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Whole Grain Products, Fish and Bilberries Alter Glucose and Lipid Metabolism in a Randomized, Controlled Trial: The Sysdimet Study

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1 **Abstract**

2 ***Background:*** Due to the growing prevalence of type 2 diabetes, new dietary solutions are needed to
3 help improve glucose and lipid metabolism in persons at high risk of developing the disease.
4 Herein we investigated the effects of low-insulin-response grain products, fatty fish, and berries on
5 glucose metabolism and plasma lipidomic profiles in persons with impaired glucose metabolism.

6 ***Methodology/Principal Findings:*** Altogether 106 men and women with impaired glucose
7 metabolism and with at least two other features of the metabolic syndrome were included in a 12-
8 week parallel dietary intervention. The participants were randomized into three diet intervention
9 groups: (1) whole grain and low postprandial insulin response grain products, fatty fish three times
10 a week, and bilberries three portions per day (HealthyDiet group), (2) Whole grain enriched diet
11 (WGED) group, which includes principally the same grain products as group (1), but with no
12 change in fish or berry consumption, and (3) refined wheat breads (Control). Oral glucose tolerance,
13 plasma fatty acids and lipidomic profiles were measured before and after the intervention. Self-
14 reported compliance with the diets was good and the body weight remained constant. Within the
15 HealthyDiet group two hour glucose concentration and area-under-the-curve for glucose decreased
16 and plasma proportion of (n-3) long-chain PUFAs increased (False Discovery Rate p-values <
17 0.05). Increases in eicosapentaenoic acid and docosahexaenoic acid associated curvilinearly with
18 the improved insulin secretion and glucose disposal. Among the 364 characterized lipids, 25
19 changed significantly in the HealthyDiet group, including multiple triglycerides incorporating the
20 long chain (n-3) PUFA.

21 ***Conclusions/Significance:*** The results suggest that the diet rich in whole grain and low insulin
22 response grain products, bilberries, and fatty fish improve glucose metabolism and alter the
23 lipidomic profile. Therefore, such a diet may have a beneficial effect in the efforts to prevent type 2
24 diabetes in high risk persons.

25 The study was registered to the ClinicalTrials.gov (Identifier: NCT00573781).

26 http://clinicaltrials.gov/ct2/results?term=NCT00573781

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27 **Introduction**

28 The beneficial health effects related to consumption of whole grain [1-3], fish or fish oil
29 supplements [4,5] and polyphenol rich foods such as berries [6] are well documented. However, the
30 synergistic effects of these foods on lipid and glucose metabolism in persons at risk for type 2
31 diabetes have not yet been investigated.

32 In epidemiological studies the intake of whole grain has been associated with lower risk of obesity,
33 insulin resistance, elevated fasting glucose and the incidence of diabetes [7]. Rye bread induces
34 post-prandially lower insulin response than wheat independently of the fiber content [8-10].
35 Additionally, a twelve-week consumption of low insulin response diet (rye bread and pasta) has
36 been shown to enhance early insulin secretion in persons with metabolic syndrome [2]. This diet
37 also modulated gene expression profile of abdominal subcutaneous tissue by down-regulating genes
38 involved in insulin signaling and apoptosis [11]. However, while the rye bread and pasta diet did
39 not alter the lipidomic profile of plasma, the high insulin response diet (oat-wheat bread and potato)
40 led to increased concentrations of proinflammatory lysophosphatidylcholines (LPCs) [12]. This
41 suggests that even a moderate dietary carbohydrate modification may affect the lipid metabolism.

42 Bilberries are particularly abundant in polyphenols, especially anthocyanins [13]. Growing evidence
43 from animal studies suggests that polyphenols as well as foods and beverages rich in polyphenols
44 may positively influence carbohydrate metabolism by attenuating postprandial glycemic responses
45 and fasting hyperglycemia as well as by improving acute insulin secretion and insulin sensitivity
46 [6]. Human intervention studies using berries or anthocyanin extracts have also demonstrated
47 significant improvements in low density lipoprotein (LDL) oxidation, lipid peroxidation, total
48 plasma antioxidant capacity and dyslipidemia [14].

49 Epidemiological studies have demonstrated associations between the (n-3) long-chain
50 polyunsaturated fatty acids (PUFA), found mainly in fish, and lower prevalence of insulin
51 resistance and type 2 diabetes [15]. However, clinical trials on (n-3) PUFA-enriched diets have so
52 far led to conflicting results [15-18]. The mechanisms behind the potential beneficial effect of (n-3)
53 PUFA on glucose metabolism are poorly understood. The evidence so far points to the role of
54 insulin receptor signaling [19], inflammation [20,21], cell membrane fatty acid composition [22,23],
55 circulating hormones and adipocytokines [18] or G protein-coupled receptor 120 [24]. By applying
56 a lipidomics approach we have recently shown that an eight-week consumption of fatty fish four to
57 five times per week led to decreased plasma concentrations of potential mediators of lipid-induced
58 insulin resistance and inflammation, including ceramides, diacylglycerols and LPCs [25]. Lipids are
59 known to play a central role in the progression of glucose metabolism towards diabetes [26]. The
60 emergence of lipidomics has enabled the global study of lipids in cells, tissues and biofluids, and
61 revitalized the study of lipids in the context of nutrition research and clinical biomarker discovery
62 [27].

63 Herein we investigate the effects of whole grain and low insulin response grain products, fatty fish,
64 and bilberries on glucose metabolism and plasma lipidomic profile in individuals with the impaired
65 fasting glucose (IFG) or impaired glucose tolerance (IGT) and features of metabolic syndrome. We
66 also aimed to study whether the increase in plasma eicosapentaenoic acid (EPA) and
67 docosahexaenoic acid (DHA) content is related to the improved glucose metabolism.

68 Materials and methods

69 The protocol for this trial and supporting CONSOT checklist are available as supporting
70 information; see Checklist S1 and Protocol S1.

71 **Participants and study design**

72 Participants volunteered to the study and gave their written informed consent. The study plan was
73 approved by the Research Ethics Committee, Hospital District of Northern Savo. The intervention
74 was performed in accordance of Helsinki Declaration.

