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Aglago, Elom K.; Rinaldi, Sabina; Freisling, Heinz; Jiao, Li; Hughes, David J.; Fedirko, Veronika; Schalkwijk, Casper G.; Weiderpass, Elisabete; Dahm, Christina C.; Overvad, Kim; Eriksen, Anne Kirstine; Kyrø, Cecilie; Boutron-Ruault, Marie Christine; Rothwell, Joseph A.; Severi, Gianluca; Katzke, Verena; Kühn, Tilman; Schulze, Matthias B.; Aleksandrova, Krasimira; Masala, Giovanna; Krogh, Vittorio; Panico, Salvatore; Tumino, Rosario; Naccarati, Alessio; Bueno-De-Mesquita, Bas; Van Gils, Carla H.; Sandanger, Torkjel M.; Gram, Inger T.; Skeie, Guri; Quirós, J. Ramón; Jakszyn, Paula; Sánchez, Maria Jose; Amiano, Pilar; Huerta, José María; Ardanaz, Eva; Johansson, Ingegerd; Harlid, Sophia; Perez-Cornago, Aurora; Mayén, Ana Lucia; Cordova, Reynalda; Gunter, Marc J.; Vineis, Paolo; Cross, Amanda J.; Riboli, Elio; Jenab, Mazda

Published in:
Cancer Epidemiology Biomarkers and Prevention

DOI (link to publication from Publisher):
10.1158/1055-9965.EPI-20-0855

Publication date:
2021

Document Version
Accepted author manuscript, peer reviewed version

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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Running title: sRAGE concentrations and colorectal cancer

Keywords: sRAGE, colorectal cancer, advanced glycation end-product, inflammation, polymorphism
Financial support: Funding [WCRF 2015/1391, Principal Investigator, Mazda Jenab] was obtained from Wereld Kanker Onderzoek Fonds (WKOF), as part of the World Cancer Research Fund International grant programme.

Conflict of interest statement: The authors declare no potential conflicts of interest.

Abbreviations used: ADAM10, A Disintegrin And Metalloproteinase Domain 10; AGE, advanced glycation end-products; AGER, Advanced Glycosylation End-Product Specific Receptor ; BMI, body mass index; CRC, colorectal cancer; CRP, C-reactive protein; CV, coefficients of variation; GLO1, Glyoxalase I; EPIC, European Prospective Investigation into Cancer and Nutrition; IARC, International Agency for Research on Cancer; mRNA, messenger ribonucleic acid ; NF-κB, nuclear factor kappa B; OR, odds ratio; RAGE, receptor for AGE; RNF5, Ring Finger Protein 5 ; SD, standard deviation; SNP, single nucleotide polymorphism; sRAGE, soluble receptor for AGE; TNFα, tumor necrosis factor alpha; WC, wait circumference; WHR, waist-to-hip ratio

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Data sharing statement: For information on how to submit an application for gaining access to EPIC data and/or biospecimens, please follow the instructions at http://epic.iarc.fr/access/index.php

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Conflict of interest statement: The authors have declared no conflicts of interest.

Word count: 4,000

Number of tables and figures: 4 tables and 2 figures

Abstract

Background: Overexpression of the Receptor for Advanced Glycation End-product (RAGE) has been associated with chronic inflammation, which in turn has been associated with increased colorectal cancer (CRC) risk. Soluble RAGE (sRAGE) competes with RAGE to bind its ligands, thus potentially preventing RAGE-induced inflammation.

Methods: To investigate whether sRAGE and related genetic variants are associated with CRC risk, we conducted a nested case-control study in the European Prospective Investigation into Cancer and Nutrition (EPIC). Plasma sRAGE concentrations were measured by ELISA in 1,361 CRC matched case-control sets. Twenty-four single nucleotide polymorphisms (SNPs) encoded in the genes associated with sRAGE concentrations were available for 1,985 CRC cases and 2,220 controls. Multivariable-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were computed using conditional and unconditional logistic regression for CRC risk and circulating sRAGE and SNPs, respectively.

