

1 **Prospective evaluation of antibody response to *Streptococcus***  
 2 ***gallolyticus* and risk of colorectal cancer**

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#### Abbreviations

CI – Confidence interval

CRC – Colorectal cancer

EPIC – European Prospective Investigation into Nutrition and Cancer

MFI – Median fluorescence intensity

OMP – Outer membrane protein

OR – Odds ratio

SGG – *Streptococcus gallolyticus* subspecies *gallolyticus*

#### Novelty and Impact

This study presents the serological analysis of an association of *Streptococcus gallolyticus* sub-species *gallolyticus* (SGG) with CRC in a prospective setting with samples from the large European Prospective Investigation into Nutrition and Cancer (EPIC) study. We found for the first time an association of antibody responses to SGG proteins with CRC risk already detectable with pre-diagnostic samples. The presented results support that SGG serology might provide a specific marker for risk of developing CRC.

## 4 Abstract

5 The gut microbiome is increasingly implicated in colorectal cancer (CRC) development. A  
6 subgroup of patients diagnosed with CRC show high antibody responses to *Streptococcus*  
7 *gallolyticus* subspecies *gallolyticus* (SGG). However, it is unclear whether the association is  
8 also present pre-diagnostically. We assessed the association of antibody responses to SGG  
9 proteins in pre-diagnostic serum samples with CRC risk in a case-control study nested within  
10 a prospective cohort.

11 Pre-diagnostic serum samples from 485 first incident CRC cases (mean time between blood  
12 draw and diagnosis 3.4 years) and 485 matched controls in the European Prospective  
13 Investigation into Nutrition and Cancer (EPIC) study were analyzed for antibody responses to  
14 eleven SGG proteins using multiplex serology. Odds ratios (OR) and 95 % confidence  
15 intervals (CI) were estimated using multivariable conditional logistic regression models.

16 Antibody positivity for any of the eleven SGG proteins was significantly associated with CRC  
17 risk with 56% positive controls compared to 63% positive cases (OR: 1.36, 95% CI: 1.04-  
18 1.77). Positivity for two or more proteins of a previously identified SGG 6-marker panel with  
19 greater CRC-specificity was also observed among 9% of controls compared to 17% of CRC  
20 cases, corresponding to a significantly increased CRC risk (OR: 2.17, 95% CI: 1.44-3.27).

21 In this prospective nested case-control study we observed a positive association between  
22 antibody responses to SGG and CRC development in serum samples taken pre-diagnostically.  
23 Further work is required to establish the possibly etiological significance of these  
24 observations and whether SGG serology may be applicable for CRC risk stratification.

## 25 Introduction

26 Colorectal cancer (CRC) is among the most frequently diagnosed cancers worldwide with an  
27 incidence of 746,000 new cases among men and 614,000 new cases among women in 2012<sup>1</sup>.  
28 Inflammation is thought to be among the major etiological risk factors for the development of  
29 CRC, and is a possible mechanism through which bacterial infections might contribute to  
30 carcinogenesis<sup>2</sup>. Changes in the local intestinal tissue can compromise the colonic barrier  
31 integrity resulting in a “leaky gut”<sup>3</sup>. Certain bacteria may opportunistically infect the intestinal  
32 tissue and potentially induce an immune response, although they usually act as commensals<sup>4</sup>.

33 An interesting candidate in this respect might be the intestinal commensal *Streptococcus*  
34 *gallolyticus* subspecies *gallolyticus* (SGG), formerly known as *Streptococcus bovis* biotype I.  
35 In the 1970’s it was found that infective endocarditis caused by bacteria belonging to the *S.*  
36 *bovis* complex<sup>5-7</sup>, and later more specifically by the subspecies SGG<sup>8</sup>, co-occurred with the  
37 presence of colorectal adenoma. A systematic review of CRC case series by Boleij et al. in  
38 2011 showed that 60% of *S. bovis*-infected individuals in the reviewed studies had a  
39 concomitant colorectal adenoma/carcinoma and that SGG-infection was specifically  
40 responsible for this association compared to other *S. bovis* subtypes<sup>9</sup>. It is hypothesized that  
41 intestinal lesions are the entry port for the commensal SGG to the blood stream enabling the  
42 bacterium to turn pathogenic and cause bacteremia or endocarditis<sup>10</sup>. Antibodies against the  
43 infecting SGG may serve as markers for the presence of colorectal neoplasia. A significant  
44 association between SGG antibody response and presence of CRC has been observed in  
45 several studies, but to date these have been exclusively case-control designs with prevalent  
46 CRC cases<sup>11-14</sup>.

