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Soluble receptor for advanced glycation end-products (sRAGE) and colorectal cancer risk

A case-control study nested within a European prospective cohort

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1 **Soluble Receptor for Advanced Glycation End-products (sRAGE) and colorectal cancer risk: a**

2 **case-control study nested within a European prospective cohort**

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2

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75   **Abbreviations used:** ADAM10, A Disintegrin And Metalloproteinase Domain 10; AGE, advanced

76   glycation end-products; AGER, Advanced Glycosylation End-Product Specific Receptor ; BMI, body

77   mass index; CRC, colorectal cancer; CRP, C-reactive protein; CV, coefficients of variation; GLO1,

78   Glyoxalase I; EPIC, European Prospective Investigation into Cancer and Nutrition; IARC,

79   International Agency for Research on Cancer; mRNA, messenger ribonucleic acid ; NF-κB, nuclear

80   factor kappa B; OR, odds ratio; RAGE, receptor for AGE; RNF5, Ring Finger Protein 5 ; SD,

81   standard deviation; SNP, single nucleotide polymorphism; sRAGE, soluble receptor for AGE; TNFα,

82   tumor necrosis factor alpha; WC, wait circumference; WHR, waist-to-hip ratio

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85   **Data sharing statement:** For information on how to submit an application for gaining access to EPIC

86   data and/or biospecimens, please follow the instructions at http://epic.iarc.fr/access/index.php

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98   **Abstract**

99   **Background:** Overexpression of the Receptor for Advanced Glycation End-product (RAGE) has

100   been associated with chronic inflammation, which in turn has been associated with increased

101   colorectal cancer (CRC) risk. Soluble RAGE (sRAGE) competes with RAGE to bind its ligands, thus

102   potentially preventing RAGE-induced inflammation.

103

104   **Methods:** To investigate whether sRAGE and related genetic variants are associated with CRC risk,

105   we conducted a nested case-control study in the European Prospective Investigation into Cancer and

106   Nutrition (EPIC). Plasma sRAGE concentrations were measured by ELISA in 1,361 CRC matched

107   case-control sets. Twenty-four single nucleotide polymorphisms (SNPs) encoded in the genes

108   associated with sRAGE concentrations were available for 1,985 CRC cases and 2,220 controls.

109   Multivariable-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were computed using

110   conditional and unconditional logistic regression for CRC risk and circulating sRAGE and SNPs,

111   respectively.

112

113   **Results:** Higher sRAGE concentrations were inversely associated with CRC ($OR_{Q5vs.Q1}$=0.77,

114   95%CI=0.59-1.00). Sex-specific analyses revealed that the observed inverse risk association was

115   restricted to men ($OR_{Q5vs.Q1}$=0.63, 95%CI=0.42-0.94) whereas no association was observed in women

116   ($OR_{Q5vs.Q1}$=1.00, 95%CI=0.68-1.48, $P_{heterogeneity}$ for sex=*0.006*). Participants carrying minor allele of

117   rs653765 (promoter region of *ADAM10*) had lower CRC risk (C vs. T, OR=0.90; 95%CI=0.82-0.99).

118

119   **Conclusion**: Pre-diagnostic sRAGE concentrations were inversely associated with CRC risk in men

120   but not in women. A SNP located within *ADAM10* gene pertaining to RAGE shedding, was associated

121   with CRC risk.

122

123   **Impact:** Further studies are needed to confirm our observed sex difference in the association and

124   better explore the potential involvement of genetic variants of sRAGE in CRC development.

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4

Introduction

Advanced glycation end-products (AGEs) are a heterogeneous group of molecules formed by non-enzymatic reactions between reducing sugars and proteins, lipids or nucleic acids (1). AGEs are produced endogenously, but diet and lifestyle are likely the largest contributors to the overall AGEs pool particularly from high-temperature processed food products which contain high amounts of AGEs and/or their precursors (2-4). Glycated proteins tend to become dysfunctional and agglutinate with other reacting molecules to create cross-links and aggregates which can accumulate within diverse tissues in the body (5). The accumulation of AGEs throughout the life course is thought to contribute to intracellular signalling alterations, chronic low-level inflammation and a decrease in tissue functionality (6).

AGEs are recognized by a multi-ligand cell-surface protein receptor, known as the Receptor for Advanced Glycation End-products (RAGE). RAGE consists of an extracellular N-terminal, a transmembrane helix, and an intracellular C-terminal tail (7). RAGE is expressed at low levels in most tissue types except the lung in which the expression is generally high (8). Overexpression of RAGE and its high activity have been demonstrated in various cancers including in the colon, breast, brain, prostate and in the ovaries (9). Binding of AGEs to their receptor triggers a signalling cascade leading to intracellular inflammation with activation of nuclear factor kappa B (NF-κB), increased secretion of cytokines and chemokines, and elevated production of reactive oxygen and nitrogen species (10).

Soluble RAGE (sRAGE) is a free circulating isoform of RAGE that also binds AGEs and acts as a decoy for RAGE. In contrast to RAGE, binding of AGEs to sRAGE does not induce inflammation and oxidative stress (8). Although the concentration of sRAGE is likely insufficient to bind all circulating AGEs (11), higher sRAGE levels had been associated with low inflammation and lower risk of several chronic diseases, including cancers (12). The variability in sRAGE concentrations is considerably affected by a combination of genetic and environmental factors (13). sRAGE levels have been reported to be elevated in women *vs.* men, younger *vs.* older individuals, and individuals with normal weight *vs.* with overweight and obesity (14-17). Furthermore, genetic determinants of sRAGE expression have also been identified and include single nucleotide polymorphisms (SNPs) located within Advanced Glycosylation End-Product Specific Receptor (*AGER*), A Disintegrin And Metalloproteinase Domain 10 (*ADAM10*), Glyoxalase I (*GLO1*), and Ring Finger Protein 5 (*RNF5*) genes (17-21).

We hypothesised that higher circulating sRAGE levels are inversely associated with colorectal cancer (CRC) development. Previously, only two prospective studies have investigated the association, and showed an inverse association of high sRAGE concentrations with CRC risk among Finnish male smokers (22) and women with overweight and obesity (23). However, there is sparse data from other prospective studies, and there is a need to carefully investigate possible differences in the association by sex or lifestyle factors. To address these gaps, we studied the association between pre-diagnostic levels of circulating sRAGE and risk of CRC in a large, multinational European

166   prospective cohort. We also investigated whether SNPs, reported to be related to sRAGE levels or

167   RAGE function, are associated with CRC risk.

168

169

170   **Materials and methods**

171   Study population and data collection

172   We used a case-control design nested within the European Prospective Investigation into Cancer and

173   Nutrition (EPIC) cohort. EPIC is an ongoing multicentre prospective cohort with 521,324 participants

174   (70% women) recruited from 23 study centres located in 10 European countries (Denmark, France,

175   Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden, and the United Kingdom). The

176   rationale and methods of the EPIC study, including information on the recruitment of the participants

177   as well as data collection have been described previously (24). Participants gave written informed

178   consent before joining the EPIC study. Participant's health history, anthropometry, socio-

179   demographic and standardised lifestyle variables including education, smoking, and physical activity

180   were collected by questionnaire at baseline, prior to disease onset or diagnosis. Physical activity was

181   based on the Cambridge physical activity index: inactive (sedentary job and no recreational activity),

182   moderately inactive (sedentary job with <0.5 h recreational activity per day/or standing job with no

183   recreational activity), moderately active (sedentary job with 0.5 to 1 h recreational activity per day/ or

184   standing job with 0.5 h recreational activity per day/ or physical job with no recreational activity) or

185   active (sedentary job with >1 h recreational activity per day/or standing job with >0.5 h recreational

186   activity per day/or physical job with at least some recreational activity/or heavy manual job) (25).