Results: Higher sRAGE concentrations were inversely associated with CRC ($OR_{Q5vs.Q1}$=0.77, 95%CI=0.59-1.00). Sex-specific analyses revealed that the observed inverse risk association was restricted to men ($OR_{Q5vs.Q1}$=0.63, 95%CI=0.42-0.94) whereas no association was observed in women ($OR_{Q5vs.Q1}$=1.00, 95%CI=0.68-1.48, $P_{heterogeneity}$ for sex=*0.006*). Participants carrying minor allele of rs653765 (promoter region of *ADAM10*) had lower CRC risk (C vs. T, OR=0.90; 95%CI=0.82-0.99).

Conclusion: Pre-diagnostic sRAGE concentrations were inversely associated with CRC risk in men but not in women. A SNP located within *ADAM10* gene pertaining to RAGE shedding, was associated with CRC risk.

Impact: Further studies are needed to confirm our observed sex difference in the association and better explore the potential involvement of genetic variants of sRAGE in CRC development.

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.
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Materials and methods

Study population and data collection

We used a case-control design nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort. EPIC is an ongoing multicentre prospective cohort with 521,324 participants (70% women) recruited from 23 study centres located in 10 European countries (Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden, and the United Kingdom). The rationale and methods of the EPIC study, including information on the recruitment of the participants as well as data collection have been described previously (24). Participants gave written informed consent before joining the EPIC study. Participant's health history, anthropometry, socio-demographic and standardised lifestyle variables including education, smoking, and physical activity were collected by questionnaire at baseline, prior to disease onset or diagnosis. Physical activity was based on the Cambridge physical activity index: inactive (sedentary job and no recreational activity), moderately inactive (sedentary job with <0.5 h recreational activity per day/or standing job with no recreational activity), moderately active (sedentary job with 0.5 to 1 h recreational activity per day/ or standing job with 0.5 h recreational activity per day/ or physical job with no recreational activity) or active (sedentary job with >1 h recreational activity per day/or standing job with >0.5 h recreational activity per day/or physical job with at least some recreational activity/or heavy manual job) (25). Dietary intake was assessed at recruitment by validated centre-specific questionnaires. In each of the study centres, blood samples were drawn at recruitment (≈80% of participants provided blood samples) and stored in liquid nitrogen (−196°C, liquid nitrogen) at the International Agency for Research on Cancer (IARC) biobank, or in local biobanks (at -150°C in nitrogen vapour in Denmark; -80°C freezers at Malmö and Umeå centres in Sweden) (24).

Follow-up for cancer incidence and vital status

Vital status follow-up (98.4% complete) is collected by record linkage with regional and/or national mortality registries in all countries except Germany and Greece, and the Italian centre of Naples, where data are collected actively. Incident cancer cases were identified through record linkage with regional cancer registries or using a combination of methods, including health insurance records, cancer and pathology registries, and active follow-up through participants and their relatives. CRC cases were eligible if they were first incident and histologically-confirmed. Cases were defined using the International Classification of Diseases for Oncology (ICD-O). Colon cancers were defined as tumours that occurred in the cecum, appendix, ascending colon, hepatic flexure, transverse colon, splenic flexure, descending and sigmoid colon (C18.0-C18.7), and overlapping and or unspecified origin tumours (C18.8 and C18.9). Rectal cancers were defined as tumours that occurred at the recto-sigmoid junction (C19) or rectum (C20). Cancers of the anal canal were excluded.

Case-control design

From baseline onwards, 1,413 first incident CRC cases with available blood samples were identified (until June 2003 as endpoint) among all the total 2,476 CRC cases ascertained **(Figure 1)**. For each identified case, one control was matched by incidence density sampling from all cohort members alive and cancer-free at the time of diagnosis of the index case. Cases and controls were matched by age (±1 year), sex, centre, and blood collection details including time (±3 hours), fasting pre-venepuncture (<3, 3-6, and >6 hours); and additionally among women only, by menopausal status (pre-, peri-, and postmenopausal), and hormone replacement therapy (HRT) use at the time of blood collection (yes/no). After exclusion of participants with incomplete matched case sets (n=16), those with extreme sRAGE levels (n=3 controls and 1 case with sRAGE concentrations unusually high i.e. >mean+4 standard deviation), and 32 cases and matched controls from Greece due to unforeseen data restriction issues, 1,361 cases and 1,361 matched controls were included in the sRAGE analysis. Among EPIC participants, 4,487 participants (until December 2012 as endpoint, 2,148 CRC cases and matched 2,339 controls) have been previously genotyped. After exclusion of 100 CRC cases and 100 matched controls from Greece, and 82 participants with missing lifestyle variable, 1,985 CRC cases and 2,220 matched controls were included in the genetic analysis. Among the participants who have been genotyped, 972 CRC cases and 767 non-cases overlap with case-control sets in whom sRAGE measurements were conducted.

