Antibody responses to *Helicobacter pylori* and risk of developing colorectal cancer in a European cohort

Running title (50/60 characters): *Helicobacter pylori* serology and colorectal cancer

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ABSTRACT

Background: While *Helicobacter pylori* (*H. pylori*) is the major cause of gastric cancer, it has also been suggested to be involved in colorectal cancer (CRC) development. However, prospective studies addressing *H. pylori* and CRC are sparse and inconclusive. We assessed the association of antibody responses to *H. pylori* proteins with CRC in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort.

Methods: We applied *H. pylori* multiplex serology to measure antibody responses to 13 *H. pylori* proteins in pre-diagnostic serum samples from 485 CRC cases and 485 matched controls nested within the EPIC study. Odds ratios (OR) and 95% confidence intervals (CI) were calculated using multivariable conditional logistic regression to estimate the association of *H. pylori* overall and protein-specific sero-positivity with odds of developing CRC.

Results: Fifty-one percent of CRC cases were *H. pylori* sero-positive compared to 44% of controls resulting in an OR of 1.36 (95% CI: 1.00-1.85). Among the 13 individual *H. pylori* proteins, the association was driven mostly by sero-positivity to *Helicobacter* cysteine-rich protein C (HcpC) (OR: 1.66, 95% CI: 1.19-2.30) and Vacuolating cytotoxin A (VacA) (OR: 1.34, 95% CI: 0.99-1.82), the latter being non-statistically significant only in the fully adjusted model.

Conclusion: In this prospective multi-center European study, antibody responses to *H. pylori* proteins, specifically HcpC and VacA, were associated with an increased risk of developing CRC.

Impact: Biological mechanisms for a potential causal role of *H. pylori* in colorectal carcinogenesis need to be elucidated, and subsequently whether *H. pylori* eradication may decrease CRC incidence.

Keywords (5): *Helicobacter pylori*, serology, colorectal cancer, prospective cohort, Europe

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a well-established cause of non-cardia gastric cancer and has been classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC) (1). In addition to gastric cancer, *H. pylori* infection has been investigated for an etiological role in other cancers of the digestive tract, including colorectal cancer (CRC) (2).

The most recent meta-analysis of 27 published case-control studies reported a pooled odds ratio (OR) of 1.27 (95% confidence interval (CI): 1.17-1.37) for the association of *H. pylori* with CRC (3). However, only a few prospective studies have assessed the risk of developing CRC with *H. pylori* sero-positivity, which moreover reported inconclusive results: Three studies conducted in Germany, Finland, and a Caucasian population in the United States (US) showed null findings (4-6), whereas two other US studies reported a positive association with sero-positivity to specific *H. pylori* proteins, including Vacuolating cytotoxin A (VacA), Chaperonin GroEl, *Helicobacter* cysteine-rich protein C (HcpC) and hypothetical protein HP1564 (7,8).

While a causality and potential biological mechanism for the observed association has not been verified yet, a study by Hu et al. further supports the hypothesis of an involvement of *H. pylori* in CRC development (9). These investigators found that the individuals either successfully eradicated for *H. pylori* infection or uninfected were at significantly lower risk of developing adenomas than those with persistent *H. pylori* infection (9).

To comprehensively assess the association of *H. pylori* infection with risk of developing CRC we analyzed antibody responses to 13 *H. pylori* proteins in a case-control study nested within a large European multi-center prospective cohort. We hypothesized that in addition to overall *H.*

pylori sero-positivity, protein-specific sero-positivity, including positivity to *H. pylori* virulence factors and toxins, would be specifically associated with CRC development.

