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Basic Study

***Helicobacter pylori vacA* genotype is a predominant determinant of immune response to *Helicobacter pylori* CagA**

Alexander Link, Cosima Langner, Wiebke Schirrmeister, Wiebke Habendorf, Jochen Weigt, Marino Venerito, Ina Tammer, Dirk Schlüter, Philipp Schlaermann, Thomas F Meyer, Thomas Wex, Peter Malfertheiner

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Abstract**AIM**

To evaluate the frequency of *Helicobacter pylori* (*H. pylori*) CagA antibodies in *H. pylori* infected subjects

and to identify potential histopathological and bacterial factors related to *H. pylori* CagA-immune response.

METHODS

Systematic data to *H. pylori* isolates, blood samples, gastric biopsies for histological and molecular analyses were available from 99 prospectively recruited subjects. Serological profile (anti-*H. pylori*, anti-CagA) was correlated with *H. pylori* isolates (*cagA*, EPIYA, *vacA s/m* genotype), histology (Sydney classification) and mucosal interleukin-8 (IL-8) mRNA and protein expression. Selected *H. pylori* strains were assessed for *H. pylori* CagA protein expression and IL-8 induction in co-cultivation model with AGS cells.

RESULTS

Thirty point three percent of microbiologically confirmed *H. pylori* infected patients were seropositive for CagA. Majority of *H. pylori* isolates were *cagA* gene positive (93.9%) with following *vacA* polymorphisms: 42.4% *vacA s1m1*, 23.2% *s1m2* and 34.3% *s2m2*. Anti-CagA-IgG seropositivity was strongly associated with atrophic gastritis, increased mucosal inflammation according to the Sydney score, IL-8 and *cagA* mRNA expression. *VacA s* and *m* polymorphisms were the major determinants for positive (*vacA s1m1*) or negative (*vacA s2m2*) anti-CagA serological immune response, which also correlated with the *in vitro* inflammatory potential in AGS cells. *In vitro* co-cultivation of representative *H. pylori* strains with AGS cells confirmed functional CagA translocation, which showed only partial correlation with CagA seropositivity in patients, supporting *vacA* as major co-determinant of the immune response.

CONCLUSION

Serological immune response to *H. pylori cagA+* strain in *H. pylori* infected patients is strongly associated with *vacA* polymorphism, suggesting the crucial role of bacterial factors in immune and clinical phenotype of the infection.

Key words: *Helicobacter pylori*; Seropositivity; Virulence factors; CagA; VacA; Immune response

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Core tip: *Helicobacter pylori* (*H. pylori*) related diseases are commonly associated with *cagA+* strains, although seropositivity against CagA varies among different studies. In this prospective study, we evaluated potential factors related to the *H. pylori* CagA-immune response. We show that anti-CagA-IgG seropositivity was strongly associated with histopathological and inflammatory factors. Most importantly, we identified *H. pylori vacA* polymorphism as one of the main determinants of immune response to CagA and inflammatory potential of *H. pylori* strains *ex vivo* and *in vitro*. Our data support the crucial role of bacterial factors that co-determine the complex interaction with *H. pylori* and define the immune and clinical phenotypes of the

infection.

Link A, Langner C, Schirrmeyer W, Habendorf W, Weigt J, Venerito M, Tammer I, Schlüter D, Schlaermann P, Meyer TF, Wex T, Malfertheiner P. *Helicobacter pylori vacA* genotype is a predominant determinant of immune response to *Helicobacter pylori* CagA. *World J Gastroenterol* 2017; 23(26): 4712-4723 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i26/4712.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i26.4712>

INTRODUCTION

Infection with *Helicobacter pylori* (*H. pylori*) causes chronic inflammation of the gastric mucosa with progression to severe complications in a subset of patients^[1-3]. The determinants for the magnitude of inflammation and progression to complication include *H. pylori* with its bacterial virulence factors, host genetic background and environmental factors. *H. pylori* virulence factors facilitate colonization (urease, flagella and catalase) and induce inflammation (OipA, NapA, DupA, IceA, VacA and CagA) of the gastric mucosa^[4,5]. CagA and VacA are the most relevant pro-inflammatory factors and are closely related to peptic ulcer disease (PUD) as well as gastric cancer (GC)^[6-9].

CagA is the principal protein encoded in the complex of the cytotoxin associated gene pathogenicity island (*cag PAI*), which is shuttled from *H. pylori* into gastric epithelial cells through the type IV bacterial secretion system^[7,8]. Intracellularly, CagA undergoes tyrosine phosphorylation by Src and Abl kinases to interact with several host proteins, influence their activity and subsequently alter morphological properties of the host cells^[10-13]. CagA protein stimulates expression of inflammatory cytokine interleukin-8 (IL-8) in gastric epithelial cells by activating nuclear factor- κ B and leads to increased inflammation of the gastric mucosa^[14]. Overall, *H. pylori cagA+* strains are associated with an increased risk of gastric cancer compared to *cagA-* strains^[15]. The oncogenic role of CagA is further supported by *in vivo* experiments in mice, where transgenic *cagA* expression in stomach leads to gastric epithelial hyperplasia, adenocarcinoma, myeloid leukemia and B-cell lymphoma^[16].