75 Altogether 131 participants were recruited for a 12-week parallel controlled dietary intervention
76 study from Kuopio area (**Figure 1**). The inclusion criteria were age 40-70 years, impaired glucose
77 metabolism (fP-gluc 5.6-6.9 mmol/l (IFG) or in oral glucose tolerance test (OGTT) 2 hour P-gluc
78 7.8-11.0 mmol/l (IGT)) and at least two of the following: BMI 26-39 kg/m^2, waist circumference ≥
79 102 cm in men and ≥ 88 cm in women, serum triglycerides ≥ 1.7 mmol/l, HDL< 1.0 mmol/l in men
80 and < 1.3 mmol/l in women or blood pressure ≥ 130 / ≥ 85 mmHg or use of medication for
81 hypertension. The cut-off values were based on NCEP Adult Treatment Panel III, 2001. The
82 participants were enrolled in the study sequentially. Of all participants, 86 % had at least one
83 medication. In the HealthyDiet group, 5.4 % of the participants did not have any medication. The
84 respective proportions were 14.7 % and 22.9 % in the whole grain enriched diet (WGED) group and
85 Control group (χ^2-test, p = 0.1). Most commonly used medications were statins (27 %), angiotensin
86 II inhibitors (27 %), beta blockers (23 %), calcium antagonists (20 %), anticoagulants (17 %) and
87 ACE inhibitors (15 %). None of the subjects used glucose lowering drugs and uses of statins, beta
88 blockers or diuretics, and hormonal replacement therapy were very similar between the groups
89 (Table 1). Besides some minor exceptions, participants continued their medications unchanged
90 throughout the study.

91 The participants were randomly assigned by the study nurse to one of the following groups:
92 HealthyDiet, WGED or Control. Randomization was performed by matching according to gender
93 and medians of age, BMI and fasting plasma glucose of the study population at screening. The
94 matching produced equal amounts of the certain strata classes among the groups. Altogether 106
95 participants completed the study. Majority of the drop-outs did not state the reason for drop-out.

96 The reported reasons were busy at work, difficulty to follow the diet instructions, bad experience of
97 biopsy and other physical or personal reasons. Drop-outs were significantly younger than
98 completers (mean age was 55 ± 8 vs. 59 ± 7), but otherwise did not differ based on their clinical
99 characteristics.

100 In the HealthyDiet group (*n*=37) the participants replaced their habitually used cereal products with
101 breads having a low postprandial glucose and insulin response, contributing up to 20-25 % of total
102 energy intake (40 % share of endosperm rye bread, 10 % share of sourdough whole meal wheat
103 bread, and 50 % share of a selection of commercial rye breads). The recommended intake of whole
104 meal pasta was at least 3.5 deciliter (uncooked) a week. The fiber content (g/100g) of the study
105 cereal products was: endosperm rye bread 6.9, whole meal wheat bread 6.4, commercial rye breads
106 10-14.4 and pasta 6. Besides the above mentioned cereal products, one portion of their habitually
107 used cereal product was allowed to be eaten daily, e.g. porridge, cereals or pastries. Participants
108 were also instructed to eat fatty fish meals (100-150 g of fish /meal) three times a week. The
109 following fish species were recommended: salmon, rainbow trout, bream, Baltic herring, roach,
110 vendace, white fish, char, trout, red-fish, mackerel and anchovy. In fish preparation, participants
111 were advised to avoid sources of saturated fat, such as butter and cream. Bilberries were eaten 3
112 portions per day. Bilberries were served as frozen, puree and dried powder. The total amount of
113 three portions is equivalent to 300 grams of fresh bilberries. Habitual use of other berries was
114 allowed with a max 3-4 portion a week.

115 In the WGED group (*n*= 34), the participants consumed the same cereal products as in the
116 HealthyDiet group. Additionally, they were given whole grain oat biscuits that they were allowed to
117 consume one portion per day on a voluntary basis. Biscuits contained 8-8.5g/100g of dietary fiber
118 and 16-18g/100g of fat, of which 4.3-7.7g was saturated. Participants in the WGED group were
119 asked not to change their fish and berry consumption.

120 In the Control group (*n*=35) participants replaced their habitually used breads with refined wheat
121 breads (dietary fiber 3-4.3 g/100g) and other cereal products, *e.g*. porridge or pasta, with low fiber
122 products (<6 g/100g dietary fiber). Participants were allowed to eat maximum of 1-2 portions of rye
123 products per day. The intake of bilberries was not allowed and other berries were allowed maximum
124 of 3-4 times per week with maximum of 1 deciliter at a time. Fish was allowed to be eaten no more
125 than once a week. Otherwise, the habitual diet and living habits were kept unchanged in all groups.

126 Participants recorded daily the use of the test breads (all groups), pasta (HealthyDiet and WGED),
127 oat biscuits (WGED), bilberries (HealthyDiet), and fish (HealthyDiet). Four-day food records
128 (consecutive days) that included one weekend day were kept by the study persons during the run-in
129 period and three times during the intervention period in all groups.

130 **Two-hour oral-glucose-tolerance test**

131 OGTT was performed at baseline and after the 12-week intervention. After the fasting blood sample
132 was drawn, the participants drank a glucose solution (75 g glucose / 3 dl) within 3 min. Blood
133 samples were drawn through the catheter 30, 60, 120 min after the beginning of the glucose solute
134 ingestion for the measurement of plasma glucose and insulin concentrations. The glucose and
135 insulin areas under the curve (AUCs) were calculated.

136 Disposition index (DI) is a product of measures of insulin sensitivity and first-phase insulin
137 secretion which is predictive of conversion to diabetes [28,29]. Insulinogenic index (IGI) and
138 quantitative insulin sensitivity check index (QUICKI) were calculated as following: IGI= (insulin
139 30 min - insulin 0 min, pmol/L) / (glucose 30 min – glucose 0 min, mmol/L), and QUICKI= 1 /
140 (lg10(insulin 0 min, mU/L) + lg10(glucose 0 min, mg/dl)). Disposition index was calculated as a
141 product of IGI and QUICKI. Homeostasis model of insulin resistance (HOMA-IR) was calculated
142 as following: (fasting glucose mmol/l x fasting insulin mU/L) / 22.5.

