RESEARCH ARTICLE

Bilberries reduce low-grade inflammation in individuals with features of metabolic syndrome

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Scope: Low-grade inflammation is a hallmark of cardiometabolic risk. Bilberries (*Vaccinium myrtillus*) are rich in polyphenols with potential anti-inflammatory properties. We studied the impact of bilberries on inflammation and gene expression profile in peripheral blood mononuclear cells in subjects with metabolic syndrome.

Methods and results: In randomized, controlled dietary intervention, the participants consumed either a diet rich in bilberries ($n = 15$) or a control diet ($n = 12$). The bilberry group consumed daily an equivalent dose of 400 g fresh bilberries, while the control group maintained their habitual diet. No differences were found between the groups in body weight, glucose, or lipid metabolism, but bilberry supplementation tended to decrease serum high-sensitivity C-reactive protein, IL-6, IL-12, and LPS concentrations. An inflammation score was significantly different between the groups ($p = 0.024$). In transcriptomics analyses (three participants with improved oral glucose tolerance test in the bilberry group), Toll-like receptor signaling, cytoplasmic ribosomal proteins, and B-cell receptor signaling pathways were differently regulated. QPCR analyses ($n = 13$ and 11 in the bilberry and control groups, respectively) showed decreased expression of *MMD* and *CCR2* transcripts associated with monocyte and macrophage function associated genes.

Conclusion: Regular bilberry consumption may reduce low-grade inflammation indicating decreased cardiometabolic risk in the long term.

Keywords:
Bilberry / Gene expression / Inflammation / Metabolic syndrome

1 Introduction

Comorbidities of obesity, such as metabolic syndrome, type 2 diabetes, and cardiovascular diseases are increasing in epidemic proportions. Many of these health problems are preventable by lifestyle changes including weight control,

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Abbreviations: CCR2, chemokine (C-C motif) receptor 2; **CD**, cluster of differentiation; **CD19**, CD19 molecule; **CD72**, CD72 molecule; **COX7B**, cytochrome c oxidase subunit VIIb; **DAPP1**, dual adaptor of phosphotyrosine and 3-phosphoinositides; **hsCRP**, high-sensitivity C-reactive protein; **FSIGT**, frequently sampled intravenous glucose tolerance test; **IFN-γ**, interferon gamma; **Ly96 (MD2)**, lymphocyte antigen 96; **MCP**, monocyte chemoattractant protein; **MMD**, monocyte to macrophage differentiation associated; **OGTT**, oral glucose tolerance test; **PBMCs**, peripheral blood mononuclear cells; **RIPK-1**, receptor (TNFRSF)-interacting serine-threonine kinase 1 (receptor interacting protein kinase 1); **RGS18**, regulator of G-protein signaling 18; **TAB-2**, TGF-beta activated kinase 1/MAP3K7 binding protein 2; **TGF**, transforming growth factor; **TICAM1**, Toll-like receptor adaptor molecule 1; **TLR**, Toll-like receptor; **TNFRSF12A**, tumor necrosis factor receptor superfamily, member 12A; **WDSUB1**, tryptophan-aspartate (WD) repeat, sterile alpha motif and U-box domain containing 1

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increased exercise, and beneficial dietary changes [1, 2]. However, it is not fully understood which dietary patterns, foods, and food components have the highest potential in reducing the risk of chronic diseases.

Low-grade inflammation has been suggested as a mechanistic link between obesity and its consequences on cardiometabolic health [3, 4]. Several markers of inflammation, such as high-sensitive C-reactive protein (hsCRP), IL-6, tumor necrosis factor-α, and IL-1β, have been studied in association with obesity, metabolic syndrome, and the risk of chronic diseases, but their role is thus far not fully established. However, hsCRP has been accepted for clinical applications to evaluate the individual risk for chronic diseases [5–7], and many inflammatory markers have also been evaluated for their response to lifestyle modification, such as dietary changes [3, 8].

How the inflammatory state develops is currently under debate. One of the current theories is that the inflammation is caused by enhanced leakage of LPS, characteristic cell wall components of gram-negative bacteria, from gut microbiota to circulation in the state of obesity [9, 10]. Dissociated LPS can translocate to the circulation by active transport through enterocytes by chylomicrons or passive extracellular leakage through tight junction in epithelial lining [9, 11]. Animal studies have shown that LPS induces inflammation as well as weight gain, fasting hyperglycemia, and insulin resistance [10]. In humans, serum LPS is associated with an increased risk of type 2 diabetes [12]. Therefore, lowering plasma LPS concentration could be a potent strategy for the control of metabolic diseases.

