoter region were related to categories such as transcriptional regulation, morphogenesis and regulation of metabolic processes (Fig. 9E).

4. Discussion

The liver plays a central role in energy homeostasis and maintenance of insulin sensitivity, and disturbance of its function by nutritional overload such as HF feeding may result in steatosis, inflammation and insulin resistance. We have previously described the prevention of steatosis and obesity by lingonberries, bilberries and blackcurrants [10]. In the present study, we extend these findings and show that intake of berries modulates the liver response to a HF diet. The protective effects of lingonberries and bilberries result in a strong inhibition of acute-phase reaction and inflammation. Blackcurrant supplementation inhibits hepatic lipid synthesis, thereby contributing to the lean phenotype. In contrast, the addition of açai berry to a HF diet results in a pronounced upregulation of pathways of lipid and cholesterol metabolism, explaining the exacerbation of fatty liver development by this berry.

4.1. Trends in gene expression correlate with mouse phenotype

As expected, the LF diet group had the highest number of differentially expressed genes compared to the HF diet control group. However, it is interesting to note that the lingonberry diet, which had the same fat content as the HF control diet, mirrored the LF diet by affecting nearly as many genes. On the same note, the obese mice in the açai group, with large steatotic livers [10], had the lowest number of changed genes compared to the HF control mice, indicating that açai is less efficient than other berries in providing protection against HF diet-induced metabolic changes. Further supporting this notion is the finding that, out of the 10 genes identified as being affected by all diets, Cidea and Anxa2 were upregulated only in the group receiving açai berries. Cidea induction is closely related to fat accumulation in hepatic steatosis [29] and Cidea-null (−/−) mice are resistant to diet-induced obesity and insulin resistance [30]. Anxa2 may be upregulated by oxidative stress [31] and has been proposed to be a marker of adipose tissue dysfunction and insulin resistance that can be targeted by dietary interventions [32]. In contrast to açai, supplementation with lingonberry, blackcurrant and bilberry resulted in mice with similar body weight and liver status to mice fed a LF diet [10]. However, as shown in the present study, hepatic gene expression and regulated pathways differed between the groups, implying that the berries exert their beneficial metabolic effects by different mechanisms.

4.2. Lingonberry and bilberry intake have antiinflammatory effects

Inflammation is a key factor in the progression of fatty liver into more severe stages, and it appears to be required for development of hepatic insulin resistance [33–35]. Furthermore, obesity is closely associated with a state of chronic low-grade inflammation [2]. From this perspective, it is interesting to observe that pathways related to acute-phase and inflammatory responses were inhibited and several inflammatory genes were several-fold downregulated in the lingonberry and bilberry groups. SAA proteins are proposed mediators of inflammation with increased levels being associated with obesity and insulin resistance [36]. SAA1 is produced in the liver and the levels rise during inflammation and upon LPS challenge [37]. The fact that three isoforms of Saa were considerably downregulated by lingonberries and that also the plasma levels of SAA were decreased suggest that the mice receiving HF diet supplemented with lingonberries were protected against HF diet-induced inflammation. Neutrophil chemottractant CXCL1 levels are elevated in obese db/db mice and T2DM patients [38,39] and were affected by berry supplementation in our study. Similar to Saa, the mRNA expression as well as the plasma levels of CXCL1 was decreased by lingonberries, bilberries and LF diet.

Another highly regulated gene was Lcn2, encoding lipocalin 2, which was 10- to 15-fold downregulated in groups receiving lingonberries, bilberries and LF control. The lipocalin family plays a role in antimicrobial and antiinflammatory response and Lcn2 has been shown to be expressed mainly in liver upon acute-phase response [40]. Lcn2 has been linked to obesity and insulin resistance and was recently proposed to be a key modulator of hepatic lipid homeostasis by controlling intracellular lipid droplet formation [41]. Furthermore, Lcn2 is an indicator of liver damage and Lcn2 expression in liver correlates with steatosis in obese human subjects [42]. The finding that Lcn2 was highly downregulated in mice receiving lingonberries and bilberries further strengthens that these berries attenuate HF-induced hepatic steatosis and inflammation, resulting in a liver phenotype similar to the LF-fed mice.