Materials and methods

Study population, case ascertainment and control selection

This CRC case-control study is nested within the European Prospective Investigation into Nutrition and Cancer (EPIC) cohort study investigating the association between diet, lifestyle and environmental factors and cancer incidence. The rationale and methods of the EPIC design have been published previously (10). Briefly, more than 520,000 individuals, aged 35 to 70 years, were enrolled from 10 Western European countries between 1992 and 2000. The present analysis is based on participant data from 7 of these countries (France, Italy, Spain, United Kingdom, The Netherlands, Greece, and Germany). Dietary and lifestyle data, as well as serum samples, were collected at baseline, with standardized blood collection and processing protocols across the study centers. Serum samples were stored at the International Agency for Research on Cancer (IARC, Lyon, France) at -196°C and shipped on dry ice to the German Cancer Research Center (DKFZ), Heidelberg, Germany. The study has been approved by the IARC Ethics Committee and the ethics committees of all local EPIC centers.

The nested case-control study included pre-diagnostic serum samples from 492 CRC cases (primary tumors coded C18-C20 according to the 10th revision of the International Statistical Classification of Diseases, Injury and Causes of Death). The median time between blood draw and diagnosis was 3.4 years with a range of 0.4 to 8.5 years. Controls were selected by incidence density sampling from all cohort members alive and free of cancer at the time of

matching and matched to cases 1:1 by age at blood collection (± 6 months to ± 2 years), sex, study center, time of the day at blood collection (± 2 to 4 hours interval), fasting status at blood collection (<3/3-6 hours); among women by menopausal status, and among premenopausal women by phase of menstrual cycle and hormone therapy use at time of blood collection. Seven case-control pairs were excluded from the analysis due to technical errors during multiplex serology measurement, resulting in a final sample set of 485 CRC cases and 485 controls for analysis.

H. pylori multiplex serology

Serum samples were analyzed for antibodies against *H. pylori* proteins in a 1:1,000 dilution using multiplex serology. The methodology has been described in detail elsewhere (11,12). Briefly, *H. pylori* proteins (**Supplementary Table S1**) were recombinantly expressed as GST-X-tag fusion proteins and affinity purified on glutathione-casein coated beads, where each bead type has a different internal fluorescent color (SeroMap, Luminex Corp., Austin, TX, USA) to identify the loaded antigen. A mixture of bead sets carrying different antigens was incubated with pre-diluted serum. A Luminex xMAP analyzer (Luminex Corp., Austin, TX, USA) was used to identify the bead type and simultaneously quantify the amount of bound serum antibody by biotinylated goat anti-human IgA/IgM/IgG antibody and a fluorescent reporter conjugate Streptavidin-R-Phycoerythrin. The level of antibody response is given as median fluorescence intensity (MFI) of at least 100 beads per type measured. Background values against the N-terminal GST, the C-terminal tag, and the bead-surface were subtracted to generate net MFI values.

Antigen-specific cut-offs (**Supplementary Table S1**) were defined as described previously at the approximate inflection point of frequency distribution curves (8). Overall sero-positivity to *H. pylori* was assigned to individuals sero-positive for at least 4 out of the 13 *H. pylori* proteins included in the multiplex serology panel, as these had previously been shown to offer the best specificity and sensitivity when the assay was validated against a commercially available ELISA (12).

Statistical analysis

Wilcoxon Mann-Whitney test was applied to compare continuous antibody responses (MFI) to *H. pylori* proteins between controls and cases.

Conditional logistic regression models were applied to compute ORs, and the corresponding 95% CIs, for the association of antibody responses to *H. pylori* overall sero-positivity and sero-positivity to individual *H. pylori* proteins with CRC development. To increase specificity, study participants were only considered *H. pylori* antigen positive to any single antigen when simultaneously classified as overall *H. pylori* sero-positive.

The following variables were considered as potential confounders and therefore included in the model for adjustment: body mass index (BMI; <25, 25-29.9, ≥30 kg/m^2), smoking status (never, former, current), alcohol consumption (<6, 6-20, ≥20 g/day), and highest education attained at baseline (≤primary school, technical/professional, ≥secondary school) (model 1). We also performed an exploratory assessment of the impact of dietary variables and physical activity on the association. Using backward elimination only total daily energy intake [kcal], as well as daily intake of fish [g] and dairy products [g] contributed significantly to the model in our population

and were included in a second model in addition to the above-mentioned potential confounders (model 2). We furthermore performed a stratified analysis by country since *H. pylori* sero-prevalence widely varied geographically (**Supplementary Table S2**).