47 We previously applied multiplex serology, a fluorescent bead-based high-throughput  
48 technology allowing serological typing of several antigens in one reaction<sup>15</sup>, to analyze  
49 antibody responses to eleven SGG proteins in a German CRC case-control study we showed

50 that positivity to two or more proteins of a SGG 6-marker panel (Gallo0272, Gallo0748,  
51 Gallo1675, Gallo2018, Gallo2178 and Gallo2179) was associated with a 1.8-fold (95% CI:  
52 1.07-3.06) increased risk for CRC (n=318) compared to controls (n=228)<sup>16</sup>. The 6-marker  
53 panel demonstrated a higher specificity for CRC risk compared to positivity towards any one  
54 of the eleven SGG proteins included in the multiplex serology panel.<sup>16</sup> These previous  
55 findings were based on traditional case-control designs where blood samples were obtained  
56 post-diagnosis. It is currently unknown whether any antibody responses to SGG are associated  
57 with CRC development at various time points prior to diagnosis, i.e. whether SGG infection is  
58 merely a consequence of the disease or is in some way involved in CRC etiology<sup>17</sup>.

59 In the current study, we evaluated whether antibody responses to SGG proteins, as measured  
60 by multiplex serology, in pre-diagnostic serum samples were associated with the risk of CRC,  
61 using serum samples of a case-control subset (485 cases and 485 matched controls) of  
62 participants of the European Prospective Investigation into Cancer and Nutrition (EPIC)  
63 study.

## 64 **Methods**

### 65 *Study population, case ascertainment and control selection*

66 EPIC is a multinational cohort to investigate the relation between diet, lifestyle and  
67 environmental factors with cancer incidence. A detailed description of study design has been  
68 published elsewhere<sup>18</sup>. Briefly, 521,468 participants, aged 35 to 70 years, were enrolled from  
69 10 different European countries (Denmark, France, Greece, Germany, Italy, Netherlands,  
70 Norway, Spain, Sweden, United Kingdom) between 1992 and 2000.

71 Dietary and lifestyle data as well as biological samples, including serum, were collected at  
72 enrollment. The blood collection and processing protocols were standardized across the study  
73 centers and blood processing and separation was conducted prior to freezing. Serum samples

74 were stored at the International agency for research on cancer (IARC, Lyon, France) at -  
75 196°C. For multiplex serology analyses, serum samples were shipped on dry ice to the  
76 German Cancer Research Center, Heidelberg.

77 The nested CRC case-control study analyzed here included pre-diagnostic serum samples  
78 from 492 incident CRC cases (primary tumors, C18-C20 as by the 10<sup>th</sup> Revision of the  
79 International Statistical Classification of Diseases, Injury and Causes of Death) and 492  
80 matched controls. Controls were selected by incidence density sampling from all cohort  
81 members alive and free of cancer at the time of matching. Cases and controls were matched  
82 1:1 by age at blood collection ( $\pm 6$  month to  $\pm 2$  years), sex, study center, time of the day at  
83 blood collection ( $\pm 2$  to 4 hours interval), fasting status at blood collection (<3/3-6 hours);  
84 among women by menopausal status, and among premenopausal women, by phase of  
85 menstrual cycle and hormone replacement therapy use at time of blood collection. After  
86 exclusion of 7 case-control pairs due to technical errors during detection, a total of 485 first  
87 incident CRC cases (colon n=432, rectum n=53) were identified that had a mean time  
88 between blood draw and diagnosis of 3.4 years (range 0.4 to 8.5 years)

#### 89 *SGG multiplex serology*

90 Serum samples were analyzed for antibody responses against SGG in a final sample dilution  
91 of 1:1000 using multiplex serology. The method is described in detail elsewhere<sup>15, 16</sup>. Briefly,  
92 eleven SGG antigens (strain UCN34, Table 1) were bacterially expressed as recombinant  
93 GST-X-tag fusion proteins and each antigen was affinity-purified on a different bead set  
94 marked with a distinct internal fluorescent color (SeroMap, Luminex Corp., Austin, TX,  
95 USA). These differently loaded bead sets were mixed to form a suspension antigen array for  
96 serum presentation. A Luminex xMAP (Luminex Corp., Austin, TX, USA) analyzer  
97 identified the bead set and simultaneously quantified bound serum IgA, IgM and IgG  
98 antibodies by a reporter fluorescent conjugate, Streptavidin-R-Phycoerythrin. The level of

99 antibody response was given as median fluorescence intensity (MFI) on at least 100 beads per  
100 set. Net MFI values were generated by subtraction of bead-background and GST-tag  
101 background MFI values.

