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2 **A prospective diet-wide association study for risk of colorectal cancer in EPIC**

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85 **Abstract** (max 260 words, structured)

86 **BACKGROUND & AIMS:** Evidence regarding the association of dietary exposures with
87 colorectal cancer (CRC) risk is not consistent with a few exceptions. Therefore, we conducted
88 a diet-wide association study (DWAS) in the European Prospective Investigation into Cancer
89 and Nutrition (EPIC) to evaluate the associations between several dietary exposures with CRC
90 risk.

91 **METHODS:** The association of 92 food and nutrient intakes with CRC risk was assessed in
92 386,792 participants, 5,069 of whom developed incident CRC. Correction for multiple
93 comparisons was performed using the false discovery rate, and emerging associations were
94 examined in the Netherlands Cohort Study (NLCS). Multiplicative gene-nutrient interactions
95 were also tested in EPIC based on known CRC-associated loci.

96 **RESULTS:** In EPIC, alcohol, liquor/spirits, wine, beer/cider, soft drinks, and pork were
97 positively associated with CRC, whereas milk, cheese, calcium, phosphorus, magnesium,
98 potassium, riboflavin, vitamin B6, beta-carotene, fruit, fibre, non-white bread, banana, and total
99 protein intakes were inversely associated. Of these 20 associations, 13 were replicated in
100 NLCS, for which a meta-analysis was performed, namely alcohol (summary HR per 1 SD
101 increment in intake: 1.07; 95%CI:1.04-1.09), liquor/spirits (1.04; 1.02-1.06), wine (1.04;1.02-
102 1.07), beer/cider (1.06;1.04-1.08), milk (0.95;0.93-0.98), cheese (0.96;0.94-0.99), calcium
103 (0.93;0.90-0.95), phosphorus (0.92;0.90-0.95), magnesium (0.95;0.92-0.98), potassium
104 (0.96;0.94-0.99), riboflavin (0.94;0.92-0.97), beta-carotene (0.96;0.93-0.98), and total protein
105 (0.94;0.92-0.97). None of the gene-nutrient interactions were significant after adjustment for
106 multiple comparisons.

107 **CONCLUSIONS:** Our findings confirm a positive association for alcohol and an inverse
108 association for dairy products and calcium with CRC risk, and also suggest a lower risk at

109 higher dietary intakes of phosphorus, magnesium, potassium, riboflavin, beta-carotene and
110 total protein.

111 **KEYWORDS:** nutrition; cohort study; colorectal cancer; epidemiology

112 **ABBREVIATIONS:** BMI: Body mass index; CI: Confidence interval; CRC: Colorectal
113 cancer; EPIC: European Prospective Investigation into Cancer and Nutrition; FDR: False
114 discovery rate; GWAS: Genome-wide association study; HR: Hazard ratio; MR: Mendelian
115 Randomization; NLCS: the Netherlands Cohort Study; DWAS: Diet-wide association study;
116 SNP: Single nucleotide polymorphism; WCRF: World Cancer Research Fund; WGS: Whole-
117 genome sequencing.

118 Colorectal cancer (CRC) is the third most common type of cancer worldwide with over
119 1.8 million new cases and over 800,000 deaths in 2018¹. The incidence rates are higher in high
120 income countries, but there has been a recent large increase in the rates in low- and middle-
121 income countries potentially due to the “westernization” of these societies¹. Several aspects of
122 the Western lifestyle such as obesity and lack of physical activity are well-established risk
123 factors of CRC^{2, 3}, but evidence regarding diet, and in particular the association of specific
124 foods and nutrients with CRC is not consistent, with a few exceptions⁴. The World Cancer
125 Research Fund (WCRF) third Expert Report identified strong evidence that consuming
126 processed meat, red meat, and alcohol increases risk of CRC, whereas consumption of whole-
127 grains, foods containing dietary fibre, and dairy products lowers CRC risk⁴. Associations for
128 other foods and nutrients and CRC risk exist, but are inconsistent and currently provide limited
129 evidence according to WCRF⁴.

130 The aim of this study was to systematically examine the associations between a wide
131 set of dietary factors and risk of CRC in the European Prospective Investigation into Cancer
132 and Nutrition (EPIC) and the Netherlands Cohort Study (NLCS), by conducting a diet-wide
133 association study (DWAS)⁵. The DWAS takes an analogous strategy to that of a genome-wide
134 association study (GWAS) by separately estimating associations for each food and nutrient,
135 using adjustments for multiple comparisons, and replicating promising associations in an
136 independent study.