All statistical analyses were performed with SAS version 9.4 (SAS Institute, Cary, NC, USA). A *p*-value below 0.05 was considered significant.

Results

Study characteristics and risk factors for H. pylori sero-positivity

Cases and controls did not differ in any of the baseline socio-demographic characteristics (**Table 1**). When assessing dietary variables, cases had a higher daily energy intake as well as lower intake of dairy products than controls at baseline (**Table 1**). Sero-positivity to *H. pylori* at baseline was more frequent in individuals with higher BMI (≥30), lower education (≤primary school), and lower physical activity index (≤moderately inactive) (**Supplementary Table S2**). Furthermore, baseline *H. pylori* sero-prevalence varied by country with highest rates in Spain (73%) and Italy (54%), followed by Germany (42%), and lowest values in the Netherlands (30%) and the UK (27%). Sample sizes from France and Greece (each contributing 11 controls) were too small to be considered for comparison (**Supplementary Table S2**). Regarding dietary variables, *H. pylori* sero-positive individuals exhibited a lower daily intake of dairy products at baseline (**Supplementary Table S2**).

Association of antibody responses to H. pylori proteins with CRC development

We first assessed whether incident CRC cases and controls differed in their level of antibody response, given by continuous MFI, to *H. pylori* proteins (**Figure 1**). MFI level tended to be

higher among CRC cases than among controls to all the antigens, except for NapA. Continuous MFI to GroEl, HcpC, and VacA were significantly higher among CRC cases than controls (**Figure 1**).

Applying cut-offs for sero-positivity, overall 51% of cases were *H. pylori* sero-positive (positive to ≥4 out of 13 *H. pylori* proteins) as opposed to 44% of controls, which resulted in significantly increased odds of developing CRC (OR: 1.41, 95% CI: 1.06-1.89, *P*=0.02). The association remained significant after adjustment for potential socio-demographic confounders (OR: 1.43, 95% CI: 1.06-1.93, *P*=0.02) and dietary variables (OR: 1.36, 95% CI: 1.00-1.85, *P*=0.05) (**Table 2**). For individual *H. pylori* proteins, sero-positivity to HcpC (28% of cases versus 20% of controls) and VacA (36% of cases versus 30% of controls) was significantly associated with increased odds of developing CRC in the model adjusting for socio-demographic variables (OR: 1.66, 95% CI: 1.19-2.30, *P*<0.01 and OR: 1.38, 95% CI: 1.02-1.86, *P*=0.04 respectively) (**Table 2**). Adjustment for dietary variables did not change the OR substantially, however, the association of CRC with VacA sero-positivity lost significance (OR: 1.34, 95% CI: 0.99-1.82, *P*=0.06), while the association with HcpC remained significant (OR: 1.66, 95% CI: 1.19-2.31, *P*<0.01) (**Table 2**). Odds for CRC were increased with sero-positivity to most of the other 11 proteins, although these results were not significant, with OR point estimates ranging from 1.39 (HpaA) to 0.93 (NapA) (**Table 2**).

H. pylori sero-prevalence is known to vary by European region. In our data, sub-group analyses by country did not differ significantly for *H. pylori* overall or to the two most strongly associated antigens (VacA and HcpC) (**Supplementary Figure S1**).

Discussion

In this case-control study nested within a large prospective European cohort, we observed that sero-positivity to at least 4 out of the 13 assessed *H. pylori* proteins was associated with significant 36% increased odds of developing CRC, after adjustments for potential confounding. Among these 13 individual *H. pylori* proteins, higher antibody levels to HcpC and VacA showed the strongest associations with CRC development.

