ANTIBODY REACTIVITY AGAINST H. PYLORI PROTEINS IN A SAMPLE OF THE SPANISH ADULT POPULATION IN 2008-2013

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Antibody reactivity against *H. pylori* proteins in a sample of the Spanish adult population in 2008-2013

Running title: Antibody reactivity against *H. pylori* proteins

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Abstract

Background: Differences in *H. pylori* protein expression have been related to the risk of severe gastric diseases. In Spain, a marked geographical pattern in gastric cancer mortality has long been reported. Objective: To characterize antibody reactivity patterns against 16 *H. pylori* proteins, by age, sex and region of birth, in a large sample of the Spanish adult population.

Materials and Methods: Antibody reactivity was quantified by *H. pylori* multiplex serology in a sample from the control group of the multicase-control study MCC-Spain. For this analysis, 2555 population-based controls were included. Each participant was classified as seropositive or seronegative for each protein according to specific cut-offs. Overall *H. pylori* seroprevalence was defined as positivity against ≥4 proteins. Descriptive analyses by age, sex and region of birth were performed for both, seroprevalence and seroreactivity (continuous measure). Differences among groups were tested by logistic and linear regression models.

Results: Overall *H. pylori* seroprevalence increased with age in both sexes. For ages 55 to 74, seroprevalence was lower in women than in men (84% vs. 92%, $p<0.001$). Region of birth explained 7% of the variability in seroprevalence. Among *H. pylori* seropositive subjects, proteins with the highest seroprevalence were GroEL, NapA, HP231 and Omp. Seropositivity for most of the proteins increased or remained stable with age, rising mainly for CagA, GroEL and HyuA in women. A clear cohort effect was not observed.

Conclusions: This is the first study to describe the antibody patterns against 16 *H. pylori* proteins in the Spanish population. We found variability in the *H. pylori* antibody profiles according to both individual factors such as age and sex, and environmental factors such as the region of birth. The slightness of the reduction in seropositivity with decreasing age highlights the ongoing importance of this infection.

Introduction

Helicobacter pylori (*H. pylori*) infection is one of the most frequent bacterial infections in humans. The discovery of *H. pylori* in 1983[1] and its implication in gastroduodenal ulcer and non-cardia gastric cancer in 1994[2] meant to be a break in the aetiological understanding of gastric diseases.

H. pylori infection is usually acquired during childhood and, if untreated, it is thought to persist throughout lifetime, causing chronic gastritis. The bacterium and the host are in a constant interaction, and *H. pylori* genome experiences modifications to adapt to the changing conditions in the gastric mucosa and to escape the host immune response[3]. In addition, reinfections may occur during lifetime, leading to even higher variability of bacterial strains infecting each person[4].

Prevalence of infection varies greatly between countries. A recent systematic review of studies with national coverage reporting data for the late 1990s/early 2000s found that prevalence of infection at around the age of 60 years ranged from 22% in Australia to 90% in Mexico[5]. In Spain, there is not a nationwide prevalence study, but regional estimates have reported high prevalences, around 70%[6–9].

Geographic variations have been described not only in the frequency of *H. pylori* infection, but also in the predominant strains in different parts of the world[10]. Importantly, bacteria expressing certain proteins have been related to higher risk of developing severe diseases, such as peptic ulcer or non-cardia gastric adenocarcinoma[11–13].

In Spain, regional differences have been reported in gastric cancer mortality rates[14]. A persistent and unique geographical pattern, similar in women and men, has been described, suggesting the implication of environmental exposures that could affect both sexes[15]. Differences in the prevalence of *H. pylori* infection and type of infecting strain could partially explain differences in gastric cancer mortality, but have not been systematically addressed up to now.

The determination of antibody reactivity against *H. pylori* proteins is an indirect way to ascertain bacterial protein expression patterns. Analysing the patterns of seroreactivity in population groups based on sex and age can provide insight into possible interactions between the bacterium and host characteristics. *H. pylori* multiplex serology is a fluorescent bead-based assay that allows simultaneous quantification of antibody reactivities against several antigens[16]. Using this technique, we analysed the antibody response against 16 *H. pylori* proteins, including the virulence factors CagA and VacA, in a sample of population-based controls from the MCC-Spain multicase-control study[17], by age, sex and geographic region of birth.

