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ORIGINAL CONTRIBUTION

Bilberry juice modulates plasma concentration of NF-κB related inflammatory markers in subjects at increased risk of CVD

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Abstract
Purpose Bilberries are abundant in polyphenols. Dietary polyphenols have been associated with strategies for prevention and treatment of chronic inflammatory diseases. We investigated the effect of bilberry juice on serum and plasma biomarkers of inflammation and antioxidant status in subjects with elevated levels of at least one risk factor for cardiovascular disease (CVD).

Methods In a randomized controlled trial, participants consumed either bilberry juice ($n = 31$) or water ($n = 31$) for 4 weeks.

Results Supplementation with bilberry juice resulted in significant decreases in plasma concentrations of C-reactive protein (CRP), interleukin (IL)-6, IL-15, and monokine induced by INF-γ (MIG). Unexpectedly, an increase in the plasma concentration of tumor nuclear factor-α (TNF-α) was observed in the bilberry group. CRP, IL-6, IL15, MIG, and TNF-α are all target genes of nuclear factor- kappa B (NF-κB), —a transcription factor that is crucial in orchestrating inflammatory responses. Plasma quercetin and *p*-coumaric acid increased in the bilberry group, otherwise no differences were observed for clinical parameters, oxidative stress or antioxidant status. Furthermore, we studied the effect of polyphenols from bilberries on lipopolysaccharide (LPS)-induced NF-κB activation in a monocytic cell line. We observed that quercetin, epicatechin, and resveratrol inhibited NF-κB activation.

Conclusions These findings suggest that supplementation with bilberry polyphenols may modulate the inflammation processes. Further testing of bilberry supplementation as a potential strategy in prevention and treatment of chronic inflammatory diseases is warranted.

Keywords Bilberry · NF-κB · Cytokines · Polyphenols · Human intervention · Cell culture

Abbreviations

CVD	Cardiovascular disease
CRP	C-reactive protein
IL	Interleukin
MIG	Monokine induced by INF-γ

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RANTES	Regulated upon activation, normal T cell expressed and secreted
TNF-α	Tumor nuclear factor-α
NF-κB	Nuclear factor-kappa B
LPS	Lipopolysaccharide
BP	Blood pressure
LDL	Low-density lipoprotein
HDL	High density lipoprotein
SRM	Standardized reference materials
FRAP	Ferric reducing/antioxidant power
TRAP	Total peroxyl-radical trapping activity
TE	Trolox equivalents
ORAC	Oxygen radical absorbance capacity
PCA	Perchloric acid
AA	Ascorbic acid
DHAA	Dehydroascorbic acid
TAA	Total ascorbic acid
HPLC	High performance liquid chromatography
GSH	Glutathione
D-ROM	Diacrons reactive oxygen metabolites
WBC	White blood cells
γ-GT	γ-glutamyltransferase

Introduction

Inflammation is a complex series of responses to stimuli or agents that are perceived as harmful to the organism. A prolonged state of inflammation may, however, contribute to the pathogenesis of several chronic diseases such as cardiovascular disease (CVD), diabetes, neurodegenerative diseases, and several cancers [1, 2]. Thus, dampening of inflammation may potentially delay development of such diseases.

The transcription factor nuclear factor-kappa B (NF-κB) plays a critical role in cellular stress-, immune- and inflammatory responses [3]. Activation of NF-κB mediates a coordinated regulation of target genes, followed by secretion of pro-inflammatory signaling molecules such as acute phase proteins, chemokines, and cytokines [4]. A balanced regulation of NF-κB is essential in normal physiology, and aberrant regulation is involved in a number of chronic human diseases as CVD and several cancers [5–10].

A chronic state of sub-clinical inflammation, as recognized by slightly elevated levels of pro-inflammatory mediators such as C-reactive protein (CRP) and certain pro-inflammatory chemokines and cytokines may be especially important in the pathogenesis of chronic diseases. The evidence is especially strong for CRP and interleukin (IL)-6 which independently of other risk factors, have been demonstrated to be predictive of CVD in apparently healthy subjects [11–13].

On the other hand, small, repetitive episodes of NF-κB activation may play an important role in disease prevention. It has been demonstrated that such so-called preconditioning mediated through activation of NF-κB is protective against several diseases such as coronary heart disease and cerebral diseases [14–16].

Bilberries from the bush *Vaccinium Myrtillus* (also called European blueberries) and related species of the *Vaccinium* family are particularly abundant in polyphenols, of which anthocyanins account for 50–80% of the total polyphenol content [17–19]. Other groups of polyphenols that are found in bilberries are flavonols, phenolic acids, stilbenes, catechins, and lignans [20–26].

Experimental studies in animals and cultured human cell lines support a role of polyphenols in the prevention of chronic inflammatory diseases, including CVD [27]. In particular, beneficial effects of dietary polyphenols have been observed on endothelial function and haemostasis in human intervention studies and support a role of dietary polyphenols in prevention of CVD [28].