102 Due to lack of an appropriate serological gold standard for comparison with our assay, the  
103 cut-off definition for SGG antibody positivity was arbitrarily defined, as described  
104 elsewhere<sup>16</sup>. The distribution of antibody responses (MFI) to all eleven SGG proteins among  
105 controls was skewed towards low MFI, especially when compared to the outer membrane  
106 protein (OMP) of *Helicobacter pylori* (*H. pylori*), analyzed in the same experimental setting  
107 (Fig 1): the upper quartile of antibody responses does not exceed 100 MFI for any of the SGG  
108 antigens, whereas this antibody level was exceeded by 50% of the control sera to *H. pylori*  
109 OMP. Among controls, we compared the frequency of individuals with the highest antibody  
110 responses (upper 10<sup>th</sup> percentile) to each protein with the frequency of individuals exceeding  
111 the same level of antibody response among cases. The technical minimum cut-off was 30 MFI  
112 (Table 1). Overall SGG positivity was defined as samples giving a high response to any of the  
113 eleven SGG proteins to allow for individual immune responses and infection with different  
114 strains. In a previous case-control study, we showed that refining the algorithm for overall  
115 SGG positivity to two or more proteins in a 6-marker panel (Gallo0272, Gallo0748,  
116 Gallo1675, Gallo2018, Gallo2178, Gallo2179) strengthened the association with CRC<sup>16</sup>. This  
117 algorithm was also applied here as a second definition for SGG positivity.

#### 118 *Statistical analyses*

119 Risk factors for SGG positivity among controls were analyzed using Chi-squared-tests. We  
120 estimated the association of incident CRC with antibody responses to individual SGG  
121 proteins, positivity to any of the eleven SGG proteins, or 2 or more proteins of the 6-marker  
122 panel<sup>16</sup> using conditional logistic regression models to compute odds ratios (OR) and 95%  
123 confidence intervals (95% CI). A p-value below 0.05 was considered statistically significant.



124 Statistical models were first run conditioned on the matching factors, and subsequently with  
125 multivariable adjustment for the following variables: level of education attainment, BMI,  
126 smoking status and level of alcohol consumption [g/day] at baseline assessment. Missing  
127 observations in these variables were included in the model as individual category to save  
128 statistical power. The resulting risk estimates did not substantially differ from those calculated  
129 without further adjustment (supplementary table S1). Sensitivity analyses were carried out  
130 excluding cases diagnosed within two years after blood draw to assess the potential for  
131 reverse causation.

132 Explorative sub-group analyses were conducted by sex, age at blood draw applying  
133 interaction analyses, as well as by anatomical sub-site.

134 All statistical analyses were performed with SAS version 9.4 (SAS Institute).

## 135 **Results**

### 136 *Study characteristics and risk factors for SGG positivity*

137 There were no significant differences between cases and controls in any of the baseline  
138 characteristics (Table 2).

139 The comparison of SGG positive versus negative control subjects did not identify any major  
140 determinants of SGG positivity (Supplementary table S2).

### 141 *Association of antibody responses to SGG with CRC risk*

142 The risk of developing CRC was significantly increased with positivity to any of the eleven  
143 SGG proteins (OR: 1.36, 95% CI: 1.04-1.77), and also positivity to individual SGG proteins  
144 Gallo0272 (OR: 1.59, 95% CI: 1.06-2.40), Gallo0748 (OR: 1.49, 95% CI: 1.02-2.16) and  
145 Gallo2178 (OR: 3.01, 95% CI: 1.49-6.08) (Table 3). Positivity for two or more proteins of the

146 previously identified 6-marker panel (Gallo0272, Gallo0748, Gallo1675, Gallo2018,  
147 Gallo2178 and Gallo2179)<sup>16</sup> was also significantly associated with increased CRC risk (OR:  
148 2.17, 95% CI: 1.44-3.27) with 9% positive controls compared to 17% positive cases.