These results concur with previous findings from two independent populations in the US, which found significant associations with sero-positivity to *H. pylori* HcpC and VacA using the same serological method (7,8). However, significant findings in the latter study of diverse populations in the US were predominantly found with African American CRC cases (n=399) and not the Caucasian American cases (n=3067). Two recent reports found an increased risk of developing advanced colorectal neoplasia among individuals with persistent *H. pylori* infection compared to non-infected individuals and/or individuals with successful *H. pylori* eradication (9,13). In line with this hypothesis, previous studies showed a higher risk of having colorectal adenomas for individuals exhibiting *H. pylori*-related chronic atrophic gastritis or more severe gastric conditions (14,15). We recently reported that antibody responses to HcpC and VacA are among the four antigens of our 13-plex serology panel that best indicate an active *H. pylori* infection (16). The toxin VacA is an important *H. pylori* virulence factor, which damages host cells by causing vacuolation (17), while the function of HcpC is unknown. Moreover, concordant with the previous US study (8), we here showed that not only sero-prevalence but also level of antibody response was higher among incident CRC cases than controls. These findings suggest that CRC risk may be increased when *H. pylori* infection is more severe. The severity of *H. pylori*

304 infection is thought to be dependent on the virulence of the bacterial strain, the individual host
305 response to the bacterium, and other environmental factors, including diet (18), potentially
306 explaining the observed difference in association among distinct populations.
307 The mechanism by which *H. pylori* might contribute to colon carcinogenesis remains unclear. A
308 direct effect would require the presence of *H. pylori* or its toxins in the colon. Presence of *H.*
309 *pylori* in the feces or tumor tissue of CRC patients is rarely reported (19-21). Thus, it needs to be
310 elucidated whether the bacterium is present in and induces a deleterious effect on the gut
311 epithelium. Another possibility is that *H. pylori* could exert indirect effects promoting colonic
312 carcinogenesis, e.g., by 1) creating a systemic inflammatory environment beyond the stomach
313 (22); 2) through inducing gut microbiome dysbiosis that in turn may exert a carcinogenic effect
314 (23); or 3) by inducing the production of effector molecules in the host like gastrin that could
315 have mitogenic effects in the colon (24-29). These hypotheses, however, need mechanistic
316 studies to show a causal relationship between *H. pylori* infection and CRC development. The
317 potential involvement of *H. pylori* infection in CRC etiology may depend on other co-factors and
318 its role may be stronger in populations where infection is more prevalent. We did not observe
319 significant heterogeneity by region or country, which could be a consequence of our limited
320 number of cases and thus a larger study would be important.
321 Our study has several limitations and strengths. As mentioned above, we applied a serological
322 assay to determine antibody responses to *H. pylori*. This is an indirect and systemic measure
323 and does not provide information on whether *H. pylori* may also locally infect the colorectal
324 tissue. Furthermore, we cannot distinguish between current and past infection, also due to the
325 lack of information on antibiotic treatment history. However, referring to the study by Hu et al.

(9), this would lead to a bias towards the null hypothesis, since the authors of this study reported that only persistent infections are associated with an increased risk of colorectal adenoma. Another limitation is the potential of residual confounding, which cannot completely be ruled out, although most EPIC data has been validated. A strength of the applied multiplex serology assay is its ability to analyze several antigens per infectious agent, allowing assessment of a more detailed host immune response to the bacterium beyond just overall sero-positivity. Due to the assessment of multiple antigens, it may be argued that correction of our findings for multiple comparisons is required. We contend against this for two reasons. First, the number of antigens tested was modest and, second, our study was based on a clear a priori hypothesis derived from previous findings for the same antigens (8). Nevertheless, even when applying the conservative Bonferroni-correction for the 13 *H. pylori* proteins included (*P*-value for significance = 0.004), the association of sero-positivity to HcpC with CRC risk would still retain statistical significance (*P*=0.003). Multiplex serology furthermore allows quantification of antibody response level to the respective antigens. As reported in a previous observational study, higher antibody levels in addition to sero-positivity were associated with increased odds of developing CRC (8), potentially referring to the severity of *H. pylori* infection and/or strength of the host's response to the infection. A major strength of the study design is that it is based on a large multi-center cohort covering most of Western Europe with detailed prospective data collection and serum samples taken several years prior to diagnosis allowing for a comprehensive adjustment for potential confounders including dietary variables. However, information on medication use, including antibiotics and non-steroidal anti-inflammatory drugs,