The aim of this study was primarily to investigate the effect of bilberry juice supplementation on biomarkers of inflammation in men and women with elevated levels of at least one CVD risk factor. Thus, we conducted a 4-week parallel-group randomized clinical trial where participants ($n = 62$) were supplemented with either bilberry juice (bilberry group, $n = 31$) or water (control group, $n = 31$). Additionally, markers of oxidative stress and antioxidant status were measured. We observed that NF-κB-related inflammatory mediators were altered in the bilberry group. We further hypothesized that these alterations were mediated by a modulation of the NF-κB activation by bilberry polyphenols. To test this hypothesis, we tested the effects of polyphenols found in bilberries on lipopolysaccharide (LPS)-induced NF-κB activation in a human monocytic cell line.

Materials, subjects, and methods

Subjects

The participants were recruited through an advertisement in a local newspaper or from the preventive cardiology outpatient clinic at Ullevål University Hospital. Men aged between 30–70 years and women between 45–70 years and at least 12 months postmenopausal were invited to participate. Subjects were defined to be at elevated risk of CVD if at least one of the following criteria was fulfilled: systolic blood pressure (BP) between 135 and 160 mmHg, diastolic BP between 90 and 100 mmHg, low-density lipoprotein

(LDL) cholesterol ≥ 3.4 mmol/l, total/high-density lipoprotein (HDL) cholesterol ratio > 4, elevated hematocrit (≥0.40 from women or ≥0.42 for men) or smoking a minimum of three cigarettes daily. The exclusion criteria included clinically recognized chronic diseases such as impaired renal function, diabetes mellitus, CVD, liver or gastrointestinal disease or cancer within the last 5 years, or use of lipid-lowering drugs, diuretics, or hormone replacement therapy for women. Subjects with a BMI > 31, whose alcohol consumption was >3 units/day for men or >1 unit/day for women and subjects who had donated blood within the last 6 months were not eligible.

Bilberry juice and laboratory chemicals

Bilberry juice was purchased from Corona Safteri (Rotvoll, Norway). The juice was prepared by steam-processing of fresh bilberries (*Vaccinium Myrtillus*) collected in Norway. No sugar or other additives were added during the preparation process.

Vacutainer tubes for blood sampling were purchased from BD Vacutainer (Franklin Lakes, USA) and Biopool (Trinity Biotech plc, Ireland). Cyanidin, cyanidin 3-O-β-glucopyranosides, delphinidin, and petunidin were donations from Medpalett Pharmaceuticals AS, Sandnes, Norway. Quercetin, enterolactone, *p*-coumaric acid, m-coumaric acid, syringic acid, protocatechuic acid, resveratrol, epicatechin, myricetin, RPMI-1640 medium, L-gluthamine, penicillin/streptomycin, hygromycin, and fetal bovine serum were purchased from Sigma–Aldrich, Norway. Other solvents and reagents were HPLC or Optima grade; common laboratory reagents were purchased from Sigma (St Louis, MO, USA), Carlo Erba (Milan, Italy), BDH Laboratory Supplies (Dorset, UK) and Fluka (Buchs, Switzerland) and were the highest grade available. Standardized reference materials (SRM) for determination of plasma vitamin C and carotenoids were purchased from NIST (Gaithersburg, MD, USA).

Study design and intervention

The study was approved by the regional committee for medical research ethics (REK Sør-Øst) and all participants gave written, informed consent. Participants were enrolled between March 2003 and March 2004. Easter holiday, summer vacation, and Christmas holiday were avoided to prevent possible confounding factors due to changes in the habitual diet.

Prior to the randomization, a 3-week wash-out period was included, where the participants were asked to minimize the intake of dietary items especially rich in antioxidants, and avoid intake of berry and berry products. At the time of inclusion, the study coordinator opened pre-sealed, consecutively numbered, envelopes containing group assignment. For the study as described herein, participants were randomized to one of two groups: one group that consumed 330 ml bilberry juice/day (diluted to 1 L using tap water, bilberry group, $n = 31$, twenty-one men and ten women) and one group that consumed 1 l water/day (water group, $n = 32$, twenty-five men and seven women). One participant in the water group was excluded due to a missing blood sample leaving $n = 31$. The participants were asked to consume the bilberry juice or water in addition to their habitual fluid consumption. For the water group, bottled water was provided (Imsdal, Ringnes AS, Oslo, Norway) and consumed by all but four subjects who consumed regular tap water. A 7-day registration of fluid intake was performed during the week before inclusion and the last week of the intervention period. Additionally, compliance with the fluid consumption was confirmed by measures of urine volume after 24-h collection of urine before, during, and following the intervention period. Another aim of the study was to examine the short-term effect of increasing water intake by 1 l/day on blood viscosity. Thus, an additional group of 31 participants following their regular habitual fluid intake served as a control group for the water group (control-water). The results related to changes in blood viscosity have been described and published elsewhere [29]. Furthermore, samples from the control-water group were not investigated as a part of this study.

Overnight fasting blood samples were collected at the time of randomization and following the 4-week intervention period. Plasma and serum were immediately prepared and stored at −70 °C unless immediately analyzed.

Cytokines, chemokines, and inflammatory mediators in plasma

The inflammatory mediators IL-1β, IL-1α, IL-1 receptor antagonist (IL-1Ra), IL-2, IL-2R IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, TNF-α, interferon (IFN)-α, IFN-γ, granolyte/macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein 1-α (MIP-1α), MIP-1β, immunoprotein (IP)-10, monocyte chemoattractant protein-1 (MCP-1), monokine induced by IFN-γ (MIG), Eotaxin and "regulated upon activation, normal T cell expressed and secreted" (RANTES) were measured in heparin plasma by a sandwich immunoassay based protein array system (Biosource International, Camarillo, CA, USA) as described elsewhere [30]. High-sensitivity CRP was determined in serum by routine laboratory methods at the Clinical Chemical Department at Ullevål University Hospital (Oslo, Norway). With the exception of CRP, inflammatory mediators in plasma were measured for a randomly selected subset of the study

population, $n = 22$ in the water group and $n = 18$ in the bilberry group.