Dietary polyphenols may influence glucose metabolism at many levels including modulation of intracellular signaling pathways and gene expression [13]. The evidence on the effects of polyphenols on lipid metabolism is, however, inconsistent [14]. Polyphenols may also harbor anti-inflammatory properties [15]. Polyphenol-rich foods may, therefore, have implications for the prevention of insulin resistance, metabolic syndrome, and type 2 diabetes.

Polyphenols are phytochemicals ubiquitous in fruits, berries, and vegetables and in plant-derived beverages including tea, coffee, wine, and cocoa [16, 17]. In the Finnish diet, berries and berry products are important sources of polyphenols [18]. Among the commonly consumed berries, bilberries (*Vaccinium myrtillus*, wild European blueberries) have the highest level of polyphenols, particularly anthocyanins [19].

The aim of the present study was to examine the effects of a bilberry-rich diet on inflammation, gene expression in peripheral mononuclear cells (PBMCs), and on glucose and lipid metabolism in subjects with features of metabolic syndrome.

2 Materials and methods

2.1 Study participants

Study participants were recruited with the announcements in local newspapers. At screening, the health status and medical history of the volunteers were assessed by an interview and by laboratory measurements including routine hematological measures as well as the measures of thyroid, liver, and kidney functions. Height, weight, waist circumference, blood pressure, and fasting plasma glucose and lipids were measured. Volunteers with lipid lowering medication were excluded. The inclusion criteria were overweight (BMI 26–39 kg/m^2), and two of the following: elevated fasting plasma glucose in the absence of diabetes (5.6–6.9 mmol/L), abnormal fasting serum lipids (triglycerides $\geq$1.7 mmol/L, HDL cholesterol $<$1.0 mmol/L (males) or $<$1.3 mmol/L (females)), waist circumference $>$102 cm (males) or $>$88 cm (females), and blood pressure $\geq$130/85 mmHg [20, 21].

Table 1. Basic characteristics of the study participants in the bilberry (BB) and control (C) diet groups[a)]

	BB	C
n (M/F)	15 (5/10)	12 (3/9)
Age (year)	53 ± 6	50 ± 7
Body weight (kg)	85.4 ± 12.1	93.1 ± 10.8
BMI (kg/m^2)	31.4 ± 4.7	32.9 ± 3.4
Waist circumference (cm)	101.7 ± 11.0	105.0 ± 8.1
Body fat (%)	35.6 ± 7.8	36.0 ± 6.8
Fasting plasma glucose (mmol/L)	6.1 ± 1.1	5.8 ± 0.5
Fasting serum cholesterol (mmol/L)	5.52 ± 0.88	5.50 ± 0.54
Fasting serum LDL cholesterol (mmol/L)	3.57 ± 0.68	3.66 ± 0.50
Fasting serum HDL cholesterol (mmol/L)	1.31 ± 0.26	1.34 ± 0.26
Fasting serum triglycerides (mmol/L)	2.22 ± 0.66	2.07 ± 0.88
Systolic blood pressure (mmHg)	147 ± 18	144 ± 12
Diastolic blood pressure (mmHg)	93 ± 9	92 ± 8

a) All values are means ± SD. General linear model for univariate analysis showed no statistical differences between the groups.

Altogether 44 individuals were randomized in the control (C) and bilberry (BB) groups for age, gender, BMI, and fasting glucose. Matching produced equal number of subjects in the strata classes among the two groups. Five participants from both groups did not start the trial or dropped out during the run-in period (control: $n = 3/2$ and bilberry: $n = 2/3$, respectively). Thus, 34 participants started the actual intervention, of which 27 participants completed the study (Table 1).

The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human participants were approved by the Research Ethics Committee of the Hospital District of Northern Savo (Finland). Written informed consent was obtained from all participants.

2.2 Study design and sampling

The study included 4-week run-in, 8-week dietary intervention, and 4-week recovery periods. During the run-in period,

the participants were asked to maintain their habitual diet, but restricting their daily consumption of berries to 1 dL (corresponding to 80 g) of fresh berries, at maximum. During the recovery period, they returned to their habitual diet with berry restriction. At the end of the run-in period and dietary intervention, the study participants underwent an oral glucose tolerance test (OGTT) and frequently sampled intravenous glucose tolerance test (FSIGT), their blood pressure was measured and blood samples were collected for measurements of serum lipids and markers of cholesterol metabolism and inflammatory responses. In addition, blood samples were collected at the end of the recovery period for the analyses of possibly sustained inflammatory responses for hsCRP and IL-6. Blood samples for the isolation of PBMCs were taken before and after the dietary intervention period. In addition to the basic anthropometric measurements (body weight, height, and waist circumference), the body composition was assessed with bioelectrical impedance (STA/BIA Body Composition Analyzer, Akern Bioresearch Srl, Florence, Italy).