149 To assess the potential impact of reverse causation, we performed a sensitivity analysis  
150 excluding those cases diagnosed within 2 years after blood draw and their respective controls  
151 (Table 3). The association for positivity to any of the eleven SGG proteins (OR: 1.38, 95%  
152 CI: 1.02-1.87) as well as positivity to two or more proteins of the 6-marker panel (OR: 2.07,  
153 95% CI: 1.29-3.31) with CRC risk was generally unaltered. Positivity to individual proteins  
154 Gallo0272 (OR: 1.87, 95% CI: 1.15-3.05) and Gallo2178 (OR: 3.28, 95% CI: 1.25-8.57)  
155 retained statistical significance while Gallo0748 lost significance but with little change in the  
156 magnitude of the risk estimate (OR: 1.40, 95% CI: 0.90-2.18).

#### 157 *Explorative subgroup analyses*

158 Positivity for two or more proteins of the 6-marker panel was associated with only a minor  
159 fraction of CRC cases (17%). We assessed whether particular subgroups showed different risk  
160 associations for CRC. Analyses stratified by age at blood draw and sex did not reveal any  
161 statistically significant difference between the subgroups.

162 Separate analyses by colon or rectal sub-site showed different associations (Fig 2). Positivity  
163 to two or more proteins of the 6-marker panel was associated with a 10-fold increased risk of  
164 rectal cancer (95% CI: 1.05-95.78) and a much lower, but also statistically significant, near  
165 two-fold higher risk for colon cancer (OR: 1.96, 95% CI: 1.28-3.00). However, it is important  
166 to note that the number of rectal cancers was small (n=53) resulting in wide confidence  
167 intervals and imprecision of the risk estimate.

## 168 Discussion

169 In this CRC case-control study nested within the prospective multinational EPIC cohort we  
170 found that antibody responses to SGG proteins, in particular to two or more proteins  
171 seropositive among a 6-marker panel (Gallo0272, Gallo0748, Gallo1675, Gallo2018,  
172 Gallo2178 and Gallo2179) were significantly associated with risk of developing CRC.

173 These findings replicate and expand previous findings from two case-control studies with  
174 CRC cases from Spain (multicenter case-control study (MCC Spain))<sup>14</sup> and an independent  
175 German study<sup>16</sup>. In MCC Spain, an association of prevalent CRC with antibody responses to  
176 SGG protein Gallo2178 alone and Gallo2178 in combination with Gallo2179 was found<sup>14</sup>. In  
177 the German case-control study, the SGG multiplex serology panel was extended to eleven  
178 SGG proteins. Positivity to any of these proteins was associated with prevalent CRC.  
179 Seropositivity for at least two proteins from a 6-marker panel subset (Gallo0272, Gallo0748,  
180 Gallo1675, Gallo2018, Gallo2178 and Gallo2179) was more specifically associated with CRC  
181 (19% SGG positives) compared to controls (11% of SGG-positives)<sup>16</sup>.

182 It is currently unknown whether SGG infects colon tissue before or after initiation of tumor  
183 development. However, it is hypothesized that the commensal SGG enters the bloodstream  
184 through a leaky epithelium, arising due to various environmental exposures, or along the  
185 processes of CRC development<sup>4</sup>. This hypothesis is supported by observations showing the  
186 presence of SGG already in early colorectal lesions, including polyps and adenoma<sup>11, 12, 16, 19</sup>.  
187 Here, we offer the first prospective observational evidence to support early involvement of  
188 SGG in colon carcinogenesis by showing that antibody responses to SGG were more  
189 frequently present in subjects who later developed CRC even more than two years after blood  
190 draw than those who remained disease-free during the same time-frame. The natural history  
191 of CRC is characterized by the progressive development of neoplasia of the colon mucosa and  
192 can take up to 10-15 years from an initial polyp to tumor diagnosis. Therefore, it is likely that

193 a number of individuals in this study, who developed CRC, already had a precancerous lesion  
194 at the time of recruitment into the cohort, but were undiagnosed and likely asymptomatic.

