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Research Article

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Antibody Responses to *Streptococcus Gallolyticus* Subspecies *Gallolyticus* Proteins in a Large Prospective Colorectal Cancer Cohort Consortium

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Abstract

Background: Antibody responses to *Streptococcus gallolyticus* subspecies *gallolyticus* (SGG) proteins, especially pilus protein Gallo2178, have been consistently associated with colorectal cancer risk. Previous case–control studies and prospective studies with up to 8 years of follow-up, however, were unable to decipher the temporality of antibody responses to SGG in the context of the long-term multistep development of colorectal cancer. In this study, we analyzed a large U.S. colorectal cancer cohort consortium with follow-up beyond 10 years for antibody responses to SGG.

Methods: We applied multiplex serology to measure antibody responses to 9 SGG proteins in participants of 10 prospective U.S. cohorts (CLUE, CPSII, HPFS, MEC, NHS, NYUWHS, PHS, PLCO, SCCS, and WHI) including 4,063 incident colorectal cancer cases and 4,063 matched controls. Conditional logistic regression was used to assess whether antibody responses to SGG were associated with colorectal cancer risk, overall and by time between blood draw and diagnosis.

Results: Colorectal cancer risk was increased among those with antibody responses to Gallo2178, albeit not statistically significant [OR, 1.23; 95% confidence interval (CI), 0.99–1.52]. This association was stronger for cases diagnosed <10 years after blood draw (OR, 1.40; 95% CI, 1.09–1.79), but was not found among cases diagnosed ≥10 years after blood draw (OR, 0.79; 95% CI, 0.50–1.24).

Conclusions: In a large cohort consortium, we reproduced the association of antibody responses to SGG Gallo2178 with colorectal cancer risk for individuals diagnosed within 10 years after blood draw.

Impact: This timing-specific finding suggests that antibody responses to SGG are associated with increased colorectal cancer risk only after tumorigenesis has begun. *Cancer Epidemiol Biomarkers Prev; 27(10); 1186–94. ©2018 AACR.*

Introduction

The intestinal commensal *Streptococcus gallolyticus* subspecies *gallolyticus* (SGG), formerly known as *Streptococcus bovis* type I, has been frequently associated with the presence of colorectal adenoma and cancer (1). The connection was first identified when cases of SGG-caused infective endocarditis presented with a concomitant adenoma/cancer in the gut (2, 3).

Previous cross-sectional studies have shown that SGG is associated with colorectal cancer as well as with precursor lesions. These studies were performed either directly by detecting bacterial DNA in tumor tissue or indirectly by serological methods (4–12). In our laboratory, we established a multiplex serology assay (13) to measure antibody responses to up to 11 SGG antigens. These antigens were selected on the basis of either known immunogenicity or predicted localization on the surface of the bacterium

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

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(12). Of specific interest were the proteins Gallo2178 and Gallo2179 that build a pilus structure, which has been shown to be important for adhesion and virulence of SGG (14, 15). Our laboratory group has previously applied SGG multiplex serology to analyze two colorectal cancer case–control studies conducted in Spain and Germany and found a reproducible association between antibody responses to SGG pilus protein Gallo2178, potentially detecting present as well as past exposure of SGG to the host's immune system, and the presence of colorectal cancer, with significant OR ranging from 1.58 to 4.13 (11, 12). Our laboratory group further analyzed serum samples from newly diagnosed colorectal cancer cases in the European Prospective Investigation into Nutrition and Cancer (EPIC) study and showed that antibody responses to Gallo2178 were statistically significantly associated with a 3-fold higher risk of colorectal cancer in prediagnostic blood samples taken up to 8 years before diagnosis with 2% of controls and 6% of cases affected. In addition, we identified two other proteins included in SGG multiplex serology (Gallo0272 and Gallo0748) to be significantly associated with colorectal cancer risk in the EPIC study; however, with these two proteins the risk was increased by only up to 60% (16).

The cross-sectional findings involving adenomas and the finding with the prospective design demonstrated that SGG infection occurred prior to clinical colorectal cancer diagnosis. The limitation, however, of the previous prospective study was a relatively short duration of follow-up (8 years). This is important in the background of the long-term multistep process of genetic and morphologic changes in the gut epithelial tissue that lead to colorectal cancer development. The process of development from an initial polyp to colorectal cancer is estimated to last on average 10–15 years but may vary between 5 and 25 years, dependent on the type of polyp/adenoma and personal risk factor background (17). Thus, for colorectal cancer cases diagnosed within 10 years of antibody assessment, carcinogenesis has probably already begun. Current literature suggests that colorectal cancer–specific conditions promote colonization of SGG in the gut (18, 19) and that SGG may contribute directly to the process of carcinogenesis (10).