143 **Biochemical analyses**

144 Concentrations of serum total, LDL and HDL cholesterol and triglycerides were analyzed using
145 commercial kits (981813, 981656, 981823 and 981786, respectively, Thermo Electron Corporation,
146 Vantaa, Finland) and Thermo Fisher Konelab 20XTi Analyzer (Thermo Electron Corporation,
147 Vantaa, Finland). Plasma insulin concentration was analyzed with a chemiluminescent
148 immunoassay (Advia Centaur Immunoassay System, Siemens Medical Solution
149 Diagnostics,Tarrytown, NY, USA). Plasma glucose was analyzed by using Konelab 20XTi Clinical
150 Chemistry Analyzer and Enzymatic photometric (glucose hexokinase) method (Konelab System
151 Reagents, Thermo Fisher Scientific, Vantaa, Finland).

152 **Fatty acids**

153 Fasting blood samples for fatty acids and lipidomics analyses were taken before and after the
154 intervention. Both samples were available from 105 participants (HealthyDiet group *n*=37, WGED
155 group *n*=34, Control group *n*=34). Lipids from plasma samples (50 µl) were extracted by a mixture
156 of chloroform and methanol (2:1, 400 µl) after addition of 40 µl NaCl 0.9 % (0.15M) and 10 µl
157 internal standard (heptadecantrienoate TG C17:0 1659.24 mg/l + FFA C17:0 584.1 mg/l). The
158 mixture was vortexed 1 min and incubated 30 min at room temperature. The lower layer was
159 separated (centrifuged 10 000 rpm 5 min) and evaporated into dryness under nitrogen flow.

160 The residue was dissolved into petroleumether (700 µl, boiling point 40-60° C) and vortexed.
161 Bound fatty acids were transmethylated with NaOME (250 µl, sodium methoxide in dry methanol
162 0.5 M) by vortexing and boiling at 45° C for 5 minutes. The mixture was acidified by adding 15 %
163 solution of $NaHSO_4$ (500 µl) and evaporated petroleumether was substituted by adding 200 µl of it
164 (vortexed). The mixture was transferred into microtubes (glass tubes washed with 200 µl of
165 petroleum ether) and centrifuged 10 000 rpm, 5 min. The petroleumether layer containing FAME
166 and FFA was separated into a GC vial, evaporated under nitrogen flow and redissolved into hexane
167 (500 µl) and vortexed.

168 1 µl aliquots were used for GC injection at 260 ° C (splitless). The Agilent 7890 Gas
169 Chromatography equipped with HP-FFAP Polyethylene Glycol TP column (25 m x 200 µm x 0.3
170 µm) was used. H_2 was used as carrier gas at a total flow of 44.5 ml/min. The initial oven
171 temperature was 70 ° C for 1.5 min and the temperature was increased at rate of 15° C/min until
172 240° C. The fatty acids were detected by flame ionization detector at 300 ° C.

173 **Lipidomics**

174 Plasma samples (10 µl) were diluted with 10 µl sodium chloride (0,9%) and 20 µl of an internal
175 standard mixture containing 10 lipid classes and 100 µl chloroform/ methanol (2:1) were added.
176 The mixture was homogenised by vortexing 2 minutes and the extraction time was around 40
177 minutes at room temperature. After centrifugation (10000 RPM, 3 minutes), an exact aliquot (60 µl)
178 of the lower layer was transferred into an HPLC vial insert and 10 µl an external standard mixture
179 containing 3 labelled standards was added to the lipid extract. The sample order for LC/MS analysis
180 was randomised.

181 Lipid extracts were analyzed on a Waters Q-Tof Premier mass spectrometer combined with ultra
182 performance liquid chromatography (UPLC). The column was an Acquity UPLC™ BEH C18
183 2.1×100 mm with 1.7 µm particles. Column temperature was kept at 50 °C and the temperature of
184 the sample organizer was set at 10 °C. The binary solvent system consisted of A. water (1% 1M
185 NH_4Ac, 0.1% HCOOH) and B. LC-MS grade isopropanol:acetonitrile (1:1, 1% 1M NH_4Ac, 0.1%
186 HCOOH). The gradient started from 65% A / 35% B and reached 80% B in 2 min, 100% B in 7
187 minutes and remained at this level for next 7 minutes. The total run time including a 4 min re-
188 equilibration step was 18 min. The flow rate was 400 µl/min and the injection volume 2.0 µl.

189 The data was collected in centroid form by using electro spray ionization + mode at mass range of
190 m/z 300-1200 and with scan duration of 0.2 sec. The voltages of the sampling cone and capillary
191 were 40.0 V and 3.0 kV, respectively. The source temperature was set at 120 °C and nitrogen was

192 used as desolvation gas (795 L/h) at 270 °C. Reserpine (200 µg/L) was the lock spray reference
193 compound at a flow rate of 8 µl/min and the scan was done at 10 s frequency.

194 The data was processed by using MZmine 2 software [30] and the lipid identification was based on
195 an internal spectral library. The MZmine 2 data processing included chromatogram building, peak
196 deconvolution, deisotoping, alignment, filtering and gap filling. The quality of data was first
197 estimated by checking the shifts in retention times and peaks in the blank control samples. There
198 were the maximum of 0.02 minute shifts in the retention times of standard compounds and only a
199 few peaks were documented in the blanks.

200 Lipids were identified by comparing with an internal spectral library including MS and MS/MS
201 data. All monoacyl lipids except cholesteryl esters, such as monoacylglycerols and monoacyl-
202 glycerophospholipids, were normalized with PC(17:0/0:0), all diacyl lipids except ethanolamine
203 phospholipids with PC(17:0/17:0), all ceramides with Cer(d18:1/17:0), all ethanolamine
204 phospholipids with PE(17:0/17:0), and all TG and cholesteryl esters with TG(17:0/17:0/17:0).
205 Unidentified lipids were calibrated with PC(17:0/0:0) for retention times less than 300 seconds,
206 with PC(17:0/17:0) for retention times between 300 and 410 seconds, and with
207 TG(17:0/17:0/17:0) for higher retention times. Identifications and exact fatty acid compositions
208 of all of the most interesting lipids were confirmed with MS/MS analyses.

