Effects of sea buckthorn and bilberry on serum metabolites differ according to baseline metabolic profiles in overweight women: a randomized crossover trial[1–4]

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ABSTRACT

Background: Berries are associated with health benefits. Little is known about the effect of baseline metabolome on the overall metabolic responses to berry intake.

Objective: We studied the effects of berries on serum metabolome.

Design: Eighty overweight women completed this randomized crossover study. During the interventions of 30 d, subjects consumed dried sea buckthorn berries (SBs), sea buckthorn oil (SBo), sea buckthorn phenolics ethanol extract mixed with maltodextrin (SBe+MD) (1:1), or frozen bilberries. Metabolic profiles were quantified from serum samples by using ^{1}H nuclear magnetic resonance spectroscopy.

Results: All interventions induced a significant ($P < 0.001$–0.003) effect on the overall metabolic profiles. The effect was observed both in participants who had a metabolic profile that reflected higher cardiometabolic risk at baseline (group B: $P = 0.001$–0.008) and in participants who had a lower-risk profile (group A: $P < 0.001$–0.009). Although most of the changes in individual metabolites were not statistically significant after correction for multiplicity, clear trends were observed. SB-induced effects were mainly on serum triglycerides and very-low-density lipoprotein (VLDL) and its subclasses, which decreased in metabolic group B. SBo induced a decreasing trend in serum total, intermediate-density lipoprotein (IDL), and low-density lipoprotein (LDL) cholesterol and subfractions of IDL and LDL in group B. During the SBe+MD treatment, VLDL fractions and serum triglycerides increased. Bilberries caused beneficial changes in serum lipids and lipoproteins in group B, whereas the opposite was true in group A.

Conclusion: Berry intake has overall metabolic effects, which depend on the cardiometabolic risk profile at baseline. This trial was registered at clinicaltrials.gov as NCT01860547. *Am J Clin Nutr* 2013;98:941–51.

INTRODUCTION

Reducing effects of sea buckthorn (*Hippophaë rhamnoides*) berries on inflammatory markers (1, 2) have been observed in clinical trials. The intake of sea buckthorn berries (SBs)[5] increased concentrations of circulating flavonols in healthy adults (3, 4). In humans, supplementation with sea buckthorn oil (SBo) extracted with supercritical carbon dioxide attenuated the signs and symptoms of dry eye (5), reduced platelet aggregation (6), had beneficial effects on atopic dermatitis (7), increased the serum concentration of HDL cholesterol (7), and reduced the concentration of vascular cell adhesion molecule-1 (VCAM-1) (2). Animal and in vitro studies have suggested that SBo (8–10) and alcohol extracts and flavonoid preparations (11–14) have antioxidant and antiinflammatory activities and may beneficially affect serum glucose and triglyceride concentrations (15). These results have been supported by recent clinical studies (16, 17).

Bilberry (the native European blueberry *Vaccinium myrtillus*) juice modulated the concentrations of inflammatory markers regulated by the nuclear transcription factor κB in men and women at elevated risk of cardiovascular diseases (18). A dietary supplement that contained a combination of blueberry and sea buckthorn concentrates improved the activity of antioxidant enzymes, increased the amount of C-peptide, and reduced concentrations of glycated hemoglobin in the circulation of type 1 diabetic children (19).

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[5] Abbreviations used: IDL, intermediate-density lipoprotein; NMR, nuclear magnetic resonance; SB, sea buckthorn berry; SBe+MD, sea buckthorn phenolics ethanol extract mixed with maltodextrin; SBo, sea buckthorn oil; SOM, self-organizing map; VCAM-1, vascular cell adhesion molecule-1. *See* Supplemental Table 1 under "Supplemental data" in the online issue for a complete list of abbreviations used in figures.

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Bilberries and sea buckthorn tended to induce a slight decrease in waist circumference, body weight, and serum concentration of VCAM-1 in slightly overweight women (2).

The objective of this study was to investigate the effects of SB, its fractions, and bilberry on concentrations of circulating lipids, lipoproteins, and low-molecular-weight metabolites in women with slightly elevated concentrations of risk markers of cardiovascular disease and type 2 diabetes. In particular, we aimed to investigate the effect of the baseline metabolic profile on the response to berry intake, which, to the best of our knowledge, has not previously been reported by using holistic approaches. We used a new nuclear magnetic resonance (NMR) metabolomics platform to get a holistic view of the effects in systemic circulation (20, 21).

SUBJECTS AND METHODS

Participants and design

A total of 110 overweight or slightly obese women were recruited in this randomized crossover trial. Each participant gave her written informed consent to the study procedures, which were approved by the Ethics Committee of the Hospital District of Southwest Finland. The inclusion criteria were as follows: BMI (in kg/m^2) of 26–34, serum total cholesterol concentration of 4.5–8 mmol/L, LDL-cholesterol concentration >2.5 mmol/L, triglyceride concentration <4 mmol/L, glucose concentration <6 mmol/L, insulin concentration <25 mU/L, blood pressure <160/99 mm Hg, hemoglobin concentration >120 g/L, thyroid stimulating hormone concentration of 0.3–4.2 mU/L, alanine aminotransferase concentration <60 U/L, and creatinine concentration <115 μmol/L. Methods for the analysis of the above-mentioned markers were described previously (2). Exclusion criteria were as follows: pregnancy; menopause; regular smoking; previously diagnosed diabetes (other than gestational); thyroid, renal, hematologic, or hepatic dysfunction; previous myocardial infarction; ongoing inflammatory disease; use of cardiovascular medication; treatment with lipid-lowering drugs; or use of any other regular medication with the exception of allergy medication and joint lubricates. The mean age of participants was 44.2 ± 6.2 y.

Study visits and sampling were conducted at the Turku University of Applied Sciences, Turku, Finland, during the period from June 2008 to June 2009. This trial was registered at clinicaltrials.gov as NCT01860547. During intervention periods of 33–35 d, participants consumed the following 4 berry diets in a randomized order: dried SBs, sea buckthorn phenolics ethanol extract mixed with maltodextrin (SBe+MD) (1:1), SBo, or frozen bilberries. Each participant was assigned to receive each berry diet once during the study in an independently randomized order. A simple unrestricted random assignment by using a random-number table was carried out. Different persons were in charge of the random assignment and enrollment of participants. Intervention periods were separated by washout periods of 30–39 d. Serum samples after a 12-h fast were collected at the beginning and end of each intervention. The main outcome measure of the study was the change in the serum alanine aminotrasferase concentration during berry interventions. Secondary outcome measures were the effects of berry interventions to other indicators of lipid and glucose metabolism and inflammation, several of which were reported previously (2). In this study, an NMR analysis of metabolic profiles of serum samples was performed to investigate the hypothesis that the berry interventions would affect metabolic profiles of participants, and the effect may differ according to baseline metabolic profiles of participants.

To keep the daily energy intake constant during interventions and washout periods, participants were asked to replace part of their normal diet with berry products provided for them. Otherwise, the diet of participants was free besides the intervention. Nutrient and energy intakes during intervention and washout periods were estimated by using 3-d food records during each period.

A total of 80 participants completed the study according to the protocol. Analysis results from all sampling points were obtained from 77 participants. Reported reasons for dropouts during the study were difficulties of participants to use the time for the whole duration of several months or other personal reasons. The prestudy sample-size estimation was based on an assumption that a change (±SD) of 0.14 ± 0.23 mmol/L in the concentration of LDL cholesterol could take place during a berry intervention. With a sample size of 85 participants, this study would have a power of ~80% to detect the change (2-sided test; 0.05 significance level). Participants, prestudy power calculations, and the study protocol were described in detail previously (2).