137 **Methods**

138 **Study populations**

139 EPIC is a large European multicentre prospective cohort that consists of 521,324
140 participants, mostly aged between 35 and 70 years, recruited between 1992 and 2000 from 23

141 centres across 10 European countries⁶. A total of 386,792 participants (71% women) were
142 included in the present analysis after pertinent exclusions (supplementary methods).

143 NLCS is a prospective cohort study of 120,852 participants, aged between 55 and 69
144 years and recruited in 1986 from 204 computerised population registries across the Netherlands
145 that uses a case-cohort approach⁷. Of the 5,000 subcohort participants, 3,893 were included in
146 the current analysis after pertinent exclusions (supplementary methods).

147 **Assessment of dietary factors**

148 In EPIC, consumption of foods over the last 12 months was assessed at baseline using
149 validated country-specific food questionnaires⁶. In total, 92 dietary factors (63 foods and 29
150 nutrients) were included in the current analysis.

151 In NLCS, information on dietary intake over the preceding 12 months was assessed at
152 baseline using a semi-quantitative 150-item food frequency questionnaire, which has been
153 validated and tested for reproducibility⁸ (supplementary methods).

154 **Identification of colorectal cancer cases**

155 In EPIC and NLCS, incident CRC cases were identified by record linkage with
156 population-based cancer registries or a combination of registries, insurance records and active
157 follow up of the study participants or their relatives. More details are provided in the
158 supplementary methods.

159 **Statistical analyses**

160 In EPIC, separate Cox proportional hazards regression models were used to investigate
161 the associations between each of the dietary factors with CRC risk. In NLCS, given the case-
162 cohort design, Prentice weighted Cox proportional hazards regression models with robust
163 standard error estimation were implemented⁹. All of the models were adjusted for: total energy
164 intake; smoking; body mass index (BMI); physical activity; diabetes history; level of education

165 and family history of CRC (in NLCS only) and further stratified by sex, age and in EPIC also
166 by centre.

167 To account for multiple comparisons, the false discovery rate (FDR) adjusted *P* values
168 (or *q* values) were estimated for each association analysed¹⁰. The dietary factors with an FDR
169 less than 0.05 were subsequently selected for replication in NLCS, and fixed-effects meta-
170 analysis was performed to combine the results from the two cohorts when heterogeneity was
171 low or moderate (*P* value for heterogeneity >0.1 and/or $I^2 \leq 50\%$). To further investigate the
172 robustness of the associations that were replicated in NLCS, a mutual adjustment model was
173 used. Presence of non-linear associations was investigated using restricted cubic spline models.
174 More details on the statistical analyses methods are provided in the supplementary methods.

175

176 **Results**

177 **Study characteristics**

178 After a mean follow up of 14.1 (SD:3.9) years, a total of 5,069 (56.8% in women)
179 incident malignant CRC cases were identified among the 386,792 included EPIC participants,
180 of which 3,143 were identified as colon (1,495 proximal; 1,435 distal; 213 unspecified) and
181 1,715 as rectal cancers. In NLCS, 3,765 cases (42.8% female) with incident and
182 microscopically confirmed CRC were included in the present analysis, of which 2,612 were
183 colon (1,348 proximal; 1,187 distal) and 801 were rectal cancers.

184 The main baseline characteristics of the study participants are shown in **Supplementary**
185 **Table 1**. In EPIC, approximately 30% of the participants were men, and 47% were overweight
186 or obese. About 50% of the participants were never smokers, and 47% were physically active.
187 More than half of the NLCS subcohort participants were male (54%), 47% were overweight or
188 obese, one third (33%) were never smokers, and 48% spent more than 60 minutes/day on non-
189 occupational physical activities.

190 **DWAS in EPIC**

191 Of the 92 dietary factors that were examined in EPIC, 20 were associated with CRC
192 risk (FDR<0.05) (**Figure 1; Supplementary Table 2**). Higher intakes of alcohol, liquor/spirits,
193 wine, beer/cider, soft drinks, and pork were positively associated with CRC, whereas higher
194 milk, cheese, calcium, phosphorus, magnesium, potassium, riboflavin, vitamin B6, beta-
195 carotene, fruit, fibre, non-white bread, banana, and total protein intakes were associated with a
196 lower CRC risk.