Laboratory analyses

Circulating sRAGE concentrations were measured in citrated plasma samples by ELISA (Quantikine, R&D Systems, MN, USA), following the manufacturer's instructions. Previous studies have reported that sRAGE is stable in plasma over a long period of time (26). Analyses were run with case-control sets randomized across batches (n=40 batches, with an average of 35 case-control pairs analysed per batch). Intra- and inter-batch coefficients of variation (CV) were assessed by measuring 3 different samples used as quality controls in duplicate in each. Mean intra- and inter-batch CVs were 1.25% and 6.0%, respectively. C-reactive protein (CRP) concentrations were determined using a high-sensitivity assay (Beckman-Coulter, Woerden, The Netherlands).

DNA genotyping and genetic variants selection

DNA was extracted from buffy coats from citrated blood samples at the Center for Inherited Disease Research (CIDR, Johns Hopkins University) using the HumanOmniExpressExome-8v1–2 array as described elsewhere (27). All SNPs met criteria for quality control for genotyping call rate (above 95%). Candidate SNPs selected for our study were those previously associated with sRAGE levels. Most of these SNPs appear to be located within the *AGER* gene, with rs2070600 being the most important and explaining 22% of the variability in sRAGE concentrations in Caucasians (17). In addition to *AGER*, four additional genes contain SNPs associated with sRAGE: *RNF5*, a neighbouring

gene which encodes for RAGE (28), *ADAM10* encodes for metalloproteinases involved in the shedding of RAGE ectodomain to form sRAGE (29), and *GLO1* encodes for glyoxalase enzyme responsible to metabolise methylglyoxal and prevent aberrant AGEs formation (30). The main SNPs are from *AGER* (rs2070600, rs1800625, rs1800624, rs184003, rs2854050), *ADAM10* (rs653765) and *RNF5* (rs9469089) (17-21,31-38). We additionally considered less-studied SNPs located within *AGER* (rs1035798, rs1800684, rs3131300, rs3134940, rs2269422, rs2853807, rs9391855, rs17846798), *ADAM10* (rs514049), *RNF5* (rs57409105, rs41268928, rs17493811), and *GLO1* (rs4746, rs1130534, rs1049346, rs6932648, rs10484854). The choice of this supplementary group of SNPs was based on the potential influence and interactions they may have in modulating sRAGE levels directly or through AGEs (13,17,21,31,39-41).

Genotype distributions were in Hardy-Weinberg equilibrium (cutoff of *P*-value=$1x10^{-3}$) for all the SNPs considered, with the exception of rs6932648 which was consequently excluded from the analysis. The selected SNPs and their characteristics are detailed in **Supplementary Table 1**. To select the independent variants, Linkage Disequilibrium (LD) pruning (LD≤1%) was performed using NCI LDlink tools (https://ldlink.nci.nih.gov). We found the following independent variants (highly correlated variants are in brackets): rs2070600 (rs41268928, rs9391855, rs2854050), rs1800625 (rs3131300, rs3134940), rs1800624 (rs17846798), rs4746 (rs1130534, rs10484854), rs17846798 (rs57409105), rs9469089, rs1800684, rs2269422, rs2853807, rs1049346, rs17493811, and rs653765 (rs514049). A flowchart outlining the selection of the independent SNPs is detailed in **Supplementary Figure 1**.

Among the 767 control subjects who had both sRAGE and genetic data, we assessed the association between the independent genetic variants and log-transformed sRAGE levels using linear regression models (**Supplementary Table 2**). The SNPs in the following genes were significantly associated with sRAGE levels: *AGER* (rs2070600, rs1800625), *RNF5* (rs9469089), and *GLO1* (rs4746). Although rs653765 (*ADAM10*) was not associated with sRAGE levels, we decided to conserve it in our analysis for two main reasons: first, as a major variant of metalloproteinases which are involved in the shedding of the ectodomain of RAGE to produce sRAGE; second, this variant was previously associated with sRAGE levels in other populations (21). Overall, five SNPs (rs2070600, rs1800625, rs9469089, rs4746, rs653765) were examined for the association with CRC risk.