To further support our investigation into the timing of the SGG antibody association with colorectal cancer development, we also sought to assess a potential difference in the association by p53 autoantibody status. Loss of function of the tumor suppressor p53 has been found to drive the transition from late adenomas to cancer (20). Missense mutations in the *p53* gene lead to inactivation of the protein's function, and the mutated protein then accumulates for unknown reasons in the cancer cells (21). A minority of these patients (20%–40%) develop autoantibodies against the accumulating p53, which have been shown to be a specific but insensitive marker for presence of colorectal cancer (22). For example, a recent prospective study in the Cancer Prevention Study-II (CPS-II) found a statistically significant association between anti-p53 antibodies and colorectal cancer risk within 3 years of diagnosis with 13% sero-positive colorectal cancer cases and 6% sero-positive controls (23). Thus, assuming that p53 autoantibodies serve as a surrogate for presence of undiagnosed (pre-)cancerous colorectal lesions, if the association between SGG and colorectal cancer is strongest for individuals with detectable p53 autoantibodies, this would also suggest that the role of SGG in colorectal cancer development begins after the initiation of the carcinogenesis process.

In summary, in this study, we sought to examine the temporality of the association between prediagnostic antibody responses to SGG and colorectal cancer by analyzing data and samples from a large U.S. cohort consortium with median follow-up times ranging from 4 to 18 years in the participating studies, enabling us to assess how long before colorectal cancer diagnosis antibody responses to SGG can be associated with colorectal cancer risk.

We further explored the interaction of p53 and SGG serology under the assumption that p53 autoantibodies indicate colorectal lesions already present at baseline blood draw. Both aims seek to address the association of SGG and colorectal cancer under the *a priori* hypothesis that development of antibody responses against SGG is dependent on the presence of a (pre-)cancerous lesion.

Materials and Methods

Study population

This colorectal cancer cohort consortium comprises 10 prospective U.S. cohorts: Campaign Against Cancer and Stroke (CLUE; ref. 24), CPS-II (25), Health Professionals Follow-up Study (HPFS; ref. 26), Multiethnic Cohort Study (MEC; ref. 27), Nurses' Health Study (NHS; ref. 28), NYU Women's Health Study (NYUWHS; ref. 29), Physicians' Health Study (PHS; ref. 30), Prostate, Lung, Colorectal, and Ovarian Screening Study (PLCO; ref. 31), Southern Community Cohort Study (SCCS; ref. 32), and Women's Health Initiative (WHI; ref. 33).

Participating cohorts contributed prediagnostic blood samples and baseline sociodemographic information from colorectal cancer cases and controls for the current study. Cases were defined on the basis of the International Classification of Diseases for Oncology (ICD-O-3) and included all cancers of colon and rectum coded as C180-189, C199, and C209. Controls were randomly selected and matched at a 1:1 ratio to each colorectal cancer case by cohort, sex, race, date of birth ($\pm$1 year, relaxed up to $\pm$5 years for sets without available controls), and date of blood collection ($\pm$1 month, relaxed up to $\pm$3 months, and further to $\pm$6 months for sets without available controls). All controls were alive and free of cancer (except nonmelanoma skin cancer) at the time of diagnosis of the index case. Study-specific information on cohort size, age at blood draw, number of contributed cases, sex distribution, and range of follow-up time is given in Supplementary Table S1.

In total, samples from 4,210 colorectal cancer cases and their 4,210 matched controls were assayed by multiplex serology. One hundred samples and therefore their 100 matched case or control counterparts had to be excluded due to technical issues, including insufficient volume ($n = 27$), pipetting errors ($n = 52$), or invalid measurements due to insufficient bead counts ($n = 21$). Furthermore, 47 pairs were excluded due to mismatches in race and sex, resulting in a final sample number of 4,063 cases and their respective 4,063 controls.

Multiplex serology

Serum samples were sent on dry ice to the German Cancer Research Center (DKFZ, Heidelberg, Germany) and analyzed in a 1:1,000 final serum dilution. Multiplex serology was performed as described previously (12, 13, 16, 34). Briefly, multiplex serology is a fluorescent bead-based assay allowing for analysis of antibody responses to several antigens in one reaction. Antigens were expressed as Glutathione-*S*-transferase (GST)-tagged fusion

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proteins and affinity purified on polystyrene beads (Luminex Corp) coupled to glutathione–casein. Different antigens were purified on different bead sets as defined by the bead's internal fluorescence. The antigen-loaded bead sets were mixed and incubated with serum. A Luminex flow cytometer then distinguished between the bead set, and therefore the loaded antigen, as well as quantified the amount of bound serum antibody by a secondary antibody detecting human IgG, IgA, and IgM and a fluorescent reporter conjugate (Streptavidin-R–phycoerythrin). The output was the median fluorescence intensity (MFI) measured on at least 100 beads per set per sample. Net MFIs were generated by subtracting two background values resulting from a well containing no serum but antigen-loaded beads and all secondary reagents as well as from a bead set loaded with GST-tag only.

SGG antigens included in the multiplex serology were expressed from strain UCN34. Previous analyses with this assay applied in total eleven SGG antigens (12, 16); however, we excluded two previously noninformative antigens for the current study (Supplementary Table S2). P53 serology was applied as described previously (34).

Antigen-specific cutoffs were defined arbitrarily by visual inspection of percentile plots at the approximate inflection point of the curve to dichotomize antibody responses as sero-positive and -negative as described previously for other antigens (Supplementary Table S2; refs. 35–37).