Analysis of plasma biomarkers associated to antioxidant status

Total antioxidant capacity assays

Ferric Reducing/Antioxidant power (FRAP) was determined as described elsewhere [31]. The total peroxyl-radical trapping activity (TRAP) was measured in plasma by a modified version of a previously described assay [32]. Briefly, plasma was added to the reaction mixture and the oxidation reaction was initiated by the addition of 2,2′-azobis(2-amidinopropane). The decay of fluorescence (FL) was monitored every min for 60 min (at 495 nm (ex) and 570 nm (em)) on a FL microplate reader (GENios Standard TECAN Italia, Milano Due, Segrate, Italy). The lag phase obtained by Trolox served as assay calibrator, and results are expressed as trolox equivalents (TE).

Plasma samples for the oxygen radical absorbance capacity (ORAC) assay were handled as described elsewhere [33], and the oxidation reaction was initiated with 2.2′-azobis (2-amidinopropane) dihydrochloride. To isolate the protein-free fraction, plasma proteins were precipitated by the addition of 0.5 mol/l perchloric acid (PCA) to an equal volume of plasma before centrifugation at 13,000*g* at 4 °C for 5 min. Plasma (total) or protein-free supernatant (PCA treated) were used for ORAC assay. Fluorescence was recorded every 3 min (485 nm (ex) and 520 nm (em)) with the use of a FLUOstar OPTIMA FL microplate reader (BMG LABTECH, Offenburg, Germany). ORAC values were calculated using the difference between areas under FL decay curves for blank (phosphate buffer) versus sample (net area). Data are expressed as TE.

Water-soluble antioxidant compounds

Samples for plasma vitamin C isoforms (ascorbic acid (AA), dehydroascorbic acid (DHAA), and total ascorbic acid (TAA)) were prepared and analyzed by high-performance liquid chromatography (HPLC) as described elsewhere [34, 35]. Plasma calibrators quantified against the NIST 970 SRM served as standards. Samples for the determination of reduced and oxidized glutathione (GSH) in plasma were prepared and analyzed as described elsewhere [36]. Determination of total GSH was performed according the "homocysteine by HPLC" kit provided by Biorad Laboratories GmbH (Munchen, Germany), previously validated for the measurement of additional plasma thiols [37]. Standard solutions prepared in PBS served as calibrators.

Fat-soluble antioxidant compounds

Tocopherols were determined by HPLC as described elsewhere [38]. Proteins were precipitated by the addition of 3 volumes of isopropanol, followed by centrifugation at 3,000*g* at 4 °C for 15 min. The internal standard tocol was added with the isopropanol. 5 µl of the clear supernatant was used for analysis. Standard solutions prepared in 1% bovine serum albumin in PBS were used for quantification. Carotenoids were determined in plasma by HPLC. Proteins were precipitated and removed by the addition of a 4.5 volume of isopropanol followed by centrifugation at 3,000*g* at 4 °C for 15 min. The internal standard astaxanthin was added with the isopropanol. 25 µL of the clear supernatant was used for analysis. The mobile phases consisted of A: 20% water and 24% acetone in ethanol and B: acetone. The gradient conditions were as follows: From 2 to 100% B within 20 min, followed by 100% B for 15 min. Detection was performed at 453 nm using a variable wavelength detector. Plasma calibrators quantified against the NIST 968c SRM served as standards.

Phenolic compounds

Quercetin was determined by HPLC and electrochemical detection after enzymatic hydrolysis as described elsewhere [39]. Spiked plasma samples served as calibrators. *p*-coumaric acid, *m*-coumaric acid, protocatechuic acid, caffeic acid, and enterolactone were determined by gas chromatography and mass spectrometry after enzymatic hydrolysis using a modification of a previously described method [40].

Oxidative stress-related parameters in plasma

Lipid peroxidation was measured by Diacrons reactive oxygen metabolites (D-ROMs test) according to manufacturer's instructions (Diacron International, Grossetto, Italy). The oxidized forms of vitamin C and GSH were determined along with the reduced forms, as described in the following.

Other laboratory measurements

Additional clinical parameters (white blood cells (WBC), fibrinogen, uric acid, γ-glutamyltransferase (γ-GT), total proteins, cholesterol, and triacylglycerol) were determined using routine laboratory methods at the Clinical Chemical Department at Ullevål University Hospital (Oslo, Norway).