197 After conducting the analysis by tumour subsite, evidence of heterogeneity between
198 colon and rectal cancer was observed for intakes of magnesium, potassium, vitamin B6 and
199 banana, with associations being inverse for colon cancer and null for rectal cancer
200 (**Supplementary Table 3**). Regarding proximal versus distal colon subsites, only total alcohol
201 and wine had heterogeneous results, whereby the associations were positive only for distal
202 colon cancer (**Supplementary Table 4**). Additionally, heterogeneous associations were
203 observed by sex, for total alcohol and spirits, magnesium, fibre, and non-white bread, where
204 the associations were only observed in men (**Supplementary Table 5**). When we investigated
205 the association of red and processed meat with CRC risk by follow-up duration, a trend towards
206 smaller HRs was observed as follow-up increased (**Supplementary Figure 1**). There was some
207 evidence for non-linearity (P value = 0.028) in the association of alcohol intake and CRC risk
208 (**Supplementary Figure 2**).

209 **Replication analysis in NLCS**

210 Of the 20 associations with an FDR<0.05 in EPIC, four associations reached nominal
211 statistical significance (P value < 0.05) in the NLCS cohort in the analysis for CRC (***Figure 2;***
212 **Supplementary Table 6**), namely alcohol and liquor/spirits (positively), milk and calcium
213 intake (inversely). An additional four associations, namely phosphorus, magnesium, riboflavin

214 and total protein, had a borderline inverse association in NLCS, and the point estimates were
215 almost identical to the ones calculated in EPIC.

216 In a separate analysis by tumour subsite in the NLCS, we found that most associations
217 were consistent across the different subsites with heterogeneous associations only evident for
218 phosphorus, potassium, vitamin B6, beta-carotene and total protein in the analysis for colon
219 versus rectal cancer (*Supplementary Tables 7-8*). Little heterogeneity was observed by sex for
220 CRC risk (*Supplementary Table 9*).

221 **Meta-Analysis of EPIC and NLCS**

222 The associations for most of the 20 dietary variables with CRC risk were homogeneous
223 between EPIC and NLCS, except for soft drinks, vitamin B6, fruit, fibre, non-white bread,
224 banana, and pork (P value for heterogeneity < 0.1 and/or $I^2 > 50\%$), where the associations were
225 null in NLCS and therefore a meta-analysis was not performed. The remaining 13 associations
226 yielded a nominally significant summary finding: alcohol (HR per 1 SD increment in intake
227 per day: 1.07; 95% CIs: 1.04-1.09), liquor/spirits (1.04; 1.02-1.06), wine (1.04; 1.02-1.07),
228 beer/cider (1.06; 1.04-1.08), milk (0.95; 0.93-0.98), cheese (0.96; 0.94-0.99), calcium (0.93;
229 0.90-0.95), phosphorus (0.92; 0.90-0.95), magnesium (0.95; 0.92-0.98), potassium (0.96; 0.94-
230 0.99), riboflavin (0.94; .92-0.97), beta-carotene (0.96; 0.93-0.98), and total protein (0.94; 0.92-
231 0.97) (*Figure 2; Supplementary Table 6*).

232 **Pairwise correlations and Mutual-adjustment analysis**

233 Most of the pair-wise correlation coefficients for the 20 FDR-significant foods/nutrients
234 in EPIC were weak and ranged from -0.25 to 0.79 (*Figure 3*).

235 When alcohol, milk, cheese, calcium, phosphorus, magnesium, potassium, riboflavin,
236 beta-carotene and total protein were included in a single multivariable-adjusted model in EPIC,

237 only alcohol remained significantly associated with CRC risk (1.05; 1.03-1.11) Supplementary
238 Table 10).

239 **Gene-Nutrient interaction analysis**

240 Of the 73×20 gene-nutrient multiplicative interactions that were tested, using the
241 Bonferroni adjusted *P* value threshold of 3.4×10^{-5} , no interaction remained significant
242 (Supplementary Table 11).

243 **Discussion**

244 We used the DWAS approach to systematically evaluate the association between
245 dietary intakes of 92 foods and nutrients and risk of CRC in EPIC and NLCS. We confirmed
246 well-described associations in the literature for alcoholic beverages (positive), milk and
247 calcium (inverse) with risk of CRC. In addition, our analysis showed that higher intakes of
248 phosphorus, magnesium, potassium, riboflavin, beta-carotene, and total protein were
249 associated with a lower risk of CRC.