187   Dietary intake was assessed at recruitment by validated centre-specific questionnaires. In each of the

188   study centres, blood samples were drawn at recruitment (≈80% of participants provided blood

189   samples) and stored in liquid nitrogen (−196°C, liquid nitrogen) at the International Agency for

190   Research on Cancer (IARC) biobank, or in local biobanks (at -150°C in nitrogen vapour in Denmark;

191   -80°C freezers at Malmö and Umeå centres in Sweden) (24).

192

193   Follow-up for cancer incidence and vital status

194   Vital status follow-up (98.4% complete) is collected by record linkage with regional and/or national

195   mortality registries in all countries except Germany and Greece, and the Italian centre of Naples,

196   where data are collected actively. Incident cancer cases were identified through record linkage with

197   regional cancer registries or using a combination of methods, including health insurance records,

198   cancer and pathology registries, and active follow-up through participants and their relatives. CRC

199   cases were eligible if they were first incident and histologically-confirmed. Cases were defined using

200   the International Classification of Diseases for Oncology (ICD-O). Colon cancers were defined as

201   tumours that occurred in the cecum, appendix, ascending colon, hepatic flexure, transverse colon,

202   splenic flexure, descending and sigmoid colon (C18.0-C18.7), and overlapping and or unspecified

203   origin tumours (C18.8 and C18.9). Rectal cancers were defined as tumours that occurred at the recto-

204   sigmoid junction (C19) or rectum (C20). Cancers of the anal canal were excluded.

205

206   Case-control design

207   From baseline onwards, 1,413 first incident CRC cases with available blood samples were identified

208   (until June 2003 as endpoint) among all the total 2,476 CRC cases ascertained **(Figure 1)**. For each

209   identified case, one control was matched by incidence density sampling from all cohort members alive

210   and cancer-free at the time of diagnosis of the index case. Cases and controls were matched by age

211   (±1 year), sex, centre, and blood collection details including time (±3 hours), fasting pre-venepuncture

212   (<3, 3-6, and >6 hours); and additionally among women only, by menopausal status (pre-, peri-, and

213   postmenopausal), and hormone replacement therapy (HRT) use at the time of blood collection

214   (yes/no).   After exclusion of participants with incomplete matched case sets (n=16), those with

215   extreme sRAGE levels (n=3 controls and 1 case with sRAGE concentrations unusually high i.e.

216   >mean+4 standard deviation), and 32 cases and matched controls from Greece due to unforeseen data

217   restriction issues, 1,361 cases and 1,361 matched controls were included in the sRAGE analysis.

218   Among EPIC participants, 4,487 participants (until December 2012 as endpoint, 2,148 CRC cases and

219   matched 2,339 controls) have been previously genotyped. After exclusion of 100 CRC cases and 100

220   matched controls from Greece, and 82 participants with missing lifestyle variable, 1,985 CRC cases

221   and 2,220 matched controls were included in the genetic analysis. Among the participants who have

222   been genotyped, 972 CRC cases and 767 non-cases overlap with case-control sets in whom sRAGE

223   measurements were conducted.

224

225   Laboratory analyses

226   Circulating sRAGE concentrations were measured in citrated plasma samples by ELISA (Quantikine,

227   R&D Systems, MN, USA), following the manufacturer's instructions. Previous studies have reported

228   that sRAGE is stable in plasma over a long period of time (26). Analyses were run with case-control

229   sets randomized across batches (n=40 batches, with an average of 35 case-control pairs analysed per

230   batch). Intra- and inter-batch coefficients of variation (CV) were assessed by measuring 3 different

231   samples used as quality controls in duplicate in each. Mean intra- and inter-batch CVs were 1.25%

232   and 6.0%, respectively. C-reactive protein (CRP) concentrations were determined using a high-

233   sensitivity assay (Beckman-Coulter, Woerden, The Netherlands).

234

235   DNA genotyping and genetic variants selection

236   DNA was extracted from buffy coats from citrated blood samples at the Center for Inherited Disease

237   Research (CIDR, Johns Hopkins University) using the HumanOmniExpressExome-8v1–2 array as

238   described elsewhere (27). All SNPs met criteria for quality control for genotyping call rate (above

239   95%). Candidate SNPs selected for our study were those previously associated with sRAGE levels.

240   Most of these SNPs appear to be located within the *AGER* gene, with rs2070600 being the most

241   important and explaining 22% of the variability in sRAGE concentrations in Caucasians (17). In

242   addition to *AGER*, four additional genes contain SNPs associated with sRAGE: *RNF5*, a neighbouring

8

243 gene which encodes for RAGE (28), *ADAM10* encodes for metalloproteinases involved in the

244 shedding of RAGE ectodomain to form sRAGE (29), and *GLO1* encodes for glyoxalase enzyme

245 responsible to metabolise methylglyoxal and prevent aberrant AGEs formation (30). The main SNPs

246 are from *AGER* (rs2070600, rs1800625, rs1800624, rs184003, rs2854050), *ADAM10* (rs653765) and

247 *RNF5* (rs9469089) (17-21,31-38). We additionally considered less-studied SNPs located within

248 *AGER* (rs1035798, rs1800684, rs3131300, rs3134940, rs2269422, rs2853807, rs9391855,

249 rs17846798), *ADAM10* (rs514049), *RNF5* (rs57409105, rs41268928, rs17493811), and *GLO1*

250 (rs4746, rs1130534, rs1049346, rs6932648, rs10484854). The choice of this supplementary group of

251 SNPs was based on the potential influence and interactions they may have in modulating sRAGE

252 levels directly or through AGEs (13,17,21,31,39-41).

253 Genotype distributions were in Hardy-Weinberg equilibrium (cutoff of *P*-value=1×10^{-3}) for

254 all the SNPs considered, with the exception of rs6932648 which was consequently excluded from the

255 analysis. The selected SNPs and their characteristics are detailed in **Supplementary Table 1**. To

256 select the independent variants, Linkage Disequilibrium (LD) pruning (LD≤1%) was performed using

257 NCI LDlink tools (https://ldlink.nci.nih.gov). We found the following independent variants (highly

258 correlated variants are in brackets): rs2070600 (rs41268928, rs9391855, rs2854050), rs1800625

259 (rs3131300, rs3134940), rs1800624 (rs17846798), rs4746 (rs1130534, rs10484854), rs17846798

260 (rs57409105), rs9469089, rs1800684, rs2269422, rs2853807, rs1049346, rs17493811, and rs653765

261 (rs514049). A flowchart outlining the selection of the independent SNPs is detailed in

262 **Supplementary Figure 1**.