Cell culture experiments

A human monocytic cell line (ATCC CRL-1593.2), stably transfected with a luciferase reporter containing three NF-κB binding sites (U937 3xκB-LUC cells) [41] was cultured in RPMI-1640 medium with L-gluthamine (2 mmol/l), penicillin (50 U/ml), streptomycin (50 mg/ml), hygromycin (75 μg/ml), and 10% fetal bovine serum at 37 °C and 5% CO_2. Cells were transferred to medium with 2% fetal bovine serum (to minimize binding of polyphenols to serum proteins) and incubated with the individual bilberry polyphenols (1–50 μmol/l) diluted in dimethylsulfoxide or vehicle control for 30 min. NF-κB activity was induced by the addition of LPS (1 μg/ml) and the cells were incubated for additional 6 h. Each experiment was performed in triplicate, and repeated three times. Cell viability was determined by trypan blue exclusion, with a cut-off value of 10% non-viable cells, and epicatechin at 50 μmol/l was excluded due to these criteria.

Luciferase activity was measured by imaging in an IVIS Imaging System 100 (Caliper Life Science, Hopkinton; MA, USA). 0.2 mg D-luciferin/ml was added to the cell medium and incubated for 4 min. Luminescence was recorded for 1 min. The number of photons emitted from each well of the cell culture plate was calculated by use of the Living Image Software ver. 2.50 (Caliper Life Science, Hopkinton; MA, USA). Gray scale images were used for reference of position. To limit the possible inhibitory effects of resveratrol on the firefly luciferase enzyme [42], the luciferase activity in U937 3xκB-LUC cells after treatment with resveratrol was measured in cell lysates as previously described [41].

Statistical analysis

Student's *t* test (for normally distributed data) and Mann–Whitney non-parametric test (for non-normally distributed data) were performed to compare the mean and median baseline values and the change during the intervention period, between the bilberry and water groups in the human intervention study. Baseline values and changes in parameters from baseline to after the intervention (change) are presented as mean (range) for normally distributed data.

Outliers were observed for several of the inflammatory mediators (defined as >4 standard deviations from median value). We excluded those samples that were defined as outliers at both baseline and after intervention. No more than two observations were excluded per variable. Plasma IFN-γ was analyzed, but the data are not presented, as IFN-γ was only detectable in samples from six participants (two in the water group and four in the bilberry group).

For the cell culture experiments, one-way ANOVA was used to examine possible effects of bilberry polyphenols on LPS-induced NF-κB activity in U937 3xκB-LUC cells, and differences were identified using Dunnett comparisons.

All statistics were performed using SPSS ver. 14.0 (SPSS Inc., Chicago, IL, USA). A *p*-value of 0.050 and below was considered statistical significant for all analyses.

Table 1 Baseline characteristics and observed changes for participant descriptive and routine laboratory parameters after intervention with bilberry juice for 4 wks presented as baseline mean (range) or median (range) and observed change reported as mean (±SD) or median[b] (95% CI for median). *Change* the value after intervention minus baseline value. *p* values refer to comparison of the observed changes, between the groups

Parameter	Water ($n = 31$)		Bilberry ($n = 31$)		*p* (Change)
	Baseline	Change	Baseline	Change	
Age (year)	53 (30–68)		53 (34–68)		
BMI (kg/m^2)	25.5 (17.8–31.5)		25.6 (19.9–31.7)		
Number of smokers	10		14		
Weight (kg)	81.3 (56–112)	−0.2 ± 1.0	79.4 (58–105)	−0.1 ± 1.2	0.890
WBC[b], × 10^9 cells/l	5.4 (2.9–9.0)	−0.3 (−0.5, 0.2)	5.5 (4.0–11.0)	−0.1 (−0.3, 0.3)	0.784
Fibrinogen[a, b] (g/l)	2.9 (2.3–5.9)	0.10 (−0.26, 0.23)	3.3 (2.0–4.2)	0.00 (−0.21, 0.14)	0.425
Uric acid (mmol/l)	333 (234–427)	17 ± 34.4	354 (241–477)	18 ± 49.6	0.907
γ-GT[a, b] (U/l)	27.1 (11.0–77.0)	−2.0 (−5.4, 4.2)	18.0 (13.0–74.0)	−0.1 (−3.2, 0.4)	0.637
Total proteins (g/l)	69.6 (63.0–78.0)	−1.0 ± 2.9	70.9 (63.0–79.0)	−1.0 ± 2.3	0.294
Total cholesterol[a] (mmol/l)	6.1 (3.8–9.2)	0.05 ± 0.70	6.7 (4.7–8.2)	−0.1 ± 0.59	0.345
Triglycerides (mmol/l)	1.37 (0.48–3.40)	−0.08 ± 0.55	1.31 (0.48–4.89)	0.05 ± 0.36	0.331

[a] Baseline values are significantly different between groups, $p < 0.050$

[b] Data are not normally distributed, *p*-values are calculated using the Mann–Whitney test

Results

Human intervention study

Baseline values and changes during the intervention period for participant descriptive- and routine laboratory parameters are presented in Table 1. No significant differences between the groups were observed at baseline for age, weight, BMI, no of smokers, WBC, uric acid, total proteins, or triglycerides. At baseline the bilberry group had statistically higher fibrinogen and total cholesterol, and lower γ-glutamyltransferase (γ-GT) as compared with the control group. No effects were observed during the intervention period for WBC, fibrinogen, uric acid, γ-GT, total proteins, total cholesterol, and triglycerides.

Table 2 summarizes baseline values and changes during the intervention period for inflammatory mediators measured in plasma. Plasma concentrations of CRP, IL-6, IL-15, and MIG decreased in the bilberry group. These changes were statistically significantly different between the groups ($p = 0.027$, $p = 0.037$, $p = 0.008$ and $p = 0.047$, respectively). Additionally, a near-statistically significant reduction was observed for RANTES ($p = 0.053$). In contrast, an increase of TNF-α was observed in the bilberry group ($p = 0.017$).