One of the interesting features related to CagA is the induction of a systemic immune response to CagA and this in fact led to the discovery of this protein^[17]. Infection with *cagA+* strains and serological detection of anti-CagA antibodies have been associated with increased risk for PUD as well as for GC^[18,19]. A meta-analysis of 16 studies concludes that seropositivity for anti-CagA-IgG is associated with a 2.87-fold higher risk for gastric cancer development^[20]. In earlier studies, Ando *et al.*^[21] found a significant correlation between anti-CagA-IgG and IL-8 expression in biopsy culture supernatant and described an association of

anti-CagA-IgG with increased neutrophil infiltration and *H. pylori* density. Therefore, it has been suggested that screening for the *cagA* status of *H. pylori* may provide an additional advantage for identifying patients at high risk for gastric cancer development^[20]. However, low levels of anti-CagA-IgG in subjects infected with *cagA*+ strains have been reported^[22,23]. *H. pylori* IgG seroprevalence in a large study in our center was 44.4%, and proportion anti-CagA-IgG positive was 43.3%^[22]. In another prospective study on patients undergoing screening colonoscopy, we observed an even lower proportion (36.6%) of anti-CagA-IgG positivity^[23]. In studies performed in various geographic regions of the world the CagA-seropositivity ranges from 35% to 80%^[22-24]. The low number of CagA-seropositivity in spite of the high prevalence of *H. pylori cagA*+ strains has not been explained. At present only few studies addressed this observation, however, systematic data are not yet available^[25-27]. In the present prospective study, we aimed to identify the factors related to serological reactivity or immune response to CagA.

MATERIALS AND METHODS

Study design

In a prospective study 413 patients were recruited between July 2011 and April 2014. Among those, 99 patients (98 patients of European descent) in total fulfilled the inclusion criteria such as microbiologically confirmed *H. pylori* infection with successful isolation and characterization of *H. pylori* strains and known *H. pylori* anti-CagA status (Figure S1). Patients, with current or past history of non-gastric cancers or stomach surgery, acute bleeding, oral anticoagulation, immunosuppressive or antibiotic therapy (within the last 2 wk before entering the study) were excluded. The study was conducted according to the "World Medical Association Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects" and approved by the ethical board of the Otto-von-Guericke University (Study Number 80/11). All patients provided written informed consent. Blood samples were drawn and systematic biopsy protocol was completed during upper GI endoscopy at the Department of Gastroenterology, Hepatology and Infectious Diseases at the Otto-von-Guericke University of Magdeburg, Germany.

Biopsy protocol and histopathological assessment

During upper GI endoscopy, biopsies from antrum and corpus were collected for microbiology cultivation of *H. pylori*, rapid urease test (CLOtest, Kimberly Clark, United States), histological assessment and further molecular analyses. Histological evaluation was performed according to the updated Sydney protocol from five biopsies (two from each antrum and corpus and one from incisura angularis)^[28,29]. Following

fixation, slides were stained with hematoxylin, eosin, PAS and modified Giemsa stain for *H. pylori* detection. Gastric cancer tumor tissues were characterized according to the International Classification of Diseases for Oncology and Lauren criteria.

Serological assessment of anti-*H. pylori* IgG and anti-CagA-IgG

Serological assessment for *H. pylori* was performed using *H. pylori* IgG ELISA Kit (Biohit, Helsinki, Finland) and CagA IgG ELISA Kit (GENESIS Diagnostics, Cambridgeshire, Great Britain). Both tests exhibited a high sensitivity for detection of *H. pylori* infection in our region and have been validated in multiple studies in the past^[22,23]. All tests were performed according to manufacturer's instructions with internal and external validation. Cut-off values for positive testing were ≥ 30.0 EIU or ≥ 6.25 U/mL for *H. pylori* IgG ELISA and CagA IgG ELISA, respectively. To confirm the data on anti-CagA-IgG we performed immunoblot testing using Helicobacter ViraStripe[®] IgG immunoblot (Viramed Biotech AG, Planegg, Germany). The test result was considered positive if following criteria were fulfilled: quantitative evaluation of the blots using an automated scanning system provided by the manufacturer (positivity values $\geq 80\%$ in comparison to control), and two researchers independently and blinded to results, confirmed the positivity.

H. pylori cultivation

Gastric biopsies were collected in 1.5 mL 0.9 vol% isotonic sodium chloride solution (Berlin-Chemie AG, Berlin, Germany) and immediately transported to the Institute of Medical Microbiology for further cultivation. Cultivation and identification of *H. pylori* was performed as described previously^[30]. Positive cultures were harvested in 0.9 vol% isotonic sodium chloride solution, centrifuged at 13.000 rpm for 3 min and cell pellets were stored at -30 °C until further analysis.