was not included in the baseline questionnaire of EPIC and could therefore not be assessed as potential confounders in the present analysis.

In conclusion, antibody responses to *H. pylori*, specifically to proteins HcpC and VacA, in pre-diagnostic serum samples were significantly associated with increased risk of developing CRC in the EPIC study. Our observations need verification in different populations, particularly in those with high *H. pylori* prevalence. Furthermore, it remains to be elucidated whether *H. pylori* infection is causally related to colorectal carcinogenesis and what the underlying biological mechanisms are. Direct pathogenic evidence would then advance *H. pylori* eradication or control as a potential beneficial effect for CRC prevention.

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464 **Tables**

465 **Table 1: Baseline characteristics of the CRC case-control study nested within the EPIC study**

	Total (n=970)	Controls (n=485)	Cases (n=485)
Sex, n (%)			
Female	494 (51)	247 (51)	247 (51)
Male	476 (49)	238 (49)	238 (49)
Age at blood draw [years], mean (SD)	60 (8)	60 (8)	60 (8)
Country, n (%)			
France	22 (2)	11 (2)	11 (2)
Italy	202 (21)	101 (21)	101 (21)
Spain	164 (17)	82 (17)	82 (17)
United Kingdom	268 (28)	134 (28)	134 (28)
The Netherlands	140 (14)	70 (14)	70 (14)
Greece	22 (2)	11 (2)	11 (2)
Germany	152 (16)	76 (16)	76 (16)
Education, n (%)			
≤Primary school	427 (46)	212 (45)	215 (46)
Technical/professional	210 (23)	115 (25)	95 (21)
≥Secondary school	295 (32)	142 (30)	153 (33)
Missing	38	16	22
BMI [kg/m^2], n (%)			
<25	327 (34)	167 (34)	160 (33)
25-29.9	458 (47)	238 (49)	220 (45)
≥30	185 (19)	80 (16)	105 (22)
Smoking status, n (%)			
Never	436 (42)	234 (48)	202 (42)
Former	337 (35)	154 (32)	183 (38)
Current	191 (20)	95 (20)	96 (20)
Missing	6	2	4
Alcohol intake [g/day], n (%)			
<6	442 (46)	229 (47)	213 (44)
6-20	254 (26)	127 (26)	127 (26)
≥20	273 (28)	129 (27)	144 (30)
Missing	1	0	1
Physical activity[a], n (%)			
Inactive	326 (34)	158 (33)	168 (35)
Moderately inactive	296 (31)	138 (29)	158 (33)
Moderately active	178 (19)	92 (19)	86 (18)
Active	162 (17)	93 (19)	69 (14)
Missing	8	4	4
Dietary Variables, intake/day, mean (SD)			
Total energy [kcal]	2111 (729)	2062 (615)	2160 (826)
Total Vegetables [g]	198 (128)	200 (123)	196 (132)
Total fruits [g]	258 (192)	255 (181)	261 (203)

Dairy [g]	324 (238)	336 (229)	312 (246)
Cereals [g]	222 (133)	226 (145)	219 (120)
Fish [g]	33 (33)	34 (34)	32 (32)
Red meats [g]	44 (34)	42 (29)	45 (39)
Processed meats [g]	32 (49)	30 (28)	35 (64)