An interesting observation was that Cfd, encoding complement factor D, was downregulated by lingonberries and LF diet, whereas it was upregulated 4.4 times by açai intake compared to mice receiving the HF control diet. Complement factor D, also known as adipsin, plays a role in humoral suppression of infectious agents and it has been shown that Cfd mRNA is dramatically induced by HF diet in the liver of C57BL/6 mice concomitant with the development of steatosis [34,43,44]. Upregulation of Cfd by açai supplementation to HF diet further strengthens the notion that açai promotes, rather than prevents, hepatic steatosis.

Based on the pathway analysis and regulation of specific genes, we conclude that HF-induced hepatic inflammation is counteracted by supplementation with lingonberries and also bilberries. In our previous study [10], we showed that mice receiving a LF diet had smaller livers with lower triacylglycerol content than the bilberry-supplemented group. However, the pathway analysis of the livers from the LF group did not indicate inactivation of inflammatory pathways, even if several genes related to inflammation were downregulated in liver of these mice compared to the HF diet control group. This implies that supplementation with lingonberries and bilberries to HF diet have antiinflammatory effects beyond reduction of fat accumulation.

4.3. Lingonberries, blackcurrants, bilberries and açai exert their effects on lipid metabolism by different mechanisms

Several factors may contribute to the development of liver steatosis, such as increased dietary lipid intake, impaired VLDL secretion, increased hepatic lipid synthesis (de novo lipogenesis), impaired hepatic fatty acid oxidation and increased free fatty acid flux.
from adipose tissue [7,8,45]. In this study, we observed that HF diet downregulates expression of genes in lipid and cholesterol biosynthesis, fatty acid synthesis and lipogenesis compared to mice receiving the normal chow LF diet. Similar results have been reported by others [34] and are expected since intake of LF diet results in an increased demand for endogenous lipid synthesis, and the observed upregulation of Fasn could be an attempt to optimize fatty acid uptake. Furthermore, we found that HF diet-induced fatty liver occurred concurrently with increased expression of several genes previously shown to be associated with steatosis (e.g., Cfd, Acau1b, Cidea, Cidec, Anxa2 [5,34]).

Supplementing the HF diet with different berries significantly altered hepatic gene expression, even though the dietary fat content was kept constant. Compared to the HF control group, the decreased steatosis of the lingonberry and blackcurrant groups [10] was accompanied by reduced expression of genes involved in lipid biosynthesis pathways, such as Elovl6 and Scd1, whereas the reduction in Acau in these groups could imply reduced inhibition of β-oxidation. Scd1 knock-out mice are protected against diet-induced obesity and hepatic steatosis [46] and Elovl6 knock-out mice are resistant to diet-induced insulin resistance [47]. Based on these results and phenotype, we suggest that lingonberries and blackcurrants protect against HF-induced steatosis at least in part by downregulating lipid synthesis to a level where harmful lipid accumulation is prevented.

The upregulation of lipid biosynthetic pathways observed in the bilberry group may represent a compensatory mechanism to the increased fecal excretion of triacylglycerol and cholesterol previously observed in this group [10]. In addition, the mice in this group had a significantly higher food intake compared to the HF control. Based on this, we conclude that the prevention of hepatic steatosis as well as adiposity and body weight gain by bilberries is at least in part due to reduced intestinal lipid absorption. The prominent upregulation of a large number of genes involved in cholesterol biosynthesis and bile acid synthesis, including Hmgcr and Cyp7a1, provides further support for this conclusion. The upregulation of Cyp7a1 (3-fold) implies an increased need for bile acid synthesis to compensate for reduced lipid absorption and/or loss of bile acids, likely due to increased bulk in the intestine hindering bile acid reuptake. Polyphenol-rich extracts from berries have been shown to inhibit pancreatic lipase in vitro [48], and it is possible that this mechanism plays a role also in vivo. Interestingly, a dietary bilberry extract reduced liver and serum triglycerides and cholesterol as well as liver weight in T2DM mice, without affecting body weight gain or food intake [49]. In a human study, daily consumption of bilberry puré and dried bilberries upregulated markers of cholesterol synthesis, and a marker of cholesterol absorption tended to be downregulated [50]. Furthermore, a recent study by Mykkänen et al. [51] validates our previous findings that supplementation with 20% of freeze-dried bilberries prevents weight gain in a HF diet mouse model [10]. Mykkänen et al. show that 10% of freeze-dried whole bilberries supplemented to HF diet prevent body weight gain in C57BL/6j mice [51]. Based on our results and these findings, we propose that intake of whole bilberries in larger doses reduces cholesterol and lipid absorption, potentially contributing to prevention of weight gain.