2.3 Dietary intervention

Dietary intervention lasted 8 weeks (weeks 0–8). The participants in BB group ($n = 15$) consumed daily an equivalent (eq.) dose of 400 g fresh bilberries comprising of 200 g of bilberry purée and 40 g of dried bilberries (eq. 200 g of fresh bilberries), substituting evenly the other sources of carbohydrates (such as fruits, other berries, breads, and other cereal products) in the participants' habitual diet by the provided bilberry products. Consumption of other berries was not allowed during the intervention. The participants in the C group ($n = 12$) were asked to maintain their habitual diet, but the use of berries was allowed occasionally at maximum of 1 dL/day (corresponding to 80 g/day). A clinical nutritionist gave detailed individual counseling concerning the use of the bilberry products and the maintenance of the caloric intake at the level of the habitual diet to prevent undesired changes in body weight. Otherwise, the participants were asked to maintain their habitual lifestyle throughout the intervention.

The study products were two commercial sweetened whole-berry products: a frozen bilberry purée (manufactured by Pakkasmarja Oy, Suonenjoki, Finland) and dried bilberries (Kiantama Oy, Suomussalmi, Finland). The purée consisted of bilberries (84%), sucrose (15%), and guar gum (1%). Frozen berries, sucrose, and the thickener were mashed and the purée was immediately frozen. The dried bilberries contained 1% added sucrose and <1% added vegetable oil. The purée is a healthier choice for a jam or juice, with higher content of berries and lower content of sugar. The dried berries served as a healthier choice to replace sweets and other sugary snacks.

Anthocyanin and flavonol contents of the bilberry products were analyzed by HPLC [22, 23]. The anthocyanin contents of the purée and dried berries were 524 and 832 mg/100 g, respectively, and the flavonol contents were 12 and 31 mg/100 g, respectively (expressed as the weight of the aglycone moiety of these flavonoid glycosides).

To assess the daily nutrient intake, participants kept 4-day food records including one weekend day before the intervention and during weeks 4 and 8. Daily nutrient intakes were analyzed based on the food records using the Micro Nutrica® program version 3.1 (Finnish Social Insurance Institute, Turku, Finland), which includes a database of Finnish foods. To assess compliance with the use of the test products, the participants kept daily records of the number of portions of test berries consumed and the quantity, quality, and frequency of other berries that were eaten.

Descriptions of the inflammatory markers and adipokines, glucose tolerance tests, serum lipids and cholesterol metabolism, isolation of PBMCs, and creating the gene expression data have been described in the Supporting Information 1.

2.4 Statistical analyses of the clinical data

The clinical data were analyzed using the SPSS software for Windows versions 14.0 and 17.0 (SPSS Inc., Chicago, IL, USA). Data are given as means ± SD, unless otherwise indicated. The normality of distributions of the study variables was tested with the Kolmogorov–Smirnov test with Lilliefors' significance correction. Logarithmic transformation was used to achieve normal distribution. General linear model for univariate analysis was used to test the differences in the basic characteristics between the study groups at the beginning of the intervention. The same test was also used for studying the differences in the change values (a value at week 8 − a value at week 0) between the groups. An "inflammation score" was formed by taking the sum of the *Z*-scores of hsCRP, IL-6, IL-12, and LPS. General linear model for repeated measures was used for analyzing the interaction of time and group for nutrient intake data, the inflammation score, and IL-6 and hsCRP from week 0 to week 12. For statistical testing the data were adjusted for age, gender, and body weight in the beginning of the intervention when appropriate. Paired samples *t*-test was used for comparing the measurements in the beginning and at the end of the intervention within the study group, when the variables were normally distributed or normality was achieved with the transformation. When logarithmic transformation was not appropriate, nonnormally distributed variables were analyzed using Wilcoxon nonparametric test for paired comparisons or by Mann–Whitney test to compare the results between the groups. Correlation analyses were done using Pearson's method. For all measurements $p < 0.05$ was considered as statistically significant.