209 **Statistical analyses**

210 The primary aim of this study was to see changes in glucose metabolism. Power calculations were
211 based on fasting glucose. By using test significance α-level 0.05, 80 % power and aim to see 5 %
212 difference between the groups the appropriate sample size was 37 / group. Statistical analyses were
213 performed using the SPSS statistical software (version 14.0, SPSS Inc., Chicago, IL) and R Project
214 for Statistical Computing version 2.7.2 [31] and nlme R-package version 3.1-96 [32]. The normality
215 of the distributions of the variables was estimated based on histograms. Linear mixed-effect models

216 were used to analyze group differences in the baseline and during the intervention. Quantitative
217 dependent variables were transformed to base-10 logarithmic scale to account for non-normal
218 distributions. Selected confounding phenotypes were included in the models as covariates (age,
219 gender, BMI, insulin and glucose), and participant's identifiers were included as a grouping random
220 effect. Interaction term between group and intervention time-point (before or after intervention) was
221 used to examine group related changes during the intervention. The Control group was used as a
222 reference group when comparing group differences. Benjamini-Hochberg false discovery rate
223 (FDR) was used to adjust results for multiple comparisons [33]. FDR p-value < 0.05 was considered
224 as statistically significant. The association of EPA and DHA with DI was tested using Spearman
225 rank correlation and linear regression model (age, gender and weight changes were included in the
226 model). The results from regression model need to be discussed with reservation, since the
227 distributions of the variables were not normal. Kruskal-Wallis test was performed in order to test
228 differences in changes in DI between different EPA and DHA quartiles.

229 Results

230 Clinical characteristics and dietary intake

231 There were no statistically significant differences between the groups at baseline (**Table 1**). The
232 results of the basic clinical and biochemical parameters (apart from glucose and insulin) during the
233 intervention periods will be reported elsewhere. Body weight remained constant during the study in
234 all groups.

235 Self-reported compliance with the diets was good. The mean test bread consumption during the
236 intervention period was 7.7, 7.9 and 6.8 portions per day in the HealthyDiet, WGED and Control
237 groups, respectively. In the HealthyDiet group, the mean fish consumption was 3.3 fish meals per
238 week. Mostly used fish species were salmon, rainbow trout, vendace and Baltic herring. The mean
239 bilberry consumption in the HealthyDiet group was 3.2 portions per day.

240 At the baseline, the intake of dietary fiber was higher in the HealthyDiet group than in the Control
241 and WGED groups (FDR *p*=0.013). Otherwise, dietary intake did not differ at baseline among the
242 groups. Energy intake did not change during the intervention. The intake of EPA, DHA, α-linolenic
243 acid and fiber increased in the HealthyDiet group compared with the Control group (**Figure 2**). In
244 the WGED group, the intake of total fat decreased and fiber increased compared with the Control
245 group. Within the Control group the intake of PUFA, EPA, DHA and fiber decreased during the
246 intervention.

247 **Glucose and insulin metabolism**

248 There were no statistical differences in changes of glucose and insulin metabolism between the
249 groups (**Table 2**). In the HealthyDiet group, the within-the-group comparison revealed decreases in
250 the 2-hour glucose concentration and AUC for glucose (Table 2, **Figure 3**). There was a trend
251 towards improved IGI (*p*=0.016 and FDR *p*=0.076) and DI (*p*=0.019 and FDR *p*=0.076) within the
252 HealthyDiet group (Table 2, Figure 3).

253 **Fatty acids and lipidomics**

254 There were no differences in plasma fatty acids between the groups at baseline. Proportions of di-
255 homo-γ-linolenic acid decreased and polyunsaturated long chain fatty acids (docosapentaenoic acid,
256 EPA, DHA) increased in the HealthyDiet group compared with the Controls (**Table 3**). Within the
257 Control group, DHA decreased during the intervention. There were no significant changes in
258 plasma fatty acid composition in the WGED group.

259 A total of 364 lipids were identified and quantified by the UPLC-MS platform including lipid
260 classes such as TGs, LPCs, phophatidylcholines (PCs), phosphatidylserines (PSs), phosphatidyl
261 ethanolamines (PEs), sphingomyelins (SMs) and ceramides. There were no significant differences
262 among the groups at baseline. Using the within-the-group comparisons, 152, 91 and 178 lipids
263 changed significantly (FDR $p < 0.05$) in the HealthyDiet, WGED and Control groups, respectively.

264 Mixed model analysis revealed 25 significantly changed lipids when comparing the HealthyDiet
265 and the WGED groups to the Control group (**Table 4**). All the significant changes occurred in the
266 HealthyDiet group. Multiple TGs with the long chain PUFAs, including TG(60:12), TG(60:13),
267 TG(58:11), TG(56:10), increased in the HealthyDiet group. Also LPC(20:5), PC(36:5), PC(38:7e),
268 PC(40:7e) and TG(55:7) increased, while the PS(38:2) and LPC(20:3) decreased. LPC(22:6)
269 increased within the HealthyDiet group (FDR p=0.005), but in a comparison of all three groups
270 together this increase did not reach the level of statistical significance (FDR p=0.088). Odd chain
271 TGs, except TG(55:7), or ceramides did not change during the intervention.

272 **Association between plasma EPA and DHA and glucose metabolism**

273 Regression model revealed significant association between the changes in plasma EPA and DHA
274 with changes in IGI (R=0.366, p=0.009 and R=0.379, p=0.006) and DI (R=0.366, p=0.009 and
275 R=0.382, p=0.005, respectively). The inclusion of changes in the fiber intake in the regression
276 model did not alter associations. There were also positive correlations between the changes in EPA
277 and DHA and changes in IGI (r=0.309, p=0.002 and r=0.413, p<0.001, respectively) and DI
278 (r=0.313, p=0.002 and r=0.341, p=0.001, respectively). IGI and DI improved most in the highest
279 quartiles of changes in EPA and DHA during the 12-week intervention (**Figure 4**).

280 Discussion

281 We studied the effects of whole grain and low insulin response grain products, fatty fish, and
282 bilberries on glucose metabolism, plasma fatty acids and lipidomic profile in individuals with
283 features of the metabolic syndrome. We found that diet with high intake of whole grain and low
284 insulin response grain products, fatty fish and bilberries (HealthyDiet) appeared to improve glucose
285 metabolism and altered plasma lipidomic profile markedly, while exclusive carbohydrate
286 modification caused only minor changes. Interestingly, we identified an association between the
287 increases in plasma EPA and DHA contents and improvement in glucose metabolism.