Methods

Study subjects

The study population derives from the MCC-Spain study (http://www.mccspain.org/). This is a multicase-control research conducted in 12 Spanish provinces where cases of five cancer types (at least two types in each province, among breast, colorectal, prostate, stomach cancer, and chronic lymphocytic leukemia) and population-based controls were included between 2008 and 2013. A common pool of controls was gathered for all tumors. Selection of controls was performed randomly from the lists of the insured people of the primary care centers participating in the study. The National Health System has universal coverage in Spain and these lists include virtually all the population in the corresponding areas. Controls were frequency-matched to cases according to the age and sex distribution of cases included in each province. Participation rate among controls was 53%. For the present analysis, we studied the sample of controls corresponding to gastric or colorectal cancer cases that donated a blood sample that could be processed for *H. pylori* serological determinations. Among the 4098 controls included in the MCC-Spain study, 2555 fulfilled the selection criteria for entering in this analysis. Reasons for exclusion were as follows: lack of any matched case of colorectal or stomach cancer in a certain sex and age group in the corresponding province of recruitment (N= 125), no consent for blood sample collection (N= 775) and blood sample not processed by *H. pylori* multiplex serology (N= 643). Participants were interviewed by trained personnel, using a specifically designed questionnaire collecting detailed information on socio-demographics, diet,

environmental exposures and clinical variables. More details about the MCC-Spain methodology have been reported elsewhere[17].

Laboratory assays

Blood samples were processed and aliquoted locally in less than 48 hours (maintained refrigerated in the meantime). Then, they were stored at –80°C until being sent to the laboratory for serological determinations. *H. pylori* multiplex serology[16] was used to quantify antibody reactivity against 16 *H. pylori* proteins: Cad (cinnamyl-alcohol-dehydrogenase ELI3-2), CagA (cytotoxin-associated antigen A), Cagδ (cag pathogenicity island protein 3), CagM (cag pathogenicity island protein 16), Catalase, GroEL (chaperonin GroEL), HcpC (Helicobacter cysteine-rich protein C), HomB (*Helicobacter* outer membrane B), HpaA (neuraminyllactose-binding hemagglutinin homolog), HP0231 (hypothetical protein HP0231), HP0305 (hypothetical protein HP0305), HyuA (hydantoin utilization protein A), NapA (neutrophil activating protein A), Omp (outer membrane protein), UreA (urease alpha subunit) and VacA (vacuolating cytotoxin). Multiplex serology is a technique based on glutathione S-transferase (GST) capture immunosorbent assay in combination with fluorescent–bead technology. Briefly, bacterially expressed recombinant GST-*H .pylori* fusion proteins were used as antigens. The fusion proteins were loaded and affinity-purified directly on individual sets of spectrally distinct glutathione-casein-coupled fluorescence-labelled polystyrene beads (SeroMap, Luminex, Austin, TX). Bead sorts, each with a different antigen, were mixed and incubated with human sera at 1:100 dilutions. Antibodies bound to the beads were stained with biotinylated anti-human-IgA, IgM, IgG (Dianova, Hamburg, Germany) and streptavidin-R-phycoerythrin. A Luminex 200 analyzer was used to quantify the antibody bound to bacterial antigen via the median R-phycoerythrin fluorescence intensity of at least 100 beads of the same internal color. Bead and GST background subtracted median reporter fluorescence intensity (net median reporter fluorescence intensity) values were calculated.

To classify each serum as seropositive or seronegative, a median reporter fluorescence intensity cut-off value was defined for each of the 16 antigens based on the median reporter fluorescence intensity values obtained from 17 sera known to be *H. pylori* negative analysed in the same assay. These cut-offs were fixed at the mean plus three standard deviations of the median reporter fluorescence intensity of each antigen

(excluding positive outliers). As previously established[16], *H. pylori* overall positivity was defined as seropositivity against four or more of the analysed proteins (excluding HomB, because this protein was included in addition into the multiplex serology assay after the *H. pylori* positivity criterion had been validated). This definition was the one that maximized the concordance between *H. pylori* multiplex serology and commercial assays performed in a sample of the German population, and reduced the overall false positivity rate derived from isolated seropositivity for *H. pylori* individual proteins, leading to a sensitivity of 89% and a specificity of 82%.

Ethical considerations

The study protocol was approved by the Ethical Committees of the collaborating institutions and by the Comité de Ética de la Investigación y de Bienestar Animal of the Instituto de Salud Carlos III and the study was performed according to the Declaration of Helsinki. All patients gave written informed consent to participate.

Statistical analysis

H. pylori overall seropositivity, and seropositivity for each protein among *H. pylori* seropositive subjects were described for age- and sex-groups by calculating percentages and 95% confidence intervals. Differences in *H. pylori* seropositivity by age, sex and region of birth were tested using the Chi-squared test. Number of proteins recognised and median reporter fluorescence intensity values were summarised by calculating medians and interquartile ranges in the *H. pylori* seropositive group.