263 Among the 767 control subjects who had both sRAGE and genetic data, we assessed the

264 association between the independent genetic variants and log-transformed sRAGE levels using linear

265 regression models (**Supplementary Table 2**). The SNPs in the following genes were significantly

266 associated with sRAGE levels: *AGER* (rs2070600, rs1800625), *RNF5* (rs9469089), and *GLO1*

267 (rs4746). Although rs653765 (*ADAM10*) was not associated with sRAGE levels, we decided to

268 conserve it in our analysis for two main reasons: first, as a major variant of metalloproteinases which

269 are involved in the shedding of the ectodomain of RAGE to produce sRAGE; second, this variant was

270 previously associated with sRAGE levels in other populations (21). Overall, five SNPs (rs2070600,

271 rs1800625, rs9469089, rs4746, rs653765) were examined for the association with CRC risk.

272

273 Statistical analysis

274 Case-control differences in baseline characteristics were evaluated using Student's paired t-test and

275 Wilcoxon's signed-rank test for continuous variables and Kruskal–Wallis test for categorical

276 variables. Spearman rank correlation was used to correlate sRAGE levels to anthropometry, dietary

277 intakes and other biomarkers. We divided sRAGE concentrations into quintiles based on the

278 distribution in the control group. Conditional logistic regression was used to compute odds ratios

279 (ORs) and 95% confidence intervals (CIs) for the associations between circulating levels of sRAGE

9

280 and CRC risk. We ran two different models by including for each successive model additional
281 adjustment variables incrementally. Model 1 (crude) was conditioned on the matching factors. Model
282 2 was additionally adjusted for body mass index (BMI), height, education (none, primary, technical
283 and professional, secondary, higher), physical activity (inactive, moderately inactive, moderately
284 active, active), smoking status, duration, and intensity (never; cigarettes/day 1-<=15, 16-<=25, >26;
285 former smokers <=10, 11-<=20, >20 years, occasional), dietary energy, and intakes of alcohol, red
286 and processed meat, dietary fibre, and dairy products. Dietary factors included as adjustment factors
287 have been previously associated with CRC and/or sRAGE levels (42). P-values for the linear trend (P
288 for trend) were obtained by including the median value of each quintile as a continuous variable in the
289 model. We also examined sRAGE levels as a continuous variable, per standard deviation (SD)
290 increment.

291　　　　Stratified analyses were performed by anatomical sub-sites (colon $vs.$ rectal cancers, proximal
292 colon $vs.$ distal colon cancers), sex (men $vs.$ women), age groups (<50, >=50-<55, >=55-<60, >=60-
293 <65, >=65), smoking (never, former, ever), alcohol intake (tertiles), physical activity (inactive,
294 moderately inactive, moderately active, active), BMI (<25, >=25-<30, >=30 kg/m²); and below or
295 above sex-specific recommended cut-offs for waist circumference (WC, men, 94 cm, women, 80 cm)
296 and waist-to-hip ratio (WHR, men, 0.90, women, 0.85), and in women by menopausal status (pre-,
297 post and perimenopause). The cut-offs for WC and WHR were based on the WHO's definitions of
298 central adiposity in European men and women (43). Additional stratified analyses were conducted for
299 CRP (tertiles) as a marker of inflammation. P-values for heterogeneity were calculated using the Wald
300 test. For sub-group analyses by anthropometric measures, individual models were run for BMI, WC
301 and WHR in men and women separately (model 2 without BMI). In sensitivity analyses, we excluded
302 cases diagnosed during the first 2 years of follow-up and rerun the analyses.

303　　　　We assessed the association between the genetic variants and CRC risk using data of all
304 participants genotyped in EPIC to increase the statistical power of the analysis. The associations
305 between the five independent genetic variants and CRC risk were assessed by unconditional logistic
306 regression models. Two models were run, an unadjusted model and a multivariable-adjusted model,
307 adjusted for sex, age, BMI, smoking status, alcohol, and country. Additive (major allele=0,
308 heterozygotous=1, minor allele=2), dominant (major allele=0, heterozygotous+minor allele=1) and
309 recessive models (major allele+ heterozygotous=0, minor allele=1) were run for the genetic variants.
310 In sensitivity analyses, we analysed the participants with overlapping genetic and sRAGE
311 concentrations data. All the statistical analyses were performed using Stata 14.0 (StataCorp, College
312 Station, TX, USA). P-values <0.05 was considered statistically significant.

313

10

314 **Results**

315 Baseline characteristics and sRAGE levels in cases and controls are presented in **Table 1**. Compared

316 to controls, CRC cases have higher BMI, WC, WHR and CRP concentrations, and consume more

317 alcohol and less dairy products and fruit and vegetables. sRAGE concentrations were slightly lower in

318 CRC cases than controls (1086 *versus* 1130 pg/mL) but this was mainly observed among men (982

319 versus 1066 pg/mL in male cases versus controls, respectively); whereas among women sRAGE was

320 1185 pg/mL in cases and 1191 pg/mL in controls. BMI, WC, WHR, and alcohol intake were all

321 negatively correlated with sRAGE levels whereas sugar and confectionaries, fruit and vegetable, and

322 cereals intakes showed positive correlations (**Supplementary Table 3).** Women with higher sRAGE

323 levels have lower CRP concentrations (Spearman rho=-0.156, p=0.004).

324

325 sRAGE and CRC risk

326 sRAGE concentrations were inversely associated with CRC risk in multivariable-adjusted analyses

327 (OR comparing the highest to the lowest quintile $OR_{Q5vs.Q1}$=0.75, 95%CI=0.58-0.98, P_{trend}=0.035,

328 **Table 2**). Sub-group analyses by sex showed an inverse risk association for men ($OR_{Q5vs.Q1}$=0.63,

329 95%CI=0.42-0.94, P_{trend}=0.001) but not in women ($OR_{Q5vs.Q1}$=0.94, 95%CI=0.63-1.38, P_{trend}=0.754;

330 $P_{heterogeneity}$=0.006). In men, sRAGE was associated with a lower risk of both colon cancer (OR per SD

331 increment, OR =0.84, 95%CI=0.70-0.99) and rectal cancer (OR=0.80, 95%CI=0.64-0.99) with no

332 heterogeneity across anatomical subsites ($P_{heterogeneity}$=0.607) (**Table 3**). The magnitude of the inverse

333 association appeared stronger for distal colon cancer (OR=0.61, 95%CI=0.44-0.84) compared to

334 proximal cancer (OR=0.94, 95%CI=0.69-1.29) but no heterogeneity was observed ($P_{heterogeneity}$=0.671).

335 In women, no association was found between sRAGE and colon (OR=0.99, 95%CI=0.85-1.15) or

336 rectal cancer (OR=1.06, 95%CI=0.86-1.32). Stratified analyses by age groups, BMI categories, WC

337 and WHR cut-offs, and smoking status showed no significant differences across strata (**Figure 2**).