288 A decreased AUC for glucose and a trend towards improved IGI and DI in the HealthyDiet group
289 suggest that the intake of whole grain and low insulin response grain products, fatty fish and
290 bilberries had positive effects on insulin secretion and glucose disposal in this study. There was also
291 a trend towards improved 2-hour glucose, but not for IGI and DI, in the WGED group. The results
292 for IGI and DI were very similar, which is not surprising as DI is calculated from IGI. In the entire
293 study group, a higher increase in EPA and DHA was curvilinearly associated with IGI and DI and
294 thus to improved glucose metabolism. Besides fatty fish, bilberries may also have had a role in the
295 improved glucose metabolism, but we do not have a biomarker for bilberry intake which would
296 have enabled better estimation of its effects. However, not all subjects in the 4th quartile of EPA and
297 DHA changes were in the HealthyDiet group, which suggests that changes in the EPA and DHA
298 may have an independent association with the glucose metabolism. Recently, Stull and colleagues
299 [34] showed that insulin sensitivity was enhanced after 6 wk dietary supplementation with bioactive
300 substances from bilberries in 32 obese, insulin-resistant persons.

301 Results from the intervention studies regarding the effects of (n-3) PUFA of animal origin on
302 glucose metabolism (mostly insulin resistance) have been variable and most of the studies have
303 been performed with fish oil supplements [18]. There are multiple discrepancies between the (n-3)
304 PUFA intervention studies relating to e.g. methods they have used to assess glucose metabolism,
305 health status of participants, diet or duration of the study [18]. Improvements in insulin resistance
306 have not been observed in healthy individuals or diabetic persons, but the positive effects have been
307 identified in obese individuals [16,18,35]. Our study population could have been an optimal target
308 group for the application of dietary strategy to improve glucose metabolism, since the participants
309 had IFG or IGT, but not diabetes. However, the diet rich in whole grain and low insulin response
310 grain products, bilberries and fatty fish did not alter insulin resistance but instead improved insulin
311 secretion in this study. Associations between the changes in plasma EPA and DHA content and
312 improved IGI and DI were curvilinear (Figure 4), meaning that the improved insulin secretion and

313 glucose disposal existed only in the highest quartiles of changes in EPA and DHA. This may partly
314 explain the discrepancy on the evidence related to (n-3) PUFA of animal origin and glucose
315 metabolism. Future studies are needed to confirm these results and to find the optimal amount of
316 fish needed to achieve improvement in insulin metabolism in subjects with IFG or IGT.

317 High-carbohydrate-low-GI diet has been shown to improve DI in persons with impaired glucose
318 tolerance [2,36] or type 2 diabetes [37]. In two studies the duration of the low-GI diet has been 4 to
319 12 months until the increase in DI has became evident [36,37]. However, Laaksonen and colleagues
320 detected improved DI after 12-week consumption of carbohydrate modified diet with cereal
321 products replaced with rye-based products and whole-grain pasta [2]. It is unlikely, but still
322 possible, that our intervention period of 12-weeks was too short to observe the improvement in DI
323 in the WGED group, while the effects of (n-3) PUFA appeared earlier. In addition, the improvement
324 in DI in the HealthyDiet group might have reached the level of statistical significance after the
325 longer intervention period. Moreover, there were slight differences in the cereal diets between
326 WGED group and in the study of Laaksonen *et al* [2] that might have had an impact on the
327 differences found.

328 Fatty acid analyses revealed increases in the plasma content of long chain (n-3) PUFAs in the
329 HealthyDiet group, whereas in the Control group plasma content of DHA decreased without a
330 change in the WGED group. Furthermore, lipidomics analysis revealed increases in TGs with long
331 chain (n-3) PUFAs in the HealthyDiet group. These changes confirm the good compliance with the
332 instructions of fish intake. Interestingly, we detected increases in highly unsaturated TGs in the
333 HealthyDiet group (*e.g*. TG(60:12) and TG(60:13)), which we have not identified in our previous
334 studies.

335 We observed increased plasma concentrations of LPC species in the HealthyDiet group. LPCs are
336 linked with atherosclerosis by virtue of their effects on arterial wall, smooth muscle cells and

337 macrophages, although also other putative antiatherogenic effects have been demonstrated [38].
338 LPC is a major component of oxidized LDL [38,39]. In high density lipoprotein (HDL) fraction, it
339 is more abundant in persons with high HDL cholesterol [40]. The acyl chains in LPC and
340 lysophosphatidicacid (LPA) molecules vary from saturated to highly unsaturated, and from 14 to 22
341 carbons, and their biological functions may vary depending on the acyl chain [38,41,42]. Block and
342 colleagues [42] demonstrated that dietary EPA and DHA fatty acids are incorporated into plasma
343 LPCs, but not into LPAs. Here we found increase in LPC(20:5) after a 12-week consumption of
344 fatty fish. LPC(22:6) also increased within the HealthyDiet group, but among the groups this
345 increase did not reach the level of statistical significance. The ability to increase LPC(20:5) and
346 LPC(22:6) with dietary ingestion of fatty fish may be important, since LPC are believed to be major
347 carriers of DHA to the brain [43].

348 We found an increase of odd chain triglyceride TG(55:7) in the HealthyDiet group. Other odd chain
349 TG:s did not change. Odd chain fatty acids 15:0 and 17:0 have been considered as a biomarker of
350 dairy fat intake, and thus even hypothesized to have protective effects against heart disease, stroke
351 and insulin resistance [44-47]. These observations have been based on plasma and adipose tissue
352 levels of 15:0 and 17:0 rather than dietary recording methods. Contradictory, association between
353 the intake of dairy fat and relative serum content of 17:0 have not been clear in every study [48] and
354 also inverse associations have been observed [49]. Furthermore, in the large cohort study EPIC,
355 there was a strong positive correlation ($r=0.8$, $p\leq0.01$) between the total intake of fish and plasma
356 content of 17:0 [50]. In our study, plasma 15:0 did not change during the intervention, and 17:0
357 was used as an internal standard in the fatty acid analyses, so we could not determine its plasma
358 levels. However, TGs with the odd number of carbons most likely include 15:0 and17:0 fatty acids.
359 Based on 4-day food diaries dietary intake of SAFA decreased from 12 E% to 10.7 E% within the
360 HealthyDiet group. This indicates compliance to the instruction to avoid cream and butter in fish
361 preparation. Decreased dairy fat intake should also have been shown in a decrease of odd chain

362 fatty acids, if those could be considered as good biomarkers for dairy fat intake. Fatty acid 17:0 is
363 present in the fat of fish in low amounts (0.31-2.0 % depending on fish species) [51,52]. Salmon
364 contains around 40 mg of 17:0 and 20 mg of 15:0 fatty acids per 100 g of fish [53]. Our results
365 suggest that intake of odd chain fatty acids from dairy fat decreased, but instead the same amount of
366 it came from fatty fish, keeping the plasma concentration constant. Therefore, we believe that it is
367 questionable to consider odd chain fatty acids as biomarkers of dairy fat intake and the associated
368 health effects might as well be related to high intake of fish. Further studies are needed to address
369 this issue.