To describe geographical variability, Autonomous Communities of birth were grouped based on their geographic proximity into seven regions in order to have sufficient number of people to estimate seroprevalences by sex and age-group in each of them. The Southern region included the Autonomous Communities of Andalucía, Canary Islands, Ceuta and Melilla; the North/Eastern-inland region, Aragón, La Rioja and Navarra; the North/Western-coastal region Asturias, Cantabria and Galicia; the Central region, Castilla La Mancha, Extremadura and Madrid; the North/Western-inland region, Castilla y León; the

Eastern-coastal region, Cataluña, Balearic Islands, Murcia and Valencia; and the North/Central-coastal region, the Basque Country.

Differences by age-group and by sex in the percentage of seropositivity for each *H. pylori* protein were tested through logistic regression analysis, taking as the dependent variable protein serostatus (seropositive vs. seronegative) and as independent variable age group or sex, stratifying by sex or age group, respectively. Differences by age and by sex in the number of antigens recognised were tested using Kruskal-Wallis and Mann-Whitney-U tests, respectively. Differences by age-group and by sex in the seroreactivity of each *H. pylori* protein among those seropositive for *H. pylori* and for the corresponding protein were tested through linear regression analysis, taking as the dependent variable protein seroreactivity (median reporter fluorescence intensity) and as independent variable age group and sex. Linear trend with age was assessed using a Wilcoxon-type test for trend[18] across ordered variables. A p-value <0.05 was considered as the threshold for statistical significance.

Age-cohort graphs were elaborated to inspect the effect of each of these two components in the percentage of seropositivity for *H. pylori* and for individual *H. pylori* proteins among *H. pylori* seropositive subjects. Birth cohorts were grouped in 5-year groups and the youngest age-group (25-44 years old) was excluded due to low number of participants in this range of age.

To explore the variability attributable to place of birth in the percentages of seropositivity (for *H. pylori* and for each protein), intraclass correlation coefficients were estimated from logistic regression mixed models, including the Autonomous Community of birth as a random effect term and age and sex as fixed effects. For this analysis Autonomous Community was used without grouping in order to have a higher number of categories at this level, which is more appropriate to study variability in mixed models. On the other hand, subjects born in Ceuta or Melilla were excluded for this analysis due to low numbers, as well as those born in other countries, given that the objective of this analysis was to explore the intra-country variability.

Results

Table 1 summarizes main sociodemographic characteristics of the study sample. Overall, 51% were men. Mean age was 63.2 years (±12.1), lower in women than in men (59.5±13.3 vs. 66.9±9.4). Sociodemographic

characteristics differed among age groups, mainly with respect to education, that was higher in younger groups, and number of siblings, that was greater in older groups. Differences were also observed between sexes, with respect to education, that was higher in men and to family socioeconomic level at birth, that was higher among women.

Overall *H. pylori* seroprevalence by age, sex and region of birth

H. pylori seroprevalence increased with age in both sexes. The age-effect seems to be more pronounced than the cohort effect (Figure 1). In every age-group seroprevalence was around 6% lower in women than in men, although differences were statistically significant only for the 55-64 and 65-74 years age-groups. The smallest difference between sexes was observed in the oldest age-group (Table 2). Supplementary Table 1 shows age and sex seroprevalence data stratified by region of birth. Difference in overall *H. pylori* seropositivity between regions (excluding the "other countries" region) ranged from 13% among 75-85 years old men to 30% among men and women in the youngest age group. Autonomous Community of birth explained 7.0% of the variability in *H. pylori* seroprevalence after taking into account age and sex differences (Supplementary Table 2).

Antigen-specific percentage of seropositivity in *H. pylori* seropositive subjects, by sex, age and region of birth

The percentage of seropositivity for most of the proteins increased or remained stable with age in both sexes in the group of *H. pylori* seropositive subjects (Figure 2). GroEL and Cad showed statistically significant increases in seropositivity with age in men, as it was observed for GroEL, NapA, CagA, HyuA and Cad in women. On the contrary, seropositivity against some proteins showed a decreasing pattern with age. This was observed for HP231, HP305 and Omp in both sexes, and for HcpC in women. The graphical displaying of a possible birth cohort effect showed that the age-effect prevails over the cohort effect for most of the proteins (Figure 3). In general, men had higher antigen-specific percentages of seropositivity than women, ranging from a 20% to a 74% higher percentage for those proteins with a statistically significant difference after adjusting by age group (GroEL, UreA, HP305, Cagδ, HyuA, Catalase, HcpC, Omp

and HomB). Supplementary Fig 1 presents the range of protein specific percentage of seropositivity among regions of birth, by age and sex. The largest difference between regions was observed for seropositivity for Catalase (overall difference between the highest and the lowest regions was 17%). The estimated percentage of variability in seropositivity that was explained by the Autonomous Community of birth among *H. pylori* seropositive subjects was between 0% for GroEL, UreA, HpaA, CagM, HyuA and VacA and 1.7% for NapA (adjusted by age-group and sex) (Supplementary Table 2).