338 Women in higher CRP tertiles tended to have higher CRC risk associated with sRAGE ($P_{heterogeneity}$

339 across=0.011) (**Figure 2**).

340

341 Analyses of genetic variants

342 **Table 4** presents the association of the genetic variants with CRC risk. While comparing minor allele

343 *vs.* major allele, rs1800625 (*AGER*, G *vs.* A, OR=1.15, 95%CI=1.02-1.29) was associated with an

344 increased risk of CRC whereas rs653765 (*ADAM10*, C *vs.* T, OR=0.88; 95%CI=0.80-0.97) was

345 associated with a lower CRC risk, in univariate models. After multivariate adjustments, the

346 association remained statistically significant for rs653765 (*ADAM10*, C *vs.* T, OR=0.90; 95%CI=0.82-

347 0.99), but not for rs1800625 (*AGER*, G *vs.* A, OR=1.11, 95%CI=0.99-1.25).

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349 Sensitivity analysis

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350   Exclusion of the cases that occurred within the first two years of follow-up did not change the

351   associations between sRAGE concentrations and CRC (**Table 1**). The associations between SNPs and

352   CRC in participants with overlapping genetic and sRAGE data showed similar, but no statistically

353   significant associations for rs653765 (*ADAM10*, OR=0.90, 95%CI=0.78-1.05) or rs1800625 (*AGER*,

354   G *vs.* A, OR=1.00, 95%CI=0.83-1.19) (**Supplementary Table 4**).

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Discussion

In this large, case-control study nested within a European prospective cohort, we found that pre-diagnostic circulating sRAGE levels were inversely associated with CRC risk in men but not in women. The associations observed between sRAGE and CRC did not vary by age, or by lifestyle factors including obesity and smoking status, suggesting that sex is the main effect modifier in the association between sRAGE and CRC. With respect to the SNP analyses, we found that the minor allele of rs653765 (*ADAM10*) was inversely associated with risk of CRC, whereas an increased risk was suggested for rs1800625 *(AGER)*. However, we did not observe the association between rs653765 and levels of sRAGE.

RAGE is a pattern recognition receptor that recognizes multiple ligands such as S100, high mobility group box 1 protein (HMGB1), amyloid-β peptide, in addition to the AGEs (44). RAGE is overexpressed in several diseases of the colon, including inflammatory bowel diseases (45). RAGE action in colon tissues may participate in CRC tumour initiation, progression and invasion (46-48). sRAGE by acting as a decoy of RAGE, binds to AGEs in the circulation and clears them by decreasing interaction with full-length cell-surface RAGE. The evidence from mouse studies shows that injection of sRAGE is associated with a reduction in the expression of inflammatory mediators such as TNF-α (49). Evidence from case-control studies also shows that elevated sRAGE levels are associated with a lower risk of several cancers including liver (50) and pancreatic cancer (51). This suggests that higher concentrations of sRAGE are protective against AGEs-induced inflammation which is involved in the aetiology of various chronic diseases such as diabetes and cancers, but the mechanisms need further exploration.

The underlying reasons for the observed difference between men and women in the association between sRAGE and CRC risk are unclear. Several previously published studies that compared sRAGE levels between men and women suggest higher circulating levels in women (14,15,17), which we also observed in our study. One explanation of the sex difference in sRAGE levels may be that oestrogens stimulate sRAGE expression and production (52). Oestrogens have also been reported to reduce AGEs production and AGEs-related inflammation (53). In our study, women with higher sRAGE levels have lower CRP concentrations (Spearman rho=-0.156, p=0.004) and lower CRC risk, suggesting that sRAGE may possibly reduce CRC risk in women, by mitigating overall inflammation. However, analysis by menopausal status showed no differences across strata in our study population. Our findings suggest that additional studies are needed to understand the physiological sex differences in sRAGE levels and how they may translate into the differential CRC risk associations that we have observed in this study.

Interestingly, the two previous publications on sRAGE and CRC in prospective cohorts have been conducted in men (22) and in women (23) only. The Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) study reported high serum sRAGE to be associated with low CRC risk in Finnish male smokers (22). We expanded this observation by showing that such an inverse association was

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394   also observed in male never smokers. We expected to observe a greater reduction in CRC risk in non-
395   smokers compared to smokers, but our findings did not differ by smoking status. Smoking may be a
396   source of AGEs exposure (2), but the magnitude of the contribution of smoking to overall AGEs
397   exposures remains to be explored. sRAGE levels have been reported to be higher, lower or unchanged
398   in smokers compared to non-smokers (54-56). It is still unknown whether smoking could induce an
399   adaptive mechanism of sRAGE synthesis to cope with sustained formation of AGEs from glycotoxins
400   contained in cigarettes. In a previous nested case-control study on a subsample of 1,249
401   postmenopausal women in the Women's Health Initiative (WHI) study, higher sRAGE levels were
402   observed to be associated with lower CRC risk in individuals with overweight and obesity, but not
403   among normal weight postmenopausal women (23). Overall, our findings showed that sRAGE levels
404   were associated with an inverse risk of CRC only in men, with no difference in magnitude across
405   smoking status or any other lifestyle factor.

406   We found that rs653765 located within *ADAM10* (C vs. T) was associated with lower risk for
407   CRC. However, rs653765 (*ADAM10*) was not associated with sRAGE levels in our study, in contrast
408   to previous studies in which the minor allele of rs653765 was associated with lower sRAGE levels
409   (21). Another SNP, rs1800625, located in the promoter region of *AGER* is involved in the initiation of
410   the production of the RAGE or its isomers (39). Xu et al. (57) reported in a meta-analysis of 18 case-
411   control genetic studies that the recessive model of rs1800625 was associated with an increase of
412   overall cancer risk, while analysing case-controls studies of 6246 cases of renal, lung, breast, cervical,
413   liver, oral, breast and CRC cancers. Although our findings with genetic variants are intriguing, they
414   may be attributed to the diversity of functions associated with the *AGER* and *ADAM10* genes. The
415   production of sRAGE through the shedding of RAGE is dependant of ADAM10 levels. Thus, the
416   overexpression of *AGER* coupled with lower ADAM10 activity will result in higher transmembrane
417   RAGE and lower circulating sRAGE levels. This suggests that the interactions between AGER and
418   ADAM10 may provide a better understanding of the genetic implications of RAGE and sRAGE in
419   CRC development. In addition, the associations observed with the genetic data could be explained by
420   other functions of the SNPs examined, particularly in the case of ADAM10 when considering its
421   multiple actions such as the formation of amyloid inclusions and the cleavage of a range of proteins
422   (58). We did not observe a significant association between rs2070600 (*AGER*) and CRC, albeit our
423   study showed that the major allele (C allele) of this SNP associates with higher sRAGE levels. A
424   meta-analysis of 15 case-control studies showed that homozygous minor allele of this SNP was
425   associated with an increased risk of all cancers (59). The absence of association of this SNP with CRC
426   may be due to low statistical power, particularly as carriers of the minor allele are rare. Additional
427   studies, using genetic data from larger research consortia, are needed to explore the link between the
428   expression of *AGER*, *ADAM10,* and *RNF5* genes, and levels of sRAGE and CRC initiation and
429   development.