250 **Alcohol** consumption was positively associated with risk of CRC in EPIC and NLCS,
251 and this association was not different between colon and rectal cancer subsites or by type of
252 alcoholic beverage. In agreement, the WCRF third Expert Report has graded the quality of this
253 evidence as strong¹¹. Persons with higher total alcohol consumption had a higher risk of CRC
254 (summary HR per SD increment in intake/day: 1.07; 1.04-1.09), colon, and rectal cancer in the
255 meta-analysis of EPIC and NLCS. When we evaluated this association by proximal vs. distal
256 colon cancer and by sex, we found heterogeneous associations in EPIC, with associations only
257 present for distal colon cancer and in men, but these findings were not confirmed in NLCS.
258 The majority of the literature agrees that the positive association of alcohol consumption with
259 CRC risk is consistent by anatomical subsite and sex^{12, 13}. Acetaldehyde, as a metabolite of
260 ethanol oxidation, can be carcinogenic in colonocytes¹⁴. Mendelian randomization (MR)

261 studies have failed to demonstrate an association between genetically proxied alcohol
262 consumption and CRC risk, but this analysis was underpowered to detect relatively small
263 effects¹⁵.

264 Our study also confirmed the inverse association between intake of dairy products and
265 calcium with risk of CRC, where individuals with higher calcium consumption had a 7% lower
266 risk of CRC per 334.5 mg increment in intake/day. One of the most prominent mechanisms by
267 which calcium is thought to act to reduce CRC risk is by its ability to bind unconjugated bile
268 acids and free fatty acids, diminishing their potential toxic effects on the colorectum¹⁶..
269 Heterogeneity by anatomical subsite or sex was not observed, in agreement with the WCRF
270 meta-analysis and a more recent publication in the Nurses' Health Study^{11, 12}. Dairy products
271 are also a rich source of **phosphorus**, which was also inversely associated with CRC risk in
272 our study but has been infrequently studied in other publications. A previous analysis of
273 nutrient patterns in EPIC identified a pattern characterised by total protein, riboflavin,
274 phosphorus and calcium that was associated with a 4% decreased CRC risk¹⁷. All these
275 nutrients were analysed independently in our analysis and yielded inverse associations in EPIC
276 that were robust after correcting for multiple testing and were replicated in NLCS. Since several
277 of these nutrients share common sources of intake, a correlation of approximately 0.50-0.70
278 was observed in EPIC, which makes it challenging to distinguish their independent effects¹⁸.
279 Evidence from MR studies suggests that genetically proxied milk consumption is associated
280 with a reduced CRC risk, but failed to demonstrate an association for genetically proxied
281 calcium or phosphorus concentrations^{19,20}. Additionally, although previous RCTs have showed
282 null associations for calcium supplementation in relation to CRC risk, a 13% decreased risk of
283 colorectal adenoma recurrence has been reported in a meta-analysis of four RCTs, with daily
284 doses of calcium ranging from 1200 to 2000 mg²¹.

285 Many studies have investigated the association between **red meat or processed meat**
286 consumption and risk of CRC. A dose-response meta-analysis by the WCRF third Expert
287 Report concluded that there is strong evidence that consuming red meat (including beef, pork,
288 lamb and goat from domesticated animals) or processed meat (meat preserved by smoking,
289 curing, salting or addition of chemical preservatives) increases the risk of CRC by 12% per 100
290 gram/day increment for red meat and 16% per 50 gram/day for processed meat⁴. A combination
291 of mechanisms may contribute to the higher risk of colorectal tumourigenesis among
292 individuals consuming larger amounts of red and/or processed meat. Cooking meat at high
293 temperatures may lead to the formation of heterocyclic amines (HCA) and polycyclic aromatic
294 hydrocarbons (PAHs), which have been associated with colorectal carcinogenesis in
295 experimental studies²². Red meat also contains haem iron at high levels that may stimulate the
296 endogenous formation of carcinogenic N-nitroso compounds, which promote colorectal
297 tumourigenesis²³. Additionally, processed meat can be an exogenous source of N-nitroso
298 compounds. Although accumulated evidence supports that higher intakes of red or processed
299 meat are associated with higher risk of CRC, these findings were not replicated in our analysis
300 in EPIC (HR per 36.2 grams of red meat intake daily: 1.02; 0.98-1.05; HR per 31.5 grams of
301 processed meat intake daily: 1.04; 1.00-1.08). An earlier report from EPIC in 2005, with a
302 mean follow-up of 4.8 years and 1,329 incident CRC cases, observed a positive association
303 between red and processed meat consumption with CRC risk²⁴. A potential reason for this
304 discrepancy is that EPIC, as most other cohorts, has assessed meat consumption only during
305 recruitment in the 1990s; thus, the current analysis assumes that consumption has stayed stable
306 over two decades. However, a notable decrease in bovine meat consumption between 2000 and
307 2013 has been noticed in Europe, which was accompanied by an analogous increase in cheese,
308 fish, dairy and poultry consumption. In the current paper, we observed a trend towards smaller
309 HRs in the association of red and processed meat with CRC risk as follow-up increased. A

