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Effects of increased wholegrain consumption on immune and inflammatory markers in healthy low habitual wholegrain consumers

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Abbreviations used: 3DFD(s), 3-day food diary(-ies); AR(s), alkylresorcinol(s); CFDA-SE, carboxyfluorescein diacetate succinimidyl ester; CFSE, carboxyfluorescein succinimidyl ester; ConA, concanavalin A; CRP, C-reactive protein; DCD, diet compliance diary; FSC, forward scatter; GIP, gastric inhibitory polypeptide/glucose-dependent insulintropic peptide; GLM, general linear model; GLP-1, glucagon-like peptide-1; MFI, mean fluorescence intensity; NK cells, natural killer cells; PAI-1, plasminogen activator inhibitor-1; PBMC(s), peripheral blood mononuclear cell(s); PI, propidium iodide; RG, refined grain; sIgA, secretory IgA; SSC, side scatter; T_{cm}, central memory T lymphocytes; T_{em}, effector memory T lymphocytes; T_{emra}, R_a⁺ effector memory T lymphocytes; TNF, tumor necrosis factor; WBC(s), white blood cell(s); WG(s), wholegrain(s).

1 **ABSTRACT**

2 **Purpose**

3 Wholegrain (WG) consumption is associated with reduced risk of cardiovascular disease, but
4 clinical data on inflammation and immune function is either conflicting or limited. The objective of
5 this study was to assess the impact of increasing WG consumption to at least 80 g/d on markers of
6 inflammation and glucose metabolism and on phenotypic and functional aspects of the immune
7 system, in healthy, middle-aged adults with low habitual WG intake.

8 **Methods**

9 Subjects consumed a diet high in WG (> 80 g/d) or low in WG (< 16 g/d, refined grain diet) in a
10 crossover study, with 6-week intervention periods, separated by a 4-week washout. Adherence to
11 the dietary regimes was achieved by dietary advice and provision of a range of food products, with
12 compliance verified through analysis of plasma alkylresorcinols (ARs).

13 **Results**

14 On the WG intervention, WG consumption reached 168 g/d ($P < 0.001$), accompanied by an
15 increase in plasma ARs ($P < 0.001$) and fibre intake ($P < 0.001$), without affecting other aspects of
16 dietary intake. On the WG arm there were trends for lower *ex vivo* activation of CD4⁺ T cells and
17 circulating concentrations of IL-10, C-reactive protein, C-peptide, insulin and plasminogen
18 activator inhibitor-1. The percentage of CD4⁺ central memory T cells and circulating levels of
19 adiponin tended to increase during the WG intervention.

20 **Conclusions**

21 Despite the dramatic increase in WG consumption, there were no effects on phenotypic or
22 functional immune parameters, markers of inflammation or metabolic markers.

23

24 **Keywords**

25 Alkylresorcinols, fibre, whole grain, immune, inflammation

26

28 INTRODUCTION

29 Subclinical inflammation is associated with cardiovascular diseases (CVDs) [1], metabolic
30 syndrome and type 2 diabetes [2, 3]. Circulating concentrations of inflammatory markers, such as
31 tumor necrosis factor (TNF)- α , IL-6, IL-10 and C-reactive protein (CRP) are elevated in obese
32 individuals and decrease with weight loss [4, 5] and may directly contribute to vascular injury,
33 insulin resistance, and atherogenesis [6].

34 Wholegrain (WG) foods have been suggested to reduce the risk of CVD [7, 8], metabolic syndrome
35 [8] and type 2 diabetes [7, 8]. However, the effects of WG consumption on health are still poorly
36 understood. Some epidemiological studies have demonstrated an inverse association between WG
37 consumption and CRP in non-diabetic [7, 9] and diabetic [10] individuals, and between WG intake
38 and elevated fasting levels of glucose [7], insulin [7, 11, 12], C-peptide [11] and leptin [11], but not
39 all studies are consistent [11]. Intervention studies also present mixed data regarding effects of WG
40 on CRP and other inflammatory markers. Some WG interventions (4-12 weeks) reported reductions
41 in IL-6 [13] and CRP [14], especially in overweight and diabetic individuals or subjects with
42 metabolic syndrome, while others report no effects of WG consumption ranging between 48 and
43 120 g/d on CRP, IL-6, PAI-1 [15-17], insulin [15, 16, 18, 19] or glucagon [18]. Interestingly, WG
44 intakes were similar or higher [15, 16] in the studies that reported no effects, compared to those
45 reporting positive effects, but it is notable that studies conducted in healthy volunteers tend to report
46 no effect [15-18], whereas those conducted in non-healthy subjects tend to report positive effects
47 [14, 20, 21]. This may be at least partly due to interventions in healthy subjects achieving a
48 relatively modest level of WG intake (56-120 g/d in previous studies compared with an average
49 intake of 168 g/d in the current study). Some of these studies added WG to the diet rather than
50 replacing refined grain products with WG products, or simply provided advice, whereas the current
51 study specifically replaced RG products with WG products to achieve a high intake of WG
52 compared with virtually no intake of WG during the RG period.

53 Currently, limited data is available on the impact of increased WG consumption on immune
54 function. However, WGs are important sources of phytic acid [22], which may enhance the activity
55 of natural killer (NK) cells [23, 24]. WGs also contain fructans, which have a degree of
56 polymerisation between that of fructooligosaccharides' and inulin [25] and may modulate immune
57 function [26, 27]. The objective of this randomized 6-week cross-over study was therefore to assess
58 the effect of increasing WG consumption to at least 80 g/d on markers of systemic inflammation
59 and glucose metabolism and on phenotypic and functional aspects of the immune system in healthy,
60 middle-aged adults, who had a habitual diet low in WG. The study was conducted in healthy
61 individuals with a wide range of BMI in an effort to capture a population of likely consumers who

62 span a wide range of body composition, but avoiding complications associated with diagnosed
63 disease. While previous studies have focused on effects of WG on blood lipids, the current study
64 examined immune and inflammatory markers, for which the issue of healthy vs non-healthy
65 subjects has not yet been considered with respect to WGs.