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430   The strengths of our study include the large number of cases and controls, the prospective

431   design and the availability of dietary and lifestyle factors and genetic variants. Our study was,

432   however, limited by the fact that we did not differentiate between endogenous secretory RAGE

433   (esRAGE), and proteolytically cleaved RAGE (cRAGE), the two components of sRAGE. esRAGE is

434   formed by alternative splicing of RAGE mRNA, and cRAGE is produced by the shedding of the

435   ectodomain of RAGE par metalloproteinases located at the surface of the cells. esRAGE is stable

436   throughout the life course whereas cRAGE levels vary with age and with environmental factors (60).

437   Because we have measured the total pool of plasma sRAGE we therefore cannot discern whether the

438   different variants of sRAGE have specific and potentially opposite associations with study outcomes.

439   Although the variability of cRAGE makes it a poor biomarker for a prospective study, cRAGE levels

440   data would have permitted us to explore the association between SNPs from the *ADAM10* gene, levels

441   of cRAGE and CRC risk. Our study was also limited by the fact that lifestyle factors and blood

442   samples were collected at the recruitment, and may not necessarily reflect changes over years.

443   Moreover, we cannot rule out residual confounding or unmeasured confounders such as lifetime

444   history of anti-inflammatory medication use.

445   In conclusion, we observed that pre-diagnostic circulating sRAGE levels were inversely

446   associated with CRC risk in men, but not among women. We also found that the minor allele of

447   rs653765 (*ADAM10*) was inversely associated with CRC risk. Additional studies are, however,

448   required to further investigate how genetic variation and sex may affect sRAGE levels or modify its

449   association with CRC risk.

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698 **Table 1**: Selected baseline demographic and lifestyle characteristics of study participants by
699 colorectal cancer status, EPIC study 1992-2012

	Cases (n=1,361)	Controls (n=1,361)	P-value*
Women, %	51.5	51.7	
Age, years, mean±SD	58.4±7.35	58.3±7.38	*0.877*
Anthropometry, mean±SD			
BMI, kg/m²	26.7±4.25	26.2±3.74	*0.004*
Waist circumference, cm	90.4±13.0	88.3±12.1	*<0.001*
Waist-to-hip ratio	0.88±0.10	0.87±0.10	*0.001*
Lifestyle variables, n (%)			
Smoking status and intensity			
Never	514 (37.9)	542 (39.8)	*0.703*
Current, 1-<=15 cig/day	129 (9.51)	139 (10.2)	
Current, 16-<=25 cig/day	87 (6.40)	94 (6.91)	
Current, >26 cig/day	20 (1.47)	23 (1.69)	
Former, quit <= 10 years	139 (10.3)	129 (9.48)	
Former, quit 11-<=20 years	144 (10.6)	123 (9.04)	
Former, quit >20 years	166 (12.2)	177 (13.0)	
Current, pipe/cigar/occasional	125 (9.22)	102 (7.49)	
Physical activity			
Inactive	343 (25.4)	307 (22.6)	*0.057*
Moderately inactive	439 (32.4)	446 (32.3)	
Moderately active	307 (22.7)	282 (20.8)	
Active	264 (19.5)	321 (23.7)	
Highest education level attained			
None	68 (5.01)	66 (4.85)	*0.275*
Primary school completed	453 (33.4)	490 (36.0)	
Technical/professional school	324 (23.9)	343 (25.2)	
Secondary school	217 (16.0)	184 (13.5)	
Higher education	247 (18.2)	244 (17.9)	
Dietary intake, mean (SD)			
Energy, Kcal/day	2124±620	2127±609	*0.764*
Alcohol, g/day	17.0±22.1	15.4±19.7	*0.040*
Red and processed meats, g/day	87.6±53.1	85.1±52.0	*0.215*
Fruits and vegetables, g/day	396±233	421±248	*0.007*
Cereals, g/day	216±121	216±119	*0.941*
Dairy products, g/day	331±251	351±244	*0.042*
Fish, g/day	28.2±28.8	29.6±30.6	*0.226*
Sugar and confectionaries, g/day	48.7±66.6	48.7±68.9	*0.995*
Fat, g/day	28.3±15.6	27.9±16.0	*0.536*
Protein, g/day	89.3±27.9	90.3±27.5	*0.337*
Biomarkers			
CRP, ng/mL[†]	4013±6011	3433±5607	*0.026*
sRAGE levels, mean±SD, pg/mL			
All participants	1086±469	1130±470	*0.015*
Men	982±431	1066±438	*<0.001*

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Women	1185±483	1191±490	*0.831*

700 Frequencies may not add up to 100% due to missing data
701 Abbreviations: AGE, Advanced glycation end products; BMI, body mass index; sRAGE, soluble
702 receptor for advanced glycation end-products
703 *Student's paired t-test and Wilcoxon's signed-rank test for continuous variables and Kruskal–Wallis
704 test for categorical variables
705 †CRP was available for 1103 cases and 925 controls

Table 2: Odds ratios (OR) and 95% confidence intervals for colorectal cancer risk associated with circulating sRAGE (Quintiles and continuous), EPIC study 1992-2012

	Quintiles of sRAGE (cutpoints, in pg/mL) *					P_{trend}	Continuous, per SD	Continuous, per SD[†]
	Quintile 1 (<754)	Quintile 2 (754-<941)	Quintile 3 (941-<1157)	Quintile 4 (1157- <1440)	Quintile 5 (≥1440)			
All participants								
Cases/controls	344/273	258/272	272/271	239/272	248/273		1361/1361	1101/1101
Model 1[‡]	1.00 (Ref.)	0.74 (0.58-0.94)	0.77 (0.61-0.98)	0.64 (0.50-0.83)	0.69 (0.54-0.89)	0.002	0.90 (0.83-0.97)	0.91 (0.82-1.00)
Model 2[§]	1.00 (Ref.)	0.75 (0.60-0.96)	0.83 (0.65-1.07)	0.69 (0.53-0.90)	0.75 (0.58-0.98)	0.035	0.93 (0.85-1.01)	0.92 (0.83-1.02)
Men								
Cases/controls	222/156	146/138	121/140	85/124	83/99		657/657	521/521
Model 1[‡|]	1.00 (Ref.)	0.77 (0.56-1.05)	0.62 (0.46-0.87)	0.46 (0.32-0.65)	0.57 (0.39-0.82)	<0.001	0.81 (0.72-091)	0.77 (0.65-0.91)
Model 2[§|]	1.00 (Ref.)	0.79 (0.57-1.09)	0.62 (0.44-0.87)	0.49 (0.33-0.72)	0.63 (0.42-0.94)	0.001	0.84 (0.74-0.96)	0.75 (0.63-0.90)
Women								
Cases/controls	122/117	115/134	151/131	152/148	164/174		704/704	580/580
Model 1[‡|]	1.00 (Ref.)	0.77 (0.53-1.12)	1.04 (0.73-1.50)	0.93 (0.65-1.35)	0.90 (0.63-1.35)	0.967	0.99 (0.88-1.10)	1.00 (0.88-1.13)
Model 2[§|]	1.00 (Ref.)	0.77 (0.52-1.15)	1.16 (0.79-1.70)	1.03 (0.70-1.53)	0.94 (0.63-1.38)	0.754	1.00 (0.89-1.13)	1.02 (0.89-1.16)