Of 82 duplicates within the WHI study set incorporated as blinded quality control samples, correlations for antibody responses (MFI) to SGG antigens ranged from 0.91 to 1.0 indicating a good reproducibility of the measured values.

Statistical analysis

Pearson χ^2 test was used to assess differences in baseline characteristics between SGG Gallo2178–negative and -positive controls. Conditional logistic regression was applied to analyze the association of each individual SGG protein as well as p53 antibodies with colorectal cancer risk and to determine OR and 95% confidence interval (CI) overall, and by interval between blood draw and diagnosis (<10 years vs. ≥10 years). A P value of below 0.05 was considered statistically significant. All matched case–control sets were exactly matched by study, race, and sex but we observed residual confounding by age, which, however, did not affect the strength of the estimates for the exposures of interest. Potential confounders (apart from the matching variables age, sex, and race within each cohort) were defined *a priori* and included education, smoking, body mass index (BMI), and family history of colorectal cancer. To note, among these there was a substantial amount of missing data: 13% of study participants were lacking data on BMI, and 25% on family history of colorectal cancer. Among the potential confounders a BMI greater than 30 was associated both with colorectal cancer risk and antibody positivity to Gallo2178 (Tables 1 and 2). However, adjusting for any of the potential confounding variables, although excluding participants with missing data, did not alter the SGG estimates by more than 10% and therefore results are presented without further adjustment. The observed difference in prevalence of antibody positivity to Gallo2178 by study was accounted for by matching and subsequent conditional logistic regression. However, we also analyzed the association using conditional logistic regression separately by study to see whether these differences potentially affected the association. This analysis was performed separately for cases diagnosed within 10 years after their blood draw and cases diagnosed longer than 10 years after blood draw because studies differed in their follow-up time, which was hypothesized to modify the SGG–colorectal cancer association.

Analysis of the association of antibody responses to SGG with colorectal cancer risk stratified by p53 antibody positivity was performed using an unconditional logistic regression model adjusting for matching factors age, sex, study, and race/ethnicity.

We further explored the association of SGG with colorectal cancer risk separately by different case characteristics. Specifically, we assessed the association in separate models by stage according to TNM classification (early stage I/II and late stage III/IV), site [colon (left, right, and not otherwise specified (NOS) and rectum)] and age at diagnosis (≤65, 66–75, 76–85, and >85 years).

Results

Cases and controls differed in their baseline characteristics with respect to BMI and family history of colorectal cancer: specifically, cases were more likely to be obese and to have a positive colorectal cancer family history (Table 1).

Because only antibody responses to SGG protein Gallo2178 were significantly associated with colorectal cancer, we assessed potential risk factors for Gallo2178 antibody positivity but not the other SGG proteins. Prevalence of antibody positivity to Gallo2178 among controls differed by study, with higher prevalence in MEC and SCCS and lower in WHI, as well as by race and ethnicity, with lower prevalence among whites and higher prevalence in African Americans and Latinos. In addition, obese individuals and never smokers were more likely to be Gallo2178 antibody positive (Table 2).

Overall, antibody positivity to none of the nine SGG proteins was associated with increased colorectal cancer risk. The only suggestion of an increased risk was seen for antibody positivity to Gallo2178, the one protein previously associated with colorectal cancer risk, with 4% sero-prevalence among controls compared with 5% among cases, which resulted in a 23% statistically nonsignificant increase in odds for colorectal cancer risk. When exploring the association in individuals diagnosed within 10 years after blood draw, the OR for most of the antigens remained around 1, whereas the association of Gallo2178 with colorectal cancer risk was stronger with a statistically significant OR of 1.40 (95% CI, 1.09–1.79; $P = 0.008$). Excluding those individuals diagnosed within 2 years after blood draw from this subgroup did not alter the observed association of Gallo2178 with colorectal cancer risk substantially (OR, 1.38; 95% CI, 1.05–1.82; $P = 0.020$). Among individuals diagnosed more than 10 years after blood draw, the OR for most of the SGG antigens was below 1 and there was no association of Gallo2178 with colorectal cancer risk in this subgroup (OR, 0.79; 95% CI, 0.50–1.24; $P = 0.300$) with 4% sero-positive controls compared with 3% sero-positive cases (Table 3).

Prevalence of antibody positivity to Gallo2178 differed by study among controls. We examined whether this influenced the association with colorectal cancer risk. This analysis was performed separately by time between blood draw and diagnosis to exclude the possibility that observed differences resulted from different follow-up times among studies. Within a follow-up time of less than 10 years the majority of studies found an OR above 1 for the association between Gallo2178 and colorectal cancer risk; however, this did not reach statistical significance in any of the individual cohorts alone. The association was strongest in CLUE

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Table 1. Baseline characteristics of the cohorts participating in this study