Cell culturing with *H. pylori*

Six days before the experiment, frozen stocks of several *H. pylori* isolates from patients were inoculated on Columbia-agar-based medium that contained 10 vol% washed human erythrocytes and 10 vol% heat inactivated horse serum (purchased from the NRZ, Nationales Referenzzentrum Helicobacter Freiburg, Germany). Bacteria were cultivated under microaerophilic conditions at 37 °C. The strain *H. pylori* ATCC[®] BAA-1606[™] (BCM300) was cultivated on selective agar plates (bioMérieux, Marcy l'Etoile, France) under the same conditions. After 3 d bacteria were removed into PBS and cultivated on fresh agar plates for another three days under the same conditions. For the experiments, bacteria were re-suspended in PBS (with Ca²⁺ and Mg²⁺) and concentration (bacteria/mL) was determined by measuring the optical density ($\lambda = 580$ nm). To check bacteria for viability, suspensions were

inspected microscopically for motility and shape.

AGS cells (CRL-1739; American Type Culture Collection-ATCC) were maintained in RPMI 1640 (Life Technologies, Carlsbad, CA, United States) with 10% Fetal Calf Serum, 100 U/mL Penicillin, 100 µg/mL streptomycin, and 100 µg/mL gentamycin (PAA, Cölbe, Germany) at 37 °C and 5% CO₂. Twenty-four hours prior to infection experiments, cells were seeded in 6 well plates at a concentration of 300000 cells/mL in the same medium as mentioned above. Four hours prior infection, medium was removed, cells were washed twice with PBS without Ca²⁺ and Mg²⁺ (Life Technologies, Carlsbad, CA, United States) and fresh antibiotic free medium was added. One well was harvested by trypsination (5 min, 37 °C) and cell number was determined. Cells were infected with *H. pylori* at a "multiplicity of infection" of 100 for 24 h. Cell culture supernatant was removed, centrifuged at 13.000 rpm for 5 min and transferred into a new reaction tube. After cells were washed twice with PBS, cells were harvested, washed with PBS and cell pellet was stored at -80 °C until further analysis.

Genomic DNA extraction of *H. pylori* and PCR methods

DNA extraction of *H. pylori* was performed using DNA Mini Kit (Qiagen, Hilden, Germany) following manufacturer's recommendations. Amplification of DNA was done in a T3 Thermocycler machine (Biometra, Goettingen, Germany) with 15 µL HotStar Taq Plus DNA Polymerase Mix (Qiagen, Hilden, Germany), 11.6 µL RNase-free water, 0.2 µL of each forward and reverse primer (50 µmol/L) and 3 µL *H. pylori* DNA. Seven primer sets were used for the study: *cagA*, *EPIYA*, *vacA s*, *vacA m*, *glmM*, *cagE* and *virB11*. The primer sequences and size of product are shown in Table S1. The reactions were carried out as follows: enzyme activation at 95 °C for 15 min, 40 cycles of denaturation at 95 °C for 30 s, annealing for 30 s, extension at 72 °C for 1 min followed by final extension at 72 °C for 10 min PCR products were analyzed by agarose gel electrophoresis, ethidium bromide staining and Hyperladder IV (Bioline, Luckenwalde, Germany) as molecular weight marker. E.A.S.Y RH system (Herolab, Wiesloch, Germany) was used for gel imaging.

Extraction of total RNA and quantitative RT-PCR

H. pylori total RNA was extracted using RNeasy Protect Bacteria Reagent and RNeasy Mini Kit (QIAGEN, Hilden, Germany) following manufacturer's recommendations. Total RNA of gastric specimens and AGS cells was isolated using RNeasy Plus Universal Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's recommendations (without gDNA Eliminator Solution). RNA concentration was determined spectrophotometrically by measuring absorbance at 260/280 nm (Biophotometer, Eppendorf, Hamburg, Germany). cDNA synthesis was performed in a 40 µL

reaction volume with 500 ng of total RNA of *H. pylori* or 1 µg RNA of antrum biopsies. *CagA* and *glmM* mRNA of *H. pylori* and β-actin with IL-8 of gastric tissue and AGS cells was determined with quantitative real-time PCR (qRT-PCR) using the CDX96-Cycler (BioRAD, Munich, Germany). A single 30 µL reaction contained 15 µL QuantiTect SYBR Green PCR Master Mix (QIAGEN, Hilden, Germany), 13.4 µL RNase-free water, 0.2 µL of each forward and reverse primer (50 µmol/L) and 1.2 µL *H. pylori* or antrum cDNA. For qRT-PCR programs see above (qualitative PCR program). Annealing temperature and primers are shown in supplementary data (Table S1). Quality of qRT-PCR products was verified by melt curve analysis and agarose gel electrophoresis (see above). Expression data were analyzed using the 2^{-ΔCt} method.