66
67

68 **METHODS**

69 **Subjects.** Thirty-three healthy subjects (twelve male, twenty-one female), 40-65 years of age, with
70 habitual WG consumption of less than 1 ½ serving or 24 g/d and BMI 20-35 kg/m² were recruited
71 into the study. Exclusion criteria included diagnosed diabetes, heart disease, stroke, vascular
72 disease, inflammatory disease, renal, bowel, liver or pancreatic disease, medication for
73 hyperlipidaemia, hypertension, hypercoagulation, inflammatory conditions or depression,
74 prescribed aspirin, asthma, allergies, smoking, excessive consumption of alcohol (> 21 units/week
75 for men, > 15 units/week for women), planned influenza vaccination or vaccination during the
76 previous 12 months, consumption of prebiotics or probiotics or antibiotic treatment during the
77 previous 3 months. Baseline characteristics of the subjects are shown in **Table 1**. This study was
78 conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures
79 involving human subjects were approved by the University of Reading Research and Ethics
80 Committee. Written informed consent was obtained from all subjects. The trial was registered
81 (ISRCTN36521837).

82

83 **Recruitment.** Potential subjects responding to advertisements were sent a participant information
84 sheet and those who were interested in participating completed a medical and lifestyle questionnaire
85 either online or by telephone. Subjects consuming less than 24 g/d of WG were identified using a
86 FFQ, which was modified from Ross *et al.* [28] for use in the UK (48 items, 8 frequencies, with
87 specified portion sizes for breakfast cereals, pasta and rice). Suitable subjects were then invited to
88 the Hugh Sinclair Unit of Human Nutrition (Department of Food and Nutritional Sciences,
89 University of Reading, UK) for a screening visit, where height, weight, waist circumference, and
90 blood pressure were measured and a blood sample for a full blood count was taken.

91

92 **Study design.** During a 2-week run-in period, dietary assessment was conducted using 3-day food
93 diaries (3DFDs) for any 3 consecutive days, which were also used to provide specific, tailored
94 advice to achieve the WG target intakes. In consultation with a dietitian, subjects were advised to
95 consume a diet high in WG (> 80 g/d) or low in WG (< 16 g/d, RG diet) in a crossover study, with
96 intervention periods lasting 6 week each, separated by a 4-week washout (**Fig. 1**). Subjects were

97 randomized based on age, gender and BMI by a research assistant who was not involved in the
98 analysis using covariate adaptive randomization software. Commercially available pasta, rice,
99 snacks and breakfast cereals in both the WG and RG categories [29] were provided to volunteers
100 every fortnight and specific advice given about substitution of habitually consumed products. Bread
101 from a pre-agreed list was purchased by subjects and costs reimbursed upon production of a receipt.
102 The values calculated for WG intake refer to the g amount of WG consumed (ie 20g if 50g of Uncle
103 Ben's WG rice was consumed). The intakes were calculated by a dietician on an individual basis for
104 every food included in the dietary records, using any available information relating to WG content
105 of foods, or, in some cases, by contacting manufacturers. They were also calculated (much more
106 simply) by the diet compliance diaries, which recorded consumption of the foods that were
107 provided as part of the study. Data calculated using both methods allow comparison. This
108 emphasizes the fact that during the WG intervention, almost all of the WG intake came from the
109 products provided to the subjects, with very little being contributed by other products. This is
110 perhaps not surprising, since the subjects were pre-selected to be very low habitual consumers of
111 WG. While on the study, subjects were asked not to consume products containing prebiotics or
112 probiotics. During each of the two intervention periods, subjects completed a 3DFD. All 3DFD
113 were analyzed using Dietplan6 (Forestfield Software Ltd.).

114 Fasting blood and saliva samples were collected at the beginning and end of each of the intervention
115 periods. Available data on which to perform power calculations was limited, so the sample size was
116 based on data derived from interventions investigating the effect of prebiotics on phenotypic and
117 functional immune parameters and the effect of prebiotics and WGs on the gut microbiota. A
118 sample size of 33 subjects per group was sufficient to detect an 8% difference in NK cell activity,
119 with a two-tailed significance level of 5% and a power of 80%.

120 **Saliva, plasma and serum samples.** Saliva was collected by spitting into eppendorf tubes,
121 centrifuged at 13,000 x g for 10 min at 4°C, and the supernatants stored at -20°C for < 15 months
122 before further analysis. Plasma was prepared from blood collected in EDTA tubes by centrifugation
123 at 2000 x g for 15 min at room temperature (RT) and serum was prepared from blood collected in
124 serum sep clot activator tubes by centrifugation at 1800 x g for 10 min at RT. Samples were frozen
125 at -20°C within 1 h from collection until further analysis.

126
127 **Preparation of peripheral blood mononuclear cells (PBMCs).** Heparinised whole blood (15 ml)
128 was layered onto Lympholyte-H (25 ml; Cedarlane laboratories, Texas, USA), before centrifuging
129 the samples at 550 x g for 15 min at RT and collecting the PBMC-rich interface. PBMCs were
130 washed in PBS (Oxoid, Hampshire, UK), resuspended in 2.5 ml of RPMI 1640 and layered onto 2.5

131 ml Lympholyte-H. Samples were then centrifuged once more at 550 x g for 15 min at RT, before
132 collecting the PBMC-rich interface and washing with PBS. PBMCs were resuspended in complete
133 medium (RPMI 1640 with HEPES and L-Glutamine medium containing 100 U/ml penicillin and
134 0.1 mg/ml streptomycin), counted in a Z1™ series Coulter Counter® (Beckman Coulter, High
135 Wycombe, UK) and adjusted to 5×10^6 cells/ml.

136

137 **Analysis of plasma alkylresorcinols (ARs).** To assess compliance, plasma ARs were analyzed by
138 extraction with diethyl ether and normal phase liquid chromatography/tandem mass spectrometry
139 (LC-MS/MS) after extraction with diethyl ester [30] modified to a 100 mm x 2 mm column and a
140 run time of 8 min. Matrix matched calibration was used, and two control samples were run in
141 triplicate with each batch. Control samples and calibration curve standards were run in random
142 order throughout each sequence. Intra-batch repeatability for each control plasma sample was < 10
143 % and inter-batch repeatability < 15%.

144

145 **White Blood Cell (WBC) counts.** Whole blood was diluted 1:501 with ISOTON II (Beckman
146 Coulter, High Wycombe, UK), erythrocytes were lysed using ZAP-OGLOBIN II. (Beckman
147 Coulter) and samples were counted on a Z1™ series Coulter Counter®. The average of three
148 measurements was recorded.