Variable	Total ($N = 8,126$)	Controls ($n = 4,063$)	Cases ($n = 4,063$)
Study, *n* (%)			
CLUE	982 (12)	491 (12)	491 (12)
CPS-II	722 (9)	361 (9)	361 (9)
HPFS	302 (4)	151 (4)	151 (4)
MEC	1,510 (19)	755 (19)	755 (19)
NHS	576 (7)	288 (7)	288 (7)
NYUWHS	572 (7)	286 (7)	286 (7)
PHS	360 (4)	180 (4)	180 (4)
PLCO	1,240 (15)	620 (15)	620 (15)
SCCS	252 (3)	126 (3)	126 (3)
WHI	1,610 (20)	805 (20)	805 (20)
Age at blood draw (years)			
Median (range)	64 (18–89)	64 (18–88)	64 (18–89)
Sex, *n* (%)			
Female	5,112 (63)	2,556 (63)	2,556 (63)
Male	3,014 (37)	1,507 (37)	1,507 (37)
Race/ethnicity, *n* (%)			
White	6,134 (75)	3,067 (75)	3,067 (75)
African American	798 (10)	399 (10)	399 (10)
Asian American	614 (8)	307 (8)	307 (8)
Latino	422 (5)	211 (5)	211 (5)
Other/unknown/multiracial	158 (2)	79 (2)	79 (2)
Education, *n* (%)			
Less than HS	972 (12)	468 (12)	504 (12)
Completed HS or GED	1,668 (21)	823 (20)	845 (21)
Post HS training other than college	362 (4)	183 (5)	179 (4)
Some college	1,672 (21)	845 (21)	827 (20)
College graduate	1,483 (18)	756 (19)	727 (18)
Graduate school	1,861 (23)	946 (23)	915 (23)
Missing	108 (1)	42 (1)	66 (2)
BMI[a] (kg/m^2), *n* (%)			
<30	5,360 (66)	2,781 (69)	2,579 (64)
≥30	1,691 (21)	748 (18)	943 (23)
Missing	1,075 (13)	534 (13)	541 (13)
Smoking, *n* (%)			
Never	3,628 (45)	1,853 (46)	1,775 (44)
Ever	4,409 (54)	2,168 (53)	2,241 (55)
Missing	89 (1)	42 (1)	47 (1)
Family history of CRC[b], *n* (%)			
No	5,167 (64)	2,638 (65)	2,529 (62)
Yes	907 (11)	408 (10)	499 (12)
Missing	2,052 (25)	1,017 (25)	1,035 (26)

Abbreviations: CRC, colorectal cancer; GED, General Educational Development Test; HS, high school.

[a]All studies except CLUE (variable not available).

[b]All studies except NYUWHS, CLUE (variable not available), and SCCS (<75% of variable information available).

with 0% sero-positive controls compared with 5% of cases (OR not calculable). In 2 of 10 cohorts, there was no association suggested between Gallo2178 and colorectal cancer risk: NYUWHS (OR, 1.00; 95% CI, 0.25–4.00) and SCCS (OR, 0.89; 95% CI, 0.34–2.30; Fig. 1A). When the time between blood draw and diagnosis was ≥10 years, a result of a statistically insignificant OR of 1 or below was found for 5 of 8 studies with a sample size of at least 20 cases. In contrast, 3 (PLCO, MEC, and HPFS) of the 8 studies were suggestive of an OR of above 1, although with very wide CIs (Fig. 1B).

Previous studies have shown that autoantibodies to p53 are a marker for presence of colorectal cancer (38). In this colorectal cancer cohort consortium, we found antibody positivity to p53 was statistically significantly associated with colorectal cancer risk among those individuals diagnosed within 10 years of their blood draw (OR, 1.53; 95% CI, 1.23–1.89) but not diagnosed after 10 years from blood draw (OR, 0.87; 95% CI, 0.50–1.33). Assuming that p53 autoantibodies serve as a surrogate for presence of undiagnosed colorectal lesions present at baseline blood collection, we performed a case–control analysis of antibody responses to Gallo2178 with colorectal cancer stratified by p53 autoantibody positivity. In line with results presented above by time between blood draw and diagnosis, association of antibody responses to Gallo2178 with colorectal cancer risk was stronger among p53-autoantibody–positive individuals in the overall study (OR, 2.74; 95% CI, 1.09–6.87) than in p53-autoantibody–negative individuals (OR, 1.15; 95% CI, 0.92–1.43, $P_{\text{interaction}} = 0.073$; Table 4). The difference between p53-autoantibody positives and negatives in the association of antibodies to Gallo2178 with colorectal cancer risk was smaller within 10 years after blood draw. Here, there was a statistically significant association of antibody responses to Gallo2178 with colorectal cancer risk observable also among p53-autoantibody negatives (OR, 1.33; 95% CI, 1.02–1.72). In contrast, the association among p53-autoantibody positives was slightly weaker than in the overall cohort and not statistically significant (OR, 2.37; 95%

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Table 2. Risk factors for antibody positivity to SGG protein Gallo2178 among controls in the cohort consortium