The number of proteins recognised was higher in men than in women in every age group (Figure 4). No trend was observed with age, although in women the number of antigens recognised was higher in the intermediate age-groups than in the youngest and the oldest groups (p value=0.034).

Median reporter fluorescence intensity for *H. pylori* proteins in *H. pylori* seropositive subjects, by sex, age and region of birth

Among subjects seropositive for *H. pylori* and for each protein, seroreactivity measured as median reporter fluorescence intensity did not show statistically significant differences among age groups or sex for most of the proteins (Supplementary Fig 2). Only for the seroreactivity against HP305 in men an inverse linear trend with age was identified at the statistical significance level (p=0.001). For HyuA and Omp, a higher seroreactivity was found in women with respect to men after adjusting for age (p=0.028 and p=0.009, respectively).

Discussion

This study found a relatively high variability between sexes, age groups and regions of birth not only in the overall *H. pylori* seroprevalence but also in the percentage of seropositivity against individual *H. pylori* proteins among *H. pylori* seropositive subjects, in a sample of the Spanish adult population.

H. pylori seroprevalence was slightly higher in men than in women in all the age-strata analysed. This finding has been also described in other studies, but whether it is due to differences in the risk of infection according to gender or to other related factors has not been established. In our sample, men reported to

have a lower socioeconomic level during childhood than women, which could partly explain the observed difference in seroprevalence. Among *H. pylori* seropositive subjects, women also had lower percentages of seropositivity than men for all the proteins even after adjusting by age group. Differences were highest for GroEL, HP305 and HomB proteins. No statistically significant differences were found between sexes for the seropositivity against recognised *H. pylori* virulence factors, CagA and VacA. When analysing the intensity of serological response, among positive subjects only HyuA and Omp antibody reactivities differed between sexes, being higher in men than in women.

With respect to the pattern of antibody response by age, *H. pylori* overall seroprevalence was higher in the older age groups, although in men, no further increase was observed from the age of 55 and upward, compared to women that showed a more gradual increase. Among *H. pylori* seropositive subjects, the percentage of seropositivity against some proteins increased with age, mainly among women. Noteworthy, CagA seropositivity increased with age in both sexes. Other proteins whose seropositivity also increased with age were GroEL and HyuA. These proteins participate, respectively, in the adhesion of *H. pylori* to epithelial cells in the gastric mucosa and the induction of the inflammatory response (GroEL) and in the synthesis of aminoacids (HyuA)[19], and have also been proposed as *H. pylori* virulence factors. On the other hand, seropositivity against HP305, HcpC and Omp decreased with age, which could be due to a lower survival capacity of strains expressing these proteins, which would lead to a loss of antibody levels with time. Alternatively, changes in the circulating strains over the course of the past century could be responsible for differences in serological response patterns among birth cohorts. The lack of a decreasing trend in the seroreactivity with age suggests that either antigenicity of the studied proteins is high and/or repetitive exposure to the bacterium is frequent and acts as a booster for antibody response. Although no firm conclusions can be drawn from our exploration of the age-cohort effect, for overall *H. pylori* seroprevalence the age effect appears to be stronger than the cohort effect in our sample. Meanwhile, among those *H. pylori* seropositive, a cohort effect seems to be present for the percentage of seropositivity against some proteins, such as GroEL and CagA that showed a decreasing pattern, and HP305, VacA, HcpC and Omp, that showed an increasing trend.

Regional differences in *H. pylori* overall seroprevalence and in protein-specific seropositivity among *H. pylori* seropositive individuals estimated in the age and sex stratified analysis showed a difference between the lowest and the highest regions of around 20%. Notably, the range of variability between regions of birth in the percentage of seropositivity against CagA was from 13% in the group of women 65-74 years old (43% in the region with the lowest seropositivity and 56% in the region with the highest) to 71% in the group of men 25-44 years old (from 0% to 71%). Although a straightforward comparison cannot be done from our data, they do not suggest the presence of a correlation between overall *H. pylori* seroprevalence and gastric cancer mortality rates among regions at an ecological level. N/W-inland and Central regions have both, high seroprevalences and high gastric cancer age-standardised mortality rates, but on the other hand regions such as N/Central-coast and N/W-coast, have high gastric cancer mortality and a relatively low seroprevalence. In this line, in our sample region of birth explained a little proportion of the variability in the percentage of protein-specific seropositivity, which could suggest that individual characteristics may have a greater influence on antibody response patterns than environmental factors associated with place of birth. This would be in accordance with previous findings[20]. Nevertheless, environmental factors that have a distribution in the population that does not fit well to the regional grouping captured by Autonomous Community could still be responsible for part of the variability.