Livers from mice receiving açai displayed a strong upregulation of several pathways and genes related to triacylglycerol as well as cholesterol synthesis compared to the HF diet control group. In addition, the gene encoding the transporter of cholesterol from the liver into bile (Abcg8) was downregulated. Several steatosis-associated (Crat, Cidec and Cfd) and lipid-synthesizing (Ppaq and Elovl6) genes were upregulated in the açai group compared to HF diet-fed mice. The upregulation of Acau could imply less β-oxidation of fatty acids, further aggravating the steatosis. In contrast to LF and bilberry, we propose that the increased fat and cholesterol synthesis in response to açai is maladaptive since it led to a large increase in liver size and fat content compared to the mice receiving HF diet alone [10].

Apolipoproteins are important in systemic lipid metabolism, and emerging evidence suggests a role for apolipoproteins in intracellular lipid homeostasis and crosstalk between adipose tissue and liver [52]. ApoA5 is predominantly expressed in the liver and was upregulated by lingonberry and bilberry diets. Studies have shown that plasma levels of ApoA5 are reduced in obese subjects and inversely correlated with measures of insulin resistance [33]. In hepatocytes and adipocytes, ApoA5 localizes on the surface of cytoplasmic lipid droplets, and incubation with ApoA5 has been shown to reduce cellular triglyceride content in human adipocytes [54,55]. ApoM overexpression has been shown to improve insulin sensitivity in rats [56] and ApoM was found to be upregulated by all berries, except açai, which downregulated ApoM. In a study by VerHague et al., HF-fed C57BL/6 mice developed steatosis, which was associated with increased hepatic ApoA4 mRNA expression [57]. In our study, HF diet-induced ApoA4 expression was decreased by all berries except açai.

4.4. Lingonberries and açai induce expression of genes related to oxidation/reduction processes, glutathione and xenobiotic metabolism

The liver, as the major organ for biotransformation of drugs and foreign compounds, is continuously exposed to reactive oxygen species (ROS). This may cause peroxidation of polyunsaturated fatty acids in lipid membranes, producing unstable lipid hydroperoxides that decompose into cytotoxic aldehydes [58]. Berries are among the plants with the highest content of antioxidants and have been shown to protect against oxidative stress-related pathologies in vivo [59]. Lingonberries possess high in vitro antioxidant activity and can increase glutathione reductase activity in livers of rats on HF diet [60,61]. HF diet is proposed to cause oxidative stress in liver, and we observed an upregulation of antioxidant enzymes and pathways of oxidation/reduction and drug, xenobiotic and glutathione metabolism by HF diet compared to LF diet. This presumably represents an adaptive response to protect against ROS, which is line with previous findings in the same mouse model [34].

Reactive lipid aldehydes have shown to be increased in livers of NAFLD patients [62]. ALDHs may be induced to metabolize lipid aldehydes to less toxic compounds [58]. In our study, several Aldhs had lower expression in mice receiving LF diets as well as HF diet supplemented with lingonberries and blackcurrants, suggesting less lipotoxicity, whereas Aldh mRNA was increased by açai supplementation. Another enzyme family important for protective biotransformations is the family of enzymes catalyzing N-methylation, including nicotinamide N-methyltransferase (Nnmt). A recent study states a role for Nnmt in insulin resistance and obesity as a mediator of weight gain [63]. Moreover, Nnmt expression is increased in livers of obese and diabetic mice, and Nnmt knockdown protects against diet-induced obesity. We observe that the gene expression of Nnmt was downregulated 3-fold in the lingonberry group.