Table 2. Daily intake of the nutrients in the bilberry (BB) and control (C) groups[a)]

	Study week	BB ($n = 15$[c)])	C ($n = 12$)	p[b)]
Energy (kJ)	0	7524 ± 2269	8401 ± 1571	ns
	8	8451 ± 2011	9215 ± 3082	
Protein (g)	0	77 ± 31	79 ± 14	ns
	8	78 ± 21	84 ± 24	
Fat (g)	0	58 ± 23	71 ± 17	ns
	8	65 ± 25	77 ± 36	
Carbohydrates (g)	0	204 ± 78	218 ± 44	ns
	8	242 ± 49[d)]	253 ± 78	
Sucrose (g)	0	32 ± 18	33 ± 14	ns
	8	52 ± 16[d)]	38 ± 21	
Fiber (g)	0	24 ± 12	24 ± 8	0.013
	8	32 ± 8[d)]	25 ± 8	
Alcohol (g)	0	18 ± 28	22 ± 34	ns
	8	18 ± 33	18 ± 27	

a) All values are means ± SD.
b) General linear model for repeated measures for the interaction of time and group.
c) Food record was not received from one study participant in the BB group at week 8.
d) $p < 0.01$, paired samples *t*-test for the change within the group.

3 Results

3.1 Compliance and daily nutrient intake

Based on diaries of the use of test products, 71–100% of the advised amount of bilberry purée and dried bilberries were consumed. Fourteen participants out of 15 consumed at least 95% of the recommended amount of bilberry purée, and 13 participants out of 15 consumed at least 93% of the suggested amount of dried bilberries.

Despite the added bilberry products in the BB group diet, the daily nutrient intakes remained mainly similar between the groups (Table 2). However, there was a significant time × group interaction for fiber intake, which increased significantly in the BB group ($p = 0.003$). Additionally, the carbohydrate ($p = 0.008$) and sucrose ($p = 0.002$) intakes increased in the BB group. This analysis was done based on the significant time effect, while the time × group effect was not significant, when comparing the daily carbohydrate and sucrose intakes between the groups. In the BB group, the daily intake of anthocyanins from the bilberry products was 1323 ± 99 mg and that of flavonols 36 ± 3 mg.

3.2 Body weight, body composition, and blood pressure

As expected, no differences in body weight, waist circumference, or percentage of body fat were seen between the study groups. Additionally, the blood pressure remained stable and did not differ between the groups during the intervention.

3.3 Low-grade inflammation

In two study participants the hsCRP levels were higher than 10 mg/L (either on study week 0, 4, or 8), indicative of acute inflammation, and they were excluded from the statistical analyses of inflammatory markers, leaving 13 participants in the BB and 11 in the C group.

Serum hsCRP, IL-6, IL-12, and LPS concentrations decreased in the BB group with varying statistical significance in comparison to the C group (Table 3). The inflammation score based on these markers increased slightly in the C group ($p = 0.016$) and decreased in the BB group ($p = 0.187$) resulting in significant time × group interaction when adjusted for age, gender, and baseline body weight.

hsCRP and IL-6 levels were measured also at week 12 to detect changes after cessation of the intervention. There was no significant time × group interaction when analyzing the difference between the groups during week 0–12 (general linear model for repeated measures). Reduction in IL-6 levels appeared, however, to continue from week 8 to 12 being significant within the BB group (3.81 ± 0.57 to 3.59 ± 0.63 μg/mL, $p = 0.022$, paired *t*-test with log-transformed variables). For the C group the corresponding values were 3.66 ± 0.30 to 4.17± 0.71 μg/mL from the week 8 to week 12. However, the reduction of hsCRP lasted only to the end of the intervention (week 8), and the level increased by week 12 to the values corresponding to those in the beginning of the intervention.

Adiponectin or leptin concentrations did not differ significantly between the study groups, nor changed within the groups during the intervention (Table 3).

3.4 Glucose and lipid metabolism

Glucose and insulin responses in OGTT did not differ between the groups, nor change during the intervention (Supporting Information 2). The insulinogenic index based on the acute responses of glucose and insulin in OGTT did not differ between the groups. Likewise, the values for S_I, S_G, and AIR based on the FSIGT did not differ between the groups.

Serum concentrations of free fatty acids, total, HDL and LDL cholesterols, triglycerides, and apolipoproteins A1 and B remained stable and did not differ between the groups during the intervention (data not shown).