Study products

SBs were air-dried berries of *Hippophaë rhamnoides* ssp. *turkistanica*. The sugar (fructose, sucrose, and glucose) content of the berries was 6.5 g/100 g. Sugars were analyzed as trimethylsilyl derivatives by using gas chromatography (22). The SBo was a standardized mixture of oils from soft parts and seeds of sea buckthorn extracted by Aromtech Ltd by using supercritical carbon dioxide. Main fatty acids of the oil were palmitoleic acid (16:1n−7; 24%), palmitic acid (16:0; 22%), oleic acid (18:1n−9; 20%), linoleic acid (18:2n−6; 15%), and α-linolenic acid (18:3n−3; 10%). Fatty acids were analyzed as methyl esters by using gas chromatography with a flame-ionization detector (modified from reference 23). The oil contained 0.09% carotenoids and 0.38% tocopherols, which were analyzed by using spectrophotometric and HPLC-UV/visible methods, respectively. The SBe+MD was prepared by extracting the carbon dioxide–extracted sea buckthorn residue with 50% aqueous ethanol. During spray drying of the extract, maltodextrin dextrose equivalent 6 at a ratio of 1:1 was added. The product contained 248 mg flavonols/100 g and 9.4 g sugars/100 g. Flavonols were analyzed as aglycones by using an HPLC-UV/visible method (an in-house method of MTT Agrifood Research Finland). Bilberries were given as frozen berries and thawed before intake. The sugar content of bilberries was 6.2 g/100 g. Daily doses of berry diets were 20 g SBs, 14.6 g (7.3 g sea buckthorn extract + 7.3 g dextrose equivalent 6) SBe+MD, 4 g (8 capsules) SBo, and 100 g frozen bilberries. Each dose corresponded to ~100 g fresh berries. More-detailed information concerning the manufacturing of study products has been reported previously (2).

Serum NMR metabolomics

We applied ^{1}H NMR spectroscopy to determine the serum metabolome via 3 different molecular windows (20, 21). These

windows provide information on 14 lipoprotein subclasses and lipoprotein-particle concentrations, low-molecular-weight metabolites such as amino acids, 3-hydroxybutyrate, and creatinine, and detailed molecular information on serum lipids including free and esterified cholesterol, sphingomyelin, saturation degree, and n−3 fatty acids. The methodology provides information on >100 primary metabolic measures and multiple derived variables with clear biochemical interpretation and significance (21). Data of 14 lipoprotein subclasses and lipoprotein-particle concentrations plus low-molecular-weight metabolites were run from native serum samples in the same experiment, and serum lipid data were run from lipid extracts afterward. The experimental protocol including the sample preparation and NMR spectroscopy are described in detail in Inouye et al (24). *See* online supplemental material and Supplemental Table 1 under "Supplemental data" in the online issue for a definition of lipoprotein subclasses and complete list of metabolites quantified, respectively. This quantitative platform has recently been applied in various large-scale epidemiologic and genetic studies (25–27).

Statistical analyses

To analyze the effect of the baseline metabolome on treatment responses, participants were divided into 2 groups (A and B) on the basis of their baseline metabolic profiles. This was done by first examining the baseline metabolic diversity with the self-organizing map (SOM) algorithm. The SOM is an unsupervised learning algorithm that spreads individuals (samples) on a plane on the basis of similarity in input data (serum metabolome). Samples with similar metabolic profiles end up in nearby coordinates, whereas a pair of samples with great deviation is organized far from each other. Complete baseline metabolic profiles were used as input for the SOM with the exception that, of lipoprotein subclass measures, only total lipid concentrations were included to prevent the overemphasis of the role of lipoproteins in the analysis (28). Input values were ranked, and the ranks were mapped onto a normal distribution before the application of the SOM algorithm. Samples were separated into 2 groups (A and B) by dividing the resulting SOM into 2 metabolically different areas. A clustering algorithm applied to prototype vectors that represented average local properties of map cells was used for the division. Only SOM inputs with a statistically significant ($P < 0.05$) separation on the SOM-component plane were used in the clustering.

Metabolic measures before and after treatment were compared by using Wilcoxon's signed-rank tests. The tests were first performed by including all participants and then separately for baseline metabolic groups A ($n = 47$) and B ($n = 30$). Measures were adjusted for sugar intake before testing. The significance threshold was corrected for multiple testing by dividing the 5% significance-level threshold by the number of principal components, which explained 95% of the variance in metabolite data. The resulting significance threshold was $P < 0.0028$ (18 components) for the whole data set as well as for group A, whereas for the smaller group B, the threshold was $P < 0.0033$ (15 components).

To test the hypothesis that the diet intervention would have a measurable overall effect on the metabolic profile, a univariate score that summarized the metabolic changes was created. Moreover, because of the large number of metabolites and small number of participants in the metabolic groups, there was low power to test differences in all individual measures beyond the multiple-testing threshold. In contrast, with the summary measure, only a single test was needed (29). The summary measure was calculated for each diet as a cross-product of a correlation vector and a difference vector. The correlation vector was populated with Pearson's correlation coefficients of each metabolite with the intervention status (1 if the value was measured after the given diet period and 0 otherwise). The difference vector contained median changes in (scaled and centered) concentrations of each metabolite during the given diet. A null distribution with the null hypothesis that no change in metabolism took place during the intervention was generated by calculating the score for 100,000 replicate data sets with treatment statuses randomly permuted. A P value for the score was then calculated by using a normal approximation of the null distribution. Before calculating scores, metabolic measures were first adjusted for sugar intake and then centered and scaled to the zero median and unit IQR. *See* Supplemental Figure 1 under "Supplemental data" in the online issue for details on calculating the summary measure. A threshold of $P < 0.05$ for statistical significance was used.

Energy and nutrient intakes during interventions were considered by comparing 3-d food records of berry interventions to records of the preceding washout period by using Wilcoxon's signed rank test. Thresholds for significance were adjusted for multiple testing by using Bonferroni correction again with the number of principal components that explained 95% of the variance in food-record data. The resulting significance threshold was $P < 0.0038$ (13 components) for the whole data set as well as for group A, whereas for the smaller group B, the threshold was $P < 0.0045$ (11 components).

All analyses were performed by using in-house scripts in the MATLAB R2012a programming environment (Mathworks Inc). An open-source package termed Melikerion for SOM analyses in the Matlab/Octave programming environment is freely available.