A study carried out in the German population[19] also encountered that the most frequent seropositive proteins among *H. pylori* seropositive participants were GroEL and Omp, and the least frequent Cad. For the overall study sample, percentage of antigen-specific seropositivity was similar to that found in our population for most of the proteins, with the exception of VacA and HcpC that were less frequent, and HP231 and NapA that were more frequent in our sample. In agreement with their results, in our study the number of proteins recognised was higher in men than in women for all the age groups. An increasing percentage of antigen-specific seropositivity with age among *H. pylori* seropositive subjects was also observed in both studies for most of the studied proteins. On the other hand, higher antibody reactivity was observed with increasing age in the German study for some proteins, in contrast to our results that did

not show any significant trend for most of the proteins. Given that in the German study the most prominent increase was observed under the 25-34 years age group, and that our study did not include participants younger than 25 years old, these differences may, at least in part, explain the discrepancy between the studies in the behavior of antibody response intensity with age.

There is not much information about the specific seropositivity against most of the studied proteins for age and sex groups. Compared to data reported by Michel et al using also *H. pylori* multiplex serology[19], percentage of seropositivity for CagA was nearly 10% higher in the German sample when comparing the same age groups. Also, seropositivity against VacA was around 20% higher in the German study. These differences, although not big, could be significant given the role of these cytotoxins in the risk of developing chronic atrophic gastritis and gastric adenocarcinoma. The higher seropositivity against CagA in older age groups in our sample could be a sign of a decreasing circulation of CagA positive *H. pylori* strains, but it could also be the result of the cumulative exposure to different strains during the lifetime. The graphical representation of the seropositivity for CagA by age and birth cohort suggests that a cohort effect could be present, with a slight decrease in the seropositivity for this virulence factor in more recent cohorts. This cohort effect could also explain the higher CagA seropositivity found in the German study, that analised samples collected in the early eighties of the XXth century.

The main limitation of the present study is its cross-sectional design and short recruitment period, which does not allow discerning clearly whether the observed antibody reactivity patterns are the result of changes in the bacterial characteristics along time leading to a birth cohort effect, or whether they are the result of differences in the host serological response depending on individual characteristics like age and sex. Another limitation is that the studied sample cannot be considered representative of the Spanish general population, as a consequence of the participation rate and the frequency-matching strategy used to recruit participants, in the context of the general methodology of the MCC-Spain study[17]. However, given that the results are presented stratified by these same variables, this limitation has been somewhat reduced. The small number of participants in some age-sex-region groups renders the estimated

prevalence data unstable for these groups. This involves mainly the youngest age groups and regions in the South, North/Eastern inland and North/Western coast of the country. With respect to the *H. pylori* multiplex serology technique, even if the concordance of its *H. pylori* overall positivity definition has been reported to be high with respect to the standard ELISA screening and Western blot confirmation assay in other population[16], their results for individual proteins can be affected by some limitations, such as cross-reactivity among proteins from different bacteria.

Our study has also some strengths, like the population-based recruitment of controls, the high number of participants, allowing a detailed description of age and sex subgroups, and the geographic diversity within the country. As regards to the laboratory procedures, the low storage time of serum samples, and the analysis of antibody reactivities against a wide set of *H. pylori* proteins represent also strengths of the study. Due to its high-throughput and simultaneous detection abilities, *H. pylori* multiplex serology has proved to be suitable in large epidemiologic studies[12,13,19,21–23], such as the MCC-Spain, to assess *H. pylori* seroprevalence and antibody reactivity patterns.

To conclude, the description presented here of the antibody reactivity against 16 *H. pylori* proteins shows that there is a significant variability depending on both, an environmental factor like geographical region, and on individual factors such as age and sex. Regional differences observed in this study suggest that current and/or past variability in the circulating *H. pylori* strains may exist even at an intra-country level. Reasons for differences between men and women, and among age groups are less straightforwardly derived from the present study because of potential confounding factors like education or cohort of birth. The slightness of the observed decreasing pattern in *H. pylori* overall seroprevalence and in seropositivity for the main virulence factors associated with age highlights the importance of maintaining a high level of awareness in clinical practice for early detection of gastroduodenal ulcers and gastric adenocarcinoma, as well as efforts for primary prevention of gastric cancer. The presence of differences in the characteristics of *H. pylori* infecting humans of different ages and sexes may have a significant impact on the population

health, given that the expression of some *H. pylori* proteins has been related to the risk of developing gastric diseases.

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Statement of Interests

Competing interests: The authors have no competing interests.