195 Although we have no data on CRC screening to estimate the numbers with existing polyps, it  
196 is likely to be comparable to other European population studies, such as for Germany where  
197 the detection rate of non-advanced and advanced adenoma was 22.3% and 9.0%, respectively,  
198 among males and 14.9% and 5.2%, respectively, among females above age 55 years<sup>20</sup>. As  
199 only a minority of adenomas progress to cancer, a similar proportion of the controls would  
200 also be expected to have some form of colorectal adenoma at blood draw that had not  
201 progressed to malignant disease by the end of follow-up. Thus, the finding that antibody  
202 responses to SGG appear prior to cancer diagnosis raises the question whether SGG infection  
203 is a potential etiological factor in the transition of an adenoma to malignant disease and  
204 whether its detection could help stratify the risk for tumor progression from a precancerous  
205 lesion. However, we were unable to directly address this question within the limitations of our  
206 study. Studies by Abdulmir et al. found pro-inflammatory cytokine profiles in human CRC  
207 tissue positive for SGG DNA and support the hypothesis of an involvement of SGG in tumor  
208 progression<sup>11, 21</sup>. A recent study comprehensively showed that SGG promotes proliferation of  
209 colon cancer cells in vitro and tumor development in a mouse model overall supporting a role  
210 of SGG in colonic tumorigenesis<sup>22</sup>. Our observations will hopefully stimulate further  
211 epidemiological studies with CRC screening data and mechanistic investigations of the  
212 potential SGG induced transformation of benign polyps to more advanced disease states.

213 The antigens selected for SGG multiplex serology include proteins predicted to be present at  
214 the cell wall of the bacterium or to be secreted<sup>23, 24</sup>. Pilus proteins Gallo2178 and Gallo2179,  
215 both included in the 6-marker panel, were previously shown to be potential virulence factors  
216 in endocarditis and for infection of colon tumor tissue by mediating attachment to collagen in  
217 tissue<sup>10, 25</sup>. Functions of the other proteins had been so far only predicted by sequence

218 comparison to proteins of other bacteria and include enzymatic (Gallo0112A/B, Gallo0748,  
219 Gallo0933, Gallo2018) as well as adhesion functions (Gallo0272, Gallo0577, Gallo1570).  
220 The function of Gallo1675 is unknown<sup>26</sup>. Future studies should focus on this 6-marker panel  
221 as it is a stronger marker for CRC risk than being positive to any of the eleven proteins  
222 included in the multiplex serology (OR: 2.17 vs OR: 1.36, respectively).

223 Stratification by age and sex did not reveal statistically significant differences. However, the  
224 small sub-group sample sizes may have limited the analysis. Secondary sub-group analysis by  
225 anatomical sub-site suggested a stronger cancer risk association for the rectum versus the  
226 colon with SGG antibody responses. This observation is highly interesting and warrants  
227 further investigation, but is limited due to small number of rectal cancer cases (n=53)  
228 included in the present analysis. The disparity between the number of colon and rectal cancer  
229 cases analyzed in this study are due to limited availability of biological samples for the  
230 required laboratory analyses in this sub-set of EPIC CRC cases.

231 Key advantages of this study are its prospective setting, multi-center design and the use of a  
232 detailed, validated biomarker approach to assess SGG exposures. A main limitation is the  
233 small sample size, being based on a subset of CRC cases in the EPIC cohort with available  
234 biological samples for the required SGG biomarker analyses. Furthermore, the SGG  
235 exposures assessed here reflect levels at recruitment into the cohort upon blood collection and  
236 so may not pertain to longer term exposures. An additional potential limitation applicable to  
237 all observational studies is the possibility for residual or uncontrolled confounding. Although,  
238 the EPIC data have been very well measured and validated, the possibility of residual  
239 confounding cannot ever be wholly discounted. Uncontrolled confounding is unlikely because  
240 the multivariate adjusted model presented here addressed a large number of potentially  
241 important confounding variables. Nevertheless, in the absence of further confirmation of these

242 findings from a larger series of CRC cases from EPIC or from other prospective cohorts,  
243 caution in the interpretation of the findings is necessary.