***CagA* expression in vitro using Western blotting**

Cell pellets were mixed with 2x Laemmli buffer (4% SDS, 20% glycerol, 120 mmol/L Tris-Cl (pH 6.8) and 0.02% bromphenol blue) and boiled for 10 min at 95 °C. Thereafter, samples were separated using 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Membranes were blocked with TBS buffer and incubated with antibodies as previously described^[31].

IL-8 quantification using ELISA

Interleukin 8 (IL-8) concentration in AGS co-culture supernatants was determined with quantitative sandwich enzyme-linked immunoassay (Quantikine® ELISA, R and D Systems, Abingdon, United Kingdom) according to manufacturer's recommendations. Results are displayed in pg/mL.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, United States). All data are presented as mean ± SD. χ² test and Fisher's exact test were used for contingency tables. The Mann-Whitney *U*-test and the Kruskal-Wallis analyses of variance were used to analyze the statistical significance for two unpaired groups or multiple groups, respectively. Post hoc analyses were performed using Dunn's multiple comparison tests. Correlation analyses were performed using Spearman's test. Two-sided *P*-values < 0.05 were considered as statistically significant.

RESULTS

Clinical characteristic of patients with and without *CagA*-IgG

From 99 patients with successful cultivation of *H. pylori* from the stomach, 30 (30.3%) patients had positive anti-*CagA*-IgG serology. First, we questioned if *CagA*-IgG seropositive and seronegative groups may have a difference in clinical phenotype. Clinical

Table 1 Characteristics of patients with active *Helicobacter pylori* infection regarding patient's CagA-IgG status *n* (%)

	Total	<i>H. pylori</i> + CagA-IgG-	<i>H. pylori</i> + CagA-IgG+	P value
Total	99	69 (69.7)	30 (30.3)	
Gender				NS
Female	72	51 (73.9)	21 (70)	
Male	27	18 (26.1)	9 (30)	
Age				
mean ± SD	54.1 ± 14.1	53.7 ± 13.7	55.1 ± 15.0	NS
<i>H. pylori</i> status				
Anti- <i>H. pylori</i> -IgG+	93	64 (92.8)	29 (96.7)	NS
Anti-CagA-IgG+	30	-	30 (100)	
mean CagA-IgG EIU		1.5 ± 1.7	39.6.5 ± 31.1	< 0.0001
Culture+	99	69 (100)	30 (100)	NS
Histology+	79	55 (79.7)	24 (80)	NS
Clinical phenotype				
Chronic active Gastritis (any severity)	92	63 (91.3)	29 (96.7)	NS
Chronic non-active Gastritis (grade > 2) ¹	7	6 (8.7)	1 (3.3)	NS
Corpus predominant gastritis	5	0 (0)	5 (16.7)	0.0020
Antrum-/pangastritis	87	63 (91.3)	24 (80)	NS
Chronic atrophic gastritis (any severity)	46	25 (36.2)	21 (70)	0.0023
Chronic atrophic gastritis (> 2/3) ¹	28	14 (20.3)	14 (46.7)	0.014
Intestinal metaplasia (any)	23	12 (17.4)	11 (36.7)	0.068
Gastric cancer	6	2 (2.9)	4 (13.3)	0.056
PUD or MALT-Lymphoma (any)	5	4 (5.8)	1 (3.3)	NS
Normal mucosa (no PMNs and ≤ 1 chronicity) ¹	4	4 (5.8)	0 (0)	NS

¹Score based on the Sydney classification. NS: Not significant, *P* > 0.05. PUD: Peptic ulcer disease; PMNs: Polymorphonuclear neutrophils.

and demographical data are presented in Table 1. Among different histological conditions, corpus predominant gastritis and chronic atrophic gastritis were more frequently found in the group of patients with seropositivity for anti-CagA-IgG. More patients with chronic non-active gastritis or patients without any inflammation were found in the anti-CagA-IgG negative group, suggesting the weaker Inflammation related to *H. pylori* infection. With further focus on the clinical phenotype, we observed a slightly higher polymorphonuclear neutrophil infiltration in corpus and a more severe atrophy with intestinal metaplasia in antrum of patients with anti-CagA-IgG based on the mean Sydney Scores for corpus and antrum separately (Figure 1). No difference in *H. pylori*-IgG antibody titer or *H. pylori* density was found histologically between those groups.

Characteristics of *H. pylori* strains in patients with and without anti-CagA-IgG

It has been previously suggested that immune response to CagA may be dependent on *H. pylori* strain characteristics and its virulence factors. As expected all six patients in CagA-IgG- group had *cagA*-strains. All strains from patients with CagA-IgG+ had *cagA*+ strains (Table 2). To evaluate if studied patients show immune response to *H. pylori* we compared CagA-IgG in both groups. We found that seropositivity against *H. pylori* was present in most of cases in 64 (92.8%) and 29 (96.7%) patients without and with anti-CagA-IgG, respectively, suggesting that the majority of patients are immunologically capable of showing the serological response to *H. pylori* or its virulence

factors. Correlation analyses between the *H. pylori*-IgG and CagA-IgG titers did not reveal any correlation (Figure 2A).