Dietary polyphenols have been studied for their strong antioxidative capacity and more recently for their potential to activate the endogenous antioxidant defense enzymes, which protect against lipid peroxidation under metabolic oxidative stress [64]. These, and other effects, could be mediated by polyphenols interfering with redox-sensitive thiol groups influencing transcription factors and kinases [65]. An interesting target of polyphenols is activation of the transcription factor Nrf2, which can bind to antioxidant-responsive elements and induce, for example, glutathione S-transferases, which mediate protection against reactive compounds by conjugation to glutathione [66]. Interestingly, the genes of several enzymes in this family were upregulated by lingonberry and açai intake, compared to ingestion of HF diet alone. In addition, Nr1f2 (gene name NFE2L2) was
predicted to be activated by both lingonberries and açai (Supplementary Table S4).

All berries increased pathways involved in oxidation/reduction. However, pathways related to metabolism of glutathione, drug and xenobiotics were most prominently increased by lingonberries and were decreased by blackcurrants. We speculate that the different gene expression profiles induced by the different berries reflect not only effects on hepatic redox homeostasis but also adaptations to the metabolism of the polyphenols themselves as a direct consequence of the different phenolic profiles of the berries [67].

4.5. Berries mediate effects on inflammation and steatosis by affecting STAT3, mTOR and NF-κB pathways in vivo

In an effort to elucidate the upstream regulatory factors, we used an in silico prediction program designed to cluster regulated genes under known regulatory proteins. The analysis revealed that STAT3 was predicted to be inhibited by lingonberry and bilberry supplementation. STAT3 is activated in response to various cytokines and growth factors, and it plays a role in many cellular processes, including inflammation and cell growth. STAT3 may be activated by phosphorylation, dimerization and translocation to the nucleus where it induces transcription of, for example, acute-phase proteins [68]. We could verify at the protein level that the lingonberry-fed mice had a reduced level of STAT3 phosphorylation compared to the HF control, which is well in line with the reduced expression of several STAT3 target genes (Saos, Coxl, Cct4 and Soc3; Supplementary Table S3) in this group. Hence, we propose that lingonberries inhibit the inflammatory response in the liver of HF-fed mice in part by suppressing phosphorylation and activation of STAT3. Reduced STAT3 phosphorylation has previously been reported in vitro in response to black raspberry extract and several polyphenols [69,70]; however, the underlying mechanisms are not understood. Furthermore, hepatic STAT3 overexpression results in increased circulating lipids and increased expression of lipogenic genes [71], and in humans, variants of STAT3 have been shown to be associated with NAFLD [72]. STAT3 phosphorylation is stimulated by LPS [73]. It is possible that compounds in lingonberries and bilberries modulate the gut microbiota in a beneficial manner, resulting in improved barrier function and less leakage of LPS into the blood stream. Indeed, upstream analysis predicted an inhibition of LPS-mediated gene expression in livers from mice receiving lingonberries and bilberries (Supplementary Table S4).

The proinflammatory transcription factor complex NF-κB was also predicted in silico to be inhibited by lingonberries. In agreement, we found that the nuclear protein content of NF-κB p65 was reduced by lingonberries, blackcurrants and bilberries compared to the HF control, implying that NF-κB translocation and thus activation of proinflammatory gene transcription are prevented by these berries. Studies on cancer inflammation suggest that there are interactions between NF-κB and STAT3 inflammatory signaling pathways creating a loop of prolonged NF-κB activation important for chronic inflammation [73,74]. Furthermore, NF-κB activity in liver has been shown to increase concomitant with the appearance of hepatic insulin resistance in a study investigating the time and tissue dependence of HF diet-induced insulin resistance [33].

Another interesting upstream regulator is the mTOR kinase, which was predicted to be inhibited by lingonberries, blackcurrants and bilberries. Protein expression analysis of Raptor, which forms the mTORC1 complex 1 (mTORC1) together with mTOR and other proteins, revealed that HF diet significantly upregulated Raptor compared to LF diet. This upregulation tended to be inhibited by lingonberries and exacerbated by acai supplementation. mTORC1 integrates environmental cues, such as nutrients, in the regulation of energy balance and metabolism. Overfeeding and high levels of nutrients and cytokines activate lipogenesis via mTORC1, and inhibition of hepatic mTORC1 impairs SREBP function and protects mice against HF-induced hepatic steatosis [75–77]. In addition, mice lacking Raptor in liver macrophages were protected against inflammation and insulin resistance [78]. Considering their regulatory role in nutrient, lipid and inflammatory processes, Raptor and mTOR are additional important regulators found to be targeted by berry supplementation. Previous studies have found that compounds such as flavonols can target mTOR, but the precise mechanism has not been defined [77]. SREBP1c is a major regulator of fatty acids and triacylglycerol synthesis and was among the top predicted upstream regulators. The prediction was based on the compelling number of SREBP1c target genes that were upregulated by acai and downregulated by in particular blackcurrants. The pattern of nuclear translocation of SREBP1c corresponded well to the observed phenotype and predictions, although none of the changes were significant compared to the HF control. However, it should be noted that immunoblot analysis of nuclear extracts does not take into account posttranscriptional modifications and interacting factors that mediate SREBP1c transcription activation. In view of the large effect of acai on SREBP1c target genes, it is tempting to speculate that acai contains one or several bioactive components that activate SREBP1c signaling, thereby promoting development of hepatic steatosis.