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Tables

Table 1. Sociodemographic characteristics of the study sample

		Number	(%)
Sex	Male	1293	(51%)
	Female	1262	(49%)
Age group	25-44	214	(8%)
	45-54	404	(16%)
	55-64	628	(25%)
	65-74	803	(31%)
	75-85	506	(20%)
Region of birth	Southern	327	(13%)
	N/E-inland	221	(9%)
	N/W-coast	285	(11%)
	Central	452	(18%)
	N/W-inland	559	(22%)
	Eastern-coast	371	(15%)
	N/Central-coast	222	(9%)
	Other countries	76	(3%)
	Missing	42	(2%)
Race	White/Caucasian	2509	(98%)
	Other	44	(2%)
	Missing	2	(0%)
Education	No/incomplete primary school	490	(19%)
	Primary school	898	(35%)
	Secondary school	703	(28%)
	University degree	464	(18%)

		Number	(%)
Socioeconomic level at birth	Low	1055	(41%)
	Intermediate	1247	(49%)
	High	88	(3%)
	Missing	165	(6%)
Number of siblings	Only child	110	(4%)
	1-2 siblings	948	(37%)
	3-6 siblings	1207	(47%)
	>6 siblings	284	(11%)
	Missing	6	(0%)

N: North; E: East; W: West.

Table 2. *H. pylori* seroprevalence by sex and age-group in the whole sample

	Men			Women				
Age group	n/N	%	(95% CI)	n/N	%	(95% CI)	p-value (1)	Men to women seropositivity ratio
25-44	20/26	77%	(56-91)	136/188	72%	(65-79)	0.622	1.1
45-54	82/94	87%	(79-93)	250/310	81%	(76-85)	0.144	1.1
55-64	345/373	92%	(89-95)	216/255	85%	(80-89)	0.002	1.1
65-74	463/502	92%	(90-94)	253/301	84%	(79-88)	<0.001	1.1
75-85	275/298	92%	(89-95)	187/208	90%	(85-94)	0.350	1.0
p-trend (2)			0.053			<0.001		

n/N: Number of seropositive subjects (n) among the total number of subjects (N) in each age-sex group.

(1) From Chi-squared test for the comparison of *H. pylori* seroprevalence between sexes.

(2) From Chi-squared trend test for *H. pylori* seroprevalence trend by age-groups.

Figures

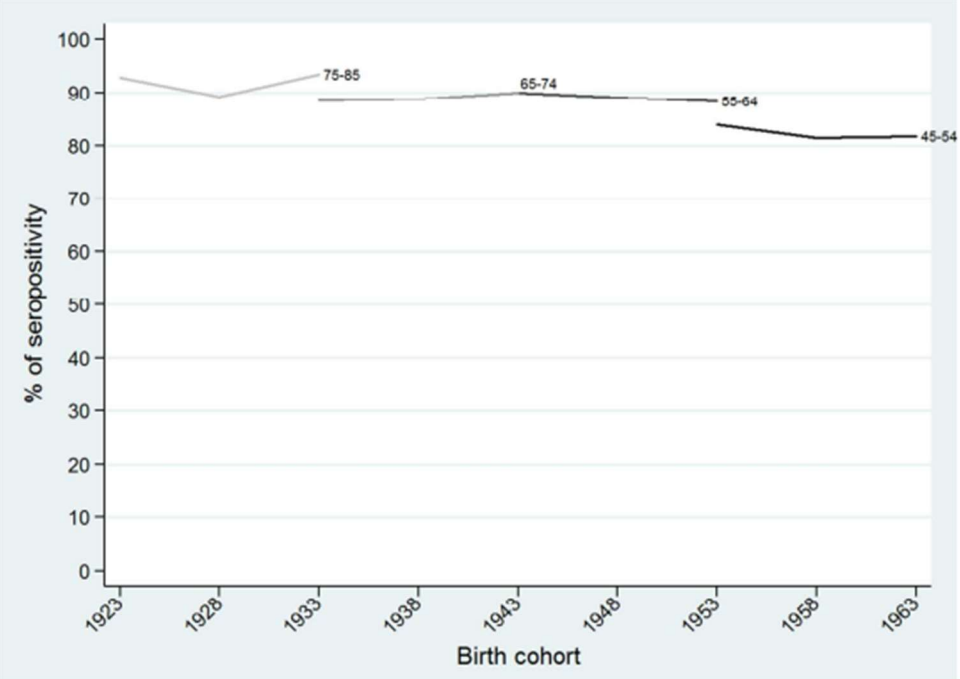

Figure 1. Vertical axis represents the percentage of *H. pylori* seropositivity and horizontal axis the first year of each birth cohort (with a range of 5 years).