Next, we speculated that CagA immune response may be further dependent on successful transcription of *cagA* mRNA. All strains in CagA-IgG+ group showed moderate or high *cagA* mRNA expression. At the same time 34 (56.7%) patients of the CagA-IgG-group had also positive *cagA* mRNA expression. We questioned if differences in EPIYA motifs or a missing Type IV secretion system could have an impact on production of CagA-IgG. A large proportion of the patients had an evidence for *H. pylori* with mixed EPIYA motifs and no specific differences were observed among CagA-IgG positive and negative groups (Table 2). As a surrogate for the presence of *cagA* pathogenicity island and type IV secretion system, we examined *cagE* (*cagPAI* marker) and *virB11* (T4SS marker) expression in 54 patients of the *cagA*+ and CagA IgG negative group. *CagE* was detectable in all tested *H. pylori* isolates, while only one strain was negative for *virB11* (data not shown) further excluding the potentially missing T4SS.

It is well known that VacA and CagA are the main pro-inflammatory bacterial factors. It has been earlier hypothesized that *vacA* polymorphism may also be related to CagA seropositivity^[25]. As shown in Table 2, all of the strains from patients with immune response had *H. pylori* with *vacA* *s1* subtype (with *m1* 76.7% and *m2* 23.3%). None of the patients with *s2m2* showed CagA seropositivity. In support, the level of anti-CagA-IgG were higher and more frequent positive in *vacA* *s1m1* (50%) and *vacA* *s1m2* (36.8%) compared to *vacA* *s2m2* (0%), further suggesting the importance

Table 2 Characteristics of *Helicobacter pylori* strains in CagA IgG dependent status of the host *n* (%)

	Total	<i>H. pylori</i> + CagA-IgG-	<i>H. pylori</i> + CagA-IgG+	<i>P</i> value
Total	99	69 (69.7)	30 (30.3)	
<i>cagA</i> gene ¹				NS
Positive	93	63 (91.3)	30 (100)	
Negative	6	6 (8.7)	0	
<i>cagA</i> mRNA ²	87	60	27	< 0.0001
Positive	61	34 (56.7)	27 (100)	
Negative	26	26 (43.3)	0 (0)	
EPIYA motifs				NS
Negative	5	5 (7.2)	0	
AB	4	2 (2.9)	2 (6.7)	
ABC	33	21 (30.4)	12 (40)	
ABCC	7	6 (8.7)	1 (3.3)	
ABCCC	2	1 (14.5)	1 (3.3)	
Mixed	48	34 (49.3)	14 (46.7)	
VacA-IgG ^{1,3}		8 (13.1)	5 (18.5)	NS
<i>vacA</i> subtype ¹				
s1	65	35 (50.7)	30 (100)	< 0.0001
s2	34	34 (49.3)	0	
m1	42	19 (27.5)	23 (76.7)	< 0.0001
m2	57	50 (72.5)	7 (23.3)	
s1m1	42	19 (27.5)	23 (76.7)	< 0.0001
s1m2	23	16 (23.2)	7 (23.3)	
s2m2	34	34 (49.3)	0	

¹Six patients with evidence for different/mixed *cagA*⁺/*cagA*⁻ or *vacA* strains in corpus and antrum have been included to the potentially more pathogenic group for simplicity; ²RNA analyses were possible only in 87 patients/strains; ³Eighty-eight samples were available for VacA-IgG analyses. NS: Not significant, *P* > 0.05.

of *H. pylori vacA* virulence factor in immune response (Figure 2B).

H. pylori-induced inflammation in mucosa and *in vitro* model

CagA with functional TSS4 is known to induce IL-8 *in vitro* and *in vivo*. Having shown increased histological inflammation in subjects with anti-CagA-IgG, we questioned if this may correlate with *H. pylori*-related cytokine IL-8 in antrum mucosa. Independently of histological phenotype, IL-8 was significantly higher (about 2 fold) in patients with anti-CagA-IgG+ compared to anti-CagA-IgG- patients with CG (0.0082 ± 0.009 vs 0.0048 ± 0.004, *P* = 0.026) (Figure 3A). This, however, was not the case in mucosa from patients with GC and PUD, although the number of the patients was very small. We observed no correlation between IL-8 expression in mucosa and the level of anti-CagA-IgG. To confirm those strain-dependent observations, we performed *in vitro* analyses using *H. pylori* co-culture with AGS cell line. We randomly selected *H. pylori* strains with different strain characteristics including *cagA* mRNA expression, CagA-IgG and *vacA* polymorphisms (Table 3). As expected, *cagA*⁺ stains and strains with *cagA* mRNA expression induced slightly higher IL-8 mRNA expression compared to controls (AGS without *H. pylori*) and *cagA*⁻ strains (Figure 3B and C). However, anti-CagA-IgG positivity