Variable	Gallo2178 neg ($n = 3900$)	Gallo2178 pos ($n = 163$)	P^a
Study, *n* (%)			
CLUE	484 (12)	7 (4)	
CPS-II	352 (9)	9 (6)	
HPFS	143 (4)	8 (5)	
MEC	701 (18)	54 (33)	
NHS	271 (7)	17 (10)	
NYUWHS	268 (7)	18 (11)	
PHS	174 (4)	6 (4)	
PLCO	607 (16)	13 (8)	
SCCS	116 (3)	10 (6)	
WHI	784 (20)	21 (13)	**<0.0001**
Age at blood draw (years)			
Median (range)	64 (18–88)	64 (31–84)	0.399
Sex, *n* (%)			
Female	2,447 (63)	109 (67)	
Male	1,453 (37)	54 (33)	0.285
Race/Ethnicity, *n* (%)			
White	2,976 (76)	91 (56)	
African American	370 (9)	29 (18)	
Asian American	293 (8)	14 (9)	
Latino	189 (5)	22 (13)	
Other/unknown/multiracial	72 (2)	7 (4)	**<0.0001**
Education, *n* (%)			
Less than HS	442 (11)	26 (16)	
Completed HS or GED	793 (20)	30 (18)	
Post HS training other than college	178 (5)	5 (3)	
Some college	814 (21)	31 (19)	
College graduate	722 (19)	34 (21)	
Graduate school	913 (23)	33 (20)	0.370
Missing	38 (1)	4 (2)	
BMI[b] (kg/m^2), *n* (%)			
<30	2,670 (68)	111 (68)	
> = 30)	704 (18)	44 (27)	**0.025**
Missing	526 (13)	8 (5)	
Smoking, *n* (%)			
Never	1,765 (45)	88 (54)	
Ever	2,096 (54)	72 (44)	**0.021**
Missing	39 (1)	3 (2)	
Family history of CRC[c], *n* (%)			
No	2,532 (65)	106 (65)	
Yes	391 (10)	17 (10)	0.887
Missing	977 (25)	40 (25)	

Abbreviations: CRC, colorectal cancer; GED, General Educational Development Test; HS, high school.

[a]χ^2 test for categorical variables; *t* test for continuous variables (age); *P* values below 0.05 are considered statistically significant and are marked in bold font.

[b]All studies except CLUE (variable not available).

[c]All studies except NYUWHS, CLUE (variable not available), SCCS (<75% of variable information available).

CI, 0.92–6.09; $P_{interaction} = 0.315$). Among those cases who were diagnosed ≥10 years after blood draw, the prevalence of Gallo2178 antibodies was higher among cases than controls only among p53-autoantibody positives (5% positive cases compared with 0% positive controls; OR not calculable) but not among p53-autoantibody negatives (OR, 0.74; 95% CI, 0.47–1.16; $P_{interaction}$ not calculable; Table 4).

Finally, we addressed potential differences in the association of Gallo2178 and colorectal cancer risk by characteristics of colorectal cancer and did not identify a substantial difference in the association by stage, site, or age at diagnosis (Supplementary Table S3).

Table 3. Antibody positivity to SGG proteins and colorectal cancer risk, overall and by time between blood draw and diagnosis (follow-up)

	Overall				<10 years follow-up				≥10 years follow-up			
SGG protein	Controls $n = 4,063$ n pos (%)	Cases $n = 4,063$ n pos (%)	OR[a] (95% CI)	*P*	Controls $n = 2,766$ n pos (%)	Cases $n = 2,766$ n pos (%)	OR[a] (95% CI)	*P*	Controls $n = 1,296$ n pos (%)	Cases $n = 1,296$ n pos (%)	OR[a] (95% CI)	*P*
Gallo0112A	320 (8)	308 (8)	0.96 (0.81–1.13)	0.610	228 (8)	219 (8)	0.96 (0.78–1.17)	0.647	92 (7)	89 (7)	0.96 (0.71–1.31)	0.815
Gallo0272	292 (7)	302 (7)	1.04 (0.88–1.23)	0.671	215 (8)	237 (9)	1.11 (0.92–1.35)	0.280	77 (6)	65 (5)	0.84 (0.60–1.17)	0.311
Gallo0577	246 (6)	256 (6)	1.05 (0.87–1.26)	0.636	175 (6)	198 (7)	1.15 (0.93–1.43)	0.207	71 (5)	59 (4)	0.80 (0.55–1.15)	0.226
Gallo0748	394 (10)	372 (9)	0.94 (0.81–1.09)	0.402	271 (10)	263 (10)	0.97 (0.81–1.16)	0.717	123 (9)	109 (8)	0.87 (0.66–1.15)	0.325
Gallo1570	266 (7)	259 (6)	0.97 (0.81–1.16)	0.748	183 (7)	191 (7)	1.05 (0.85–1.30)	0.663	83 (6)	68 (5)	0.80 (0.57–1.12)	0.201
Gallo1675	352 (9)	351 (9)	1.00 (0.85–1.17)	0.968	233 (8)	257 (9)	1.12 (0.93–1.35)	0.250	119 (9)	94 (7)	0.76 (0.57–1.02)	0.067
Gallo2018	287 (7)	264 (7)	0.91 (0.76–1.09)	0.298	226 (8)	212 (8)	0.93 (0.76–1.14)	0.476	52 (4)	61 (5)	0.84 (0.57–1.24)	0.371
Gallo2178	163 (4)	197 (5)	1.23 (0.99–1.52)	0.063	117 (4)	160 (6)	**1.40 (1.09–1.79)**	**0.008**	36 (4)	37 (3)	0.79 (0.50–1.24)	0.300
Gallo2179	209 (5)	213 (5)	1.02 (0.84–1.24)	0.840	149 (5)	158 (6)	1.07 (0.84–1.35)	0.593	60 (5)	55 (4)	0.91 (0.63–1.33)	0.632

[a]Conditional logistic regression model, controls are matched to cases by age, sex, and race/ethnicity; significant associations are marked in bold font ($P < 0.05$); and Pos = antibody positive.