Abbreviations: BMI, body mass index; sRAGE, soluble receptor for advanced glycation end-products

*Quintiles (in pg/mL) were created based on the distribution of sRAGE in the control group. All the models were run using conditional logistic regression

[†]Analysis excluding cases that occurred within two years of follow-up

[‡]Model 1 was conditioned on the matching factors

§Model 2 is Model 1 further adjusted for body mass index (BMI, continuous), height (continuous), education (none, primary, technical and professional, secondary, higher education), physical activity (inactive, moderately inactive, moderately active, active), smoking status, duration, and intensity (never, 1-<=15 cigarettes/day, 16-<=25 cigarettes/day, >26 cigarettes/day, former smokers who quit <=10 years, former smokers who quit 11-<=20 years, former smokers who quit>20 years, current pipe-cigar and occasional smokers), dietary energy (continuous) and intakes of alcohol, red and processed meat, dietary fibre, and dairy products (all as continuous variables)

[|]Heterogeneity by sex for sRAGE and colorectal cancer risk association was statistically significant for the two models (*P* for heterogeneity=*0.005*, and *0.006* for the models 1 and 2, respectively)

Table 3: Odds ratios (OR) and 95% confidence intervals (CI) for risk of colorectal cancer anatomical subsites associated with circulating sRAGE (Continuous, per SD), EPIC study 1992-2012

| | Colon cancer | | | Rectal cancer |
	All colon	Proximal colon	Distal colon	
All participants				
Cases/Controls*	854/854	372/372	414/414	502/502
OR (95% CI) [†]	0.94 (0.84 - 1.04)	0.92 (0.77 - 1.10)	0.88 (0.75 - 1.03)	0.90 (0.78 - 1.05)
Men				
Cases/Controls*	388/388	160/160	191/191	270/270
OR (95% CI) [†‡]	0.84 (0.70 – 0.99)	0.94 (0.69 - 1.29)	0.61 (0.44 - 0.84)	0.80 (0.64 - 0.99)
Women				
Cases/Controls*	466/466	212/212	223/223	232/232
OR (95% CI) [†‡]	0.99 (0.85-1.15)	0.85 (0.64 - 1.13)	1.05 (0.83 - 1.31)	1.06 (0.86 - 1.32)

*Some colorectal cancers cases were not included in the analysis as they were overlapping (5 were neither colon nor rectal tumours, 68 were neither proximal nor distal colon tumours)

[†]Conditional logistic regression models conditioned on matching factors and adjusted for body mass index (BMI, continuous), height (continuous), education (none, primary, technical and professional, secondary, higher education), physical activity (inactive, moderately inactive, moderately active, active), smoking status, duration, and intensity (never, 1-<=15 cigarettes/day, 16-<=25 cigarettes/day, >26 cigarettes/day, former smokers who quit <=10 years, former smokers who quit 11-<=20 years, former smokers who quit>20 years, current pipe-cigar and occasional smokers), dietary energy (continuous) and intakes of alcohol, red and processed meat, dietary fibre, and dairy products (all as continuous variables)

[‡]P for heterogeneity colon cancer vs. rectal cancer were 0.607, 0.091, and 0.291 for all the participants, men and women, respectively

P for heterogeneity proximal colon cancer vs. distal colon cancer were 0.307, 0.671, and 0.870 for all the participants, men and women, respectively

P for heterogeneity by sex were 0.042, 0.832, 0.004, 0.063 for all colon cancer, proximal colon cancer, distal colon cancer, and rectal cancer, respectively

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Table 4: Odds ratios (OR) and 95% confidence intervals (CI) for colorectal cancer risk associated with SNPs associated with sRAGE levels, EPIC study 1992-2012

SNP	Cases	Controls	OR (95% CI) *	P-value[‡]	OR (95% CI) [†]	P-value[‡]
rs2070600 (*AGER*)						
CC	1836	2048	1.00 (ref.)		1.00 (ref.)	
CT	148	164	1.01 (0.80-1.27)	0.955	1.06 (0.84-1.35)	0.608
TT	1	8	0.14 (0.02-1.12)	0.063	0.17 (0.02-1.36)	0.095
T *vs.* C	1985	2220	0.93 (0.75-1.16)	0.519	0.99 (0.79-1.24)	0.906
CT+TT *vs.* CC	1985	2220	0.97 (0.77-1.21)	0.768	1.03 (0.81-1.30)	0.835
TT vs. CT+CC	1985	2220	0.14 (0.02-1.12)	0.063	0.17 (0.02-1.35)	0.094
rs1800625 (*AGER*)						
AA	1350	1584	1.00 (ref.)		1.00 (ref.)	
AG	574	578	1.17 (1.02-1.34)	0.028	1.13 (0.98-1.3)	0.084
GG	61	58	1.23 (0.86-1.78)	0.261	1.17 (0.81-1.7)	0.397
G vs. A	2135	2331	1.15 (1.02-1.29)	0.020	1.11 (0.99-1.25)	0.071
AG+GG *vs.* AA	2135	2331	1.17 (1.03-1.34)	0.019	1.13 (0.99-1.3)	0.067
GG *vs.* AG+AA	2135	2331	1.18 (0.82-1.7)	0.369	1.13 (0.78-1.64)	0.513
rs9469089 (*RNF5*)						
GG	1408	1619	1.00 (ref.)		1.00 (ref.)	
GC	532	548	1.12 (0.97-1.28)	0.121	1.14 (0.99-1.31)	0.070
CC	45	53	0.98 (0.65-1.46)	0.907	0.99 (0.65-1.49)	0.948
C *vs.* G	1985	2220	1.08 (0.95-1.21)	0.231	1.09 (0.97-1.23)	0.152
GC+CC *vs.* GG	1985	2220	1.10 (0.96-1.26)	0.150	1.13 (0.98-1.29)	0.089
CC *vs.* GC+GG	1985	2220	0.95 (0.63-1.42)	0.796	0.95 (0.63-1.43)	0.813
rs4746 (*GLO1*)						
TT	651	724	1.00 (ref.)		1.00 (ref.)	
TG	965	1034	1.04 (0.90-1.19)	0.596	1.03 (0.9-1.19)	0.645
GG	369	462	0.89 (0.75-1.06)	0.179	0.89 (0.75-1.06)	0.192
G *vs.* T	1985	2220	0.95 (0.88-1.04)	0.275	0.95 (0.88-1.04)	0.282
TG+GG *vs.* TT	1985	2220	0.99 (0.87-1.13)	0.899	0.99 (0.87-1.13)	0.870