Table 3 Validation of CagA expression and cellular translocation in AGS cells

ID	Strain characterization				<i>In vitro</i>		
	<i>cagA</i> DNA	<i>cagA</i> RNA	CagA-IgG	<i>vacA</i>	<i>H. pylori</i> CagA	AGS + <i>H. pylori</i> CagA	AGS + <i>H. pylori</i> p-CagA
BCM300	+	+	-	s1m1	+	+	+
117	+	+	+	s1m1	+	+	+
6	+	+	-	s1m1	+	+	+
255	+	+	-	s1m1	+	+	+
13/1	+	+	+	s1m1	+	+	-
46	+	+	-	s1m1	+	+	-
89	+	+	-	s1m2	+	+	-
424	+	+	+	s1m2	-	-	-
21	+	-	-	s1m1	-	-	-
321	+	+	-	s2m2	-	-	-
374	+	+	-	s2m2	-	-	-
342	+	-	-	s2m2	-	-	-
314	-	-	-	s1m2	-	-	-
450	-	-	-	s1m2	-	-	-

AGS cells co-cultivated in similar conditions without *Helicobacter pylori* were considered as negative control. Strains were characterized based on *cagA* DNA/RNA/*cagA*-IgG seropositivity of the host and *vacA* polymorphism. "+": positive and "-": negative expression. p-CagA and CagA: Phosphorylated p-CagA and total CagA protein expression *in vitro*.

did not correlate with IL-8 expression suggesting that host serological immunotype/phenotype does not correlate with *in vitro* potential of *H. pylori* to induce inflammation (Figure 3D). IL-8 mRNA expression in AGS cells correlated significantly with IL-8 expression in supernatant (Figure S2A), and we observed identical pattern for IL-8 release in supernatant of AGS cells in confirmation of the results (Figure 3B-D).

The inflammatory potential of *H. pylori cagA*⁺ strains showed relatively high distribution suggesting other bacterial factors potentially responsible for the observation. Therefore, we questioned whether *vacA* s and m polymorphisms may correlate with inflammatory potential of *H. pylori in vitro*. Strains with *vacA* s1 induced higher IL-8 mRNA (Figure 4) and IL-8 expression in supernatant; however, the highest difference was related to *vacA* m polymorphism with highest values for *vacA* m1 compared to *vacA* m2. This data further confirms the highest inflammatory potential defined by IL-8 expression of *vacA* s1m1 compared to *vacA* s1m2 or s2m2 (Figure S2B and C).

CagA expression *in vitro*

Having shown that multiple factors may be related to seropositivity to CagA, we questioned if the *H. pylori* strains indeed a capable of expression of functional CagA protein (including its phosphorylated form) in AGS cells. For this purpose, we performed CagA Western blotting using bacterial pellets and AGS cells co-cultivated with *H. pylori* (Table 3). As expected, we found that the majority of *H. pylori cagA*⁺ strains with *vacA* s1m1 polymorphism indeed were capable of CagA protein expression independently to anti-CagA-IgG positivity in host (Table 3). This provides