Statistical analysis

Case-control differences in baseline characteristics were evaluated using Student's paired t-test and Wilcoxon's signed-rank test for continuous variables and Kruskal–Wallis test for categorical variables. Spearman rank correlation was used to correlate sRAGE levels to anthropometry, dietary intakes and other biomarkers. We divided sRAGE concentrations into quintiles based on the distribution in the control group. Conditional logistic regression was used to compute odds ratios (ORs) and 95% confidence intervals (CIs) for the associations between circulating levels of sRAGE

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

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

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

Results

Baseline characteristics and sRAGE levels in cases and controls are presented in **Table 1**. Compared to controls, CRC cases have higher BMI, WC, WHR and CRP concentrations, and consume more alcohol and less dairy products and fruit and vegetables. sRAGE concentrations were slightly lower in CRC cases than controls (1086 *versus* 1130 pg/mL) but this was mainly observed among men (982 versus 1066 pg/mL in male cases versus controls, respectively); whereas among women sRAGE was 1185 pg/mL in cases and 1191 pg/mL in controls. BMI, WC, WHR, and alcohol intake were all negatively correlated with sRAGE levels whereas sugar and confectionaries, fruit and vegetable, and cereals intakes showed positive correlations (**Supplementary Table 3).** Women with higher sRAGE levels have lower CRP concentrations (Spearman rho=-0.156, p=0.004).

sRAGE and CRC risk

sRAGE concentrations were inversely associated with CRC risk in multivariable-adjusted analyses (OR comparing the highest to the lowest quintile $OR_{Q5vs.Q1}$=0.75, 95%CI=0.58-0.98, P_{trend}=*0.035*, **Table 2**). Sub-group analyses by sex showed an inverse risk association for men ($OR_{Q5vs.Q1}$=0.63, 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*; $P_{heterogeneity}$=*0.006*). In men, sRAGE was associated with a lower risk of both colon cancer (OR per SD increment, OR =0.84, 95%CI=0.70-0.99) and rectal cancer (OR=0.80, 95%CI=0.64-0.99) with no heterogeneity across anatomical subsites ($P_{heterogeneity}$=*0.607*) (**Table 3**). The magnitude of the inverse association appeared stronger for distal colon cancer (OR=0.61, 95%CI=0.44-0.84) compared to proximal cancer (OR=0.94, 95%CI=0.69-1.29) but no heterogeneity was observed ($P_{heterogeneity}$=*0.671*). In women, no association was found between sRAGE and colon (OR=0.99, 95%CI=0.85-1.15) or rectal cancer (OR=1.06, 95%CI=0.86-1.32). Stratified analyses by age groups, BMI categories, WC and WHR cut-offs, and smoking status showed no significant differences across strata (**Figure 2**). Women in higher CRP tertiles tended to have higher CRC risk associated with sRAGE ($P_{heterogeneity}$ across=*0.011*) (**Figure 2**).

Analyses of genetic variants

Table 4 presents the association of the genetic variants with CRC risk. While comparing minor allele *vs.* major allele, rs1800625 (*AGER*, G *vs.* A, OR=1.15, 95%CI=1.02-1.29) was associated with an increased risk of CRC whereas rs653765 (*ADAM10*, C *vs.* T, OR=0.88; 95%CI=0.80-0.97) was associated with a lower CRC risk, in univariate models. After multivariate adjustments, the association remained statistically significant for rs653765 (*ADAM10*, C *vs.* T, OR=0.90; 95%CI=0.82-0.99), but not for rs1800625 (*AGER*, G *vs.* A, OR=1.11, 95%CI=0.99-1.25).

Sensitivity analysis

Exclusion of the cases that occurred within the first two years of follow-up did not change the associations between sRAGE concentrations and CRC (**Table 1**). The associations between SNPs and CRC in participants with overlapping genetic and sRAGE data showed similar, but no statistically significant associations for rs653765 (*ADAM10*, OR=0.90, 95%CI=0.78-1.05) or rs1800625 (*AGER*, G *vs.* A, OR=1.00, 95%CI=0.83-1.19) (**Supplementary Table 4**).