In Table 3, the baseline values and changes during the intervention period for plasma biomarkers of antioxidant status and oxidative stress status are listed. Quercetin and *p*-coumaric acid increased significantly in the bilberry group as compared with the water group ($p = 0.029$ and $p = 0.016$, respectively). No significant differences between the groups were observed for FRAP, TRAP, ORAC, d-ROM, measures of GSH, antioxidants, or antioxidant metabolites (Table 3).

Table 2 Baseline values and observed changes in inflammatory mediators after intervention with bilberry juice for 4 weeks. *p*-values refer to comparison of the observed changes, between the groups presented as baseline r median (range) and observed change reported as median (95% CI for median). All variables are non-normally distributed and *p*-values are calculated using the Mann–Whitney test. Except for CRP, inflammatory mediators were measured in a subset of the study population ($n = 22$ in the water group and $n = 18$ in the bilberry group). *Change* the value after intervention minus baseline value

Parameter	Water ($n = 31$)		Bilberry ($n = 31$)		*p* (Change)
	Baseline	Change	Baseline	Change	
CRP (mg/l)	1.00 (0.60–4.10)	0.15 (−0.2, 1.68)	1.30 (0.70–6.00)	−0.30 (−1.45, 0.75)	0.027*
IL-1β (pg/ml)	66.9 (1.50–973.2)	−10.6 (–473.6, 21.3)	91.8 (1.50–1,135)	−13.3 (−123.7, 7.4)	0.181
IL-1 Ra(pg/ml)	246 (3–1,203)	−11.2 (−75, 88)	277 (14–1,714)	−46 (−208, 36)	0.186
IL2 (pg/ml)	22.4 (6.1–900)	0.9 (−79.4, 26.1)	21.6 (0.6–1233)	−4.5 (−71.6, 45.9)	0.205
IL2r (pg/ml)	84 (3.0–1,259)	9.0 (−36, 35)	149 (5–1,097)	−18 (−71, 29)	0.116
IL4 (pg/ml)	3.4 (1.1–133)	0.0 (−3.2, 2.7)	3.4 (1.1–35.2)	0.0 (−0.9, 1.1)	0.458
IL5 (pg/ml)	0.89 (0.30–8.5)	0.4 (−0.56, 0.59)	1.18 (0.3–17.7)	0.0 (−1.37, 0.36)	0.263
IL6 (pg/ml)	11.5 (0.0–114.2)	−0.2 (–4.0, 6.1)	21.2 (3.1–255.9)	−6.0 (−35.2, −1.0)	0.037*
IL7 (pg/ml)	1.0 (0.7–31.9)	0.0 (−6.6, 8.4)	2.9 (0.7–32.6)	−1.5 (−3.4, 1.9)	0.276
IL8 (pg/ml)	1.3 (0.5–5.2)	0.1 (−0.2, 0.4)	1.5 (0.1–54.2)	0.1 (−4.9, 6.0)	0.843
IL10 (pg/ml)	32.2 (2.7–164)	0.0 (−6.5, 2.5)	42.6 (0.5–708)	−0.2 (−48, 16.3)	0.778
IL12 (pg/ml)	44.7 (26.9–161)	−4.8 (−12.0, 2.1)	45.9 (21.7–199)	1.2 (−11.7, 10.6)	0.196
IL13 (pg/ml)	2.64 (0.88–41.7)	0.0 (−0.73, 0.61)	2.64 (0.9–93.5)	0.0 (−3.0, 3.9)	0.609
IL15 (pg/ml)	11.6 (1.0–318)	5.5 (−0.0, 5.7)	17.0 (0.5–165)	−7.2 (−21.8, 19.3)	0.008*
IL17 (pg/ml)	5.3 (0.9–140)	0.0 (−4.9, 3.5)	0.9 (0.9–62.1)	0.0 (−2.13, 1.51)	0.764
TNF-α (pg/ml)	5.6 (1.0–135)	−1.0 (−5.9, −0.2)	5.6 (1.0–232)	1.9 (−18.9, 13.1)	0.017*
IFN-α (pg/ml)	6.4 (1.5–154)	0.0 (−3.8, 3.0)	1.5 (1.5–259)	0.0. (−9.7, 14.6)	0.361
GM-CSF (pg/ml)	16.8 (1.5–358)	0.0 (−3.0, 3.0)	24.3 (1.5–252)	0.0 (−18.5, 24.2)	0.488
MIP-1α (pg/ml)	63.0 (2.9–586)	−3.6 (−24.3, 7.4)	26.6 (1.0–563)	−4.0 (−68.5, 35.8)	0.563
MIP-1β (pg/ml)	65.6 (1.0–305)	3.0 (−9.6, 22.6)	64.1 (8.6,479)	−5.8 (−66.2, 55.1)	0.142
IP10 (pg/ml)	8.5 (1.7–15.1)	0.6 (−0.3, 2.1)	11.3 (1.0–19.6)	−0.58 (−8.2, 13.5)	0.530
MIG (pg/ml)	7.5 (1.2–24.9)	0.5 (−1.1, 2.5)	8.5 (3.5–43.8)	−2.6 (−4.1, 1.1)	0.047*
Eotaxin (pg/ml)	67.6 (46.7–110.6)	2.6 (−2.6, 7.4)	60.0 (47.7–156)	4.0 (−9.3, 19.7)	0.699
Rantes (pg/ml)	1,597 (454–4,553)	0.0 (−408, 625)	2,909 (350–4,769)	−132 (−1,085.17)	0.053
MCP-1 (pg/ml)	149 (107–356)	−2.2 (−7.5, 7.7)	153 (122–318)	1.8 (−29.4, 58.9)	0.918