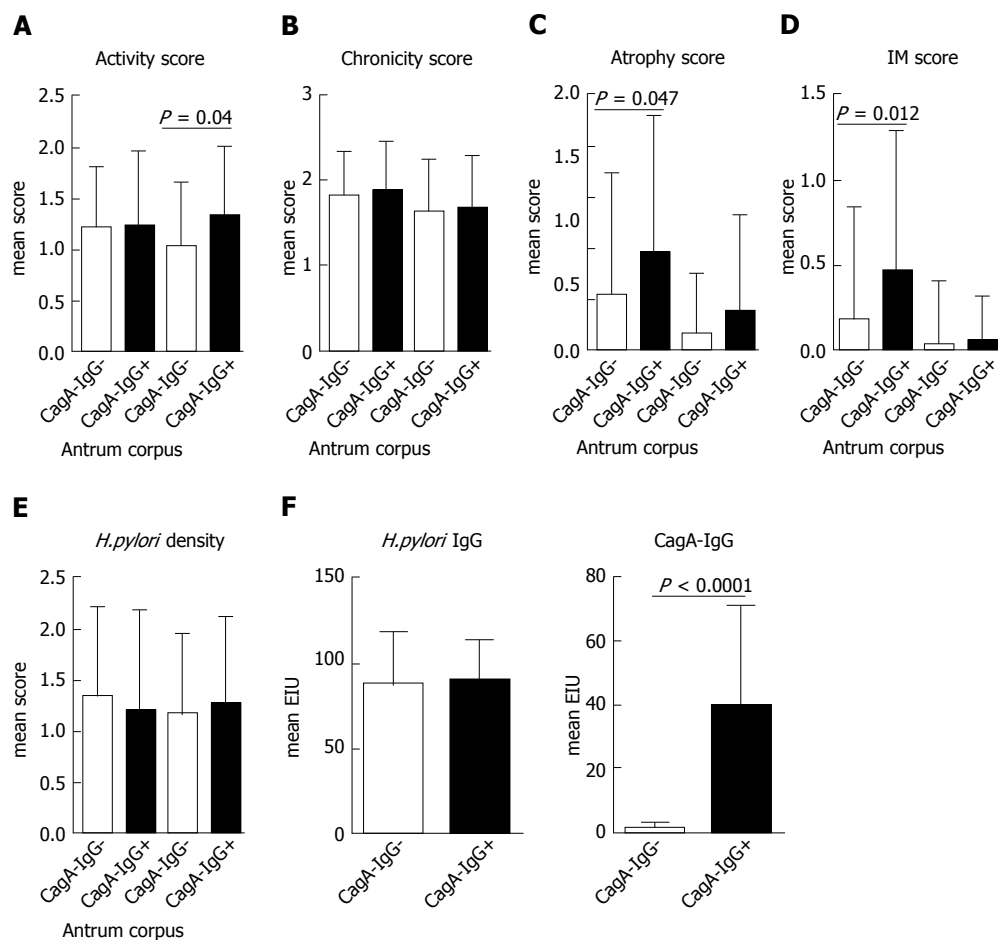


Figure 1 Difference in Sydney score and *Helicobacter pylori* seropositivity in patients with and without anti-CagA-IgG. Mean histological scores \pm SD. A: Activity; B: Chronicity; C: Atrophy; D: Intestinal metaplasia are shown; E: Anti-*Helicobacter pylori* IgG; F: anti-CagA-IgG titer were evaluated using ELISA; Statistical analyses were performed using Mann-Whitney test.

an additional level of evidence that anti-CagA-IgG is dependent on various bacterial and probably host factors but may not be useful as a biomarker for lesser pathogenic *H. pylori* infection.

Validation of CagA-IgG data

For the analysis of IgG response against CagA, we used well established ELISA-based method^[22,23]. To confirm these results and to further evaluate seropositivity, we performed an independent analysis using Immunoblot based method to evaluate the seropositivity. *Helicobacter* ViraStripe® IgG Kit includes, besides CagA, also various other Antigen-preparations such as VacA, p90, UreA, *etc.* Overall, there was a strong correlation between the two tests [$r = 0.722$ (95%CI: 0.6-0.81), $P < 0.0001$] (Figure S3A). All samples with positivity in anti-CagA-IgG ELISA test (Omega Genesis) showed very strong signal in immunoblot with values above 200 (Figure S3B). However, there were also several samples with positive signal in immunoblot and low values in ELISA, suggesting that certain samples with anti-CagA-IgG could be probably missed due to methodological issues (Figure S3C). However, the immunoblot-based method

(ViraStripe CagA-IgG Blot) was positive in some patients without evidence for past or present *H. pylori* infection and the lower specificity could be at least in part be the explanation for the higher detection rate (data not shown).

DISCUSSION

A substantial number of patients infected with *H. pylori* *cagA*-positive strains do not develop systemic immune response to CagA. In this study, we performed prospective and systematic analysis of *H. pylori* and its virulence factors CagA and VacA to find the explanation for the missing CagA-seropositivity. We confirm that the seroprevalence of CagA in unselected population with microbiologically confirmed *H. pylori* infected patients is low despite the high prevalence of *H. pylori* *cagA*+ strains. Following multilevel analyses, we found that among various potential factors *vacA* polymorphism is the most important factor associated with anti-CagA-IgG seropositivity.

The anti-CagA-seropositivity varies between different regions with highest prevalence in Asian countries and lowest in Europe. While earlier data