BMI, body mass index; g, grams; kcal, kilocalories; SD, standard deviation;
[a]Cambridge physical activity index

Table 2: Antibody responses to *H. pylori* proteins and odds of developing CRC; the EPIC study

Antigen	Positive n (%) Controls n=485	Cases n=485	Crude Model OR (95% CI)[b]	Crude Model p-value[b]	Multivariable Model 1 OR (95% CI)[c]	Multivariable Model 1 p-value[c]	Multivariable Model 2 OR (95% CI)[d]	Multivariable Model 2 p-value[d]
***H. pylori* Overall+**	214 (44)	247 (51)	**1.41 (1.06-1.89)**	**0.02**	**1.43 (1.06-1.93)**	**0.02**	**1.36 (1.00-1.85)**	**0.05**
HcpC+[a]	95 (20)	134 (28)	**1.65 (1.20-2.27)**	**<0.01**	**1.66 (1.19-2.30)**	**<0.01**	**1.66 (1.19-2.31)**	**<0.01**
VacA+[a]	145 (30)	174 (36)	**1.37 (1.02-1.83)**	**0.04**	**1.38 (1.02-1.86)**	**0.04**	1.34 (0.99-1.82)	0.06
HpaA+[a]	36 (7)	48 (10)	1.40 (0.88-2.24)	0.16	1.46 (0.90-2.36)	0.13	1.39 (0.85-2.26)	0.19
Catalase+[a]	112 (23)	134 (28)	1.31 (0.96-1.78)	0.09	1.32 (0.97-1.82)	0.08	1.24 (0.90-1.72)	0.19
GroEl+[a]	208 (43)	231 (48)	1.30 (0.96-1.74)	0.09	1.29 (0.95-1.75)	0.11	1.23 (0.90-1.68)	0.20
CagA+[a]	133 (27)	152 (31)	1.24 (0.92-1.68)	0.15	1.28 (0.94-1.74)	0.12	1.24 (0.91-1.70)	0.18
HP1564+[a]	177 (36)	201 (41)	1.28 (0.96-1.69)	0.09	1.27 (0.95-1.69)	0.11	1.26 (0.94-1.69)	0.13
UreA+[a]	102 (21)	122 (25)	1.29 (0.94-1.76)	0.12	1.27 (0.92-1.75)	0.14	1.23 (0.89-1.70)	0.22
HP0231+[a]	93 (19)	102 (21)	1.16 (0.84-1.60)	0.37	1.18 (0.85-1.65)	0.32	1.17 (0.83-1.63)	0.37
HP0305+[a]	94 (19)	106 (22)	1.16 (0.85-1.59)	0.34	1.17 (0.85-1.61)	0.35	1.12 (0.80-1.56)	0.50
Cad+[a]	67 (14)	77 (16)	1.18 (0.83-1.67)	0.37	1.15 (0.80-1.65)	0.46	1.17 (0.81-1.70)	0.41
HyuA+[a]	116 (24)	119 (25)	1.04 (0.76-1.42)	0.81	1.02 (0.74-1.41)	0.89	1.03 (0.74-1.43)	0.86
NapA+[a]	128 (26)	127 (26)	0.99 (0.74-1.32)	0.94	0.97 (0.72-1.31)	0.84	0.93 (0.68-1.26)	0.63

[a]Participants were only considered antigen-positive when simultaneously overall *H. pylori* sero-positive (i.e. ≥4 out of 13 antigens positive);

[b] Crude Model: Conditional logistic regression model based on the matching factors;