244 In conclusion, this study provides the first exploration in a prospective setting of the  
245 association between SGG infection and risk of CRC development. Our observations indicate a  
246 positive association of antibody responses to SGG proteins with CRC risk, taking into account  
247 other important confounding factors. SGG infection, possibly acquired through lifestyle  
248 exposures leading to colonic epithelial barrier dysfunction, may be an important etiological  
249 component of CRC development. Thus, antibody responses to SGG proteins may be  
250 indicative for individuals at increased risk for developing CRC.

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**Supplementary Table S1: Antibody responses to SGG proteins and protein combinations in relation to CRC risk in a nested case-control study within EPIC**

	Positive n (%)		Unadjusted model <sup>1</sup>		Adjusted model <sup>2</sup>	
	Controls n=485	Cases n=485	OR	95% CI	OR	95% CI
Gallo0112A	33 (7)	37 (8)	1.14	0.69-1.90	1.09	0.64-1.84
Gallo0112B	28 (6)	26 (5)	0.93	0.54-1.60	0.96	0.55-1.67
Gallo0272	47 (10)	67 (14)	1.49	1.00-2.21	1.59	1.06-2.40
Gallo0577	47 (10)	49 (10)	1.05	0.69-1.59	1.03	0.67-1.59
Gallo0748	50 (10)	74 (15)	1.51	1.05-2.18	1.49	1.02-2.16
Gallo0933	49 (10)	44 (9)	0.89	0.58-1.36	0.92	0.59-1.43
Gallo1570	47 (10)	52 (11)	1.13	0.73-1.74	1.13	0.72-1.76
Gallo1675	48 (10)	51 (11)	1.07	0.70-1.63	1.08	0.70-1.67
Gallo2018	47 (10)	54 (11)	1.16	0.77-1.74	1.24	0.81-1.89
Gallo2178	12 (2)	31 (6)	2.58	1.33-5.03	3.01	1.49-6.08
Gallo2179	47 (10)	64 (13)	1.43	0.95-2.14	1.48	0.97-2.24
Any SGG protein	273 (56)	306 (63)	1.32	1.02-1.71	1.36	1.04-1.77
≥ 2 of 6-marker panel <sup>3</sup>	45 (9)	83 (17)	2.03	1.37-3.01	2.17	1.44-3.27

<sup>1</sup>Conditional logistic regression model conditioned on the matching factors; <sup>2</sup> Model 1 with further adjustment for BMI, highest level of education attainment, smoking status and alcohol intake at baseline as categorical variables, missings in the variables considered as individual category; <sup>3</sup>Gallo0272, Gallo0748, Gallo1675, Gallo2018, Gallo2178, Gallo2179

**Supplementary Table S2: Comparison of SGG negative and positive individuals for demographic and other risk factors among controls.**

		Any <i>S. gallolyticus</i> protein			≥2 of 6-marker panel <sup>2</sup>		
		neg (n=212)	pos (n=273)	p- value <sup>1</sup>	neg (n=440)	pos (n=45)	p- value <sup>1</sup>
Sex	female	103 (49)	144 (53)	0.363	225 (51)	22 (49)	0.774
	male	109 (51)	129 (47)		215 (49)	23 (51)	
Age at blood draw, years	37-55	44 (21)	76 (28)	0.180	106 (24)	14 (31)	0.573
	56-60	55 (26)	69 (25)		113 (26)	11 (24)	
	61-77	113 (53)	128 (47)		221 (50)	20 (44)	
	mean (range)	60 (39-77)	59 (37-75)		60 (37-77)	59 (37-74)	
Country	France	4 (2)	7 (3)	0.907	9 (2)	2 (4)	0.497
	Italy	47 (22)	54 (20)		95 (22)	6 (13)	
	Spain	37 (17)	45 (16)		75 (17)	7 (16)	
	United Kingdom	60 (28)	74 (27)		119 (27)	15 (33)	
	The Netherlands	29 (14)	41 (59)		61 (14)	9 (20)	
	Greece	3 (1)	8 (3)		11 (3)	0 (0)	
	Germany	32 (15)	55 (58)		70 (16)	6 (13)	
Education	≤primary school	92 (45)	120 (46)	0.736	196 (46)	16 (39)	0.663
	technical/professional	54 (26)	61 (23)		103 (24)	12 (29)	
	≥secondary school	60 (29)	82 (31)		129 (30)	13 (32)	
	missing	6	10		12	4	
BMI	<25	76 (36)	91 (33)	0.177	151 (34)	16 (36)	0.739
	25-29.9	95 (45)	143 (52)		218 (50)	20 (44)	
	≥30	41 (19)	39 (14)		71 (16)	9 (20)	
Smoking status	never	94 (45)	140 (51)	0.316	212 (48)	22 (49)	0.238
	former	73 (35)	81 (30)		136 (31)	18 (40)	
	current	44 (21)	51 (19)		90 (21)	5 (11)	
	missing	1	1		2	0	
Alcohol intake at baseline (g/day)	<6	100 (47)	129 (47)	0.925	209 (48)	20 (44)	0.316
	6-20	54 (25)	73 (27)		118 (27)	9 (20)	
	>20	58 (27)	71 (26)		113 (26)	16 (36)	