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

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

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

The strengths of our study include the large number of cases and controls, the prospective design and the availability of dietary and lifestyle factors and genetic variants. Our study was, however, limited by the fact that we did not differentiate between endogenous secretory RAGE (esRAGE), and proteolytically cleaved RAGE (cRAGE), the two components of sRAGE. esRAGE is formed by alternative splicing of RAGE mRNA, and cRAGE is produced by the shedding of the ectodomain of RAGE par metalloproteinases located at the surface of the cells. esRAGE is stable throughout the life course whereas cRAGE levels vary with age and with environmental factors (60). Because we have measured the total pool of plasma sRAGE we therefore cannot discern whether the different variants of sRAGE have specific and potentially opposite associations with study outcomes. Although the variability of cRAGE makes it a poor biomarker for a prospective study, cRAGE levels data would have permitted us to explore the association between SNPs from the *ADAM10* gene, levels of cRAGE and CRC risk. Our study was also limited by the fact that lifestyle factors and blood samples were collected at the recruitment, and may not necessarily reflect changes over years. Moreover, we cannot rule out residual confounding or unmeasured confounders such as lifetime history of anti-inflammatory medication use.

In conclusion, we observed that pre-diagnostic circulating sRAGE levels were inversely associated with CRC risk in men, but not among women. We also found that the minor allele of rs653765 (*ADAM10*) was inversely associated with CRC risk. Additional studies are, however, required to further investigate how genetic variation and sex may affect sRAGE levels or modify its association with CRC risk.

Acknowledgements: The authors would like to thank the EPIC study participants and staff for their valuable contribution to this research. The authors would also like to especially thank Mr. Bertrand Hemon and Dr. Aurelie Moskal for their support in preparing the databases and providing technical support pertaining to the data analysis; along with Ms. Audrey Brunat-Manquat for her assistance with the laboratory analyses for sRAGE.

The coordination of EPIC is financially supported by the European Commission (DG-SANCO); and the International Agency for Research on Cancer. The national cohorts are supported by Danish Cancer Society (Denmark); Ligue Contre le Cancer; Institut Gustave Roussy; Mutuelle Générale de l'Education Nationale; and Institut National de la Santé et de la Recherche Médicale (INSERM) (France); German Cancer Aid, German Cancer Research Center (DKFZ), and Federal Ministry of Education and Research (BMBF) (Germany); Italian Association for Research on Cancer (AIRC); National Research Council; and Associazione Iblea per la Ricerca Epidemiologica (AIRE-ONLUS) Ragusa, Associazione Volontari Italiani Sangu (AVIS) Ragusa, Sicilian Government (Italy); Dutch Ministry of Public Health, Welfare and Sports (VWS); Netherlands Cancer Registry (NKR); LK Research Funds; Dutch Prevention Funds; Dutch ZON (Zorg Onderzoek Nederland); World Cancer Research Fund (WCRF); and Statistics Netherlands (the Netherlands); Health Research Fund (FIS); Regional Governments of Andalucía, Asturias, Basque Country, Murcia (No. 6236) and Navarra; and the Centro de Investigación Biomédica en Red en Epidemiología y Salud Pública and Instituto de Salud Carlos II (ISCIII RETIC) (RD06/0020) (Spain); Health Research Fund (FIS) - Instituto de Salud Carlos III (ISCIII), Regional Governments of Andalucía, Asturias, Basque Country, Murcia and Navarra, and the Catalan Institute of Oncology - ICO (Spain); Swedish Cancer Society; Swedish Scientific Council; and Regional Government of Skåne and Västerbotten (Sweden); Cancer Research UK; Medical Research Council; Stroke Association; British Heart Foundation; Department of Health; Food Standards Agency; and the Wellcome Trust (UK). Cancer Research UK (14136 to EPIC-Norfolk; C570/A16491 and C8221/A19170 and C8221/A29017 to EPIC-Oxford), Medical Research Council (1000143 to EPIC-Norfolk, MR/M012190/1 to EPIC-Oxford) (United Kingdom). The EPIC-Norfolk study (DOI 10.22025/2019.10.105.00004) has received funding from the Medical Research Council (MR/N003284/1 and MC-UU_12015/1) and Cancer Research UK (C864/A14136). We are grateful to all the participants who have been part of the project and to the many members of the study teams at the University of Cambridge who have enabled this research. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

This work was partially financially supported by the Fondation de France (FDF, grant, no 00081166; to HF and RC, and FDF grant no. 00089811, ALM).