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GG *vs.* TG+ TT	1985	2220	0.87 (0.75-1.01)	*0.071*	0.87 (0.75-1.02)	*0.084*
rs653765 (*ADAM10*)						
TT	1076	1125	1.00 (ref.)		1.00 (ref.)	
TC	757	887	0.89 (0.79-1.01)	*0.081*	0.90 (0.79-1.02)	*0.098*
CC	152	208	0.76 (0.61-0.96)	*0.019*	0.83 (0.66-1.04)	*0.109*
C *vs.* T	1985	2220	0.88 (0.80-0.97)	*0.008*	0.90 (0.82-0.99)	*0.038*
TC+CC *vs.* TT	1985	2220	0.87 (0.77-0.98)	*0.022*	0.88 (0.78-1.00)	*0.051*
CC vs. TC+TT	1985	2220	0.80 (0.64-1.00)	*0.048*	0.87 (0.70-1.09)	*0.219*

*Crude model (unadjusted)

†Adjusted for sex, country, age (1-year categories), BMI (continuous), smoking status (never, former, current) and alcohol intake (continuous)

‡P-values were calculated by considering genetic variant as continuous

Figure legends:

Figure 1: sRAGE and genetic data available within EPIC

Two endpoints were used for our data; the first ended in June 2003 and included 1361 colorectal cancer cases and 1361 matched controls for the analysis of sRAGE concentrations. December 2012 was considered for the second endpoint, with 1985 samples of colorectal cancer cases, and 2220 controls analysed for genetic data. The overlapping between the two samples was used for sensitivity analysis.

Figure 2: Multivariable-adjusted odds ratio and 95%CI of the associations between RAGE and colorectal cancer, stratified by lifestyle, obesity, CRP and menopause status

Multivariable-adjusted OR and 95% CI were computed for the stratified analysis. All the analyses were conditional logistic regression models conditioned on matching factors and adjusted for BMI, education, physical activity, smoking status, dietary energy and intakes of alcohol, red and processed meat, dietary fibre, and dairy products. The analyses stratified by BMI, physical activity, smoking, and alcohol were not adjusted for their respective variables.

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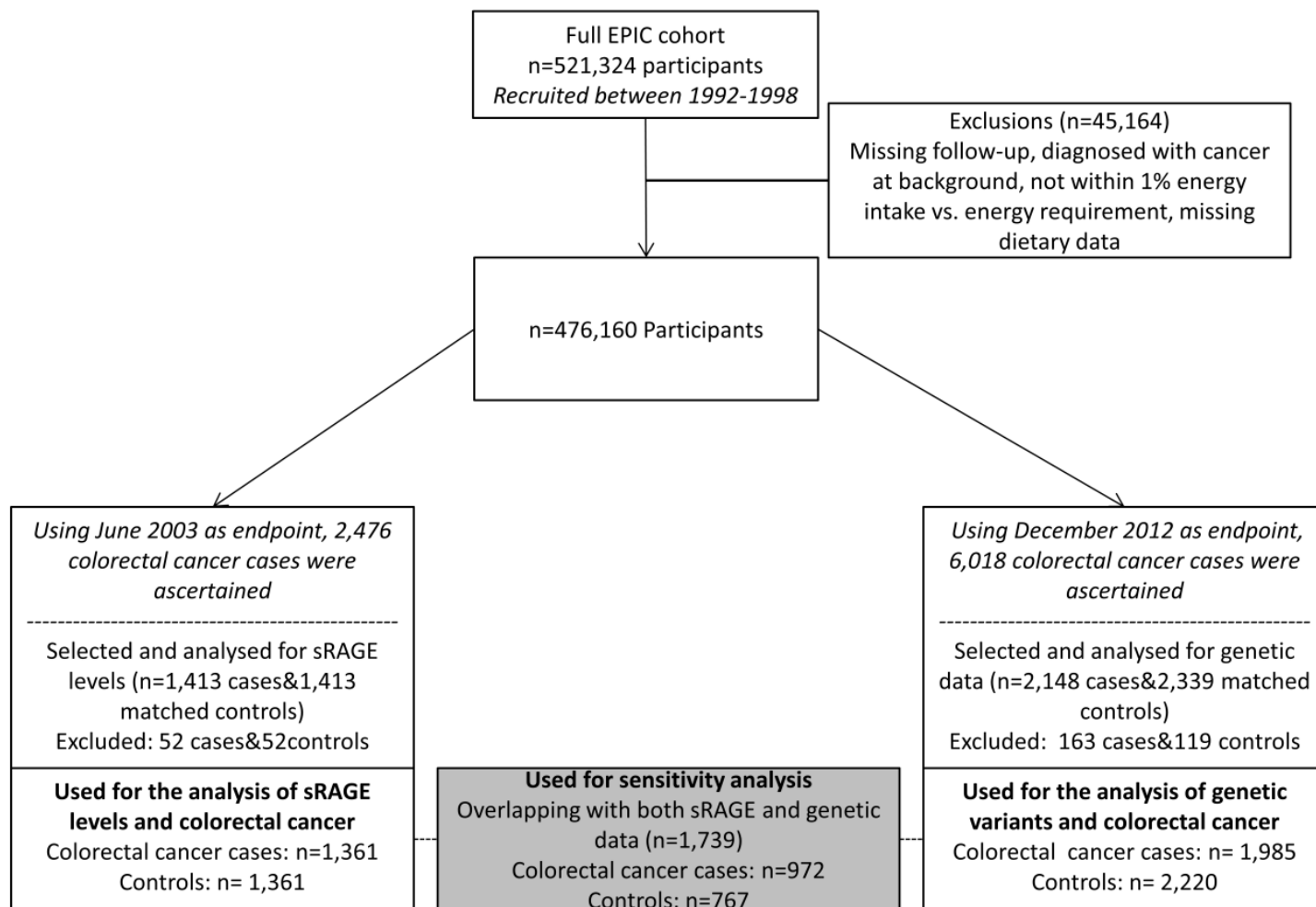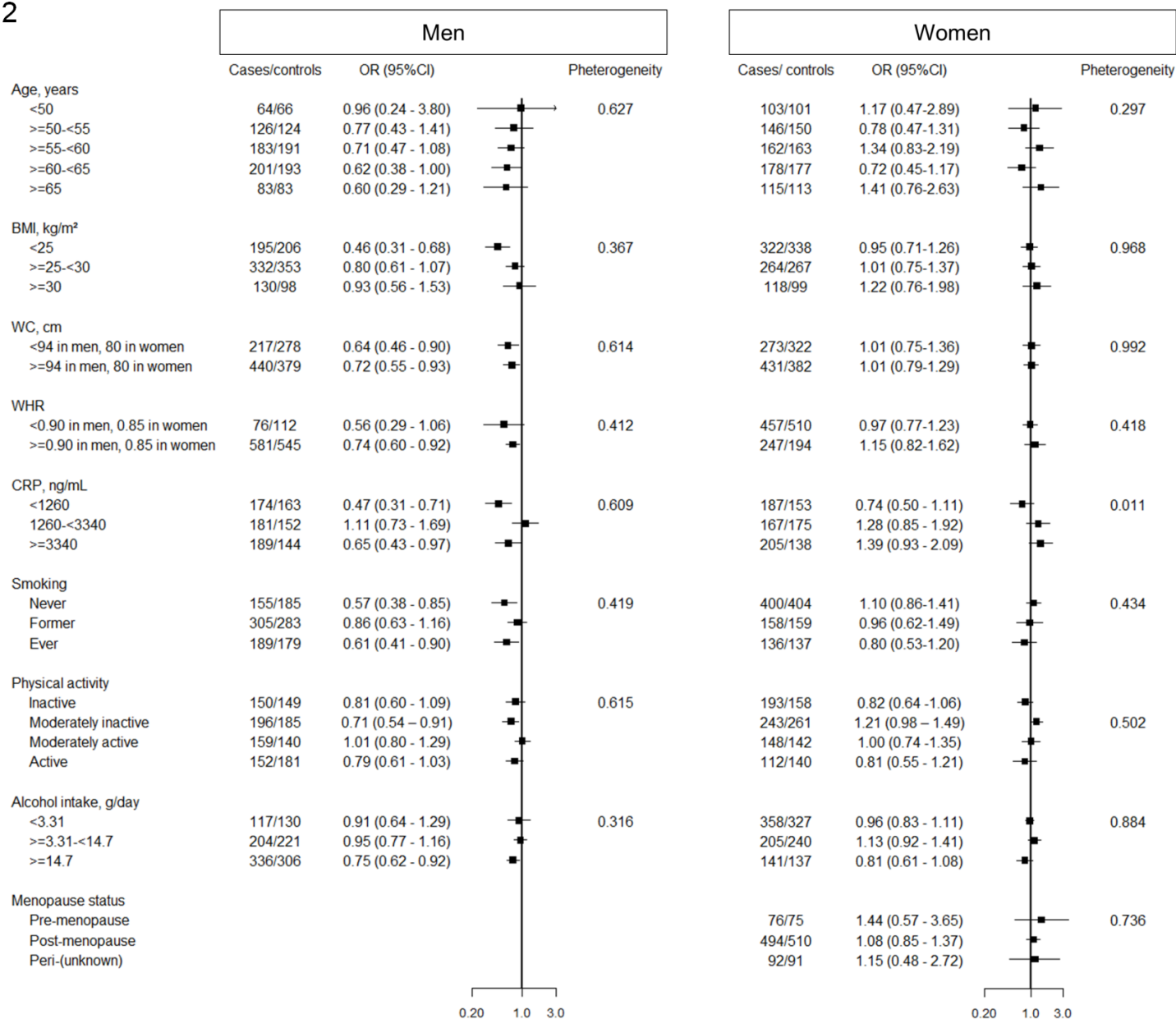# Figure 1