<sup>1</sup>Pearson's Chi-Square-test; <sup>2</sup>Gallo0272, Gallo0748, Gallo1675, Gallo2018, Gallo2178 and Gallo2179

1 **Tables:**

2 **Table 1: Antigens included in SGG (strain UCN34) multiplex serology and antigen**  
3 **specific cut-offs.**

Name	Putative function	Antigen specific cut-off (MFI)
Gallo0112A	Fructan hydrolase N-terminus	30
Gallo0112B	Fructan hydrolase C-terminus	30
Gallo0272*	Glucan binding protein C domain	192
Gallo0577	Cell-wall protein with CnaB domain	185
Gallo0748*	Cell-envelope proteinase A	96
Gallo0933	Tannase	175
Gallo1570	<i>Pil2</i> pilus subunit	185
Gallo1675*	Cell wall protein of unknown function	36
Gallo2018*	Putative cell wall protein involved in bacteriocin synthesis	95
Gallo2178*	<i>Pil1</i> pilus subunit (major pilin)	30
Gallo2179*	<i>Pil1</i> pilus subunit (collagen-binding domain)	118

4 \* antigens included in 6-marker panel

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6 **Table 2: Baseline characteristics of the CRC case-control study nested within EPIC**

		Controls (n=485) n (%)	Cases (n=485) n (%)
Sex	female	247 (51)	247 (51)
	male	238 (49)	238 (49)
Age at blood draw, years	37-55	120 (25)	121 (25)
	56-60	124 (25)	122 (25)
	61-77	241 (50)	242 (50)
	Mean (range)	60 (37-77)	59 (37-77)
Country	France	11 (2)	11 (2)
	Italy	101 (21)	101 (21)
	Spain	82 (17)	82 (17)
	United Kingdom	134 (28)	134 (28)
	The Netherlands	70 (14)	70 (14)
	Greece	11 (2)	11 (2)
	Germany	76 (16)	76 (16)
Education	≤primary school	212 (45)	215 (46)
	Technical/professional	115 (25)	95 (21)
	≥secondary school	142 (30)	153 (33)
	missing	16	22
BMI	<25	167 (34)	160 (33)
	25-29.9	238 (49)	220 (45)
	≥30	80 (16)	105 (22)
Smoking status	never	234 (48)	202 (42)
	former	154 (32)	183 (38)
	current	95 (20)	96 (20)
	missing	2	4
Alcohol intake at baseline (g/day)	<6	229 (47)	213 (44)
	6-20	127 (26)	127 (26)
	>20	129 (27)	144 (30)
	missing	0	1

7

8 **Table 2: Comparison of SGG negative and positive individuals for demographic and**  
 9 **other risk factors among controls.**