Table 3 Baseline values and observed changes in biomarkers of antioxidant and oxidative stress status after intervention with bilberry juice for 4 wks presented as baseline mean (range) or median[b] (range) and observed change reported as mean (± SD) or median[b] (95% CI for median). *P* values refer to comparison of the observed changes, between the groups. Change = the value after intervention minus baseline value

Parameter	Water ($n = 31$)		Bilberry ($n = 31$)		p (change)
	Baseline	Change	Baseline	Change	
Biomarkers of antioxidant status					
AA[b] (μmol/l)	44.8 (15.7–70.1)	−2.4 (−6.8, 1.9)	46.7 (10.5–80.4)	−3.2 (−8.2, 1.9)	0.825
FRAP (μmol/l)	1,294 (996–1,789)	98 ± 161	1,313 (982–1,611)	20 ± 222	0.109
TRAP (μmol/l TE)	1,285 (779–1,662)	25 ± 240	1,327 (841–1,682)	−48 ± 229	0.375
ORAC plasma (μmol TE/ml)	15.27 (8.97–21.13)	1.16 ± 2.74	15.25 (11.52–24.65)	1.91 ± 3.69	0.390
ORAC, PCA treated (μmol TE/ml)	0.92 (0.42–1.46)	0.020 ± 0.14	0.93 (0.51–1.68)	−0.052 ± 0.22	0.150
β-carotene[b] (nmol/l)	281 (45–1,210)	72 (29, 131)	307 (51–1,061)	35 (9, 97)	0.202
Quercetin[b] (nmol/l)	26.4 (8.6–322.8)	2.3 (−28.0, 29.7)	31.1 (9.1–215.5)	12.5 (3.0, 26.2)	0.029*
m-coumaric acid[b] (nmol/l)	13.1 (2.9–84.8)	−0.4 (−7.8, 22.9)	11.2 (3.6–50.9)	1.6 (−2.3, 4.5)	0.569
p-coumaric acid[b] (nmol/l)	13.2 (5.2–155.2)	0.1 (−8.5, 11.5)	18.7 (4.7–116.1)	8.7 (5.3, 19.9)	0.016*
Protocatechuic acid[a, b] (nmol/l)	81.2 (35.4–186.3)	2.8 (−9.2, 13.7)	96.8 (45.8–228.3)	2.6 (−12.3, 11.6)	0.863
Enterolactone[b] (nmol/l)	21.3 (1.1–262.8)	−1.0 (−26.1, 52.8)	18.3 (1.4–107.5)	7.9 (−3.0, 23.5)	0.076
α-tocopherol[a, b] (μmol/l)	23.5 (16.2–41.4)	0.4 (−0.9, 1.5)	28.5 (11.6–37.2)	0.1 (−1.5, 2.1)	0.729
Total glutathione[b] (μmol/l)	2.50 (1.6–4.2)	1.5 (1.1, 1.7)	2.58 (1.7–4.5)	1.2 (0.8, 1.6)	0.298
Reduced glutathione[b] (μmol/l)	2.0 (1.0–3.8)	−0.14 (−0.3, 0.5)	1.9 (1.0–3.2)	0.29 (−0.1, 0.4)	0.444
Biomarkers of oxidative stress status					
DHAA:TAA[b] ratio	0.109 (0.001–0.303)	0.018 (−0.013, 0.050)	0.121 (0.010–0.256)	0.017 (−0.008, 0.042)	0.948
D-ROM, Carr U	241 (171–375)	−16 ± 34	254 (167–349)	−23 ± 29	0.452
Oxidized glutathione[b] (μmol/l)	0.050 (0.000-0.302)	0.01 (−0.001, 0.059)	0.054 (0.000–0.287)	0.01 (−0.012, 0.043)	0.729
Plasma GSH redox potential[b] (mV)	−143 (−169 to 115)	4 (−4, 10)	−139 (−160 to 111)	2 (−4, 5)	0.608

[a] Baseline values are significantly different between groups, $p < 0.050$

[b] Data are not normally distributed, *p*-values are calculated from the Mann–Whitney test

Cell culture experiments

The human monocytic cell line U937 3xκB-LUC was incubated with LPS (1 μg/ml) to induce NF-κB activity. LPS alone induced NF-κB activity to 312% compared with the basal NF-κB activity.

Table 4 summarizes content of the major polyphenol classes found in bilberry, and of these, 13 were tested for their ability to modulate LPS-induced NF-κB activity. When the cells were co-incubated with quercetin, the LPS-induced NF-κB activation was inhibited in a dose-dependent manner as compared with control cells (Fig. 1a, b) (77 and 95 at 25 and 50 μmol/l, respectively, both $p < 0.001$). Resveratrol (50 μmol/l) and epicatechin (25 μmol/l) inhibited the LPS-induced NF-κB activation by 79% ($p = 0.020$) and 32% ($p = 0.003$), respectively (Fig. 1b). Myricetin gave a slight, but statistically significant increase in the LPS-induced NF-κB activity to a maximum of 122% at 50 μmol/l ($p < 0.001$).