RESULTS

Effects of berries between all participants

All 4 berry diets showed a significant ($P < 0.001$–0.003) effect on overall metabolic profiles of participants (**Figure 1**). Although most of changes in individual metabolites were NS after adjustment for multiple analyses, clear trends were observed. SBs induced effects on individual metabolites focused on triglyceride and VLDL subclasses, where a decreasing trend was observed. During the SB period, a significant ($P < 0.0028$; corresponds to 5% level of significance after correction for multiple testing) decrease took place in triglycerides in small HDL particles as well as in serum creatinine and phenylalanine (**Figure 2**; *see* Supplemental Table 2 under "Supplemental data" in the online issue). The SBo period showed a significant ($P < 0.0028$) decrease in the concentrations of serum-free cholesterol, albumin, and lactate as well as in cholesterol, triglyceride, and particle concentrations in the intermediate-density lipoprotein (IDL) subclass and triglycerides in small HDL. A nonsignificant decreasing trend was observed in apolipoprotein B, LDL, and total cholesterol; particles and lipids of very small VLDL; and all subclasses of IDL and LDL, including small LDL particles

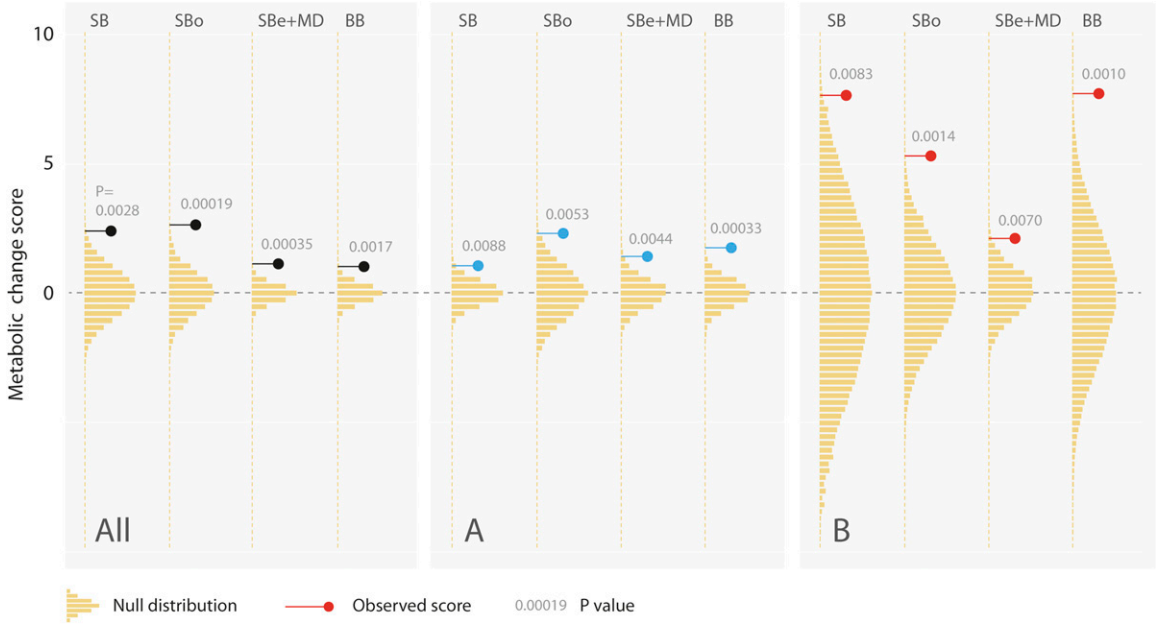

FIGURE 1. Relative magnitudes and levels of statistical significance of overall metabolic changes during the berry interventions. Group A included participants with the baseline metabolic profile indicating lower cardiometabolic risk (n = 47). Group B included participants with the baseline profile indicating higher cardiometabolic risk (n = 30). To test whether there was an observable change in metabolism during the intervention, an overall score was calculated. The score summarized the metabolic changes associated with each intervention in a single measure. The significance level was assessed with a simulated null distribution generated by randomly permuting the before and after statuses and calculating the score for 100,000 permuted data sets. *See* Supplemental Figure 1 under "Supplemental data" in the online issue for a detailed description of the summary metabolic score and statistical method. BB, bilberries; SB, sea buckthorn berries; SBe+MD, sea buckthorn phenolics ethanol extract mixed with maltodextrin (1:1); SBo, sea buckthorn oil.

(Figure 2). During the SBe+MD intervention, a significant ($P <$ 0.0028) increase in cholesterol esters in large VLDL and decrease in serum acetate took place (Figure 2). In addition, an increasing trend in serum VLDL triglycerides, serum total triglycerides, and in almost all of the VLDL subclasses was observed. The changes during the bilberries diet were less pronounced, and a significant ($P < 0.0028$) change was seen only in triglycerides in small HDL particles (Figure 2). None of the diets were associated with a significant ($P < 0.0028$) change on fasting blood glucose.

Effect of baseline metabolic profile on response to berries

The metabolic clustering of baseline samples revealed 2 distinct baseline metabolic groups of participants. Group B (30 participants) was characterized by higher serum concentrations of apolipoprotein B, a higher ratio of apolipoprotein B to apolipoprotein A-I, higher serum LDL cholesterol, higher triglycerides, lower HDL cholesterol, and smaller diameters of HDL and LDL particles compared with in participants assigned to the metabolic group A (47 participants) (**Figure 3**, **Table 1**; *see* Supplemental Figure 2 under "Supplemental data" in the online issue). Median concentrations of inflammatory markers [reported previously by Lehtonen et al (2)] TNF-α, IL-6, high-sensitivity C-reactive protein, intercellular adhesion molecule-1, and VCAM-1 tended to be higher, and adiponectin tended to be lower, in the participants in the baseline metabolic group B (data not shown). In general, metabolic profiles in group B indicated higher cardiometabolic risk than in group A.

All berry treatments had a significant effect on overall metabolic profiles of participants, regardless of their baseline metabolome (group A: $P < 0.001$–0.009; group B: $P = 0.001$–0.008) (Figure 1). The analysis of individual metabolites revealed that the trend for reduced serum triglycerides during the SB period, which was observed in all participants, occurred mainly in participants in the baseline group B, in whom serum VLDL triglycerides, several VLDL subclasses, and LDL and serum total cholesterol also showed lowering tendencies. Changes in individual metabolites because of SBs were subtle in participants in group A, with the exclusion of the decrease in serum creatinine ($P < 0.0028$), that was observed also in group B ($P < 0.0033$) (**Figures 4** and **5**; *see* Supplemental Tables 3 and 4 under "Supplemental data" in the online issue). Similarly, in general, only modest effects on individual metabolites were observed in baseline group A because of the intake of SBo. During the SBo intervention, lowering trends of serum apolipoprotein B, total and LDL cholesterol, and IDL and LDL subclasses and very small VLDL were observed in group B. None of the individual changes in group B during the SBo intervention were significant after adjustment for multiple testing, whereas in group A, the albumin concentration was decreased significantly ($P < 0.0028$). During the SBe+MD intervention, the trend for the elevation of VLDL fractions was observed in both baseline groups A and B (Figures 4 and 5).