No significant differences were found in concentrations of markers of cholesterol synthesis (squalene, cholestenol, desmosterol, and lathosterol) or absorption (cholestanol, sitosterol, and campesterol) or the respective ratios to cholesterol between the groups at the beginning of the intervention. The serum desmosterol concentration ($p = 0.070$) and the ratio to cholesterol ($p = 0.051$) tended to increase in BB group in comparison to the C group. In the BB group the mean desmosterol concentration was 131 ± 10 mg/dL at 0 and 151 ± 14 mg/dL at 8 week, and the ratio to cholesterol, respectively, 66 ± 5 and 76 ± 7 × 10^2 mmol/mol of cholesterol,

Table 3. Inflammatory markers and adipokines at baseline (0 week) and the changes during the intervention in the bilberry (BB) and control (C) groups[a)]

		BB ($n = 13$[c)])	C ($n = 11$[c)])	p[b)]
hsCRP (mg/L)	0 week	2.85 ± 1.70	2.80 ± 2.77	
	Δ[d)]	−0.71 ± 1.98	0.06 ± 1.37	0.054
IL-6 (μg/mL)	0 week	4.13 ± 1.63	3.65 ± 1.47	
	Δ	−0.32 ± 1.12	0.02 ± 0.78	0.074
IL-12 (pg/mL)	0 week	1.80 ± 4.89	0.15 ± 0.20	0.063
	Δ	−0.98 ± 3.30	0.19 ± 0.24	0.015
LPS (EU/mL)	0 week	1.97 ± 0.71	2.18 ± 2.01	
	Δ	−0.18 ± 0.20	0.32 ± 0.79	0.094
Adiponectin (μg/mL)	0 week	10.29 ± 5.31	7.51 ± 4.14	
	Δ	−0.1 ± 3.2	−0.8 ± 1.2	ns
HMW-adiponectin (μg/mL)	0 week	7.40 ± 5.11	6.56 ± 3.30	
	Δ	−0.57 ± 1.56	−0.62 ± 3.32	ns
Leptin (ng/mL)	0 week	22.3 ± 20.5	22.4 ± 13.6	
	Δ	0.61 ± 5.85	−0.07 ± 5.42	ns
Inflammation score[e)]	0 week	0.29 ± 2.17	−0.35 ± 2.59	
	8 week	−0.14 ± 2.38	0.17 ± 2.25	0.024

a) All values are means ± SD.
b) General linear model for univariate analyses of the change values between the groups adjusted for age, gender, and the weight in the beginning of the study. IL-12 was tested using Mann–Whitney test without the adjustment.
c) Three study participants ($n = 2$/1, BB/C) were excluded due to hsCRP concentration >10 mg/L.
d) Δ = value at 8 week − value at 0 week.
e) Inflammation score was calculated based on the values of hsCRP, IL-6, IL-12, and LPS. The difference between the groups was tested using general linear model for repeated measures for time × group interaction adjusted for age, gender, and weight in the beginning of the study.

while in the corresponding values in the C group were 123 ± 13 versus 118 ± 15 mg/dL and 62 ± 6 versus 60 ± 7 × 10^2 mmol/mol of cholesterol.

3.5 Gene expression at mRNA level

Transcriptomic gene expression analyses in PBMCs of the subset of BB group participants ($n = 3$) showed differential expression of 50 genes due to the intervention with uncorrected $p < 0.005$. A total of 11 pathways had Z-scores >2 when including genes with $p < 0.01$. In search of up- or downregulated pathways, only three pathways were identified with $p < 0.05$ and with Z-scores >2. The genes related to Toll-like receptor (TLR) signaling, cytoplasmic ribosomal proteins, and the genes related to B-cell receptor signaling pathways were differentially regulated.

Five genes (*WDSUB1, COX7B, RGS18, DAPP1, TICAM1*) (Table 4) were selected to be verified whether the whole genome transcriptomic analyses could be confirmed in all participants of the BB group. The expression of these genes was also analyzed in the C group. The decreased mRNA expressions of *DAPP1* ($p = 0.018$) and *RGS18* ($p = 0.010$) were verified in the BB group.

In addition, on the basis of the pathway analyses, we determined the mRNA expression of a number of other genes with QPCR (Table 5). These genes were related to TLR and B-cell receptor pathways, or individual genes associated with monocyte and macrophage function, namely membrane receptors in macrophage differentiation or activation pathways. The expression of the *MMD* ($p = 0.007$) and *CCR2* ($p = 0.003$) genes were significantly decreased during the intervention in the BB group. In the C group, the expression of the *Ly96* gene (also known as *MD-2*), decreased significantly ($p = 0.049$).