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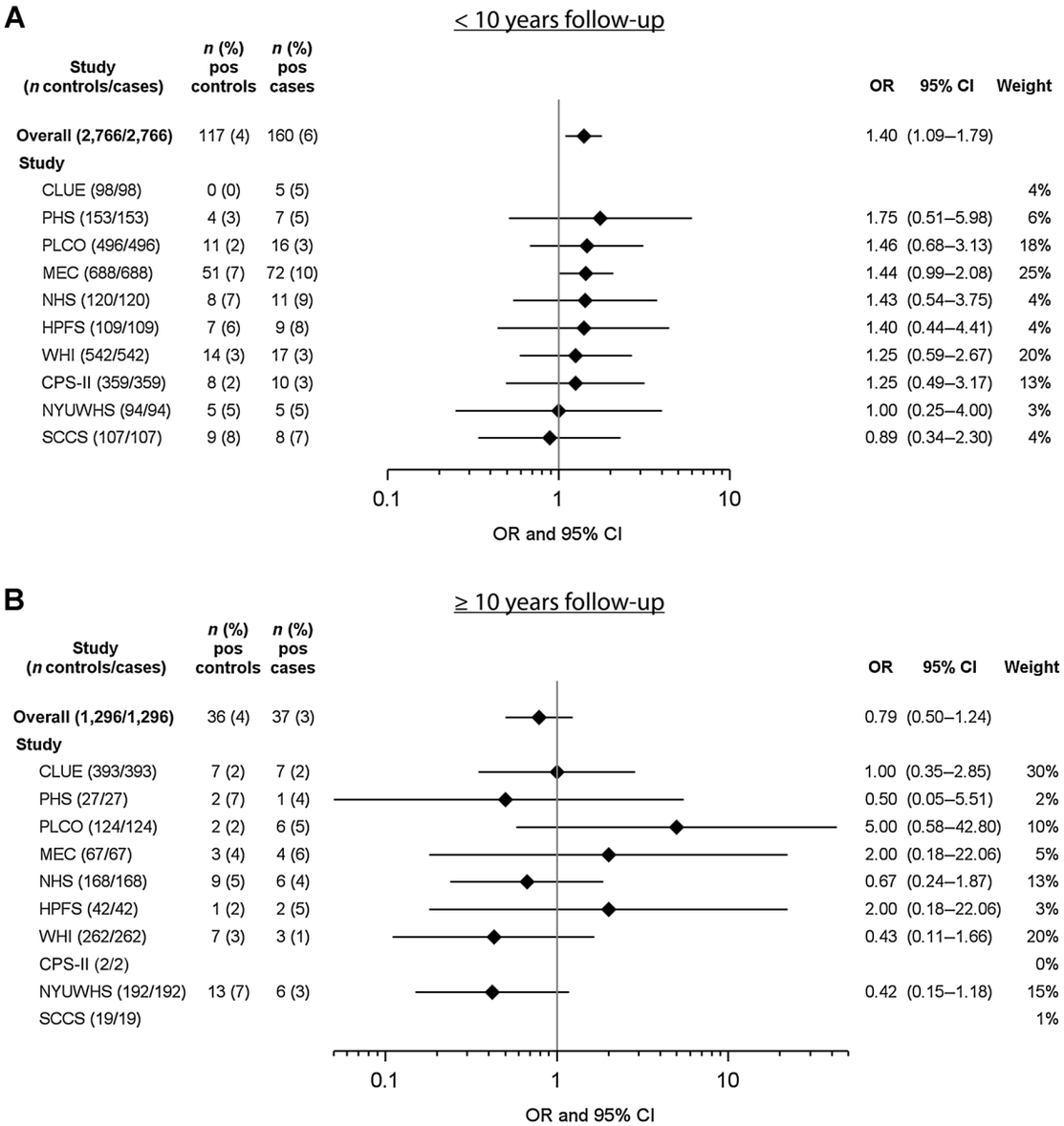

Figure 1.

Forest plot of antibody positivity to Gallo2178 and colorectal cancer risk by study, within 10 years of blood draw (**A**) and (**B**) with diagnosis after more than 10 years of blood draw. Conditional logistic regression models were applied to determine OR (diamonds) and 95% CI (horizontal lines); controls are matched to cases by study, age, sex and race/ethnicity. **A,** No OR is given for CLUE because the denominator was 0. **B,** No values are given for CPSII and SCCS because total case numbers are below 20. Weight shows the contribution of each study to the overall number of participants in percentage. The vertical line at an OR of 1 serves as a reference for null association. Pos, antibody positive.