The analysis of individual metabolites revealed a trend for opposing changes in VLDL subclasses in group A (an increasing trend) compared with in group B (a decreasing trend) during the bilberry intervention (Figures 4 and 5). In group A participants, increases in most VLDL subclasses were observed, whereas most VLDL subclasses, as well as IDL and LDL subclasses, and serum triglycerides and total cholesterol showed decreasing trends in group B. Of changes in the individual metabolites during the

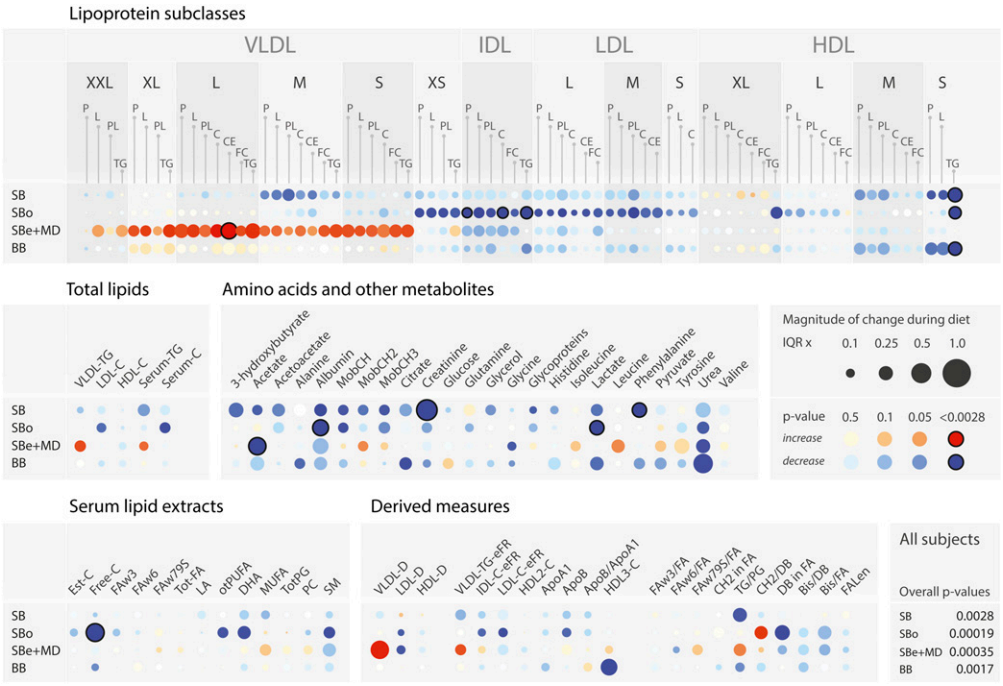

FIGURE 2. Wilcoxon's signed-rank test was used to compare concentrations of metabolic measures before and after the intervention ($n = 77$). Red and blue dots indicate a detected increase and decrease, respectively, in the target variable. The size of the dot shows the magnitude of the change. Color saturation increases with the statistical significance. Significance at the 5% level (corrected for multiple testing by using a modified Bonferroni method) is indicated by a black border. Measures were adjusted for sugar intake. *See* Supplemental Table 2 under "Supplemental data" in the online issue for a table of exact results with *P* values. In the following categories, values that were estimated by using the extended Friedewald's method (50) are denoted with a superscript *1*. Amino acids and other metabolites (mmol/L): MobCH, double-bond protons of mobile lipids; MobCH2, CH_2 groups of mobile lipids; MobCH3, CH_3 groups of mobile lipids; derived measures: ApoA1 (g/L), apolipoprotein A-I[1]; ApoB (g/L), apolipoprotein B[1]; ApoB/ApoA1, apolipoprotein B by apolipoprotein A-I[1]; BIS/DB, ratio of bisallylic groups to double bonds; BIS/FA, ratio of bisallylic groups to total fatty acids; CH2/DB, average number of methylene groups per a double bond; CH2 in FA, average number of methylene groups in a fatty acid chain; DB in FA, average number of double bonds in a fatty acid chain; FALen, description of average fatty acid chain length and not the actual carbon number; FAw79S/FA (%), ratio of n−7, n−9, and SFAs to total fatty acids; FAw6/FA (%), ratio of n−6 fatty acids to total fatty acids; FAw3/FA (%), ratio of n−3 fatty acids to total fatty acids; HDL-D (nm), mean diameter for HDL particles; HDL2-C (mmol/L), total cholesterol in HDL_2[1]; HDL3-C (mmol/L), total cholesterol in HDL_3[1]; IDL-C-eFR (mmol/L), total cholesterol in IDL[1]; LDL-C-eFR (mmol/L), total cholesterol in LDL[1]; LDL-D (nm), mean diameter for LDL particles; TG/PG, ratio of triglycerides to phosphoglycerides; VLDL-D (nm), mean diameter for VLDL particles; VLDL-TG-eFR (mmol/L), triglycerides in VLDL[1]; lipoprotein subclasses (mmol/L, unless otherwise indicated): IDL-C, total cholesterol in IDL; IDL-FC, free cholesterol in IDL; IDL-L, total lipids in IDL; IDL-P (mol/L), concentration of IDL particles; IDL-PL, phospholipids in IDL; IDL-TG, triglycerides in IDL; L-HDL-C, total cholesterol in large HDL; L-HDL-CE, cholesterol esters in large HDL; L-HDL-FC, free cholesterol in large HDL; L-HDL-L, total lipids in large HDL; L-HDL-P (mol/L), concentration of large HDL particles; L-HDL-PL, phospholipids in large HDL; L-LDL-C, total cholesterol in large LDL; L-LDL-CE, cholesterol esters in large LDL; L-LDL-FC, free cholesterol in large LDL; L-LDL-L, total lipids in large LDL; L-LDL-P (mol/L), concentration of large LDL particles; L-LDL-PL, phospholipids in large LDL; L-VLDL-C, total cholesterol in large VLDL; L-VLDL-CE, cholesterol esters in large VLDL; L-VLDL-FC, free cholesterol in large VLDL; L-VLDL-L, total lipids in large VLDL; L-VLDL-P (mol/L), concentration of large VLDL particles; L-VLDL-PL, phospholipids in large VLDL; L-VLDL-TG, triglycerides in large VLDL; M-HDL-C, total cholesterol in medium HDL; M-HDL-CE, cholesterol esters in medium HDL; M-HDL-FC, free cholesterol in medium HDL; M-HDL-L, total lipids in medium HDL; M-HDL-P (mol/L), concentration of medium HDL particles; M-HDL-PL, phospholipids in medium HDL; M-LDL-C, total cholesterol in medium LDL; M-LDL-CE, cholesterol esters in medium LDL; M-LDL-L, total lipids in medium LDL; M-LDL-P (mol/L), concentration of medium LDL particles; M-LDL-PL, phospholipids in medium LDL; M-VLDL-C, total cholesterol in medium VLDL; M-VLDL-CE, cholesterol esters in medium VLDL; M-VLDL-FC, free cholesterol in medium VLDL; M-VLDL-L, total lipids in medium VLDL; M-VLDL-P (mol/L), concentration of medium VLDL particles; M-VLDL-PL, phospholipids in medium VLDL; M-VLDL-TG, triglycerides in medium VLDL; S-HDL-L, total lipids in small HDL; S-HDL-P (mol/L), concentration of small HDL particles; S-HDL-TG, triglycerides in small HDL; S-LDL-C, total cholesterol in small LDL; S-LDL-L, total lipids in small LDL; S-LDL-P (mol/L), concentration of small LDL particles; S-VLDL-C, total cholesterol in small VLDL; S-VLDL-FC, free cholesterol in small VLDL; S-VLDL-L, total lipids in small VLDL; S-VLDL-P (mol/L), concentration of small VLDL particles; S-VLDL-PL, phospholipids in small VLDL; S-VLDL-TG, triglycerides in small VLDL; XL-HDL-C, total cholesterol in very large HDL; XL-HDL-CE, cholesterol esters in very large HDL; XL-HDL-FC, free cholesterol in very large HDL; XL-HDL-L, total lipids in very large HDL; XL-HDL-P (mol/L), concentration of very large HDL particles; XL-HDL-PL, phospholipids in very large HDL; XL-HDL-TG, triglycerides in very large HDL; XL-VLDL-L, total lipids in very large VLDL; XL-VLDL-P (mol/L), concentration of very large VLDL particles; XL-VLDL-PL, phospholipids in very large VLDL; XL-VLDL-TG, triglycerides in very large VLDL; XS-VLDL-L, total lipids in very small VLDL; XS-VLDL-P (mol/L), concentration of very small VLDL particles; XS-VLDL-PL, phospholipids in very small VLDL; XS-VLDL-TG, triglycerides in very small VLDL; XXL-VLDL-L, total lipids in chylomicrons and extremely large VLDL; XXL-VLDL-P (mol/L), concentration of chylomicrons and extremely large VLDL particles; XXL-VLDL-PL, phospholipids in chylomicrons and extremely large VLDL; XXL-VLDL-TG, triglycerides in chylomicrons and extremely large VLDL; serum lipid extracts (mmol/L, unless otherwise indicated): Est-C, esterified cholesterol; FAw3, n−3 fatty acids; FAw6, n−6 fatty acids; FAw79S, n−7, n−9 and SFAs; Free-C, free cholesterol; LA, 18:2, linoleic acid; MUFA, monounsaturated fatty acids with 16 or 18 carbon atoms; otPUFA (the value not given as mmol/L because of several overlapping PUFAs that have different amounts of bisallylic CH_2-groups), PUFAs other than 18:2; PC, phosphatidylcholine and other cholines; SM, sphingomyelins; TotFA, total fatty acids; TotPG, total phosphoglycerides; total lipids (mmol/L): HDL-C, total cholesterol in HDL; LDL-C, total cholesterol in LDL; Serum-C, serum total cholesterol; Serum-TG, serum total triglycerides; VLDL-TG, triglycerides in VLDL. Abbreviations used: BB, bilberries; SB, sea buckthorn berries; SBe+MD, sea buckthorn phenolics ethanol extract mixed with maltodextrin (1:1); SBo, sea buckthorn oil; IQR, change from baseline to the end of intervention as numbers of IQRs.