310 recent time-varying exposure analysis in the Nurses' Health Study and the Health Professionals
311 Follow-up Study showed that a decrease in red meat consumption and simultaneous increases
312 in healthy alternative food choices over time were associated with a lower risk of all-cause
313 mortality²⁵.

314 The current DWAS study observed an inverse association of **magnesium** intake with
315 risk of CRC, which agreed with the results of a recent meta-analysis of seven observational
316 studies²⁶. One purported mechanism by which magnesium may be implicated in lower CRC
317 risk is by its potential to inhibit *c-myc* oncogene expression in colon cancer cells²⁷.
318 Furthermore, magnesium has been shown to improve insulin sensitivity and lower plasma
319 insulin concentrations, which may have an impact on CRC development²⁸. No association of
320 genetically proxied circulating concentrations of **magnesium** was found in a recent MR study²⁰

321 We also observed an inverse association between intake of **beta-carotene** and risk of
322 CRC, but few other studies have investigated this association²⁹. Our findings agree with a
323 previous report from EPIC in 2014²⁹. However, a cohort analysis in the Alpha-Tocopherol,
324 Beta-Carotene Cancer Prevention (ATBC) trial, comprising of 26,951 middle-aged male
325 smokers, showed no association between dietary beta-carotene and risk of CRC³⁰. No evidence
326 of association between genetically proxied circulating concentrations of **beta-carotene** and
327 CRC have been reported in MR studies²⁰.

328 Vitamins B2 and B6 are among the micronutrients that play a pivotal role in one-carbon
329 metabolism, which has been related to carcinogenesis because of its involvement in the
330 synthesis of purines and pyrimidines for subsequent DNA synthesis and in the synthesis of
331 methionine for DNA methylation³¹. Inverse associations between **riboflavin** (vitamin B2) and
332 **vitamin B6** intake and CRC risk were observed in EPIC, but only the association with
333 riboflavin was replicated in the NLCS. Previous studies on the association between riboflavin
334 intake and CRC risk are scarce³². Results from the Women's Health Initiative Observational

335 Study indicated a 25% decreased CRC risk for the highest compared to the lowest quartile of
336 total riboflavin intake, but was not statistically significant when only dietary intake of
337 riboflavin was considered³². A meta-analysis of eight studies did not show an association
338 between vitamin B6 intake and CRC risk, but blood levels of its active form, pyridoxal 5'-
339 phosphate, were associated with lower CRC risk³³.

340 Little is known on the role that **potassium** may play in relation to CRC risk, and
341 epidemiological evidence thus far is limited³⁴. We cannot rule out the possibility that the
342 inverse association observed in our study may mirror the effect of other nutrients, such as
343 vitamin B6 or dietary fibre, which share common dietary sources with potassium.