Table 4. Confirmation of the gene expression in transcriptomics analyses using QPCR at baseline (0 week) and the changes during the intervention in the bilberry (BB, $n = 13$) and control (C, $n = 11$) groups[a)]

	Group[b)]	0 week	Change	p[c)]	p[d)]
TICAM1	BB	1.10 ± 0.31	−0.04 ± 0.26	ns	
	C	0.97 ± 0.13	+0.03 ± 0.16	ns	ns
DAPP1	BB	1.02 ± 0.15	−0.10 ± 0.14	0.018	
	C	1.05 ± 0.29	+0.06 ± 0.23	ns	0.022
WDSUB1	BB	0.97 ± 0.23	+0.01 ± 0.18	ns	
	C	0.88 ± 0.17	−0.02 ± 0.18	ns	ns
RGS18	BB	1.04 ± 0.37	−0.27 ± 0.32	0.010	
	C	1.16 ± 0.54	−0.11 ± 0.40	ns	ns
COX7B	BB	0.88 ± 0.23	+0.08 ± 0.32	ns	
	C	0.80 ± 0.19	−0.00 ± 0.23	ns	ns

a) All values are means ± SD. Values of each gene are expressed as relative expression to endogenous control *GAPDH*.
b) Three study participants ($n = 2$/1, BB/C) were excluded due to hsCRP concentration >10 mg/L.
c) Paired samples t-test was used for analyzing the change within the study group with logarithmic transformed variables.
d) General linear model for univariate analyses of change values between the groups adjusted for age, gender, and body weight in the beginning of the study.

Table 5. mRNA expression of genes (QPCR) chosen from the pathways determined from the transcriptomic analyses at baseline (0 week) and the changes during the intervention in the bilberry (BB, $n = 13$) and control (C, $n = 11$) groups[a)]

	Group[b)]	0 week	Change	p[c)]	p[d)]
Toll-like receptor pathway					
RIPK-1	BB	1.70 ± 0.25	−0.02 ± 0.21	ns	
	C	1.80 ± 0.60	−0.05 ± 0.26	ns	ns
Ly96 (MD-2)	BB	0.81 ± 0.15	−0.03 ± 0.16	ns	
	C	0.81 ± 0.23	+0.15 ± 0.20	0.049	0.037
TNFRSF12A	BB	1.30 ± 0.50	−0.14 ± 0.42	ns	
	C	1.46 ± 0.33	+0.10 ± 0.45	ns	ns
TAB-2	BB	1.15 ± 0.15	−0.05 ± 0.21	ns	
	C	1.34 ± 0.55	+0.05 ± 0.31	ns	ns
B-cell receptor pathway					
CD19	BB	1.10 ± 0.46	+0.10 ± 0.29	ns	
	C	1.22 ± 0.71	+0.12 ± 0.28	0.085	ns
CD72 (LYB2)	BB	1.08 ± 0.44	+0.01 ± 0.19	ns	
	C	1.16 ± 0.52	+0.09 ± 0.35	ns	ns
Monocyte or macrophage associated genes					
CCR2 (MCP1-R)	BB	1.19 ± 0.26	−0.11 ± 0.15	0.003	
	C	1.33 ± 9.29	+0.09 ± 0.28	ns	0.043
MMD	BB	1.58 ± 0.57	−0.44 ± 0.43	0.007	
	C	1.58 ± 0.45	−0.05 ± 0.39	ns	0.039

a) All values are means ± SD. Values of each gene are expressed as relative expression to endogenous control GAPDH.
b) Three study participants ($n = 2$/1, BB/C) were excluded due to hsCRP concentration >10 mg/L.
c) Paired samples *t*-test was used for analyzing the change within the study group with logarithmic-transformed variables.
d) General linear model for univariate analyses of change values between the groups adjusted for age, gender, and body weight in the beginning of the study.

Since other transcripts in the TLR or B-cell receptor signaling pathways showed no differential expression, we verified our initial gene expression analyses with a more robust method [24]. The total number of significantly expressed transcripts was still similar and only minor changes in pathway analysis were observed. In brief, in the final microarray analyses the number of differentially expressed probes was a total of 1584/816 transcripts of which 783/331 were upregulated and 801/485 downregulated (number of Illumina ID/ Entrez Gene ID probes). The five genes examined using qPCR are found within the 166/97 of transcripts with $p < 0.01$ of which 72/34 were found to be up- and 94/63 downregulated (Supporting Information 3). The enrichment revealed similar genes as the initial analyses and TLR pathway was the major pathway enriched with transcripts for *LPB, TICAM1*, and *RIPK-1* (Supporting Information 4). The highest enriched GO processes showed these genes also within the processes of LPS-mediated signaling and macrophage activation during immune responses. With applying a broad search limit ($p < 0.1$) an additional process of T-helper cell (2) differentiation was also found.

The changes of IL6, IL12, hsCRP, and LPS correlated significantly with differentially expressed transcripts: hsCRP and LPS both correlated positively with *CCR2* ($r = 0.472$ and $r = 0.479$, respectively, $p < 0.05$ for both) and IL-6 with *RGS18* ($r = 0.521$, $p < 0.05$) (Pearson's correlation analyses with log-transformed values). In the BB group, hsCRP correlated positively with *CCR2* ($r = 0.565$, $p < 0.05$), IL6 with *RGS18* ($r = 0.660$, $p < 0.05$), and IL-6 ($r = -0.636$, $p < 0.05$) as well as IL-12 ($r = -0.683$, $p < 0.05$) both with *DAPP1*. In the C group, no significant correlations were detected.