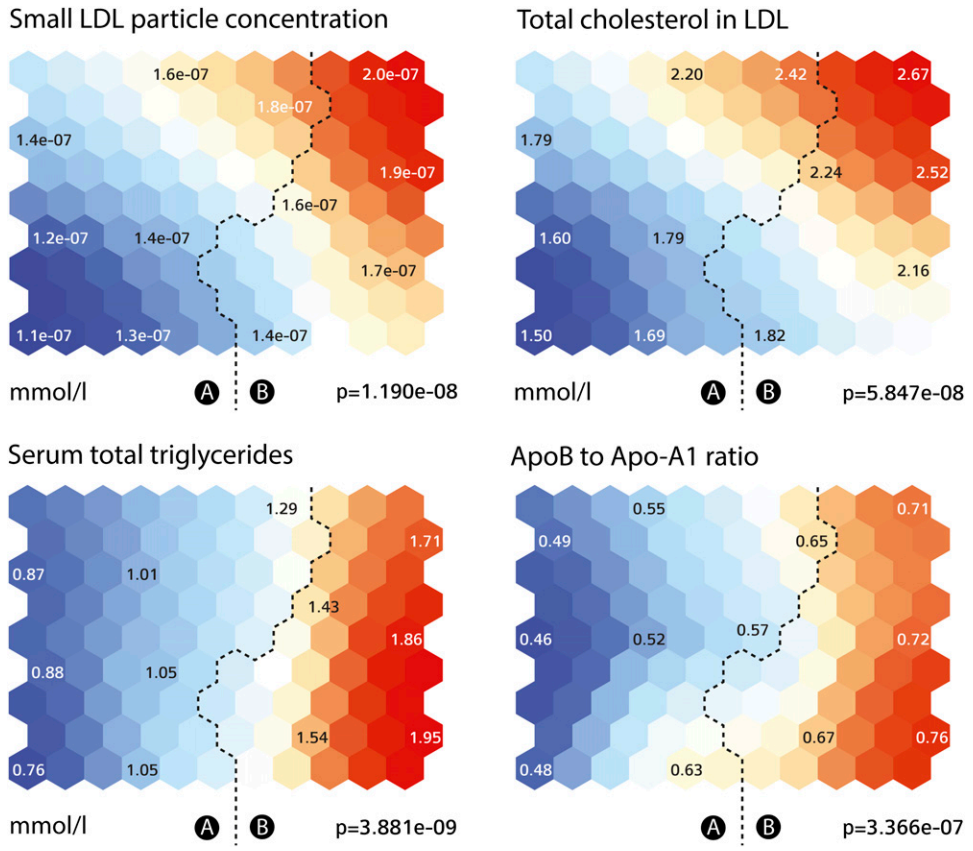

FIGURE 3. Self-organizing map component planes of selected metabolic measures at baseline. Here, the self-organizing map algorithm has been used to spread the studied subjects on a 2-dimensional map on the basis of their metabolic profile at baseline. The self-organizing map algorithm minimizes the differences between adjacent samples. Values of the component planes are visualized to show whether the unit values are above (red), at (white), or below (blue) the median of the variable. Numbers indicate the local mean value for that particular region. Empirical *P* values for the statistical colorings were calculated by comparing the observed dynamic range with a simulated set of randomly permuted component planes. The map has been divided into lower cardiometabolic risk (A; $n = 47$) and higher cardiometabolic risk (B; $n = 30$) areas by using a clustering algorithm. *See* Supplemental Figure 2 under "Supplemental data" in the online issue for an additional set of component planes including serum lipids, fatty acids, and selected low-molecular-weight metabolites. Apo-A1, apolipoprotein A1; ApoB, apolipoprotein B.

bilberry period, increases in the concentration of very large VLDL particles, total lipids in very large VLDL, and total cholesterol in small VLDL in group A and decreases in total cholesterol in IDL, triglycerides in small HDL, and double-bond protons of mobile lipids in group B were significant (group A, $P < 0.0028$; group B, $P < 0.0033$).

Reported nutrient intakes during the treatments

On the basis of 3-d food records, intakes of vitamin E, sugars, and fiber were significantly ($P < 0.0038$) increased during the SB diet than preceding washout. The increase in sugar intake was larger than expected to result from the berries, which indicated that the participants changed their diets during the SB period. During the SBo intervention, there was a reported increase ($P < 0.0038$) in intakes of MUFA, PUFA, n−3 and n−6 fatty acids, and vitamin E. All of the previously mentioned nutrients are components of SBo. During the SBe+MD treatment, no change in food records reached significance after correction for multiple testing. During the bilberry period, the intake of dietary fiber was increased ($P < 0.0038$). The intake of n−3 fatty acids as percentage of total energy intake was decreased ($P < 0.0038$) (*see* Supplemental Figure 3 under "Supplemental data" in the online issue). Detailed information of food intakes during the intervention has been reported earlier (2).

Reported nutrient intakes during treatments in baseline metabolic groups

At baseline, the reported intake of energy nutrients was slightly greater in the metabolic group A than group B, with the exception of sugar intake, which was slightly higher in group B (Table 1). In general, trends in nutrient intakes during berry interventions in metabolic groups A and B were similar. Possibly because of the greater number of participants, changes in group A more closely resembled those observed in all participants. During the SB period, significant changes in nutrient intakes were the same in group A as in all participants, with the exception of sugars, for which no significant change was present in group A. In group B, only the increase in vitamin E was significant ($P < 0.0045$) during the SB treatment, although the increase in dietary fiber was higher in magnitude (*see* Supplemental Figures 4 and 5 under "Supplemental data" in the online issue).