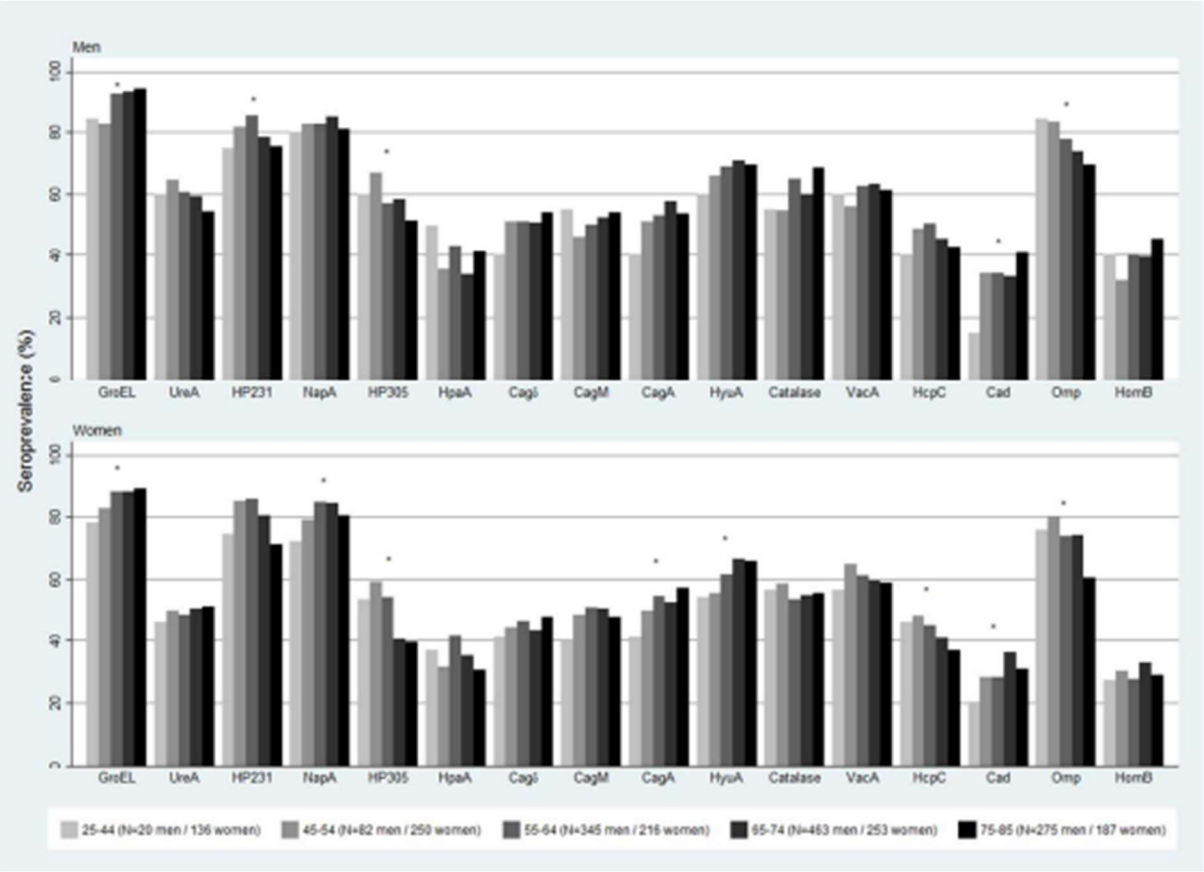

Figure 2.Seropositivity against specific antigens in *H. pylori* positive subjects by sex and age group. N: Number of subject in each age-group. *Statistically significant trend with age within sex.