Discussion

In this colorectal cancer cohort consortium, we found that antibody responses to SGG protein Gallo2178 were statistically significantly associated with a 40% increase in colorectal cancer risk among individuals diagnosed within 10 years of their blood draw and that there was no association with antibody responses to SGG for individuals diagnosed 10 or more years after blood draw. Furthermore, the association of antibody responses to Gallo2178 with colorectal cancer risk was more pronounced among p53-autoantibody–positive cases, a surrogate for the presence of (pre-) cancerous lesions at baseline. These data support the hypothesis that SGG infection of gut epithelial tissue after an initial precursor lesion has formed may act as a cancer promotor increasing colorectal cancer risk once tumorigenesis has already begun. Because this was a serologic study; however, it could not be assessed whether SGG might have colonized the gut lumen before an antibody response in serum was detectable and whether this

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Table 4. Antibody positivity to Gallo2178 and colorectal cancer risk by p53 auto-antibody positivity and time between blood draw and diagnosis

		N	N (%) Gallo2178 pos			
Follow-up time	**p53 autoantibody**	**Controls/cases**	**Controls**	**Cases**	**OR[a] (95% CI)**	**$P_{interaction}$[a]**
Overall	Neg	3865/3797	156 (4)	174 (5)	1.15 (0.92–1.43)	
	Pos	198/266	7 (4)	23 (9)	**2.74 (1.09–6.87)**	0.073
<10 years	Neg	2614/2540	110 (4)	139 (5)	**1.33 (1.02–1.72)**	
	Pos	152/226	7 (5)	21 (9)	2.37 (0.92–6.09)	0.315
≥10 years	Neg	1250/1256	46 (4)	35 (3)	0.74 (0.47–1.16)	
	Pos	46/40	0 (0)	2 (5)	—	—

Abbreviations: Neg, negative; Pos, positive.

[a]Unconditional logistic regression model with adjustment for study, sex, race, and age; significant associations ($P < 0.05$) are marked in bold font.

colonization might have had increased the risk of developing colorectal cancer already. Another interpretation of our findings would be simply that individuals with (pre-)cancerous lesions are more likely to harbor SGG antibodies than individuals farther away from cancer development.

Gallo2178 is expressed from the *pil1* operon together with proteins Gallo2177 and Gallo2179. These proteins build a SGG pilus that was shown to be important for bacterial adhesion to collagen as well as for biofilm formation, which consequently relates to SGG's virulence in infective endocarditis and infection of colorectal tissue (14, 15). Gallo2177 is a sortase that assembles Gallo2178 and Gallo2179 into a pilus structure (15). Gallo2179 was shown to have collagen-binding abilities, preferentially collagen type I, present for example in damaged heart valves, followed by collagen type IV, found in the basal lamina of epithelial tissues. This collagen-binding domain is similar to collagen-binding proteins of other bacteria, for example, *Staphylococcus aureus*. Gallo2178 has been shown to be the major pilin, the backbone structure of the pilus (14, 15). Why only antibody responses to Gallo2178 but not to other SGG proteins, including Gallo2179, were significantly associated reproducibly in this and previous studies (11, 12, 16) remains unclear. Possible factors affecting this involve host immune responses recognizing antigenic epitopes differently between individuals as well as bacterial strains expressing different sets of proteins. Supporting the latter, the prevalence of antibody positivity to Gallo2178 among controls was lower than to any of the other analyzed SGG proteins. The underlying reason could be a higher specificity of this protein due to lower similarities to proteins of other bacteria and consequently less cross-reactivity. For example, there is no homolog of Gallo2178 encoded in *Staphylococcus aureus*, which is not true for Gallo2179 (39). This could make Gallo2178 a more specific marker than the other proteins included in SGG multiplex serology leading to a stronger association with colorectal cancer risk.

In the few prior research studies, antibody responses to Gallo2178 have been consistently associated with colorectal cancer. A small study by Boleij and colleagues analyzed early-stage colorectal cancer ($n = 44$) and asymptomatic controls ($n = 47$) for antibody responses to Gallo2178 and found 9% positive cases at a predefined specificity of 100% (6). We previously reported antibody responses to Gallo2178 associated with colorectal cancer with up to 4-fold increased odds in two independent case–control studies (11, 12). The new data from the prospective analysis presented here are in line with an independent prospective study from Europe: antibody responses to Gallo2178 were associated with a 3-fold increased risk for colorectal cancer in blood samples taken up to 8 years before diagnosis (16). In both prospective studies, antibody responses to Gallo2178 were found in only 6% of colorectal cancer cases, making it a rare exposure. PCR data from Lopes and colleagues support this as they found SGG DNA in only 5 rectal swab specimens (11%) out of 54 individuals undergoing colonoscopy (40).