Trends in intakes of fatty acids during the SBo intervention were similar in groups A and B, which reflected changes observed in all participants. In baseline-divided participants, there were no

TABLE 1
Characteristics of the participants in metabolic baseline groups A (metabolic baseline profile indicating lower cardiometabolic risk) and B (metabolic baseline profile indicating higher cardiometabolic risk)[1]

	Baseline metabolic group	
	A (n = 47)	B (n = 30)
Total LDL cholesterol (mmol/L)[2]	3.1 (0.6)	3.5 (0.8)
Total HDL_2 cholesterol (mmol/L)[2]	1.1 (0.3)	0.8 (0.3)
Total HDL_3 cholesterol (mmol/L)[2]	0.5 (0.0)	0.5 (0.1)
Total serum triglycerides (mmol/L)	1.0 (0.2)	1.7 (0.5)
LDL-D (nm)	23.6 (0.1)	23.5 (0.2)
HDL-D (nm)	10.0 (0.2)	9.8 (0.2)
Apo A-I (g/L)[2]	1.7 (0.3)	1.6 (0.3)
Apo B (g/L)[2]	0.9 (0.2)	1.2 (0.2)
Apo B:Apo A-I	0.5 (0.1)	0.7 (0.2)
Energy intake (kcal/d)	1905 (481)	1750 (644)
Protein intake (g/d)	81 (25)	67 (22)
Fat intake (g/d)	64 (25)	63 (24)
Carbohydrate intake (g/d)	202 (78)	193 (69)
Sugar intake (g/d)	93 (45)	97 (47)

[1] All values are medians; IQRs in parentheses. Apo A-I, apolipoprotein A1; Apo B, apolipoprotein B; Apo B:Apo A-I, apo B to apo A-I ratio; HDL-D, diameter for HDL particles; LDL-D, diameter for LDL particles.
[2] Estimated by using the extended Friedewald's method (50).

significant changes in reported intakes during the SBe+MD intervention. During the bilberry intervention, there was a significant ($P < 0.0038$) increase in the intake of dietary fiber and decrease in n−3 fatty acids in group A. Trends in these nutrients were similar in group B, but the changes were not statistically significant ($P > 0.0045$) (*see* Supplemental Figures 4 and 5 under "Supplemental data" in the online issue).

DISCUSSION

Our study suggests that intakes of berries and berry fractions for a period of 1 mo induce significant effects on overall metabolic profiles of overweight women. Even though most of the changes that occurred in individual metabolites were not statistically significant after correction for multiple testing, clear trends accounting for the significant overall effects were observed. Changes induced by berries were different in women who had a baseline metabolic profile that indicated higher cardiometabolic risk than in women who had a baseline profile that reflected lower risk.

The intake of SBo induced reducing trends on serum total, IDL, and LDL cholesterol and apolipoprotein B in participants with the baseline metabolome of higher cardiometabolic risk. A significant effect was observed in IDL and HDL subfractions and free cholesterol in all participants. Previously, beneficial effects of sea buckthorn seed oil on HDL- and LDL-cholesterol concentrations, both in rabbits given a normal diet and in rabbits given a cholesterol-rich diet, have been reported (30). In a clinical study, an elevating effect on serum HDL cholesterol took place after an intervention of 4 mo with sea buckthorn pulp oil (7). No changes in circulating cholesterol or triglyceride concentrations were observed in a shorter intervention with combined sea buckthorn seed and pulp oils given to 12 participants (31). Several components of SBo may have contributed to the effect observed in our trial. The lowering of serum total cholesterol, LDL cholesterol, triglycerides, and other risk factors of cardiovascular disease have been associated with intakes of linoleic acid and α-linolenic acid (32), palmitoleic acid (33–35), and plant sterols (36, 37).

SBs are rich in flavonoids, particularly the glycosides of isorhamnetin (3′-methylquercetin) and quercetin flavonols (38, 39). Quercetin has been shown to induce a hypolipidemic effect in mice (40) and rabbits (41) and inhibit fatty acid and triglyceride synthesis in rat liver cells in vitro (42). Water extracts of sea buckthorn seeds, containing sea buckthorn flavonoids, induced a hypoglycemic and triglyceride-lowering effect in diabetic rats (15). A recent study indicated beneficial effects of ethanol-soluble fraction of SBs on the postprandial insulin and glucose response in humans (16). In our study, the intake of SBe+MD induced a rising trend in VLDL lipids and serum total triglycerides. The daily dose of SBe+MD contained 36 mg sea buckthorn flavonols, which was >6 times the average flavonol intake by Finns (43). Diets high in sugars, including fructose, and starches having a high glycemic index that may induce increases in fasting and postprandial serum triglycerides and VLDL concentrations in humans. The effect is modified by the dietary context, genetic background, physical activity, and other subject factors (44). The reasonably high carbohydrate content of the SBe+MD may have contributed to the VLDL elevating effect of the product.

In whole SBs, potentially all compounds in the soft parts of the berry, including flavonols, carotenoids, fatty acids, tocopherols, tocotrienos, and phytosterols of the pulp oil, were expected to affect the metabolic profile. Because seeds, for the most part, are not degraded in the digestive system if not crushed by teeth, the components of seed oil were not completely available in SBs. The increased intake of sugars and carbohydrates during SB periods may have interfered with the potential effects of the berries. SBs have a strong taste, and it is possible that sugar was added by participants to make the berries taste more pleasant. The effect of sugar as a potential confounder in treatment responses was removed by using a regression model.

Beneficial effects of whole bilberries on plasma lipids were observed in participants in the higher–metabolic risk baseline group B, whereas the opposite was shown in group A. The analysis of individual metabolites revealed an elevating trend in VLDL subclasses and serum triglycerides in participants who were in metabolic baseline group A. In participants in group B, clear reducing trends in VLDL, IDL, and LDL as well as serum triglycerides and cholesterol were observed.

There was no clear explanation for the observed opposite effects of bilberries on VLDL subgroups in metabolic baseline groups A and B. Results of earlier studies support the observation of beneficial effects of bilberry on metabolic risk factors. Anthocyanins are the main phenolic compounds in bilberries and blueberries (18, 45). Isolated bilberry anthocyanins have, in several studies, shown beneficial effects on cholesterol and triglyceride metabolism (46–49). Whole blueberries and bilberries have induced desired changes on markers of metabolic syndrome in clinical studies (18, 45). Previously, negative effects of bilberry and blueberry intakes on lipid metabolism have rarely been reported. Prior et al (49) showed the intake of whole blueberries fortified weight gain and increased body fat in epididymal fat in mice eating a high-fat diet.