149

150 **Enumeration of lymphocyte subsets using TruCount™ tubes.** Absolute counts of T
151 lymphocytes, B lymphocytes and NK cells were performed by flow cytometry using the BD
152 Multitest™ CD3 FITC/CD16⁺CD56 PE/CD45 PerCP/CD19 APC with BD TruCount™ Tubes (BD
153 Biosciences, Oxford, UK). Samples were analysed on a BD FACSCanto II flow cytometer (BD
154 Biosciences).

155

156 **Naïve and memory T lymphocyte subsets.** Whole blood in EDTA tubes was stained with a
157 mixture of APC-Cy7 mouse anti-human CD3, FITC mouse anti-human CD4, PerCP-Cy5.5 mouse
158 anti-human CD8, PE-Cy7 mouse anti-human CD45RA and PE rat anti-human CD197 (CCR7) (BD
159 Biosciences, Oxford, UK) in the dark for 45 min at RT. Erythrocytes were lysed using BD Pharm
160 Lyse™ lysing buffer (BD Biosciences, Oxford, UK), incubating in the dark for 20 min at RT,
161 before washing twice with BD CellWASH™ (BD Biosciences). Samples were analysed on a BD
162 FACSCanto II flow cytometer. Results are reported as percentages of parent CD8⁺ and CD4⁺ T
163 lymphocyte subsets.

164

165 **Ex vivo phagocytosis monocytes and granulocytes.** Monocyte and granulocyte phagocytosis was
166 assessed using the PHAGOTEST® kit (GlycoType Biotechnology, Heidelberg, Germany); both the
167 percentage of monocytes and granulocytes engaged in phagocytosis of opsonised FITC-labelled
168 *Escherichia coli* bacteria and mean fluorescence intensity (MFI), reflecting degree of phagocytosis,
169 were recorded. Samples were analysed on a BD FACSCanto II flow cytometer within 60 min.

170
171 **Ex vivo T cell activation.** Heparinised whole blood was diluted 1:10 with complete medium,
172 stimulated with concanavalin A (Sigma-Aldrich, Poole, UK) or CytoStim (Miltenyi Biotec, Surrey,
173 UK) at final concentrations of 25 µg/ml and 0.1 µl/ml respectively and incubated at 37°C, 5% CO₂
174 for 4 h. Samples were washed once with EDTA buffer (2 mM EDTA in PBS, containing 0.5% v/v
175 BSA stain buffer) before staining with a mixture of APC-Cy7 mouse anti-human CD3, FITC mouse
176 anti-human CD4, PE mouse anti-human CD8 and APC mouse anti-human CD69 (BD Biosciences,
177 Oxford, UK) in the dark for 45 min at RT. Erythrocytes were lysed using BD Pharm Lyse™ lysing
178 buffer, incubating in the dark for 20 min at RT, before washing twice with BD CellWASH™.
179 Finally, 7-AAD (BD Biosciences, Oxford, UK) was added as a viability stain and samples were
180 analysed on a BD FACSCanto II flow cytometer.

181
182 **NK cell activity assay.** K562 cells were stained with 0.2 % Trypan Blue (Sigma-Aldrich, Poole,
183 UK) and counted using a haemocytometer before removing 5 x 10⁶ live K562 cells from the
184 suspension. Cells were washed twice in PBS and stained with carboxyfluorescein diacetate
185 succinimidyl ester (CFDA-SE, 100 µg/ml, Sigma-Aldrich) in dimethyl sulfoxide (Sigma-Aldrich) at
186 37°C, 5 % CO₂ for 45 min, during which CFDA-SE was intracellularly converted to
187 carboxyfluorescein succinimidyl ester (CFSE). CFSE-labelled K562 cells were washed twice in
188 PBS, re-suspended and diluted 1:100 in complete medium. PBMCs were then incubated together
189 with the K562 cells at ratios of 100:1, 50:1, 25:1, 12.5:1 and 0:1 (PBMC:K562) at 37°C, 5% CO₂
190 for 2 h. Propidium iodide (1 mg/ml, Sigma-Aldrich) in PBS was used to stain dead K562 cells, prior
191 to flow-cytometric analysis in a FACSCanto II (BD Biosciences).

192
193 **Serum cytokine analysis.** Serum concentrations of IL-1β, IL-6, IL-8, IL-10 and TNF-α were
194 analysed by Luminex®100™ (Luminex Corporation), using a high sensitivity multiplex cytokine
195 Kit (R&D Systems, Abingdon, UK), according to the manufacturer's instructions. Standard curves
196 were generated by the Luminex® software (mean R² = 0.9991). All samples for individual subjects
197 were analysed within a single plate and the intra-assay CV for the Luminex platform was 5.9%.

198

199 **CRP analysis.** CRP concentrations were analysed by an Automatic Analyzer ILab 600 using
200 enzyme based kits and appropriate quality controls (Instrumentation Laboratory Ltd.). All samples
201 for individual subjects were analysed within a single batch and the intra-assay CV was 1.6 %.

202

203 **Salivary secretory IgA (sIgA).** Salivary sIgA was analysed using Immunodiagnostic sIgA ELISA
204 kit (Oxford Biosystems, Oxford, UK), in accordance with the manufacturer's instructions, All
205 samples for individual subjects were analysed within a single plate and the intra-assay CV was
206 9.0%.

207

208 **Metabolic and inflammatory markers.** Serum concentrations of C-Peptide, ghrelin, gastric
209 inhibitory polypeptide (GIP), glucagon-like peptide-1 (GLP-1), glucagon, insulin, leptin, PAI-1,
210 resistin, visfatin, adiponectin and adipsin were analysed by Luminex®100™, using the Bio-Plex
211 Pro Human Diabetes 10-Plex Assay and Bio-Plex Pro Human Diabetes Adipsin and Adiponectin
212 Assays (Bio-Rad Laboratories) according to the manufacturer's instructions. All samples for
213 individual subjects were analysed within a single plate and the intra-assay CV was 16.6%.