4 Discussion

In the present study, a bilberry rich diet tended to exert anti-inflammatory effects in individuals with features of metabolic syndrome. Bilberry supplementation tended to decrease hsCRP, IL-6, IL-12, and LPS, all characteristic proinflammatory markers elevated in the metabolic syndrome [12, 25]. The "inflammation score" was significantly different between the groups showing an increase in the C group. These changes were accompanied with the differential regulation of the genes related to TLR signaling, cytoplasmic ribosomal proteins, and to B-cell receptor signaling pathway as well as the differential expression of *MMD* and *CCR2* transcripts representing monocyte and macrophage function associated genes.

The amount of the bilberries eaten, in the present study, was quite large, corresponding to 400 g of fresh berries daily—the amount that can only be achieved with the use of freeze-dried berries or other condensed forms of bilberry products. The average intake of berries is 50–70 g/day within the Finnish population consuming berries regularly [26]. The amount of the berry products consumed in the present study is also at the high end of the range compared to previous studies with bilberries or blueberries [27–32].

The daily intakes of anthocyanins (1323 mg) and flavonols (36 mg) increased in the BB group, being much higher than the average intake of these polyphenols in the Finnish population estimated to be 47 and 5.4 mg/day, respectively [18]. Bilberries have uniquely high anthocyanin content, but they also contain other polyphenols such as phenolic acids (45 mg/100 g) [33], and proanthocyanidins (148 mg/100 g) [34].

The amount of the bilberries in the diet provided daily 13.2 g of fiber based on the nutrient content of the fresh bilberries. In practice, the increased daily intake of fiber was 8–10 g, and the bilberry purée contained an additional 2 g/day of guar gum. The intake of sucrose increased in the BB group, because the bilberries were eaten as commercial products preserved with sucrose. However, no adverse effects on glucose and lipid metabolism were seen due to increased sucrose intake.

The "inflammation score" based on the changes of hsCRP, IL-6, IL-12, and LPS during intervention showed an increase in the C group, while in the BB group the change was toward decreased inflammation. This finding is of importance, since these participants represented a population with low-grade inflammation prior to the intervention. Thus, this result may show the improvement in the development of the chronic disease. Our observations are partly in line with some previous clinical studies [29, 32], while others have shown no significant effects [28, 30, 31]. Karlsen and co-workers [29] showed that daily consumption of 1 L of bilberry juice for 4 weeks decreased the levels of circulating hsCRP, IL-6, IL-15, and IFN-γ and increased the levels of tumor necrosis factor-α. Previously Karlsen and co-workers [32] had shown that intake of anthocyanins from bilberries and black currant (300 mg/day for 3 weeks) was also associated with anti-inflammatory effects such as considerable decreases in the circulating levels of IL-8, RANTES and IFNα, and a modest decrease in Th2 cytokines, IL-4, and IL-13, but no reduction in CRP, plasma lipids, or increase in antioxidants [32].

Notably, we also showed that a bilberry enriched diet tended to decrease circulating LPS concentrations or, more precisely, to prevent the increase that was seen in the control group. While the effect may seem modest, it may have clinical relevance considering that both human and animal studies have linked subclinical endotoxemia, occurring after a high fat, high carbohydrate meal, with the obesity associated systemic inflammation, hyperglycemia, and insulin resistance [10, 12, 35]. The present study is one of the first reports of such an influence of berries on humans, and is supported by a previous finding with rats where blackberry anthocyanins were shown to protect from postprandial LPS burden [36]. Why bilberry consumption appeared to reduce the subclinical endotoxemia remains to be determined.

Another potential mechanism affecting circulating endotoxin levels would be enhanced LPS removal, which primarily occurs via uptake by liver Kupffer cells and splenic macrophages [37], but we are not aware of the effects of polyphenols on such activity. In addition to reduction in LPS levels per se, the body has several mechanisms to reduce the proinflammatory responsiveness to LPS including binding by LPB, sCD14, and lipoproteins [38]. These seemed to be unaffected by the bilberry consumption as shown either at protein or gene expression level. In general, the changes in LPS levels were inversely associated with the levels of LDL and total cholesterol, supporting recent observations on the subacute effects of LPS in cats and acute effects in humans [39, 40].