344 **Strengths** of this study include its large size and long follow-up duration and the
345 DWAS approach that involved a comprehensive assessment of foods and nutrients whilst
346 accounting for multiplicity of tests and replication of findings in an independent cohort.
347 Another strength was the ability to explore associations according to different anatomical
348 subsites as well as by sex. The primary **limitation** was that the analysis relied on a single dietary
349 assessment at recruitment, not allowing to capture potential changes in dietary habits over time.
350 In addition, intercorrelations between dietary exposures and overall dietary patterns were not
351 accounted for. Intercorrelations between dietary exposures may have led to low precision of
352 the estimates, even though variance inflation factors were relatively small, which might explain
353 that none of the dietary factors remained in the multivariable adjusted model. Furthermore, it
354 is possible that there might be an association for foods or nutrients that were not included in
355 this analysis. Additionally, the data derived from the Dutch food composition table were not
356 checked against the use of ENDB for nutrient calculation, so discrepancies may have occurred,
357 hence it is possible that some of the discrepancies observed between the two cohorts for some
358 dietary exposures, are in part due to poor reproducibility in measurements. Among the
359 exposures for which heterogeneity was observed between EPIC and NLCS, correlation

360 between the baseline FFQs and 24-hour diet recalls has been reported to be good for fruit, fibre,
361 vitamin B6 and beverage consumption in NLCS and fairly good for fibre and fruit across most
362 EPIC centres, but for exposures like non-white bread or vitamin B6 no information was
363 available^{8, 35}. Finally, we cannot exclude the possibility of residual confounding, although we
364 adjusted for several potential confounders.

365 In **conclusion**, our study confirmed the well-established positive association for alcohol
366 consumption and inverse association for dairy products and calcium intake with CRC risk. The
367 study further suggested that higher intakes of magnesium, phosphorus, potassium, riboflavin,
368 beta-carotene and total protein are associated with lower CRC risk.

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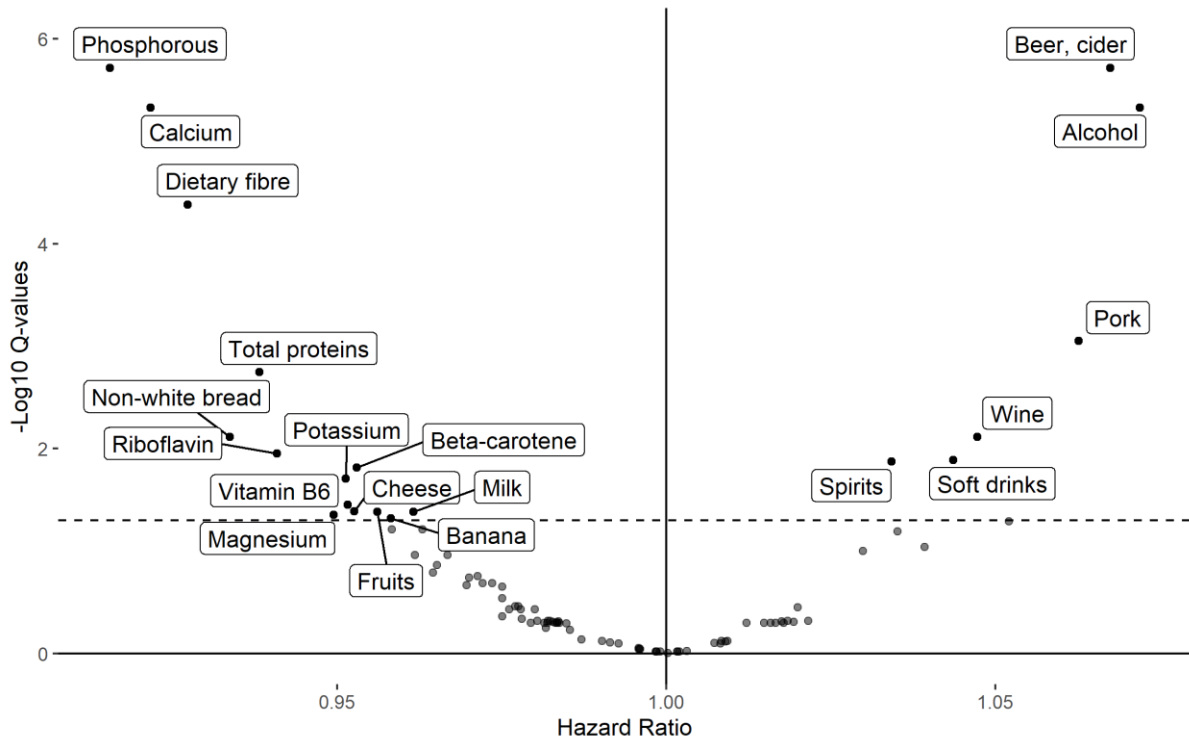
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397 views of the International Agency for Research on Cancer / World Health Organization.