214

215 **Statistical analysis.** Analysis of the data acquired by flow cytometry was performed by BD
216 FACSDiva Software (BD Biosciences) and statistical analysis was carried out on Minitab, version
217 15 (Minitab Ltd. State College, PA). Data are means and SD or SEM at baseline and end of
218 intervention (within each arm), as well as difference from the within-arm baseline (Δ intervention)
219 for both interventions. Data were normally distributed, as confirmed by the Kolmogorov-Smirnov
220 normality test. To assess whether the order of the intervention arms had an effect on the data,
221 baseline means were compared using two sample T-tests and no statistical differences were found.
222 With the exceptions mentioned below, a general linear model (GLM) was used to analyze the data,
223 in which intervention delta and gender were specified as fixed model parameters and starting diet as
224 a random parameter, while age and baseline measurements of BMI, systolic and diastolic blood
225 pressure, body fat percentage, waist circumference, alcohol consumption, plasma ARs, habitual
226 fibre and WG consumption, CRP and blood biochemistry (i.e. total cholesterol, triglycerides, LDL
227 and HDL cholesterol and glucose) were listed as covariates. The analysis of 3DFD and ARs was
228 performed using a single factor GLM and further comparisons made using Tukey simultaneous
229 tests. Spearman's ρ correlation coefficient between studied parameters and ARs, fibre intake and
230 WG intake were calculated and *P*-values reported. In all cases, the level of significance was set at
231 5% and 10% for effects and trends, respectively.

232

233 **RESULTS**

234 **Dietary analysis, assessment of WG intake and plasma ARs**

235 A habitual diet low in WG intake was a prerequisite for subjects taking part in this study and two
236 methods were used to assess it; the FFQ at screening and the 3DFD during the run-in period.
237 Subjects were recruited on the basis of the FFQ, although comparison with the 3DFD suggests that
238 the FFQ slightly but significantly underestimated habitual consumption of WG ($P < 0.001$) (data
239 not shown). Using data from the 3DFD administered during the run-in period, recruited subjects
240 consumed on average 28 g/d of WG, which was slightly higher than the threshold of 24 g/d.
241 Nevertheless, the substitution of habitual grain-based foods with the provided WG varieties during
242 the WG intervention period resulted in consumption of WG far exceeding the target of 80 g/d and
243 reaching an average of 168 g/d, with a range of 67-335 g/d (**Table 2**). In contrast, WG consumption
244 during the RG period was 0.1 g/d on average (**Table 2**). During the WG intervention, wheat was the
245 major contributor to the increased WG intake, providing 59% of the WG consumed, while oats, rice
246 and corn contributed 40% in total. Increased WG consumption was accompanied by a statistically
247 significant increase in plasma ARs and total fibre intake, but there was no effect on other aspects of
248 dietary intake (**Table 2**).

249

250 **Effect of WG intervention on absolute numbers of WBCs, B and T lymphocytes and NK cells**
251 **and on naïve and memory T lymphocyte subsets**

252 There was no effect of the WG intervention on absolute counts of WBCs, B and T lymphocytes or
253 NK cells (data not shown) or on naïve or memory cell subpopulations of CD4⁺ and CD8⁺ T
254 lymphocytes (**Table 3**). However, there was a trend towards an increase in CD4⁺ Tcm cells during
255 the WG intervention ($P = 0.06$). Modest positive correlations were observed between plasma ARs,
256 but not WG intake (Spearman's ρ , P), and absolute numbers of B (0.19, 0.03) and T (0.18, 0.05)
257 lymphocytes and NK cells (0.23, 0.01).

258

259 **Effect of WG intervention on *ex vivo* phagocytic ability of monocytes and granulocytes, T cell**
260 **activation and NK cell activity**

261 The intervention had no effect on phagocytosis by monocytes or granulocytes (data not shown).
262 There was also no effect on the expression of CD69 by CD4⁺ or CD8⁺ T lymphocyte subsets
263 following *ex vivo* stimulation of whole blood cultures (**Table 4**), or on NK cell activity towards
264 K562 cells (data not shown). ConA-stimulated expression of CD69 on CD4⁺ cells tended to be
265 higher during the RG period than the WG period ($P = 0.08$).

266

267 **Effect of WG intervention on serum cytokines, CRP and salivary sIgA**

268 There was no effect of the intervention on serum concentrations of cytokines or CRP (**Table 5**) or
269 on salivary sIgA (data not shown). There was a trend towards a reduction in IL-10 and CRP during
270 the WG intervention ($P = 0.08$ and 0.10 , respectively) and a modest negative correlation between
271 IL-1 β and total fibre intake (Spearman's $\rho = -0.21$, $P = 0.04$). Despite the inverse trend with WG
272 intake, IL-10 was positively correlated with plasma ARs (Spearman's $\rho = 0.19$, $P = 0.04$).

273

274 **Effect of WG intervention on metabolic markers**

275 There was no effect of WG on any of the metabolic markers assessed (**Table 6**), although C-
276 peptide, insulin and PAI-1 tended to be higher during the RG period than the WG period ($P = 0.10$,
277 0.08 and 0.07 , respectively). In contrast, there was a trend towards a reduction in adipsin during the
278 RG period compared with the WG period ($P = 0.07$). Adipsin was also positively correlated with
279 plasma ARs (Spearman's $\rho = 0.22$, $P = 0.01$), but not with WG.

280

281 **DISCUSSION**

282 This intervention resulted in a substantial increase in WG consumption in habitual low consumers
283 of WG to an average of 168 g/d (a 500% increase), which was associated with an increase in plasma
284 ARs. Despite the increase in WG consumption, there were no effects on phenotypic or functional
285 immune parameters, markers of inflammation or metabolic markers.

286 ARs are phenolic lipids that have been proposed as potential biomarkers of WG intake [31] and are
287 present in the bran of rye, wheat and barley, but not in the edible parts of other cereals. In line with
288 published data [32], total AR concentrations were below 60 nM at baseline and during the RG arm
289 and the mean concentration after 6 weeks of WG intervention was 161 nmol/L. This is at the lower
290 end of the previously reported range of 142-847 nmol/L following a WG intervention of a similar
291 magnitude [32].

292 In the current study, there were no effects of WG consumption on absolute counts of WBC, B and T
293 lymphocytes, NK cells, naïve and memory cell subsets of CD4⁺ and CD8⁺ T lymphocytes,
294 phagocytic ability of monocytes and granulocytes, activation (expression of CD69) of T
295 lymphocytes after *ex vivo* stimulation of whole blood, activity of NK cells or concentration of saliva
296 sIgA. While there was a trend towards increased activation of CD4⁺ T cells after stimulation with
297 ConA during the RG period relative to the WG period and a trend towards increased percentage of
298 CD4⁺ Tcm cells during the WG intervention, the lack of consistent effects on immune function
299 suggest that these are not meaningful and cannot be interpreted as either positive or negative effects.
300 There is currently very limited data on the impact of increased WG consumption on immune
301 function. Single measures of immune function are notoriously difficult to interpret with respect to
302 biological or clinical relevance, but the use of a panel of markers has the potential to identify

303 consistent effects, which is arguably a more appropriate approach to investigating the effects of
304 dietary components on immune function [33]. The primary immune outcomes in the current study
305 were immune phenotyping and functional analysis and the secondary outcomes were the circulating
306 metabolic and inflammatory markers.