		Any <i>S. galloyticus</i> protein			≥2 of 6 marker panel <sup>2</sup>		
		neg (n=212)	pos (n=273)	p- value <sup>1</sup>	neg (n=440)	pos (n=45)	p- value <sup>1</sup>
Sex	female	103 (49)	144 (53)	0.363	225 (51)	22 (49)	0.774
	male	109 (51)	129 (47)		215 (49)	23 (51)	
Age at blood draw, years	37-55	44 (21)	76 (28)	0.180	106 (24)	14 (31)	0.573
	56-60	55 (26)	69 (25)		113 (26)	11 (24)	
	61-77	113 (53)	128 (47)		221 (50)	20 (44)	
	mean (range)	60 (39-77)	59 (37-75)		60 (37-77)	59 (37-74)	
Country	France	4 (2)	7 (3)	0.907	9 (2)	2 (4)	0.497
	Italy	47 (22)	54 (20)		95 (22)	6 (13)	
	Spain	37 (17)	45 (16)		75 (17)	7 (16)	
	United Kingdom	60 (28)	74 (27)		119 (27)	15 (33)	
	The Netherlands	29 (14)	41 (15)		61 (14)	9 (20)	
	Greece	3 (1)	8 (3)		11 (3)	0 (0)	
	Germany	32 (15)	55 (20)		70 (16)	6 (13)	
Education	≤primary school	92 (45)	120 (46)	0.736	196 (46)	16 (39)	0.663
	technical/professional	54 (26)	61 (23)		103 (24)	12 (29)	
	≥secondary school	60 (29)	82 (31)		129 (30)	13 (32)	
	missing	6	10		12	4	
BMI	<25	76 (36)	91 (33)	0.177	151 (34)	16 (36)	0.739
	25-29.9	95 (45)	143 (52)		218 (50)	20 (44)	
	≥30	41 (19)	39 (14)		71 (16)	9 (20)	
Smoking status	never	94 (45)	140 (51)	0.316	212 (48)	22 (49)	0.238
	former	73 (35)	81 (30)		136 (31)	18 (40)	
	current	44 (21)	51 (19)		90 (21)	5 (11)	
	missing	1	1		2	0	
Alcohol intake at baseline (g/day)	<6	100 (47)	129 (47)	0.925	209 (48)	20 (44)	0.316
	6-20	54 (25)	73 (27)		118 (27)	9 (20)	
	>20	58 (27)	71 (26)		113 (26)	16 (36)	

<sup>1</sup>Pearson's Chi-Square test; <sup>2</sup>Gallo0272, Gallo0748, Gallo1675, Gallo2018, Gallo2178 and Gallo2179

10 | **Table 43: Antibody responses to SGG proteins in relation to CRC incidence in a nested case-control study within EPIC**

	All				Diagnosed more than 2 years after blood draw			
	Positive n (%)		OR <sup>1</sup>	95% CI	Positive n (%)		OR <sup>1</sup>	95% CI
	Controls n=485	Cases n=485			Controls n=355	Cases n=355		
Gallo0112A	33 (7)	37 (8)	1.09	0.64-1.84	22 (6)	23 (6)	1.08	0.55-2.11
Gallo0112B	28 (6)	26 (5)	0.96	0.55-1.67	15 (4)	16 (5)	1.13	0.55-2.32
Gallo0272	47 (10)	67 (14)	1.59	1.06-2.40	32 (9)	51 (14)	1.87	1.15-3.05
Gallo0577	47 (10)	49 (10)	1.03	0.67-1.59	34 (10)	36 (10)	1.05	0.64-1.72
Gallo0748	50 (10)	74 (15)	1.49	1.02-2.16	37 (10)	51 (14)	1.40	0.90-2.18
Gallo0933	49 (10)	44 (9)	0.92	0.59-1.43	37 (10)	38 (11)	1.05	0.64-1.73
Gallo1570	47 (10)	52 (11)	1.13	0.72-1.76	36 (10)	41 (12)	1.19	0.72-1.96
Gallo1675	48 (10)	51 (11)	1.08	0.70-1.67	38 (11)	39 (11)	1.09	0.67-1.76
Gallo2018	47 (10)	54 (11)	1.24	0.81-1.89	38 (11)	43 (12)	1.22	0.77-1.95
Gallo2178	12 (2)	31 (6)	3.01	1.49-6.08	7 (2)	17 (5)	3.28	1.25-8.57
Gallo2179	47 (10)	64 (13)	1.48	0.97-2.24	34 (10)	44 (12)	1.47	0.90-2.40
Any SGG protein	273 (56)	306 (63)	1.36	1.04-1.77	201 (57)	224 (63)	1.38	1.02-1.87
≥2 of 6-marker panel <sup>2</sup>	45 (9)	83 (17)	2.17	1.44-3.27	36 (10)	60 (17)	2.07	1.29-3.31

<sup>1</sup>Conditional logistic regression model with multivariable adjustment for BMI, education, smoking and alcohol intake at baseline; <sup>2</sup>Gallo0272, Gallo0748, Gallo1675, Gallo2018, Gallo2178, Gallo2179