The immunomodulatory effects of bilberry consumption were in accordance with our microarray-based gene expression results in PBMCs. There was a coherent trend toward shift to humoral responses away from the innate, cell-mediated immunity skewed responsiveness characterizing the low-grade inflammation in obesity and related comorbidities [41]. In the present study, this was shown as correlations between measured cytokines hsCRP, IL-6, and IL-12 and the transcripts linked to B-cell receptor pathway and monocyte and macrophage function associated genes. A similar trend has been previously associated with weight loss in pre- and type 2 diabetes [42]. In our study, this was observed as downregulation of genes associated with the TLR pathway, Th17 cell activity and monocyte and macrophage function associated genes, upregulation of genes in B-cell receptor signaling pathways, and with significant regulation of genes involved in Th2 differentiation. Monocyte and macrophage function associated gene, *CCR2*, a critical factor in the development of metabolic syndrome via its function as chemokine attracting monocytes in the sites of inflammation [43]. *CCR2* has also been found to be expressed especially in visceral adipose tissue to induce inflammation [44]. MMD has been linked to monocyte macrophage differentiation, but the exact function in humans is still not known.

Moreover, decreased serum levels of IL-12, the primary Th1 polarizing cytokine, attest to the observed trend at the protein level. Considering the apparent, but nonsignificant, upward fluctuation in LPS levels in the control group, it seems intriguing that the expression of *Ly-96 (MD-2)*, which is needed for activation of TLR4-mediated signaling by LPS [45], was significantly increased. One could speculate that since such increase was absent in the BB group, the increase in the control group could be due to the restrictions in berry consumption. The downregulation of TLR-mediated innate immunity and linkage to LPS following consumption of polyphenol rich foods is supported by previous studies on NFκB activation downstream to TLR ligation. In one study, red wine polyphenols were shown to prevent the increase of NFκB activation in PBMCs postprandially after a high-fat diet [46]. Moreover, anthocyanins have decreased the levels of cytokines that induce NFκB activation [32] and also inhibited LPS-induced NFκB activation in human monocytic cell line [29].

The improvement in insulin sensitivity has been shown using the hyperinsulinemic-euglycemic clamp after a 6-week blueberry diet [30]. There are, however, publications that do not show any effects on glucose metabolism after bilberry or blueberry consumption [28, 31]. The latter are in line with

our findings showing no effects on glucose metabolism after 8-week bilberry consumption. It is notable that we used both OGTT and FSIGT to study the effects of bilberry rich diet on glucose metabolism.

In the present study, the bilberry consumption did not have significant effects on serum cholesterol concentrations. However, the bilberry consumption was associated with enhanced cholesterol synthesis as indicated by the increased (16–18%) serum concentration of the cholesterol precursor, desmosterol. In humans, if cholesterol synthesis is upregulated, cholesterol absorption is downregulated according to the homeostatic regulation of cholesterol metabolism [47]. Accordingly, in the present study serum cholestanol level, a marker of cholesterol absorption, tended to be lower during the bilberry rich diet, though it did not reach statistical significance. The serum plant sterol concentrations were not significantly affected in this study despite of the calculated daily intake of 100 mg of plant sterols from bilberries.

Water-soluble fiber decreases serum cholesterol concentrations [48] by interfering with bile acid metabolism and thus increasing cholesterol synthesis [49]. However, most of the fiber content of bilberries is nonsoluble (2.6 g out of 3.3 g/100 g). The guar gum content of the bilberry purée increased the amount of water-soluble fiber in the diet. Since we did not find a correlation between fiber intake and cholesterol metabolism, the increased levels of desmosterol along with decreased hsCRP underlines further the anti-inflammatory impact of the bilberry rich diet.

The present study has some limitations. The number of study participants was relatively low, which obviously limits the statistical power. However, while this may leave the mechanistic picture incompletely characterized, all the observed effects seen, both at gene expression and circulating protein level, were logical and consistently supportive for the inflammation alleviating effects of bilberries. Regarding the gene expression analyses, the results of microarray analyses could not be fully generalized to the whole BB group when choosing random samples of differentially expressed transcripts for confirmation. One explanation for the lack of consistency in these results may have been different sensitivity of the microarray and QPCR methods. However, when choosing the genes based on the pathway findings, the results were consistent with both methods. The berry consumption before the trial was not recorded, but it is likely that the cohort population in this part of Eastern Finland consumes relatively large amounts of berries in general. This might have induced variation in the study parameters reducing, in part, the statistical power of the study.

In conclusion, regular bilberry consumption may be potential in reducing low-grade inflammation. The finding may indicate decreased cardiovascular and metabolic risk in the long term.

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