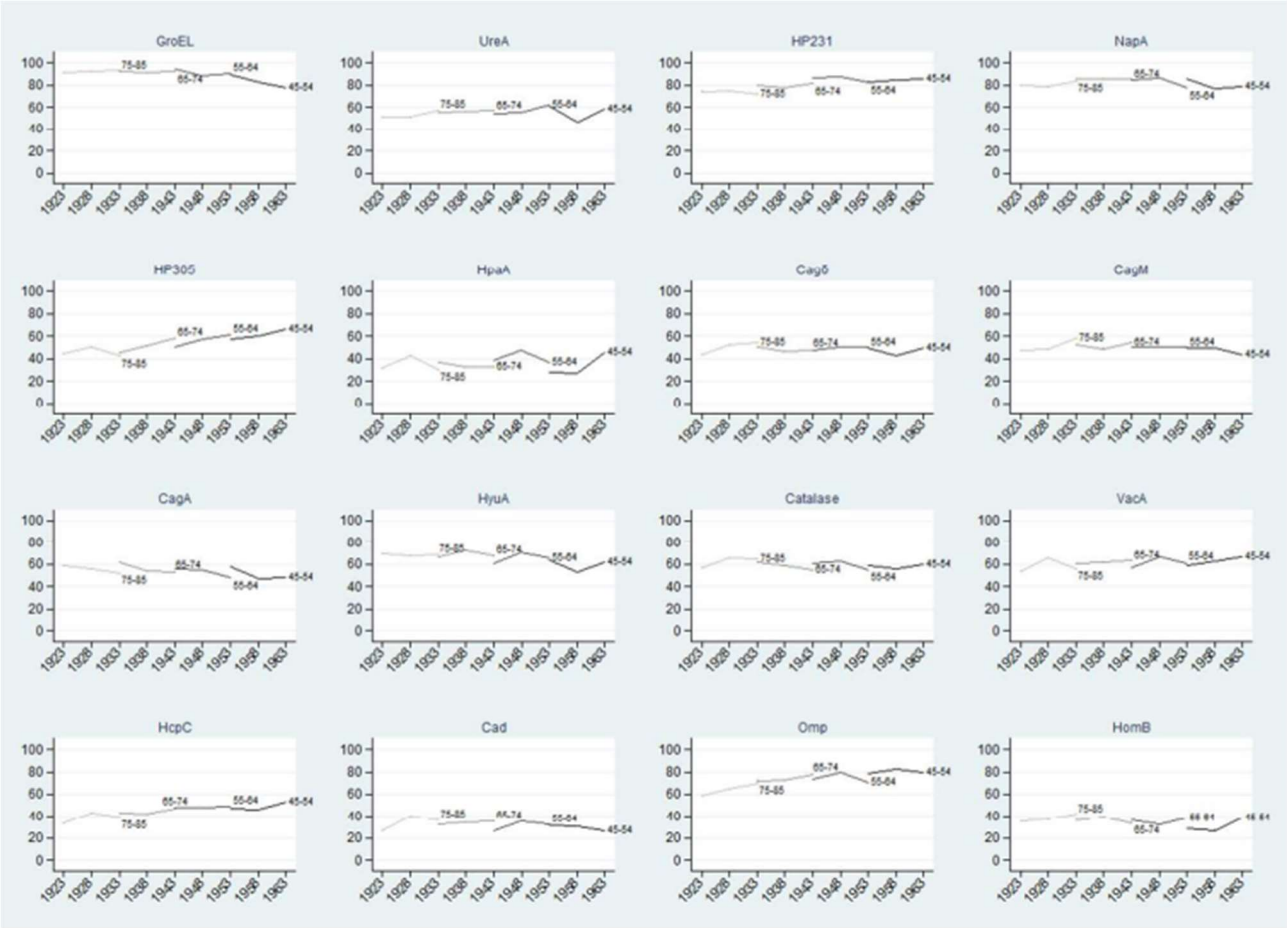

Figure 3. Vertical axes represent the percentage of seropositivity to the corresponding protein in *H. pylori* positive subjects and horizontal axes the first year of each birth cohort (with a range of 5 years).

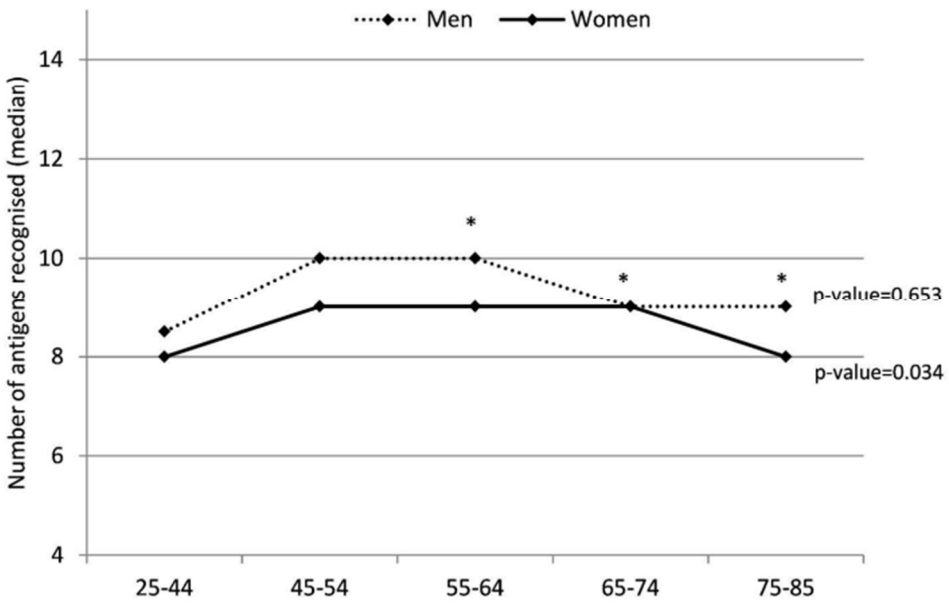

Figure 4. Median of the number of proteins recognised in *H. pylori* positive subjects, by sex and age group. p-values correspond to Kruskal-Wallis test for differences between age groups within each sex. Trend was not statistically significant neither among men nor among women. * Statistically significant difference between men and women for the corresponding age group, from the Mann-Whitney U-test.