We conducted a thorough AMPK analysis since activation of AMPK has been implicated in the antidiabetic effects of a wide variety of polyphenols and plant extracts, including lingonberry and bilberry extracts [28,49,79,80]. Eid et al. have shown that lingonberry extracts may increase the phosphorylation of the AMPK target ACC in myotubes and that mice with diet-induced obesity supplemented with lingonberry extract have higher levels of phosphorylated AMPK in the liver. In addition, pharmacological activation of AMPK has been shown to suppress IL-6-induced STAT3 phosphorylation in C57BL/6N mouse liver [81], and AMPK is proposed to negatively regulate Raptor/mTOR [82]. In the present study, we did not observe lingonberry-induced phosphorylation and activation of AMPK or its target ACC. In addition, there was no increased expression of AMPK subunits at the gene or protein level. We conclude that hepatic AMPK activation is likely not involved in the health-promoting effects of lingonberries observed in our study [10], although we cannot rule out that the AMPK pathway is affected in other tissues and/or other time points than what was studied or that the discrepancy with previous literature is explained by the use of whole lingonberries, as supposed to extracts.

4.6. Lingonberry supplementation alters genome-wide DNA methylation profile

Epigenetic modification is one way for environmental factors, such as diet, to interact with metabolism and fine-tune gene expression. A growing body of literature suggests that aberrant DNA methylation may play a role in diseases such as NAFLD and T2DM [83–85], although the understanding of these mechanisms remains limited. The effect of HF diet on hepatic methylation patterns has been studied previously in mouse models, but mainly in utero studies or in a context of deficiency of or supplementation with methyl donors [86]. We hypothesized that lingonberries may modulate HF-induced gene expression by acting on DNA methylation. Interestingly, we observed that lingonberry supplementation to an obese genetic HF diet induced a promoter-specific hypomethylation and a global shift toward DNA hypermethylation. This may be of importance as HF diet has been shown to induce DNA hypomethylation in mouse liver [87]. Furthermore, in our study, several DMRs were associated with genes in pathways that were highly affected by lingonberry supplementation, such as inflammation and lipid metabolism pathways. Ncor2, a key transcription corepressor, was found to be hypermethylated in livers from mice receiving lingonberries compared to mice receiving HF diet only. Notably, a recent human study found that Ncor2 was
hypermethylated in adipose tissue in response to exercise [88]. NCoR (also known as SMRT) is implicated in the regulation of lipid, inflammation and oxidative stress pathways. For example, NCoR/SMRT has been shown to mediate effects on Nrf2-induced Gst expression [89] and cytokine expression in macrophages by interaction with PPARγ2/NF-κB [90].