The limitation of multiplicity in the analyses compared with the number of participants in our study was addressed by

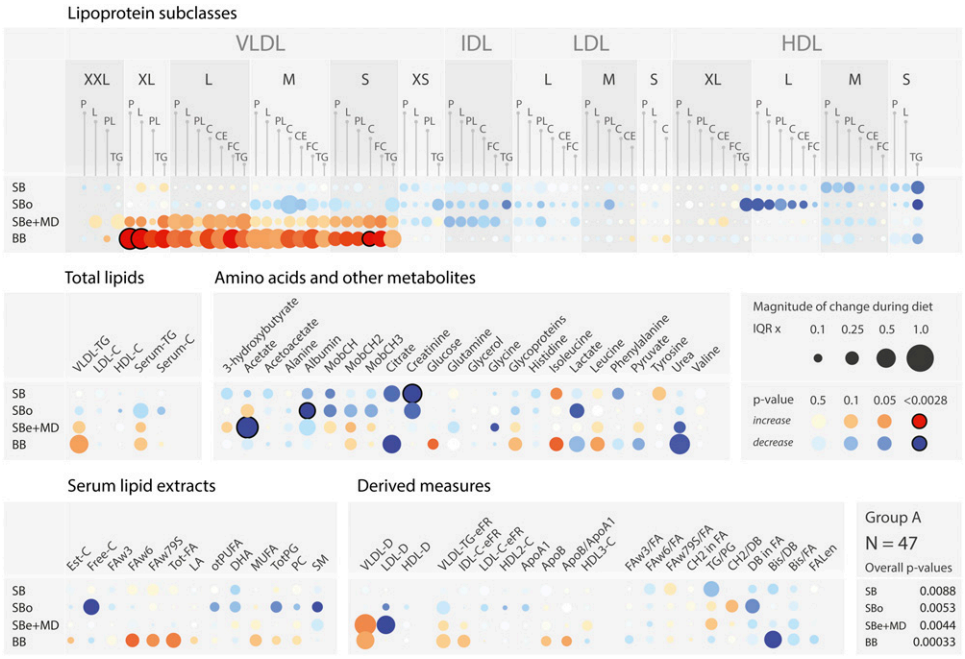

FIGURE 4. Wilcoxon's signed-rank test was used to compare concentrations of metabolite concentrations before and after the intervention in participants who had the baseline indicating lower cardiometabolic risk (baseline metabolic group A; $n = 47$). Red and blue dots indicate a detected increase and decrease, respectively, in the target variable. The size of the dot shows the magnitude of the change. Color saturation increases with the statistical significance. Significance at the 5% level (corrected for multiple testing by using a modified Bonferroni method) is indicated by a black border. Measures were adjusted for sugar intake. *See* Supplemental Table 2 under "Supplemental data" in the online issue for a table of exact results with *P* values. In the following categories, values that were estimated by using the extended Friedewald's method (50) are denoted with a superscript *1*. Amino acids and other metabolites (mmol/L): MobCH, double-bond protons of mobile lipids; MobCH2, CH_2 groups of mobile lipids; MobCH3, CH_3 groups of mobile lipids; derived measures: ApoA1 (g/L), apolipoprotein A-I[1]; ApoB (g/L), apolipoprotein B[1]; ApoB/ApoA1, apolipoprotein B by apolipoprotein A-I[1]; BIS/DB, ratio of bisallylic groups to double bonds; BIS/FA, ratio of bisallylic groups to total fatty acids; CH2/DB, average number of methylene groups per a double bond; CH2 in FA, average number of methylene groups in a fatty acid chain; DB in FA, average number of double bonds in a fatty acid chain; FALen, description of average fatty acid chain length and not the actual carbon number; FAw3/FA (%), ratio of n−3 fatty acids to total fatty acids; FAw6/FA (%), ratio of n−6 fatty acids to total fatty acids; FAw79S/FA (%), ratio of n−7, n−9, and SFAs to total fatty acids; HDL-D (nm), mean diameter for HDL particles; HDL2-C (mmol/L), total cholesterol in HDL_2[1]; HDL3-C (mmol/L), total cholesterol in HDL_3[1]; IDL-C-eFR (mmol/L), total cholesterol in IDL[1]; LDL-C-eFR (mmol/L), total cholesterol in LDL[1]; LDL-D (nm), mean diameter for LDL particles; TG/PG, ratio of triglycerides to phosphoglycerides; VLDL-D (nm), mean diameter for VLDL particles; VLDL-TG-eFR (mmol/L), triglycerides in VLDL[1]; lipoprotein subclasses (mmol/L, unless otherwise indicated): IDL-C, total cholesterol in IDL; IDL-FC, free cholesterol in IDL; IDL-L, total lipids in IDL; IDL-P (mol/L), concentration of IDL particles; IDL-PL, phospholipids in IDL; IDL-TG, triglycerides in IDL; L-HDL-C, total cholesterol in large HDL; L-HDL-CE, cholesterol esters in large HDL; L-HDL-FC, free cholesterol in large HDL; L-HDL-L, total lipids in large HDL; L-HDL-P (mol/L), concentration of large HDL particles; L-HDL-PL, phospholipids in large HDL; L-LDL-C, total cholesterol in large LDL; L-LDL-CE, cholesterol esters in large LDL; L-LDL-FC, free cholesterol in large LDL; L-LDL-L, total lipids in large LDL; L-LDL-P (mol/L), concentration of large LDL particles; L-LDL-PL, phospholipids in large LDL; L-VLDL-C, total cholesterol in large VLDL; L-VLDL-CE, cholesterol esters in large VLDL; L-VLDL-FC, free cholesterol in large VLDL; L-VLDL-L, total lipids in large VLDL; L-VLDL-P (mol/L), concentration of large VLDL particles; L-VLDL-PL, phospholipids in large VLDL; L-VLDL-TG, triglycerides in large VLDL; M-HDL-C, total cholesterol in medium HDL; M-HDL-CE, cholesterol esters in medium HDL; M-HDL-FC, free cholesterol in medium HDL; M-HDL-L, total lipids in medium HDL; M-HDL-P (mol/L), concentration of medium HDL particles; M-HDL-PL, phospholipids in medium HDL; M-LDL-C, total cholesterol in medium LDL; M-LDL-CE, cholesterol esters in medium LDL; M-LDL-L, total lipids in medium LDL; M-LDL-P (mol/L), concentration of medium LDL particles; M-LDL-PL, phospholipids in medium LDL; M-VLDL-C, total cholesterol in medium VLDL; M-VLDL-CE, cholesterol esters in medium VLDL; M-VLDL-FC, free cholesterol in medium VLDL; M-VLDL-L, total lipids in medium VLDL; M-VLDL-P (mol/L), concentration of medium VLDL particles; 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XL-VLDL-PL, phospholipids in very large VLDL; XL-VLDL-TG, triglycerides in very large VLDL; XS-VLDL-L, total lipids in very small VLDL; XS-VLDL-P (mol/L), concentration of very small VLDL particles; XS-VLDL-PL, phospholipids in very small VLDL; XS-VLDL-TG, triglycerides in very small VLDL; XXL-VLDL-L, total lipids in chylomicrons and extremely large VLDL; XXL-VLDL-P (mol/L), concentration of chylomicrons and extremely large VLDL particles; XXL-VLDL-PL, phospholipids in chylomicrons and extremely large VLDL; XXL-VLDL-TG, triglycerides in chylomicrons and extremely large VLDL; serum lipid extracts (mmol/L, unless otherwise indicated): Est-C, esterified cholesterol; FAw3, n−3 fatty acids; FAw6, n−6 fatty acids; FAw79S, n−7, n−9, and SFAs; Free-C, free cholesterol; LA, 18:2, linoleic acid; MUFA, monounsaturated fatty acids with 16 or 18 carbon atoms; otPUFA (the value not given as mmol/L because of several overlapping PUFAs that have different amounts of bisallylic CH_2-groups), PUFAs other than 18:2; PC, phosphatidylcholine and other cholines; SM, sphingomyelins; TotFA, total fatty acids; TotPG, total phosphoglycerides; total lipids (mmol/L): HDL-C, total cholesterol in HDL; LDL-C, total cholesterol in LDL; Serum-C, serum total cholesterol; Serum-TG, serum total triglycerides; VLDL-TG, triglycerides in VLDL. Abbreviations used: BB, bilberries; SB, sea buckthorn berries; SBe+MD, sea buckthorn phenolics ethanol extract mixed with maltodextrin (1:1); SBo, sea buckthorn oil; IQR, change from baseline to the end of intervention as numbers of IQRs.