The novelty of this study, however, is that we were able to analyze blood samples with a longer follow-up time than in the study described above (16), that is, taken more than 8 years before diagnosis. It is estimated that progression from a colorectal polyp to colorectal cancer lasts 10–15 years (17). Antibody responses to Gallo2178 were not associated with increased colorectal cancer risk when the blood was sampled 10 or more years prior to diagnosis. These results were in line with analysis stratified by antibody positivity to tumor suppressor p53, a putative marker for prevalent undiagnosed colorectal cancer and adenoma (38): colorectal cancer risk was increased almost 3-fold with antibody responses to Gallo2178 only among p53 antibody-positive individuals, that is, those with a suspected prevalent lesion in the gut. When regarding this interaction separately by follow-up time, antibody responses to Gallo2178 were associated with colorectal cancer risk also in the p53 autoantibody negatives within 10 years of diagnosis but not when follow up was equal to or more than 10 years. Individuals diagnosed within 10 years of their blood draw were much more likely to have already had a precursor lesion at the time of blood draw than those individuals with a longer time span between blood sampling and diagnosis as supported by the p53-autoantibody finding. As proposed by Tjalsma and colleagues, SGG could be considered a so-called passenger bacterium, a pathogen-turned commensal that is able to infect the epithelium upon decrease of the gut epithelial integrity after tumor formation, which then might or might not act as a carcinogenesis-promoting agent (41). This hypothesis is supported by several mechanistic studies including a study by Boleij and colleagues, where it was shown that SGG growth is stimulated *in vitro* under metabolic conditions of the colorectal cancer microenvironment (19). Aymeric and colleagues reproduced this finding and showed that SGG colonization in the colon is promoted by colorectal cancer–specific conditions: increased secondary bile–acids in the oncogenic context were shown to induce bacteriocin synthesis in SGG, which led to killing of other bacterial species creating a new niche for SGG colonization in the gut (18). Infection of the gut epithelial tissue by SGG was shown to be enabled by the presence of collagen-rich surfaces as present in (pre-)cancerous lesions with diminished epithelial integrity but not by adhesion to or internalization by normal epithelial cells themselves (14).

A promoting effect of SGG infection on carcinogenesis and thus on the formation of malignant cancer out of a precursor lesion cannot directly be inferred from the data presented here: we showed only that antibody responses to SGG within 10 years of diagnosis were associated with an increased risk of developing colorectal cancer. Thus, these antibodies were more frequent in

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individuals later diagnosed with malignant disease. It is instructive that a cancer-promoting effect of SGG was suggested by a mechanistic study by Kumar and colleagues, who showed that SGG treatment increased proliferation of colorectal cancer cell lines but also tumor burden in an azoxymethane-induced mouse model of colorectal cancer (10). Further studies are needed to confirm these findings and identify the underlying mechanisms.

This study does have several limitations. First, assessment of SGG infection by serologic analysis provided data on a systemic marker for past, acute, or chronic SGG infection. Thus, this method is not able to determine whether there exists a local acute or persistent infection at the site of interest. However, the advantage is that a blood draw is less invasive and easier to conduct on a large scale than a biopsy in the gut, especially in asymptomatic individuals. Unfortunately, although, the natural history of antibody responses to SGG proteins is unknown, including at what exact event antibody responses first occur (e.g., colonization of the epithelium and entry into blood stream) and how stable these antibody responses are. Serial samples from the same individual over time could give insight into these questions. Furthermore, the cut-off for sero-positivity to SGG was set arbitrarily due to a lack of reference samples because no serologic gold standard for SGG diagnostics are available. The reproducibility of the association of antibody responses to Gallo2178 with colorectal cancer risk in independent studies (11, 12, 16); however, supports the strength of the findings presented here. This reproducibility also argues against a potential misinterpretation of the results as being just a chance finding. A Bonferroni correction for multiple testing in this study would have required a P value of 0.006 for significance with 9 SGG antigens analyzed. The P value for the association of antibody responses to Gallo2178 with colorectal cancer risk within 10 years after blood draw was 0.008 and thus would not have been regarded as a statistically significant finding outside the already published context (11, 12, 16). A further limitation of the study was missing information in variables of potentially high interest, such as family history of colorectal cancer and BMI, which might have led to residual confounding. However, it should be emphasized that the large sample size of this consortium offered the possibility to comprehensively assess the association of this rare exposure with colorectal cancer risk. Finally, a more detailed analysis by follow-up time beyond 10 years might have provided a more elaborate view on the temporality of the association of SGG with colorectal cancer risk; however, we were not powered for such further stratified analyses due to the rarity of the exposure.

In conclusion, we reproduced the finding that antibody responses to SGG Gallo2178 were associated with a 1.4-fold higher risk for colorectal cancer development among individuals who were diagnosed within 10 years of blood draw. This temporality of the association together with an interaction with autoantibodies to p53, a putative marker for undiagnosed (pre-)cancerous lesions at baseline, supports the hypothesis that SGG infection of gut epithelial tissue in individuals for whom tumorigenesis has already begun may promote carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Butt, K. Visvanathan, Y. Chen, Y. Bao, H.D. Sesso, G.Y. Ho, T.L. Cover, L.H. Hendrix, L.-C. Huang, M. Pawlita, M. Epplein
Writing, review, and/or revision of the manuscript: J. Butt, W.J. Blot, L.R. Teras, K. Visvanathan, L. Le Marchand, Y. Chen, Y. Bao, H.D. Sesso, S. Wassertheil-Smoller, G.Y. Ho, L.F. Tinker, R.M. Peek, J.D. Potter, T.L. Cover, T. Waterboer, M. Pawlita, M. Epplein
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Study supervision: M. Pawlita, M. Epplein

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