370 In the WGED group, we did not find significant changes in lipids in comparison with the Control
371 group. This is consistent with our earlier findings, *i.e.,* the intake of low insulin response grain
372 products (rye bread and pasta) [12] or the intake of high-fiber rye bread [54] did not lead to changes
373 in serum lipidomic profiles. Changes in the HealthyDiet group are different than those which we
374 found in our previous study, where the study persons ate at least 4 fatty or lean fish meals per week
375 [25]. In that study, unlike in the present one, we found a decrease in plasma ceramides and LPCs as
376 a result of fatty fish diet. However, participants in that study were coronary heart disease patients
377 with multiple medications, which may have affected the results. This discrepancy also suggests that
378 even if carbohydrate modification itself does not markedly change serum lipidomic profile, it has
379 effects with bilberries and fatty fish, which cause changes in lipidomic profile. On the other hand, it
380 is possible that the intake of three fish meals per week was not enough to see all the effects, which
381 were detectable after four fish meals per week. Furthermore, the power calculations were based on
382 fasting glucose, and it is possible that there was not enough power to see all changes in lipidomics
383 or other glucose parameters. To see also the individual effects of fish or bilberries, and also
384 synergistic effects of grain products, fish and bilberries, separate intervention groups with increased
385 intake of only fish or only bilberries would have been needed.

386 In conclusion, our results suggest that diet rich in whole grain and low insulin response grain
387 products, bilberries, and fatty fish alter plasma lipidomic profiles and may be associated with
388 improved glucose metabolism. Therefore, in long-term these dietary components may have a
389 beneficial role in the prevention of type 2 diabetes in persons with impaired glucose metabolism.

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Figure legends

Figure 1. Flow diagram of the study.

Figure 2. Changes in dietary intake during the 12-week intervention in the HealthyDiet (n=36), Whole grain enriched diet (WGED) (n=34) and Control (n=35) groups. Bars represent mean of absolute changes ± SD. Means without a common letter differ in mixed model comparison, FDR $p < 0.05$.

Figure 3. Bar charts of glucose metabolism parameters in each group before and after the intervention. A) plasma 2 hour glucose, B) area under the curve (AUC) for glucose, C) Insulinogenic index (IGI) and D) Disposition index (DI) in OGTT. Values are means ± SEM, *n*=37, 34 and 35 in the HealthyDiet, Whole grain enriched diet (WGED) and Control groups, respectively, except for IGI and DI, *n*=36 before and *n*=35 after the intervention in the HealthyDiet group, and *n*=32 before and after the intervention in the WGED group.

Figure 4. Associations between glucose metabolism and plasma EPA and DHA content. Changes in insulinogenic index (IGI) (A and B) and disposition index (DI) (C and D) during the intervention period (95% Confidence interval) according to quartiles of changes in plasma EPA (A and C) and DHA (B and D) content (%). * $p< 0.01$ in Kruskal-Wallis test. All groups combined, *n*=99. Ranges of changes in EPA quartiles: 1=-2.3--0.4 % units; 2=-0.3-0.1 % units; 3=0.1-0.6 % units; 4=0.6-4.2 % units, and in DHA quartiles: 1=-2.4--0.6 % units; 2=-0.6--0.1 % units; 3=-0.04-0.7 % units; 4=0.7-3.0 % units.

Table 1. Baseline characteristics of the participants [1,2]

	HealthyDiet *n*=**37**	**WGED**[3] *n*=**34**	**Control** *n*=**35**
Gender, ***male/female***	17/20	17/17	18/17
Age, ***years***	58 ± 7	58 ± 8	59 ± 7
Body weight, ***kg***	89.8 ± 12.2	89.2 ± 15.3	89.7 ± 13.0
Body mass index, ***kg/m²***	31.1 ± 3.6	31.4 ± 3.4	31.0 ± 3.6
Waist circumference, ***cm***	105.6 ± 9.5	106.3 ± 11.1	105.8 ± 10.0
Serum cholesterol, ***mmol/l***	5.1 ± 0.9	5.1 ±1.0	5.4 ± 1.0
LDL cholesterol, ***mmol/l***	3.1 ± 0.7	3.2 ±0.8	3.4 ± 0.8
HDL cholesterol, ***mmol/l***	1.3 ± 0.3	1.2 ±0.4	1.3 ± 0.3
Serum triacylglycerols, ***mmol/l***	1.6 ± 0.6	1.5 ± 0.8	1.5 ± 0.8
Fasting plasma glucose, ***mmol/l***	6.1 ± 0.5	6.1 ±0.4	6.2 ± 0.5
Plasma glucose OGTT 2 hour, ***mmol/l***	6.7 ± 1.7	6.6 ± 1.6	6.8 ± 1.9
Systolic blood pressure, ***mmHg***	137 ± 13	135 ±16	139 ± 12
Diastolic blood pressure, ***mmHg***	89 ± 7	86 ± 8	88 ± 7
How many of the MetS characteristics fulfilled:[4]			
2 characteristics, ***%***	11	15	14
3 **characteristics,** ***%***	43	38	43
4 **characteristics,** ***%***	24	29	29
5 characteristics, ***%***	22	18	14
Use of statins, ***n***	10	10	9
Use of hormonal replacement therapy, ***n***	3	4	3
Use of beta blocker or diuretics, ***n***	11	12	9

[1]Values are mean ± SD, [2]There were no significant differences in clinical characteristics between the groups at baseline, [3]Whole grain enriched diet, [4]On top of the impaired fasting glucose or glucose tolerance.