398 **Data availability:** The EPIC study data can be accessed via an application to the EPIC
399 Steering Committee (<https://epic.iarc.fr/access/index.php>). Further information is available
400 from the corresponding author upon request.

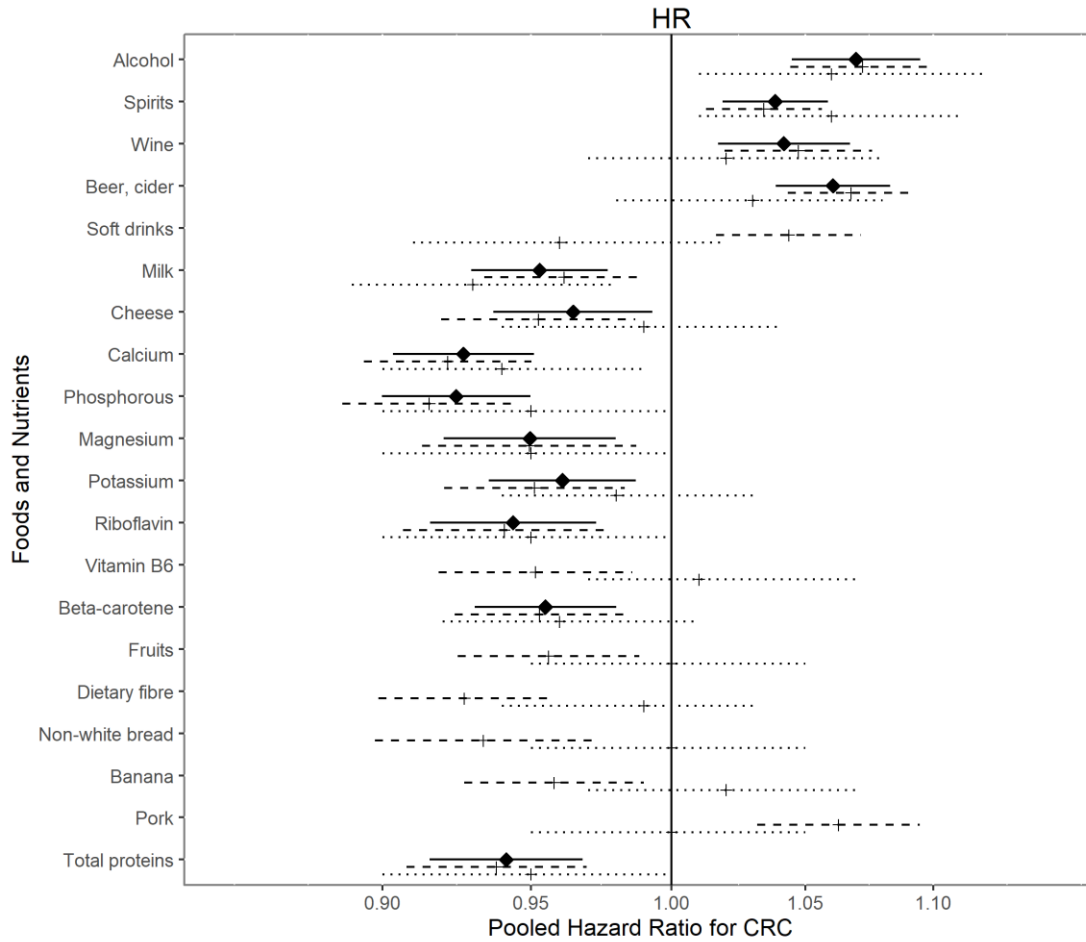
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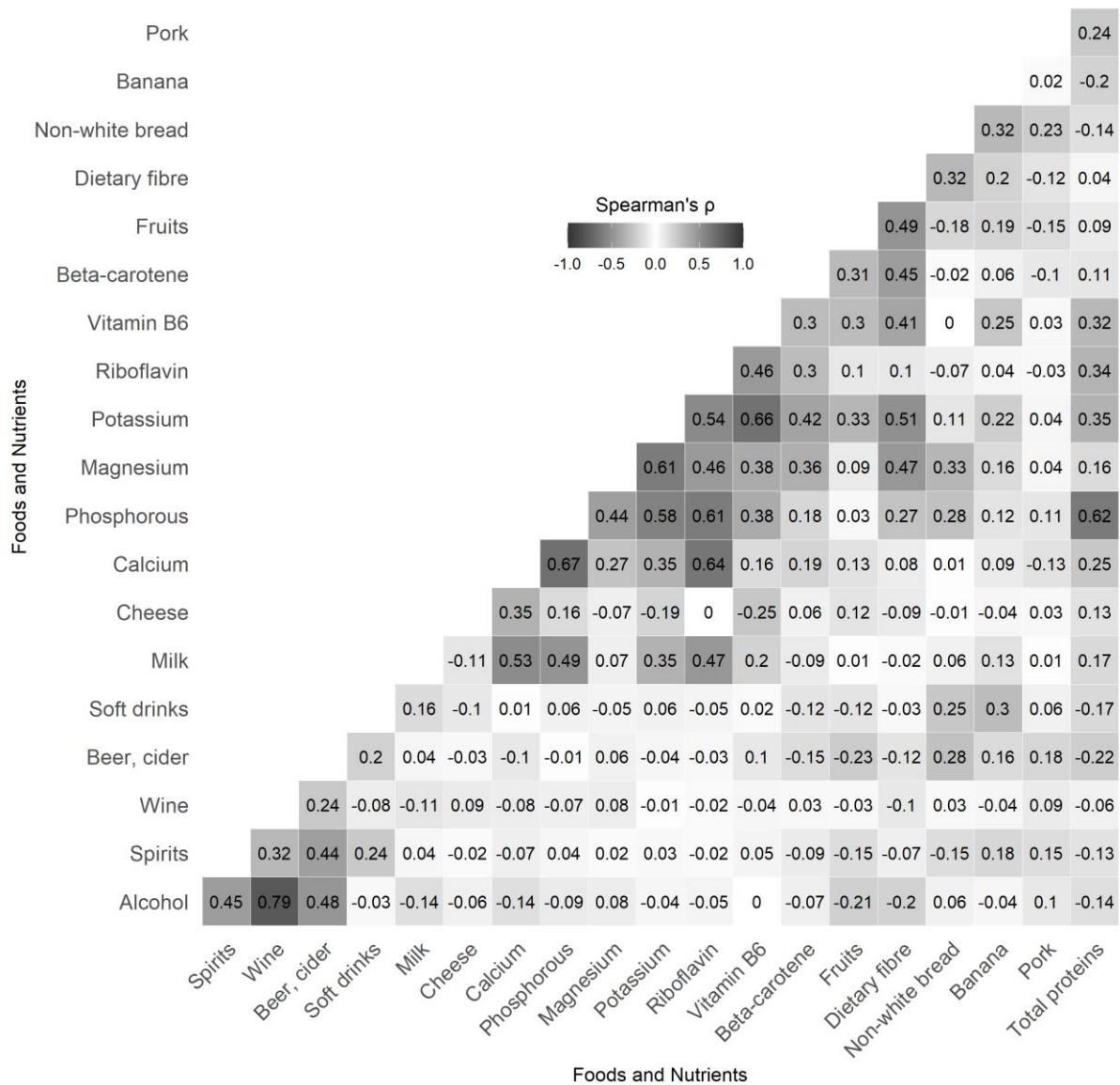
504

505 **Figure 1. Volcano plot showing results from the DWAS regarding the association between**
 506 **92 dietary factors and CRC risk in EPIC.** The Y-axis shows the false discovery rate (FDR)
 507 adjusted *P* values in $-\log_{10}$ scale from the Cox proportional hazards models for each dietary
 508 factor. The X-axis shows the estimated HR for each dietary factor per 1 SD increase in daily
 509 consumption. The dashed horizontal line represents the level of significance corresponding to
 510 FDR of 5%.



511

512 **Figure 2. Forest plot showing the hazard ratios and 95% confidence intervals for the 20**
 513 **FDR significant associations (FDR<5%), in EPIC (---) and NLCS (···), as well as the**
 514 **results from a meta-analysis (MA) (—). The X-axis shows the estimated HR for each dietary**
 515 **factor for 1 SD increase in daily consumption. The diamond and the solid line represent the**
 516 **pooled HR and 95%CI of the MA. MA was not performed when heterogeneity was high.**



517

518 **Figure 3.** Pairwise partial correlation coefficients (Spearman's ρ) of the 20 FDR-significant
 519 foods/nutrients in EPIC, adjusting for age, sex and centre.