Neither of the anthocyanidins/anthocyanin tested demonstrated ability to inhibit LPS-induced NF-κB activity in the U937 3xκB-LUC cells (Fig. 1c, d). Interestingly, petunidin (50 μmol/l) and delphinidin (50 μmol/l) increased LPS-induced NF-κB activity to, respectively, 245 and 146% of controls, ($p = 0.003$ and $p = 0.007$).

For the additional polyphenols that were tested (*m*-coumaric acid, *p*-coumaric acid, protocatechuic acid and syringic acid, cyanidin, or cyanidin 3-*O*-β-glucopyranoside), and the lignan metabolite enterolactone, no significant effects on LPS-induced NF-κB activity was observed.

Table 4 Specific content of the major polyphenol classes found in bilberry

Polyphenol class	Constituent	Content[a], mg/100 g fw
Anthocyanins	Cyanidin	18–290
	Delphinidin	29–280
	Petunidin	15–86
Flavonol	Quercetin	1.5–8
	Myricetin	nd-3
Phenolic acid	*p*-coumaric acid	1–9
	m-coumaric acid	7–30
	Protocatechuic acid	4–8
	Syringic acid	13–15
Stilbenes	Resveratrol	1–12
Catechins	Epicatechin	6–7
Lignans	Enterolactone[b]	Precursor: 13

[a] References are given in Supplementary Information

[b] Enterolactone is a lignan metabolite

Discussion

We have demonstrated that intake of bilberry juice can modulate inflammatory mediators in men and women at increased risk of CVD and increase the levels of plasma polyphenols. Interestingly, all these mediators are target genes of NF-κB [43–47], a transcription factor that is essential in orchestrating the inflammatory responses to a wide range of insults [4] and is involved in the pathogenesis of CVD [48]. These observations indicate that constituents in the bilberry juice may have mediated modulation of NF-κB activity. Decreased levels of CRP and RANTES following intervention with sweet cherries rich in anthocyanins has previously been reported [49]. Additionally, we have previously reported that supplementation with anthocyanins significantly reduces the plasma levels of NF-κB-related inflammatory mediators [30]. Thus, our observation is supported by data from other human studies where polyphenol-rich food items are able to decrease plasma concentration of inflammatory mediators associated with NF-κB activation [30, 49]. The findings of lowered levels of CRP and IL-6 is of particular interest, as these proteins are considered independent predictors of vascular events in apparently healthy subjects [11, 12, 50].

In contrast, we observed a significant increase in TNF-α in the bilberry group. Normally, an increase in TNF-α would lead to elevated levels of IL-6, CRP, and other inflammatory mediators. We observed, however, that levels of CRP, IL-6, IL-15, and MIG were lowered in the bilberry group. Also, TNF-α is a stimulus for the anti-inflammatory cytokine IL-10, and an increased TNF-α:IL-10 ratio may be a measure of an inflammatory signature in CVD [51–53]. TNF-α:IL-10 ratio was, however, not significantly changed in the bilberry group compared with the control group (data not shown).

To investigate whether the effects on NF-κB related inflammatory mediators could be mediated by bilberry polyphenols, a number of polyphenols found in bilberries (and one polyphenol metabolite) were tested for their ability to modulate NF-κB activation in a human monocytic cell line. Quercetin, epicatechin, and resveratrol were potent inhibitors of NF-κB activation in the U937 3xκB-LUC cells, while *p*-coumaric acid, enterolactone (a human lignan metabolite), and several others did not show significant effects. Similar inhibitory effects on NF-κB activation have previously been reported for quercetin and resveratrol in vitro [3]. Seemingly in contrast to the clinical findings, the anthocyanidins delphinidin and petunidin mediated an increase in the LPS-induced NF-κB activation at the highest concentration tested in vitro. It should, however, be kept in mind that the bioavailability of