Table 2. Glucose and insulin parameters at baseline and after the 12-week intervention [1]

	HealthyDiet *n*=37				**WGED[2] *n*=34**				**Control *n*=35**		
	0 wk	**12 wk**	**FDR *p* group[3]**	**FDR *p* time* group[4]**	**0 wk**	**12 wk**	**FDR *p* group[3]**	**FDR *p* time* group[4]**	**0 wk**	**12 wk**	**FDR *p* group[3]**
Fasting glucose (mmol/L)	6.1 ± 0.5	6.0 ± 0.5	0.71	0.91	6.1 ± 0.4	6.1 ± 0.5	0.99	0.90	6.2 ± 0.5	6.2 ± 0.5	0.81
Glucose 2 h (mmol/L)	6.7 ± 1.7	6.0 ± 0.5	**0.027**	0.53	6.6 ± 1.6	6.1 ± 1.9	0.058	0.68	6.9 ± 1.9	6.7 ± 2.2	0.81
Fasting insulin (mU/L)	11.7 ± 5.9	12.5 ± 6.3	0.37	0.91	12.0 ± 6.2	13.7 ± 8.0	0.16	0.73	12.8 ± 6.6	13.2 ± 6.3	0.81
Insulin 2 h (mU/L)	62.6 ± 48.2	61.3 ± 46.7	0.69	0.91	78.3 ± 65.6	65.9 ± 59.9	0.16	0.67	72.1 ± 54.2	72.3 ± 55.7	0.95
AUC[5] for glucose (mmol/L)	241 ± 130	190 ± 121	**0.027**	0.49	233 ± 128	204 ± 137	0.16	0.53	246 ± 136	246 ± 137	0.84
AUC[4] for insulin (mU/L)	6787 ± 3279	7509 ± 3772	0.64	0.91	7650 ± 6004	7523 ± 4740	0.57	0.68	7766 ± 4173	7514 ± 3861	0.89
HOMA-IR[6]	3.2 ± 1.8	3.4 ± 1.9	0.43	0.91	3.3 ± 1.7	3.7 ± 2.3	0.16	0.73	3.6 ± 2.0	3.7 ± 1.9	0.89
IGI[7]	143 ± 99[9]	212 ± 251[10]	0.076	0.49	160 ±115[11]	169 ± 113[11]	0.99	0.91	157 ± 97	149 ± 89	0.89
QUICKY[8]	0.33 ± 0.02	0.33 ± 0.02	0.37	0.91	0.33 ± 0.02	0.32 ± 0.03	0.16	0.90	0.32 ± 0.02	0.32 ± 0.02	0.81
Disposition index	46.3 ± 30.4[9]	67.8 ± 78.8[10]	0.08	0.49	51.5 ± 35.2[11]	53.7 ± 34.3[11]	0.99	0.91	50.0 ± 28.2	47.6 ± 27.2	0.82

[1]Values are mean ± SD,[2]Whole grain enriched diet, [3]False discovery rate p-value for effect of time within the groups, [4]False discovery rate p-value for interaction of time and group (time*group), [5]Area under the curve in 2-hour OGTT, [6]Homeostasis model of insulin resistance, [7] Insulinogenic index, [8]Quantitative insulin sensitivity check index, [9]*n*=36, [10]*n*=35, [11]*n*=32

Table 3. Plasma fatty acids at baseline and after the 12-week intervention [1]

	HealthyDiet *n*=37				**WGED[2] *n*=34**				**Control *n*=34**		
Fatty acid, %	**0 wk**	**12 wk**	**FDR *p* group[3]**	**FDR *p* time* group[4]**	**0 wk**	**12 wk**	**FDR *p* group[3]**	**FDR *p* time* group[4]**	**0 wk**	**12 wk**	**FDR *p* group[3]**
Myristic (14:0)	1.0 ± 0.47	1.09 ± 0.41	0.18	0.96	0.82 ± 0.43	0.93 ± 0.49	0.23	0.82	0.79 ± 0.41	0.92 ± 0.42	0.22
Myristoleic (14:1(n-5))	0.10 ± 0.04	0.10 ± 0.05	0.89	0.92	0.10 ± 0.05	0.10 ± 0.05	0.64	0.96	0.10 ± 0.05	0.10 ± 0.04	0.83
Pentadecanoic (15:0)	0.21 ± 0.05	0.21 ± 0.06	0.86	0.92	0.21 ± 0.05	0.20 ± 0.06	0.42	0.81	0.21 ± 0.05	0.20 ± 0.05	0.56
Palmitic acid (16:0)	26.90 ± 1.99	27.66 ± 2.22	0.13	0.58	26.46 ± 1.95	27.25 ± 2.17	0.07	0.56	26.57 ± 1.59	26.86 ± 2.00	0.56
Palmitoleic (16:1(n-7))	2.66 ± 0.92	2.58 ± 0.77	0.89	0.76	2.58 ± 0.72	2.79 ± 0.79	0.23	0.76	2.36 ± 0.72	2.41 ± 0.64	0.82
Stearic (18:0)	9.08 ± 0.90	9.42 ± 0.95	**0.045**	0.76	8.93 ± 0.98	8.94 ± 1.19	0.64	0.57	8.95 ± 0.85	9.14 ± 1.00	0.35
Oleic (18:1(n-9))	23.40 ± 3.19	22.31 ± 3.37	**0.009**	0.09	23.58 ± 2.93	23.80 ± 2.97	0.64	0.96	23.00 ± 2.88	23.12 ± 2.66	0.87
Cis-vaccenic (18:1(n-7))	2.38 ± 0.33	2.12 ± 0.30	**$7x10^{-4}$**	0.11	2.47 ± 0.28	2.41 ± 0.36	0.42	0.83	2.31 ± 0.28	2.21 ± 0.23	0.08
Linoleic (18:2(n-6))	19.39 ± 3.29	18.52 ± 3.09	0.13	0.32	20.13 ±2.31	19.41 ± 2.67	0.33	0.53	20.59 ±3.03	20.66 ± 3.02	0.75
γ-linolenic (18:3(n-6))	0.23 ± 0.08	0.23 ± 0.11	0.79	0.16	0.20 ± 0.07	0.22 ± 0.08	0.23	0.82	0.22 ± 0.08	0.26 ± 0.12	0.07
α-linolenic (18:3(n-3))	0.96 ± 0.26	1.10 ± 0.23	**0.002**	0.09	1.02 ± 0.36	0.94 ± 0.35	0.23	0.46	1.00 ± 0.34	1.00 ± 0.26	0.88
Di-homo-γ-linolenic (20:3(n-6))	1.79 ± 0.32	1.61 ± 0.35	**0.001**	**$7x10^{-4}$**	1.79 ± 0.45	1.77 ± 0.44	0.90	0.46	1.78 ± 0.41	1.85 ± 0.32	0.26
Aracidonic (20:4(n-6))	6.09 ± 1.25	5.45 ± 0.99	**0.004**	0.27	6.08 ± 1.58	5.79 ± 1.41	0.32	0.92	6.31 ± 1.52	6.09 ± 1.52	0.51
EPA (20:5(n-3))	1.60 ± 0.77	2.55 ± 1.21	**$9x10^{-4}$**	**$7x10^{-4}$**	1.51 ± 0.69	1.41 ± 0.91	0.42	0.92	1.66 ± 0.96	1.45 ± 0.76	0.17
DPA (22:5(n-3))	0.83 ± 0.18	0.95± 0.24	**0.001**	**$1x10^{-4}$**	0.79 ± 0.14	0.80 ± 0.18	0.64	0.46	0.84 ± 0.16	0.80 ± 0.16	0.18
DHA (22:6(n-3))	3.43 ± 1.09	4.10 ± 1.09	**$7x10^{-4}$**	**$5x10^{-7}$**	3.34 ± 0.14	3.22 ± 1.3	0.23	0.46	3.31 ± 0.98	2.92 ± 0.99	**0.009**