In general, the overlap of differentially methylated and expressed genes was low in our data and methylation of specific genes did not explain their expression changes at this time point. The analysis of differentially methylated pathways showed that genes with DMRs in the promoter region were involved in broad functional categories such as transcription and morphogenesis rather than the categories that were most transcriptionally affected. DNA methylation is typically associated with gene repression; however, crosstalk between transcription factors and methylation of CpG sites in regulatory elements may affect chromatin state and gene expression in a manner that is difficult to predict with current knowledge and tools. To the best of our knowledge, this is the first study utilizing a genome-wide technique assessing methylation of individual CpG sites in liver in response to HF diet and HF diet supplemented with a natural compound effective in preventing adiposity and NAFLD. The HELP-tagging technique provides data for approximately 1.8 million CpG sites throughout the genome covering many but not all sites. Putative CpG sites of importance, not located in HpaII/MspI recognition sites, are overlooked by this technique. Furthermore, a diet modification such as lingonberry supplementation of HF diet may not induce dramatic changes in DNA methylation such as those observed following early-life perturbation of nutrient provision during fetal and/or neonatal development [22,86]. It is also possible that a larger number of animals are required to identify with certainty small, but meaningful, changes in DNA methylation associated with lingonberry supplementation of HF diet. Also, it is possible that methylation crosstalk with other epigenetic modifications such as histone marks in the liver and/or other tissues is important in our model. Given the large number of epigenetic modifiers and marks, we still know very little about the ways in which environmental cues may alter chromatin landscapes and regulatory factors to induce global chromatin modifications and control the expression of individual genes [91].

4.7. Concluding remarks

We report that consumption of lingonberries, and also blackcurrants and bilberries, modulates the liver response to HF diet of an in vivo model by altering the expression of genes related to mainly inflammation and lipid metabolism. We propose that reduced activities of NF-κB, STAT3 and mTOR are important mechanisms by which lingonberries, in particular, but also bilberries and blackcurrants, prevent HF-induced steatosis, low-grade inflammation and insulin resistance (summarized in Fig. 10).

Given the scarce knowledge of the complex interactions of nutrients and host metabolism in vivo, we believe that this study provides valuable mechanistic insight into how chronic intake of certain foods may help prevent disease. Knowledge regarding the mechanisms behind the capacity of lingonberries, blackcurrants and bilberries to prevent steatosis can be used to better target and design future studies, which should include studies in humans, as increased intake of these berries may be a useful strategy to prevent disease development caused by an unbalanced Western-style diet.

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Authors' contribution

LHL conducted the study; extracted RNA/DNA; performed DAVID analysis, qPCR, immunoblotting and plasma measurements; and wrote the paper. LHL together with YS performed the HELP tag assay and data analysis, with invaluable advice on design and data interpretation from MJC. YS conducted MassArray and contributed to writing the paper. LHL and HAJ performed upstream analysis, and HAJ conducted database searches and took active part in all steps of interpreting results, developing and writing the manuscript. PS performed bioinformatics analysis of the microarray data and contributed to the manuscript. LHL, KB and CH designed the study. KB and CH performed bioinformatics analysis of the microarray data and contributed to the manuscript. LHL conducted the study; extracted RNA/DNA; performed DAVID analysis, qPCR, immunoblotting and plasma measurements; and wrote the paper. LHL together with YS performed the HELP tag assay and data analysis, with invaluable advice on design and data interpretation from MJC. YS conducted MassArray and contributed to writing the paper. LHL and HAJ performed upstream analysis, and HAJ conducted database searches and took active part in all steps of interpreting results, developing and writing the manuscript. PS performed bioinformatics analysis of the microarray data and contributed to the manuscript. LHL, KB and CH designed the study. KB and CH supported in interpreting data and writing of the paper.

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<table>
<thead>
<tr>
<th>Berry</th>
<th>Effect on HF-induced steatosis and obesity</th>
<th>Proposed regulators and mechanisms</th>
</tr>
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<tbody>
<tr>
<td>Lingonberry</td>
<td>Prevention</td>
<td>Anti-inflammation -reduced STAT3, mTOR and NF-κB signalling.</td>
</tr>
<tr>
<td>Blackcurrant</td>
<td>Prevention</td>
<td>Decreased lipid synthesis -likely involving inhibition of SREBP1c transcriptional activity. Anti-inflammation -reduced NF-κB translocation to nucleus.</td>
</tr>
<tr>
<td>Bilberry</td>
<td>Prevention</td>
<td>Anti-inflammation -reduced NF-κB translocation to nucleus.</td>
</tr>
<tr>
<td>Açaí</td>
<td>Aggravates hepatic steatosis and obesity</td>
<td>Increased lipid synthesis -increased mTOR signaling -likely involving activation of SREBP1c transcriptional activity.</td>
</tr>
</tbody>
</table>

Fig. 10. Summary of findings and suggested main regulators and mechanisms explaining the effects on liver of berry supplementation to a HF diet.