307 WGs, and rice, corn and wheat in particular, are important sources of phytic acid [22, 34], a metal
308 chelator which may enhance the activity of NK cells [23, 24]. WGs also contain arabinoxylans, β -
309 glucans and fructans, which have structural similarities with inulin and fructooligosaccharides [25],
310 both of which have been shown to have beneficial effects on parameters of immune function. For
311 example, clinical interventions in elderly adults (>65y) demonstrate that inulin and
312 fructooligosaccharides increase numbers of in peripheral blood B cells [26], T cells [34], and CD4⁺
313 and CD8⁺ T cell subsets [35], decrease numbers of memory CD8⁺ T cells [26] and improve NK cell
314 activity [27]. However, in similar studies in young healthy individuals [35] or cancer and
315 polypectomised patients [36], there was little or no effect of inulin, as part of a synbiotic [36] or
316 other prebiotics [35] on phagocytic activity of neutrophils [35, 36] and monocytes [36] or activity
317 [36] and numbers of NK cells [35].

318 Increased WG consumption had no effect on either serum cytokines or on CRP levels, although
319 there were trends towards decreased IL-10 and CRP concentrations during the WG compared to the
320 RG period. Based on epidemiological data, WG consumption has been inversely associated with
321 inflammatory markers, and particularly CRP [37]. However, intervention studies do not always
322 demonstrate a clear effect. Martinez *et al.* [13] reported reductions in IL-6 and CRP concentrations
323 (although the latter did not reach statistical significance) after 4 weeks of consumption of 60 g/d of
324 WG rice and barley. Katcher *et al.* [14] reported a 38 % reduction in CRP following a WG diet for
325 12 weeks. In these studies, WG products were either added to the diet instead of replacing the
326 existing RG products [13] or subjects were given dietary advice either to avoid WG or to obtain all
327 of their grain servings from WG [14]. Importantly, these studies were either conducted in obese
328 volunteers with metabolic syndrome [14] or reductions in inflammatory markers were more
329 significant in overweight subjects [13], suggesting that health status of the subject might be a key
330 factor determining the outcome. In another study, both a WG (60 g/d of WG) and a RG (4 g/d of
331 WG) diet resulted in reductions between 22 and 42 % in TNF- α , IL-1 β , and IL-6 after 6 weeks in
332 adolescents [38], but some intervention studies reported no effects of WG on CRP or IL-6 after 2
333 [39] 6 [15], 8 [16], 12 [40] or 16 [16] weeks following a WG diet (between 48 and 150 g/d of WG).
334 A number of aspects of health status may influence the impact of WG consumption on health
335 outcomes, including presence of metabolic syndrome or type 2 diabetes, initial concentration of the
336 studied inflammatory markers, intervention duration, cereal type and perhaps even particular
337 cultivar and processing. Variations in these factors may well underlie discrepancies between and

338 within epidemiological and intervention studies. In addition, glycemic index (GI) of the foods
339 selected for the intervention may be an important factor for consideration. A number of the WG
340 foods provided had a medium or high GI value [41], and GI has been demonstrated to modulate
341 inflammatory markers [42], perhaps even more so than WG. The desire to provide a wide range of
342 acceptable foods to achieve a high total WG intake therefore conflicts with the potential necessity to
343 limit the WG foods to those which are low GI, and the overall impact of WG vs GI on inflammation
344 is not yet clear.

345 WG consumption had little effect on the metabolic markers examined in the current study, although
346 there was a tendency for C-peptide, insulin and PAI-1 to decrease, while adiponectin tended to increase
347 during the WG period relative to the RG period. Reviewing observational studies and clinical trials,
348 Koh-Banerjee and Rimm [43] concluded that WG intake is inversely associated with insulin, C-
349 peptide and leptin concentrations. Moreover, based on epidemiological studies, PAI-1 has been
350 related to RG and inversely associated with WG [9]. Generally, however, WG intervention studies
351 have had mixed results in terms of markers related to glucose metabolism. Following WG
352 interventions of 6 or 12 weeks, insulin, leptin and PAI-1 were significantly decreased in overweight
353 or obese hyperinsulinaemic adults [20], patients with early-stage prostate cancer [21] and obese
354 adults with metabolic syndrome [14], respectively. In these studies WG was either based solely on
355 rye [21] or consumed in a variety of products [14, 20], similar to the current study. However, in the
356 study of Katcher *et al.* [14], total energy was restricted and the reported reduction in PAI-1 was
357 attributed to weight loss, rather than increased consumption of WG. Indeed, loss of body weight is
358 directly associated with lowering PAI-1 and also leptin and resistin concentrations [5]. Another
359 study, reported a decline in adiponectin levels in diabetic patients, after a high-carbohydrate, low-
360 fibre, RG wheat meal in comparison to a high-carbohydrate, high-fibre WG wheat meal [44]. On
361 the other hand, a number of studies in healthy individuals report no effects of WG on PAI-1 [15, 16,
362 25], insulin [15, 16, 18, 19] or glucagon [18] after 4 [17], 6 [15, 18], 8 [16], 12 [19] or 16 [16]
363 weeks of consuming whole meal rye bread [17], WG wheat sourdough bread [18], high-fibre oat or
364 wheat cereal [19] or a variety of WG products [15, 16], where WG intake ranged from 56 to 120 g/d
365 [15, 16]. Notably, most studies show no effects of WG on metabolic markers in healthy subjects (n
366 = 28-316) [15-19], while clinical interventions tend to report significant effects (n = 11-60) [14, 20,
367 21, 44]. In the current study, there were no effects of the WG intervention on body weight, BMI,
368 body fat percentage or waist circumference [29] and there was no influence of body composition on
369 immunological parameters.

370 Power is an important consideration in evaluation of the evidence for health effects of WGs;
371 inconsistency in previous data and uncertainty with respect to effect size meant that it was difficult
372 to conduct a well-justified power calculation and the sample size in the current study was based on

373 interventions investigating the effect of prebiotics on phenotypic and functional immune parameters
374 and the effect of prebiotics and WGs on the gut microbiota. The numbers of participants in
375 previously published studies have ranged from 17 to and 316, with the majority including 50
376 subjects or fewer. Thus, although the current study demonstrated no effects in healthy subjects, the
377 potential for WGs to improve inflammatory markers and markers of glucose metabolism needs to
378 be further examined, perhaps in subjects pre-selected for inflammatory status, and almost certainly
379 with larger sample sizes.