[1]Values are mean ± SD, *n*=105, [2] Whole grain enriched diet, [3]False discovery rate p-value for effect of time within the groups, [4]False discovery rate p-value for interaction of time and group (time*group).

Table 4. Lipids that change significantly different between the groups after the 12-week intervention period[1]

Lipid	**Beta HD[2] vs. Control**	**FDR *p* HD[2] vs. Control[3,4]**	**Beta HD[4]**	**FDR *p* HD[2,4,5]**	**Beta WGED[6]**	**FDR *p* WGED[4,5,6]**	**Beta Control**	**FDR *p* Control[4,5]**	**Beta HD[2] vs. WGED[6]**	**FDR *p* HD[2] vs. WGED[6,7]**
TG(60:13)	0.35	**0.007**	0.35	**$7x10^{-6}$**	0.06	0.43	-0.01	0.87	0.27	0.08
TG(60:12)	0.46	**$3x10^{-4}$**	0.42	**$3x10^{-6}$**	0.07	0.37	-0.05	0.49	0.33	0.07
TG(60:12)[8]	0.43	**$5x10^{-4}$**	0.41	**$5x10^{-6}$**	0.08	0.27	-0.03	0.66	0.31	0.07
TG(58:11)	0.33	**0.008**	0.37	**$4x10^{-6}$**	0.08	0.33	0.04	0.50	0.27	0.08
TG(58:11)[8]	0.29	**0.013**	0.34	**$4x10^{-6}$**	0.06	0.43	0.05	0.38	0.26	0.08
TG(58:10)	0.24	**0.026**	0.30	**$4x10^{-6}$**	0.05	0.42	0.06	0.29	0.23	0.08
TG(58:10)[8]	0.22	**0.041**	0.31	**$4x10^{-6}$**	0.08	0.23	0.08	0.18	0.21	0.08
TG(56:10)	0.24	**0.013**	0.31	**$4x10^{-6}$**	0.07	0.31	0.05	0.26	0.22	0.08
TG(56:9)	0.22	**0.041**	0.31	**$4x10^{-6}$**	0.06	0.38	0.09	0.14	0.24	0.07
TG(56:6)	0.17	**0.019**	0.18	**$6x10^{-6}$**	0.04	0.44	-0.001	0.97	0.13	0.12
TG(55:7)	0.27	**0.036**	0.29	**$7x10^{-5}$**	0.10	0.17	0.01	0.87	0.19	0.19
TG(54:8)	0.25	**0.020**	0.31	**$5x10^{-6}$**	0.08	0.24	0.05	0.40	0.21	0.08
PC(40:7e)	0.14	**0.013**	0.11	**$3x10^{-4}$**	0.04	0.31	-0.03	0.25	0.06	0.35
PC(40:7e) [8]	-0.15	**0.041**	-0.03	0.48	0.09	**0.044**	0.12	**0.007**	-0.13	0.08
PC(40:4)	-0.23	**0.040**	-0.04	0.58	0.12	0.07	0.18	**0.004**	-0.18	0.17
PC(38:7e)	0.13	**0.013**	0.11	**$2x10^{-4}$**	0.02	0.63	-0.02	0.39	0.08	0.19
PC(38:7)	0.15	**0.040**	0.19	**$1x10^{-5}$**	0.08	0.08	0.03	0.49	0.10	0.22
PC(38:4e)	-0.14	**0.013**	-0.03	0.33	0.03	0.22	0.10	**0.004**	-0.07	0.24
PC(36:5)	0.19	**0.013**	0.24	**$4x10^{-6}$**	0.07	0.13	0.04	0.41	0.16	0.08
PE(40:7)	-0.13	**0.040**	-0.08	**0.032**	0.04	0.24	0.06	0.10	-0.12	0.08
PE(38:7e)	0.20	**0.013**	0.20	**$2x10^{-5}$**	0.08	0.09	-0.01	0.87	0.11	0.22
PE(38:4)/PC(35:4)	-0.13	**0.013**	-0.02	0.43	0.06	**0.048**	0.10	**0.009**	-0.09	0.08
PS(38:2)	-0.14	**0.013**	-0.09	**0.010**	0.02	0.54	0.05	0.10	-0.11	0.08
LPC(20:5)	0.21	**0.007**	0.15	**0.002**	-0.001	0.97	-0.05	0.17	0.16	0.08

[1]*n*=105, [2]HealthyDiet group, [3]False discovery rate p-value for interaction of time and group (time*group), the HealthyDiet group compared with the Controls, [4]Age, gender, BMI, Insulin and glucose are used as covariates, [5]False discovery rate p-value for effect of time within the groups,[6]Whole grain enriched diet group, [7]False discovery rate p-value for interaction of time and group, the HealthyDiet group compared with the WGED group, [8]Lipids which are mentioned twice are isobaric, meaning that the fatty acid composition differs.