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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*

Women	1185±483	1191±490	*0.831*

Frequencies may not add up to 100% due to missing data

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

*Student's paired t-test and Wilcoxon's signed-rank test for continuous variables and Kruskal–Wallis test for categorical variables

[†]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

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*

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.

Figure 1

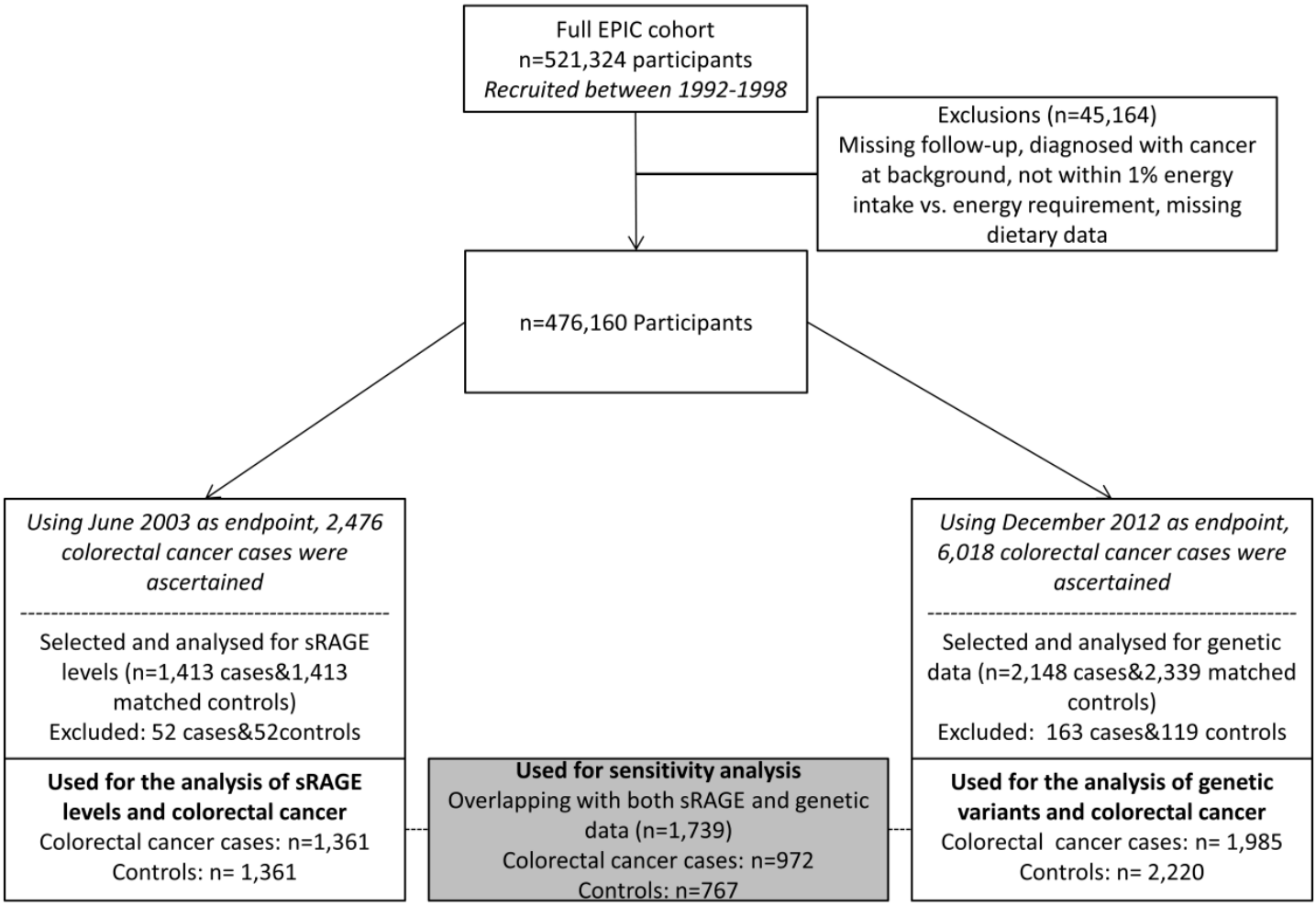
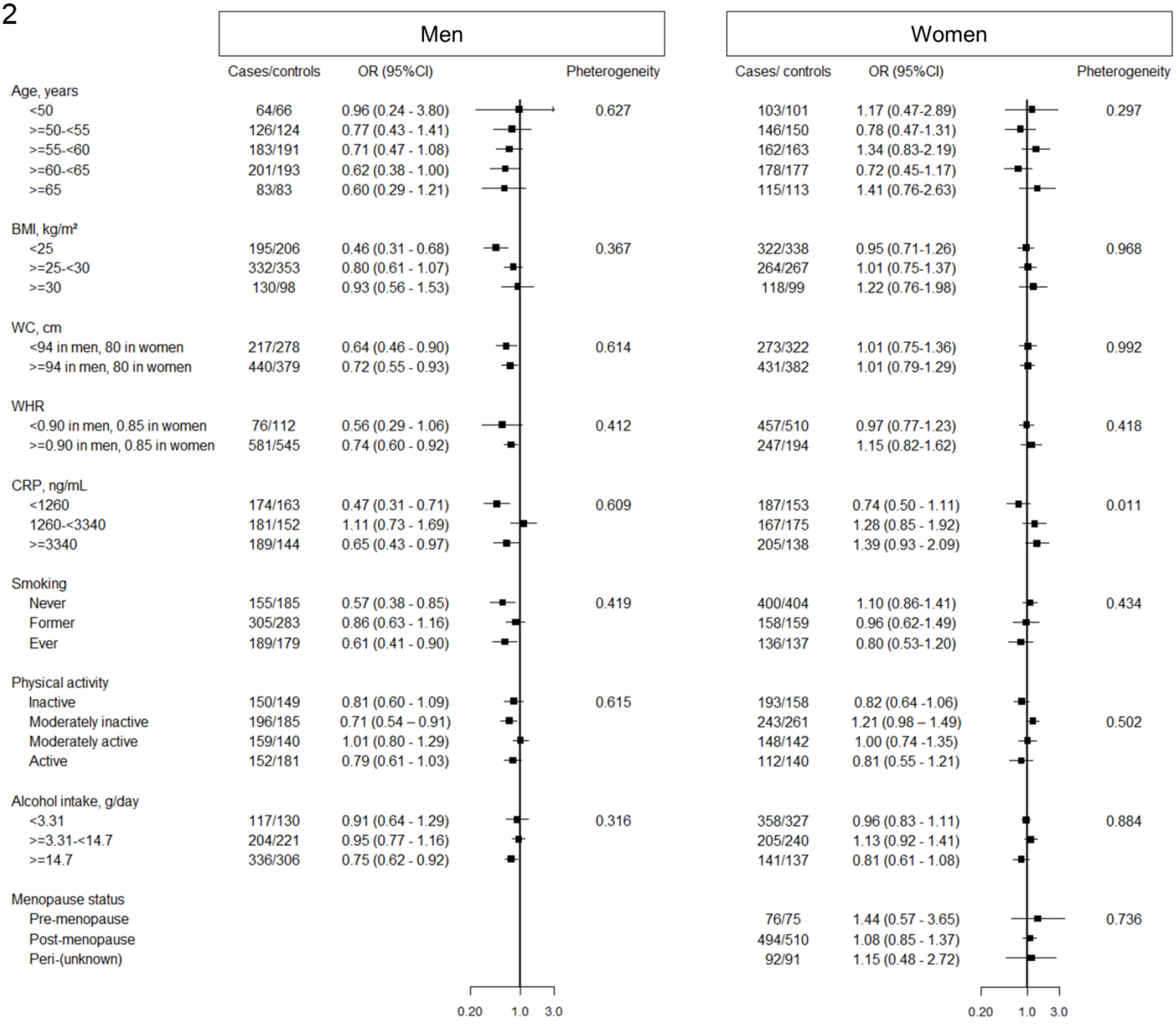


Figure 2



Cancer Epidemiology, Biomarkers & Prevention

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Cancer Epidemiol Biomarkers Prev Published OnlineFirst October 20, 2020.