380 Processing and the physical properties, structure and composition of the cereal grain, especially
381 with regard to amount and type of fibre, may be important considerations in WG intervention
382 studies. Anson *et al.* [45] demonstrated that bio-processing of bran in WG wheat bread resulted in
383 increased bio-availability of phenolic acids and consumption of the processed wheat bread
384 decreased the ratio of pro-inflammatory to anti-inflammatory cytokines in endotoxin-stimulated
385 blood *ex vivo* compared with non-processed bread. However, evidence for effects of processing is
386 generally limited. The role of the amount and type of fibre in WG foods, is better understood,
387 particularly with respect to carbohydrate metabolism [46]. It is well known, for example, that
388 soluble fibre improves control of blood glucose and insulin levels [39]. Wheat contains relatively
389 less soluble and more insoluble fibre than oats, rye or barley, which may partially explain
390 differences in the outcomes of studies where different sources of WG were used. In the current
391 study, 59% of the WG intake was derived from wheat, and it was this source of WG which
392 increased plasma ARs. It was notable that changes in some biomarkers correlated well with plasma
393 AR concentrations, but not WG intake (eg adipsin), and it is tempting to speculate that this might be
394 related to effects of wheat vs oats, rye or barley. Plasma AR concentrations may also reflect the
395 extent of absorption of phytochemicals from WG, and better represent actual exposure of the body
396 to WG components, than estimates of WG intake.

397 In conclusion, despite the dramatic increase in WG consumption in habitual low consumers of WG,
398 there were no effects on phenotypic or functional immune parameters, markers of inflammation or
399 metabolic markers. Health status of subjects, statistical power, physical and structural properties of
400 the WGs and processing conditions may be important factors to consider in future studies.

401

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404 and conducted the clinical visits. KKA and CMM analyzed the 3DFDs. AA and CLW processed
405 clinical samples and analyzed biochemical parameters and ABR analyzed plasma ARs. AA
406 performed the statistical analysis. AA, PY, ABR and FT wrote the paper and PY had primary

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409

410 **Conflict of interest**

411 This work was funded by Cereal Partners Worldwide. AA, CLM, KKA, CMM, OBK and PY have
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570

571

572

574 **FIGURE 1.** Intervention study design of a randomized controlled crossover study in which 33
575 subjects received a whole-grain (WG) diet (> 80 g/d of WG) and a refined grain diet (< 16 g/d of
576 WG) for a period of 6 weeks each. Blood and saliva samples were collected from each volunteer
577 before and after each intervention arm.

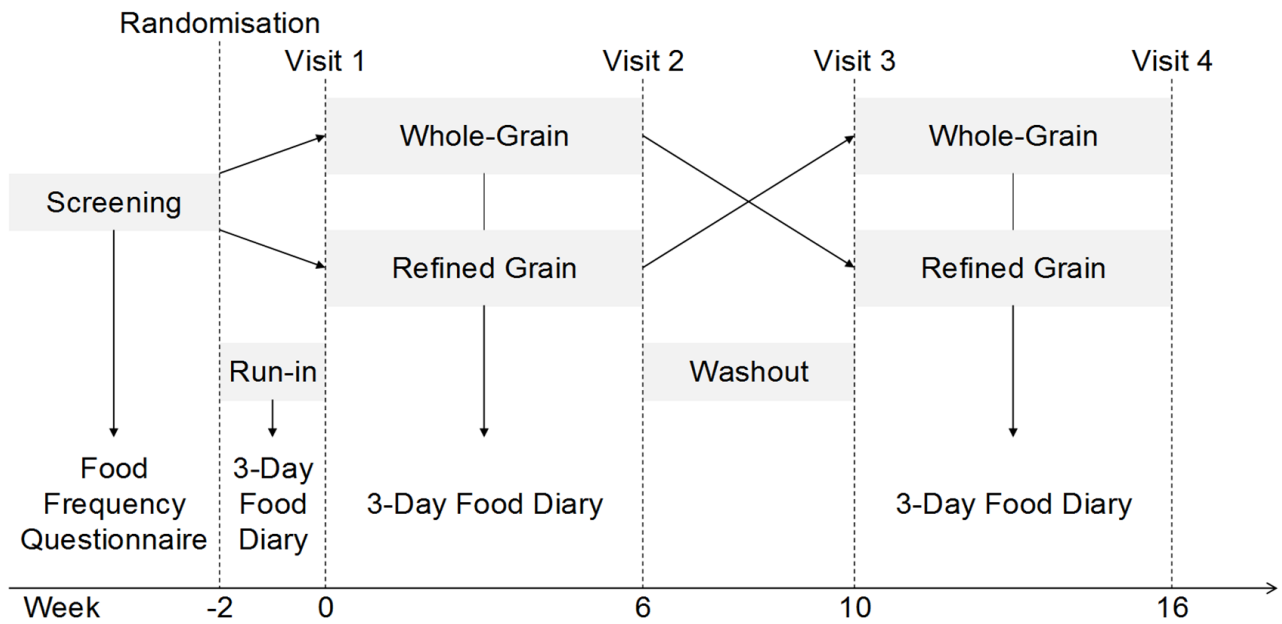


Table 1 Baseline characteristics of volunteers and baseline measurements of plasma lipids and glucose, body composition, blood pressure (BP) and alcohol consumption¹

Parameter (<i>unit</i>)	Total volunteers	Male volunteers	Female volunteers
n	33	12	21
Age (<i>years</i>)	48.8 ± 1.1	51.8 ± 2.2	47.1 ± 1.1
Cholesterol (<i>mmol/L</i>)	5.2 ± 0.2	5.5 ± 0.3	5.1 ± 0.2
LDL cholesterol (<i>mmol/L</i>)	3.6 ± 0.2	3.9 ± 0.3	3.5 ± 0.2
HDL cholesterol (<i>mmol/L</i>)	1.4 ± 0.1	1.3 ± 0.1	1.4 ± 0.1
Triglycerides (<i>mmol/L</i>)	1.1 ± 0.1	1.4 ± 0.2	1.0 ± 0.1
Glucose (<i>mmol/L</i>)	5.4 ± 0.1	5.6 ± 0.2	5.2 ± 0.1
Weight (<i>kg</i>)	77.3 ± 2.5	87.6 ± 4.3	71.4 ± 2.2
BMI (<i>kg/m²</i>)	27.9 ± 0.7	28.9 ± 1.5	27.4 ± 0.7
% Body fat	32.0 ± 1.5	25.4 ± 2.3	35.9 ± 1.4
Waist circumference (<i>cm</i>)	97.3 ± 1.9	102.7 ± 3.6	94.2 ± 2.0
Systolic BP (<i>mmHg</i>)	128.7 ± 2.1	135.4 ± 3.3	124.8 ± 2.3
Diastolic BP (<i>mmHg</i>)	81.7 ± 1.4	82.9 ± 2.5	81.0 ± 1.7
Alcohol consumption (<i>units/week</i>)	2.9 ± 0.6	4.8 ± 1.2	1.8 ± 0.6