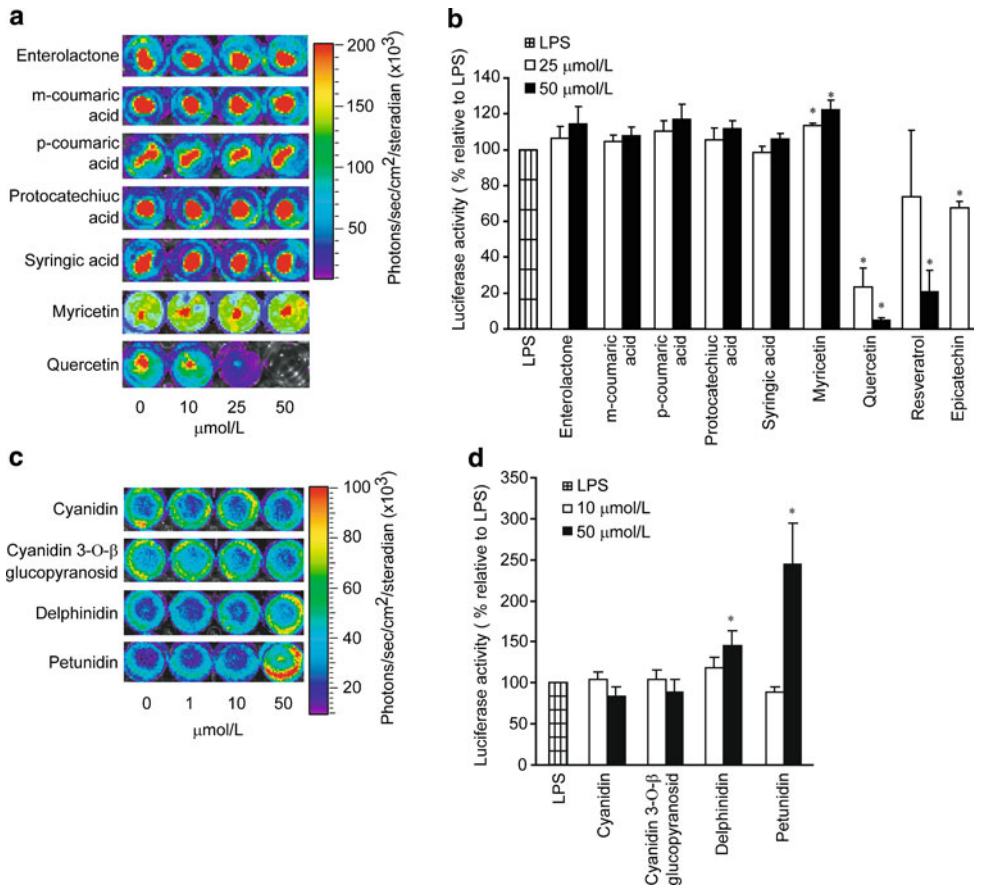

Fig. 1 Effect of bilberry polyphenols on LPS-induced NF-κB activation in U937 3xκB-LUC cells. U937-3xκB-LUC cells were pre-incubated with the indicated polyphenols and concentrations for 30 min, LPS (1 µg/ml) was added and cells incubated for an additional 6 h. Luciferase activity was measured by imaging in an IVIS 100 Imaging System. **a** and **c**: Images of cell culture plates from the IVIS100 Imaging System. One representative experiment per polyphenol is shown. Luminescence is measured in photons per sec per cm^2 per steradian, and the light intensity is presented with the *color bar*. *Gray scale images* are used for reference of position. **b** and **d** The number of photons emitted from each well was calculated by use of the Living Image Software (Caliper Life Science, Hopkinton; MA, USA). To limit the possible inhibitory effects of resveratrol on the firefly luciferase enzyme [42], the luciferase activity in U937 3xκB-LUC cells after treatment with resveratrol was measured as previously described [41]. Epicatechin at 50 µmol/l was excluded due to cell toxicity. Thus, resveratrol, and epicatechin are also only shown in (**b**). All treatments are expressed as percent change compared to control cells incubates with LPS and vehicle only. *Each bar* represents the mean of three experiments ± SD. One-way ANOVA followed by Dunnett comparisons were performed to identify statistical differences. $*p < 0.05$

anthocyanidins is low as compared with other bilberry polyphenols [54]. Little is known about distinct regulatory effects of polyphenols on specific NF-κB target genes in specific tissues. However, we have previously observed that dietary plants and phytochemicals have ability to either induce or inhibit NF-κB in the same cell type, depending on the physiological setting [55]. Even though we studied the effect on NF-κB in monocytes, other cell types such as peripheral blood mononuclear cells and endothelial cells, and tissues (e.g. liver and adipose tissue) may be important sources of pro-inflammatory mediators. Thus, such dual effects depending on dose, target gene, or target tissue may as least in part explain the seemingly conflicting results on the intervention effects on TNF-α.

We observed significant increases in plasma levels of quercetin and *p*-coumaric acid in the bilberry group. The observed immunoregulatory effects may be related to the accumulation of bilberry polyphenols in plasma, although no correlations were found between inflammatory markers and plasma concentrations of polyphenols. A number of the measured polyphenolic compounds have a rapid metabolism and elimination [54]. Thus, we did not expect to find increases in these polyphenols using overnight, fasting blood samples. These findings indicate that the 4-week intake of bilberry juice was sufficient to increase the "steady state" of quercetin and *p*-coumaric acid in plasma. Furthermore, this bear promise that permanent dietary changes may lead to persistent increases in plasma levels of

polyphenols which are not confined to the post-prandial state only.

In conclusion, supplementation with bilberry juice decreases plasma concentrations of several NF-κB regulated inflammatory mediators in adults at increased risk of CVD. These findings are supported by observations in a human monocytic cell line where bilberry polyphenols inhibited LPS-induced NF-κB activation. Our findings suggest that bilberry polyphenols may modulate inflammatory processes. Further testing of bilberry supplementation in prevention or alleviation of chronic inflammatory such as CVD, is warranted.

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Conflict of interest statement RB has interests in Cgene AS, Bioindex AS and Vitas AS. Cgene AS and Bioindex AS were established by Birkeland Innovation, the Technology transfer office at the University of Oslo while Vitas AS was established by the Oslo Innovation Center. No competing financial interests exist for AK, IP, SKB, AKS, GIB, IE, MS, PL or ST.

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