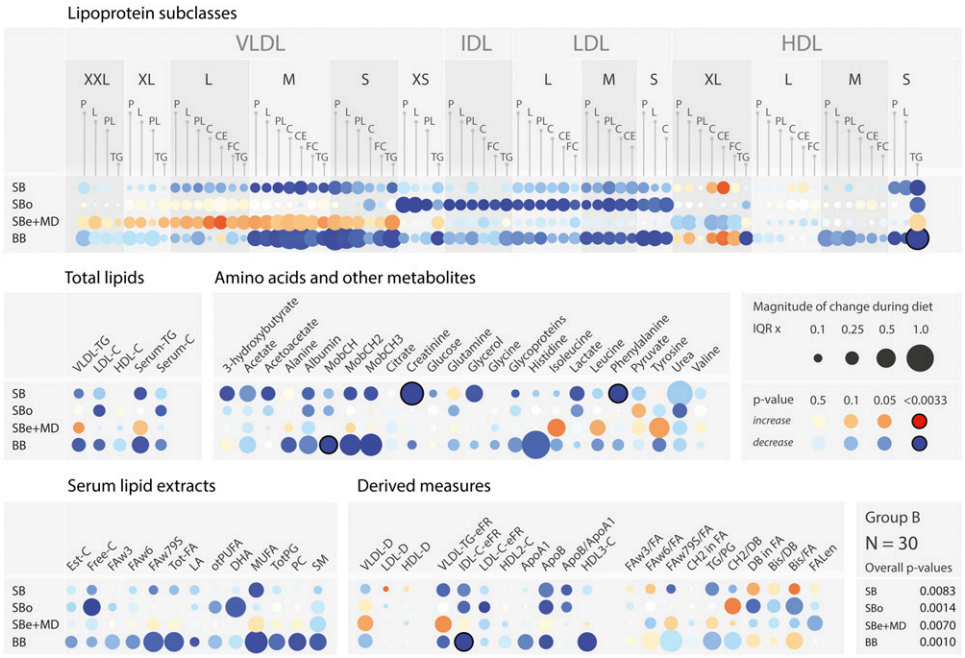

FIGURE 5. Wilcoxon's signed-rank test was used to compare concentrations of metabolite concentrations before and after the intervention in participants who had the baseline indicating higher cardiometabolic risk (baseline metabolic group B; $n = 30$). Red and blue dots indicate a detected increase and decrease, respectively, in the target variable. The size of the dot shows the magnitude of the change. Color saturation increases with the statistical significance. Significance at the 5% level (corrected for multiple testing by using a modified Bonferroni method) is indicated by a black border. Measures were adjusted for sugar intake. *See* Supplemental Table 2 under "Supplemental data" in the online issue for a table of exact results with *P* values. In the following categories, values that were estimated by using the extended Friedewald's method (50) are denoted with a superscript *1*. 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M-VLDL-PL, phospholipids in medium VLDL; M-VLDL-TG, triglycerides in medium VLDL; S-HDL-L, total lipids in small HDL; S-HDL-P (mol/L), concentration of small HDL particles; S-HDL-TG, triglycerides in small HDL; S-LDL-C, total cholesterol in small LDL; S-LDL-L, total lipids in small LDL; S-LDL-P (mol/L), concentration of small LDL particles; S-VLDL-C, total cholesterol in small VLDL; S-VLDL-FC, free cholesterol in small VLDL; S-VLDL-L, total lipids in small VLDL; S-VLDL-P (mol/L), concentration of small VLDL particles; S-VLDL-PL, phospholipids in small VLDL; S-VLDL-TG, triglycerides in small VLDL; XL-HDL-C, total cholesterol in very large HDL; XL-HDL-CE, cholesterol esters in very large HDL; XL-HDL-FC, free cholesterol in very large HDL; XL-HDL-L, total lipids in very large HDL; XL-HDL-P (mol/L), concentration of very large HDL particles; XL-HDL-PL, phospholipids in very large HDL; XL-HDL-TG, triglycerides in very large HDL; XL-VLDL-L, total lipids in very large VLDL; XL-VLDL-P (mol/L), concentration of very large VLDL particles; 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PC, phosphatidylcholine and other cholines; SM, sphingomyelins; TotFA, total fatty acids; TotPG, total phosphoglycerides; total lipids (mmol/L): HDL-C, total cholesterol in HDL; LDL-C, total cholesterol in LDL; Serum-C, serum total cholesterol; Serum-TG, serum total triglycerides; VLDL-TG, triglycerides in VLDL. Abbreviations used: BB, bilberries; SB, sea buckthorn berries; SBe+MD, sea buckthorn phenolics ethanol extract mixed with maltodextrin (1:1); SBo, sea buckthorn oil; IQR, change from baseline to the end of intervention as numbers of IQRs.

performing statistical tests on an overall metabolic measure summarizing metabolic changes associated with a given diet. Individual markers were analyzed to explore in more detail the metabolites that caused the observed overall effects. After correction for multiple testing, most of the changes that occurred in individual analytes were not statistically significant. Still, clear trends in changes of metabolites, which differed depending on the baseline metabolome, were seen. Another limitation in our study was the reasonably short duration of interventions, which, nevertheless, induced significant overall changes. The generalizability of our results is restricted by these limitations and because only women with slightly elevated concentrations of factors associated with risk of metabolic syndrome were enrolled. In general, changes in nutrient intakes during interventions were similar in metabolic groups A and B and were, therefore, unlikely to explain the observed differences in treatment responses between groups. The effect of sugar intake was taken into account in the statistical model used.

In conclusion, our study suggests significant effects of berry consumption on overall metabolic profiles of overweight women. The effects differ according to the berry or berry fraction and are more evident in individuals who have a baseline profile indicating higher cardiometabolic risk. Changes in individual metabolites in most cases were not statistically significant after correction for multiple testing but showed clear trends. Dried SBs induced beneficial effects on serum triglycerides and VLDL subclasses. SBo reduced the serum concentration of total and LDL cholesterol and apolipoprotein B. SBe+MD had an elevating effect on serum triglycerides and VLDL. The effects of bilberries were most clearly affected by the baseline. Desirable changes in serum lipids and lipoproteins were observed in the baseline group of higher cardiometabolic risk during the bilberry intervention, whereas the opposite was true for the group with the lower-risk baseline metabolome. To the best of our knowledge, the effect of the overall metabolic profile on the response to berry intake has not been reported previously. The diverse effects of berry fractions according to different baseline metabolome deserve additional studies in larger study populations to allow a more-detailed investigation of baseline-response associations.

We thank the volunteers who participated in the study.

The authors' responsibilities were as follows—PSL, H-ML, J-PS, BY, JV, and HPK: designed and conducted the study; PSL, AJK, and MA-K: analyzed and interpreted data; AJK, PS, and MA-K: designed and performed the NMR analysis; PSL and AJK: wrote the manuscript; and all authors: provided comments and read and approved the final manuscript. PSL is an employee of Aromtech Ltd. AJK, PS, H-ML, J-PS, BY, JV, MA-K, and HPK had no conflicts of interest.

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