¹ Values are means ± SEM. Six volunteers suffered from mild hay fever and occasionally took antihistamines or used corticosteroid nasal sprays and four volunteers occasionally consumed prebiotic/probiotic supplements. During the study, including the 2-week run-in period, however, no volunteers reported taking medication for hay fever or consuming prebiotic/probiotic supplements.

Table 2 Energy, macronutrient, total fibre and WG intake and plasma ARs before the start of the study (habitual) and at the end of the RG and WG interventions (n = 33)

Parameter	Energy (kJ//d), macronutrient (g/d), fibre (AOAC, g/d) and WG (g/d) intake and plasma ARs (nM)								
	Habitual		RG intervention		WG intervention		P-values (Tukey Simultaneous Tests)		
	Mean	(SD)	Mean	(SD)	Mean	(SD)	Habitual vs RG	Habitual vs WG	RG vs WG
Energy	8,765	(2,536)	8,686	(1,937)	8,556	(2,088)	0.989	0.924	0.969
Protein	86.0	(27.9)	81.1	(21.9)	82.9	(17.5)	0.677	0.853	0.950
Fat	83.9	(35.7)	80.2	(25.1)	72.9	(27.9)	0.874	0.310	0.594
Saturated fat	30.4	(14.0)	32.0	(12.6)	27.7	(13.0)	0.878	0.692	0.396
Carbohydrate	234.3	(69.9)	240.8	(56.7)	223.6	(60.6)	0.911	0.773	0.518
Sugars	103.1	(48.3)	96.4	(32.4)	95.9	(46.2)	0.803	0.778	0.999
Fibre (AOAC)	17.9	(7.1)	15.5	(6.0)	25.8	(7.6)	0.364	< 0.001	< 0.001
WG	27.7	(25.6)	0.1	(0.6)	168.4	(63.3)	0.017	< 0.001	< 0.001
Plasma ARs	59.8	(66.6)	38.1	(29.4)	161.1	(176.8)	0.712	0.001	< 0.001

WG, whole-grain; ARs, alkylresorcinols; RG, refined grain

P-values < 0.05 are highlighted in bold

Table 3 Effects of WG and RG interventions on naïve and memory cells subpopulations of CD4⁺ and CD8⁺ T lymphocytes (n = 33)

Parameter	Naïve and memory cells % of parent T lymphocyte subset														
	RG intervention					WG intervention					Δ intervention				
	Baseline		End		Comparison	Baseline		End		Comparison	ΔRG		ΔWG		Comparison
	Mean	(SEM)	Mean	(SEM)	<i>P</i> -value	Mean	(SEM)	Mean	(SEM)	<i>P</i> -value	Mean	(SEM)	Mean	(SEM)	<i>P</i> -value
CD4⁺															
Naïve	30.2	(2.1)	30.3	(2.0)	1.000	29.4	(2.1)	30.0	(2.0)	0.998	0.1	(0.8)	0.5	(0.7)	0.787
Tcm	25.5	(1.5)	24.6	(1.3)	0.879	24.4	(1.3)	24.9	(1.5)	0.968	-0.8	(0.6)	0.5	(0.5)	0.062
Tem	39.2	(2.0)	39.9	(2.0)	0.976	40.7	(2.0)	40.0	(2.0)	0.992	0.6	(1.0)	-0.7	(0.9)	0.345
Temra	5.1	(0.7)	5.2	(0.7)	0.996	5.5	(0.7)	5.2	(0.6)	0.948	0.1	(0.4)	-0.3	(0.3)	0.328
CD8⁺															
Naïve	23.0	(2.2)	21.7	(2.1)	0.968	22.0	(2.1)	22.4	(2.2)	0.998	-1.3	(0.9)	0.4	(0.8)	0.173
Tcm	4.7	(0.5)	4.6	(0.4)	1.000	4.7	(0.6)	4.8	(0.5)	0.999	0.0	(0.2)	0.1	(0.3)	0.783
Tem	56.7	(2.3)	58.0	(2.3)	0.975	57.8	(2.2)	57.2	(2.3)	0.996	1.3	(0.8)	-0.6	(0.8)	0.143
Temra	15.6	(1.7)	15.7	(1.7)	1.000	15.5	(1.7)	15.6	(1.6)	1.000	0.0	(0.5)	0.1	(0.5)	0.896

WG, whole-grain; RG, refined grain; Tcm, central memory T lymphocytes; Tem, effector memory T lymphocytes; Temra, RA⁺ effector memory T lymphocytes

Within the RG and WG interventions, comparisons were performed between baseline and end measurements, while within Δ intervention ΔRG was compared against ΔWG. *P*-values < 0.1 are highlighted in bold

Table 4 Effects of WG and RG interventions on activation (expression of CD69) of CD4⁺ and CD8⁺ T lymphocyte subsets following *ex vivo* stimulation (n = 33)

Parameter	CD69 ⁺ cells % of parent T lymphocyte subset														
	RG intervention					WG intervention					Δ intervention				
	Baseline		End		Comparison	Baseline		End		Comparison	ΔRG		ΔWG		Comparison
	Mean	(SEM)	Mean	(SEM)	P-value	Mean	(SEM)	Mean	(SEM)	P-value	Mean	(SEM)	Mean	(SEM)	P-value
CD4⁺															
Unstimulated	1.6	(0.2)	1.3	(0.2)	0.932	1.9	(0.4)	1.3	(0.1)	0.255	-0.2	(0.3)	-0.6	(0.4)	0.293
ConA	6.9	(0.9)	8.0	(1.1)	0.654	8.6	(1.3)	7.0	(0.9)	0.72	1.3	(1.0)	-1.6	(1.2)	0.079
CytoStim	54.1	(2.6)	56.3	(2.5)	0.783	53.9	(2.6)	53.4	(2.2)	1.000	1.8	(1.2)	-0.4	(1.6)	0.535
CD8⁺															
Unstimulated	2.4	(0.2)	2.4	(0.2)	1.000	2.5	(0.2)	2.6	(0.3)	0.99	0.1	(0.2)	0.1	(0.3)	0.787
ConA	11.3	(1.3)	12.4	(1.1)	0.883	11.8	(1.5)	11.5	(1.2)	0.999	1.3	(0.8)	-0.3	(0.8)	0.174
CytoStim	55.1	(2.2)	56.1	(2.2)	0.992	54.2	(2.1)	53.9	(1.9)	1.000	0.5	(1.1)	-0.3	(1.5)	0.855