Figure 2

Men	Cases/controls	OR (95%CI)		Pheterogeneity
Age, years				
<50	64/66	0.96 (0.24 - 3.80)		0.627
>=50-<55	126/124	0.77 (0.43 - 1.41)		
>=55-<60	183/191	0.71 (0.47 - 1.08)		
>=60-<65	201/193	0.62 (0.38 - 1.00)		
>=65	83/83	0.60 (0.29 - 1.21)		
BMI, kg/m²				
<25	195/206	0.46 (0.31 - 0.68)		0.367
>=25-<30	332/353	0.80 (0.61 - 1.07)		
>=30	130/98	0.93 (0.56 - 1.53)		
WC, cm				
<94 in men, 80 in women	217/278	0.64 (0.46 - 0.90)		0.614
>=94 in men, 80 in women	440/379	0.72 (0.55 - 0.93)		
WHR				
<0.90 in men, 0.85 in women	76/112	0.56 (0.29 - 1.06)		0.412
>=0.90 in men, 0.85 in women	581/545	0.74 (0.60 - 0.92)		
CRP, ng/mL				
<1260	174/163	0.47 (0.31 - 0.71)		0.609
1260-<3340	181/152	1.11 (0.73 - 1.69)		
>=3340	189/144	0.65 (0.43 - 0.97)		
Smoking				
Never	155/185	0.57 (0.38 - 0.85)		0.419
Former	305/283	0.86 (0.63 - 1.16)		
Ever	189/179	0.61 (0.41 - 0.90)		
Physical activity				
Inactive	150/149	0.81 (0.60 - 1.09)		0.615
Moderately inactive	196/185	0.71 (0.54 - 0.91)		
Moderately active	159/140	1.01 (0.80 - 1.29)		
Active	152/181	0.79 (0.61 - 1.03)		
Alcohol intake, g/day				
<3.31	117/130	0.91 (0.64 - 1.29)		0.316
>=3.31-<14.7	204/221	0.95 (0.77 - 1.16)		
>=14.7	336/306	0.75 (0.62 - 0.92)		
Menopause status				
Pre-menopause				
Post-menopause				
Peri-(unknown)				

Women	Cases/ controls	OR (95%CI)		Pheterogeneity
Age, years				
<50	103/101	1.17 (0.47-2.89)		0.297
>=50-<55	146/150	0.78 (0.47-1.31)		
>=55-<60	162/163	1.34 (0.83-2.19)		
>=60-<65	178/177	0.72 (0.45-1.17)		
>=65	115/113	1.41 (0.76-2.63)		
BMI, kg/m²				
<25	322/338	0.95 (0.71-1.26)		0.968
>=25-<30	264/267	1.01 (0.75-1.37)		
>=30	118/99	1.22 (0.76-1.98)		
WC, cm				
<94 in men, 80 in women	273/322	1.01 (0.75-1.36)		0.992
>=94 in men, 80 in women	431/382	1.01 (0.79-1.29)		
WHR				
<0.90 in men, 0.85 in women	457/510	0.97 (0.77-1.23)		0.418
>=0.90 in men, 0.85 in women	247/194	1.15 (0.82-1.62)		
CRP, ng/mL				
<1260	187/153	0.74 (0.50 - 1.11)		0.011
1260-<3340	167/175	1.28 (0.85 - 1.92)		
>=3340	205/138	1.39 (0.93 - 2.09)		
Smoking				
Never	400/404	1.10 (0.86-1.41)		0.434
Former	158/159	0.96 (0.62-1.49)		
Ever	136/137	0.80 (0.53-1.20)		
Physical activity				
Inactive	193/158	0.82 (0.64 -1.06)		
Moderately inactive	243/261	1.21 (0.98 - 1.49)		0.502
Moderately active	148/142	1.00 (0.74 -1.35)		
Active	112/140	0.81 (0.55 - 1.21)		
Alcohol intake, g/day				
<3.31	358/327	0.96 (0.83 - 1.11)		0.884
>=3.31-<14.7	205/240	1.13 (0.92 - 1.41)		
>=14.7	141/137	0.81 (0.61 - 1.08)		
Menopause status				
Pre-menopause	76/75	1.44 (0.57 - 3.65)		0.736
Post-menopause	494/510	1.08 (0.85 - 1.37)		
Peri-(unknown)	92/91	1.15 (0.48 - 2.72)		

Axis (both panels): 0.20   1.0   3.0

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Soluble Receptor for Advanced Glycation End-products (sRAGE) and colorectal cancer risk: a case-control study nested within a European prospective cohort

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