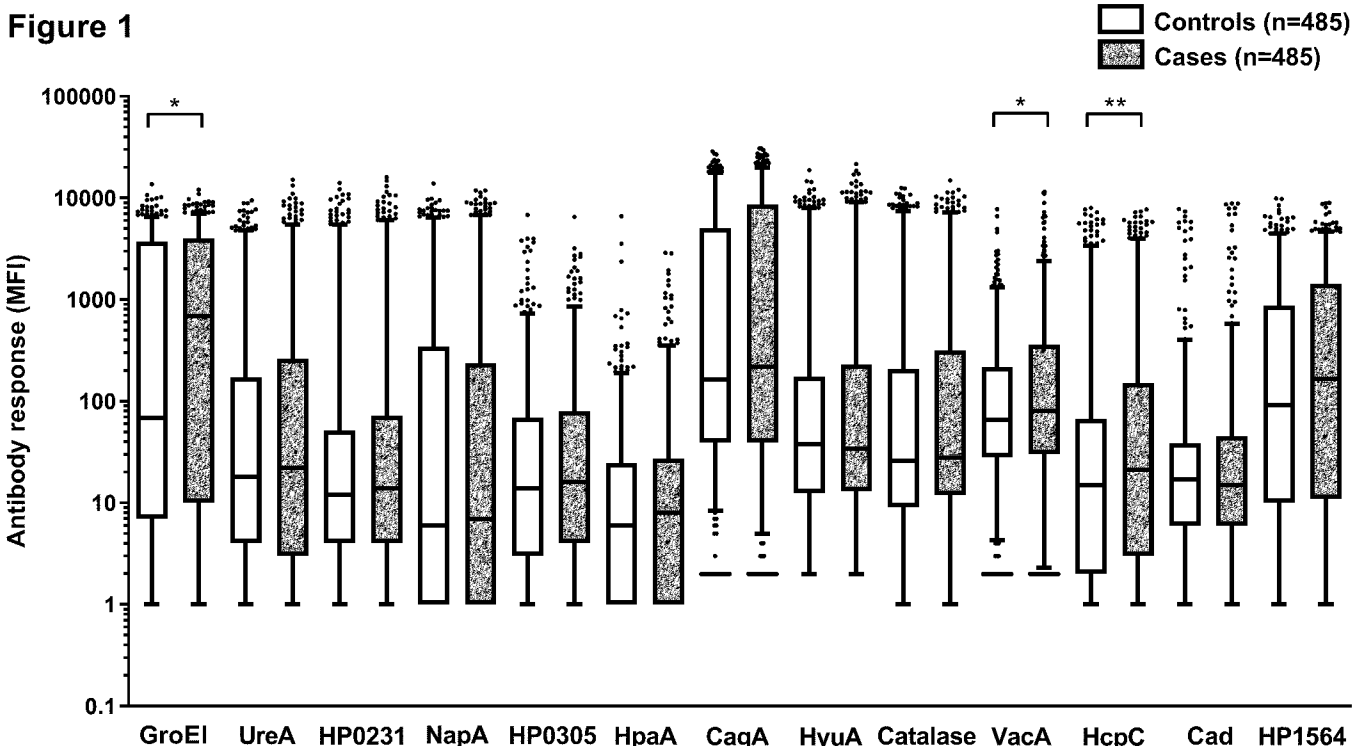
[c] Multivariable model 1: crude model plus additional adjustment for BMI (<25, 25-29.9, ≥30 kg/m^2), smoking status (never, former, current), alcohol consumption (<6, 6-20, ≥20 g/day), and highest education attained at baseline (≤primary school, technical/professional, ≥secondary school);

[d]Multivariable model 2: multivariable model 1 plus additional adjustment for daily intake levels of dairy products [g], fish [g], and total energy [kcal] (all as continuous variables); significant associations ($p < 0.05$) are marked in bold font.

Figure legends

Figure 1: Antibody responses (median fluorescence intensities, MFI) to *H. pylori* proteins in cases and controls; the EPIC study. Boxes represent 25th to 75th and whiskers the 5th to 95th percentile, solid lines show the median. Dots represent data points lying outside the 5th and 95th percentiles, respectively. Wilcoxon Mann-Whitney test was applied to compare continuous antibody responses [MFI] between controls and cases: *p-value < 0.05; **p-value < 0.01

Figure 1



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