WG, whole-grain; RG, refined grain; ConA, Concanavalin A; CytoStim, Miltenyi Biotec Ltd.-130-092-173

Within the RG and WG interventions, comparisons were performed between baseline and end measurements, while within Δ intervention ΔRG was compared against ΔWG. *P*-values < 0.1 are highlighted in bold

Table 5 Effects of WG and RG interventions on serum concentrations of cytokines and CRP (n = 33)

Parameter	Concentration in serum (ng/l)														
	RG intervention					WG intervention					Δ intervention				
	Baseline		End		Comparison	Baseline		End		Comparison	ΔRG		ΔWG		Comparison
	Mean	(SEM)	Mean	(SEM)	<i>P</i> -value	Mean	(SEM)	Mean	(SEM)	<i>P</i> -value	Mean	(SEM)	Mean	(SEM)	<i>P</i> -value
IL-10	0.8	(0.1)	0.9	(0.1)	0.996	1.1	(0.3)	0.8	(0.1)	0.326	0.1	(0.1)	-0.3	(0.2)	0.075
IL-1β	1.3	(0.1)	1.3	(0.1)	0.905	1.3	(0.1)	1.3	(0.1)	0.94	0.1	(0.1)	0.1	(0.0)	0.888
IL-6	1.3	(0.2)	1.4	(0.2)	0.99	1.2	(0.2)	1.2	(0.1)	0.999	0.1	(0.2)	-0.1	(0.1)	0.702
IL-8	9.9	(0.6)	10.2	(0.8)	0.931	10.1	(0.8)	10.4	(0.8)	0.996	0.3	(0.5)	0.3	(0.5)	0.649
TNF-α	10.5	(0.5)	10.7	(0.5)	0.937	10.8	(0.4)	10.8	(0.6)	0.995	0.2	(0.3)	0.0	(0.4)	0.381
CRP (x 10 ³)	1.7	(0.3)	1.8	(0.3)	0.948	2.2	(0.5)	1.6	(0.4)	0.248	0.1	(0.2)	-0.6	(0.4)	0.099

WG, whole-grain; RG, refined grain; CRP, C-reactive protein; TNF, tumor necrosis factor

Within the RG and WG interventions, comparisons were performed between baseline and end measurements, while within Δ intervention ΔRG was compared against ΔWG. *P*-values < 0.1 are highlighted in bold

Table 6 Effects of WG and RG interventions on serum concentration of glucose metabolism related proteins (n = 33)

Parameter	Concentration in serum (ng/l)														
	RG intervention					WG intervention					Δ intervention				
	Baseline		End		Comparison	Baseline		End		Comparison	ΔRG		ΔWG		Comparison
	Mean	(SEM)	Mean	(SEM)	<i>P</i> -value	Mean	(SEM)	Mean	(SEM)	<i>P</i> -value	Mean	(SEM)	Mean	(SEM)	<i>P</i> -value
C-Peptide	889.8	(63.9)	982.8	(83.6)	0.535	940.9	(75.5)	878.7	(70.3)	0.846	93.0	(69.0)	-62.3	(61.6)	0.099
Ghrelin	918.0	(55.4)	916.0	(58.0)	0.999	880.7	(50.2)	914.8	(54.0)	0.868	-1.9	(31.8)	34.1	(20.9)	0.193
GIP	186.1	(23.5)	193.4	(31.7)	0.999	231.8	(68.8)	183.5	(31.9)	0.745	7.3	(20.5)	-48.4	(38.8)	0.239
GLP-1	347.6	(36.2)	348.3	(36.5)	1.000	351.6	(36.7)	348.1	(36.5)	1.000	0.8	(4.4)	-3.5	(5.9)	0.516
Glucagon	552.0	(12.4)	552.5	(12.3)	1.000	564.3	(12.8)	555.2	(13.3)	0.903	0.5	(4.9)	-9.1	(4.1)	0.151
Insulin	438.2	(45.4)	491.1	(53.7)	0.583	491.6	(58.6)	434.8	(41.9)	0.614	52.9	(43.5)	-56.8	(50.6)	0.075
Leptin (x 10 ³)	12.1	(1.6)	11.6	(1.4)	0.932	12.3	(1.4)	11.0	(1.3)	0.589	-0.4	(0.5)	-1.3	(0.6)	0.489
PAI-1 (x 10 ³)	41.7	(2.0)	41.9	(1.5)	1.000	47.3	(3.1)	41.9	(1.3)	0.074	0.2	(1.9)	-5.4	(3.1)	0.068
Resistin (x 10 ³)	4.0	(0.3)	4.2	(0.3)	0.904	4.1	(0.3)	4.1	(0.3)	1.000	0.2	(0.1)	-0.1	(0.1)	0.136
Visfatin (x 10 ³)	4.4	(0.9)	4.6	(1.0)	1.000	6.8	(3.1)	4.8	(1.2)	0.74	0.2	(0.2)	-2.0	(2.0)	0.28
Adiponectin (x 10 ⁶)	2.8	(0.3)	2.7	(0.3)	0.99	2.8	(0.3)	2.6	(0.3)	0.834	-0.1	(0.2)	-0.2	(0.2)	0.538
Adipsin (x 10 ³)	660.0	(31.4)	615.5	(36.3)	0.395	643.2	(35.3)	656.7	(36.6)	0.973	-44.5	(29.5)	13.6	(25.3)	0.071

WG, whole-grain; RG, refined grain; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1; PAI-1, plasminogen activator inhibitor-1

Within the RG and WG interventions, comparisons were performed between baseline and end measurements, while within Δ intervention ΔRG was compared against ΔWG. *P*-values < 0.1 are highlighted in bold