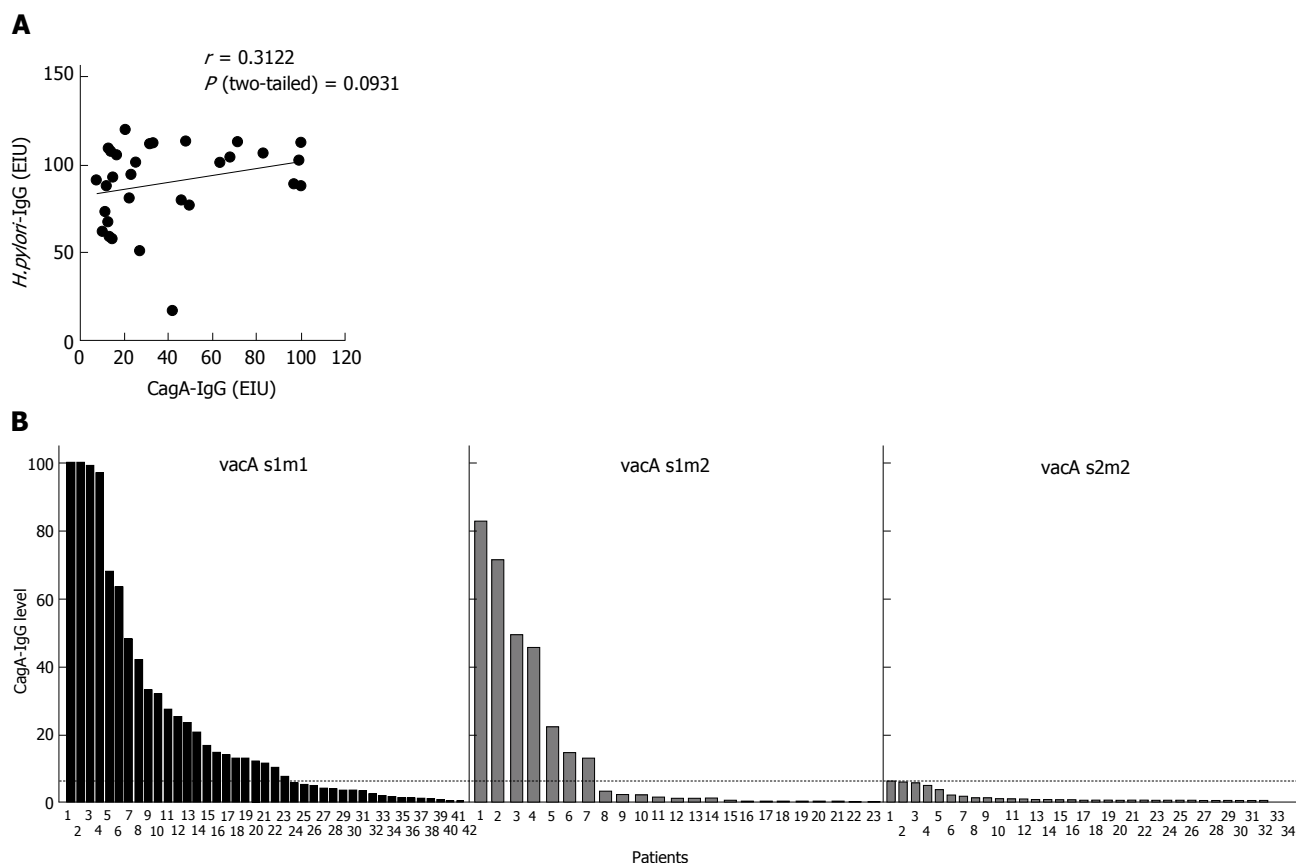


Figure 2 Correlation between anti-CagA-IgG and anti-*Helicobacter pylori* IgG and *vacA* s/m polymorphisms. A: Quantitative values for anti-*Helicobacter pylori* IgG and anti-CagA-IgG were correlated in patients with positive serology ($n = 30$) using Pearson's test; B: Patients with available data to *vacA* s1/2m1/2 polymorphisms were divided in subgroups dependent on s/m subtype. Anti-CagA-IgG values were sorted in increasing order. Dotted line shows the cut-off for seropositivity of the test (6.25 U/mL).

suggested correlation between *cagA* gene and seropositivity against CagA, our data showed that only 36%-43% patients had anti-CagA-IgG^[22,23,32]. In a recent work from Blaser's group, the prevalence of anti-CagA-IgG in a large cohort of children in Europe was 32%^[33]. The data from those studies confirm the low seropositivity in a European population with seropositivity for anti-CagA-IgG of microbiologically confirmed *H. pylori cagA+* infected subjects.

Experience from *H. pylori* vaccine trials suggests that an immune response to CagA is a common event. In the phase- I vaccine trials, intramuscular application of CagA, VacA and NAP induced strong systemic immune reactions measured *via* anti-CagA-IgG^[34]. So, basically any contact of inflammatory cells with CagA leads to antibody production in B-lymphocytes following antigen presentation. The failure in CagA presentation may happen during various steps of infection such as defective CagA expression, missing translocation due to T4SS system or missing or low cell death related to *H. pylori* infection and according low antigen presentation to immune cells. Indeed, the majority of *H. pylori* strains from subjects with anti-CagA-IgG exhibited mRNA expression *in vitro*, while a subgroup of bacteria showed no or very low *cagA* mRNA expression which further correlated

with CagA protein expression the *in vitro* AGC cell model. However, multiple factors related to host and environment (for example low acidity, predisposition to inflammation) may play very important role^[35]. Using *in vitro* model, we confirmed that the inflammatory potential of *cagA* positive strain was confirmed *in vitro* using the classical co-cultivation model of AGS cells and using CagA expression analysis in AGS cells (Table 3). To the first, direct analyses of strains with anti-CagA-IgG seropositivity did not reveal significant difference in inflammatory potential measured by IL-8 *in vitro*, suggesting that other bacterial factors could contribute to immune reaction. Second, co-cultivation analysis using AGC cells confirmed from mRNA expression showing that the multiple *H. pylori* strains from patients with negative anti-CagA-IgG have fully functional CagA and TSS4 (Table 3).

Increasing evidence highlights the role of *vacA* polymorphisms in gastric diseases^[26]. Assessment of *H. pylori vacA* and *cagA* genotypes and serological host response earlier revealed the association with *vacA* s1^[25]. Systematic analyses of *vacA* subtypes in background of anti-CagA-IgG have revealed crucial dependency of seropositivity on *H. pylori vacA* s1m1 polymorphism in our cohort. The *in vitro* data highlight the inflammatory potential of *H. pylori* strains with

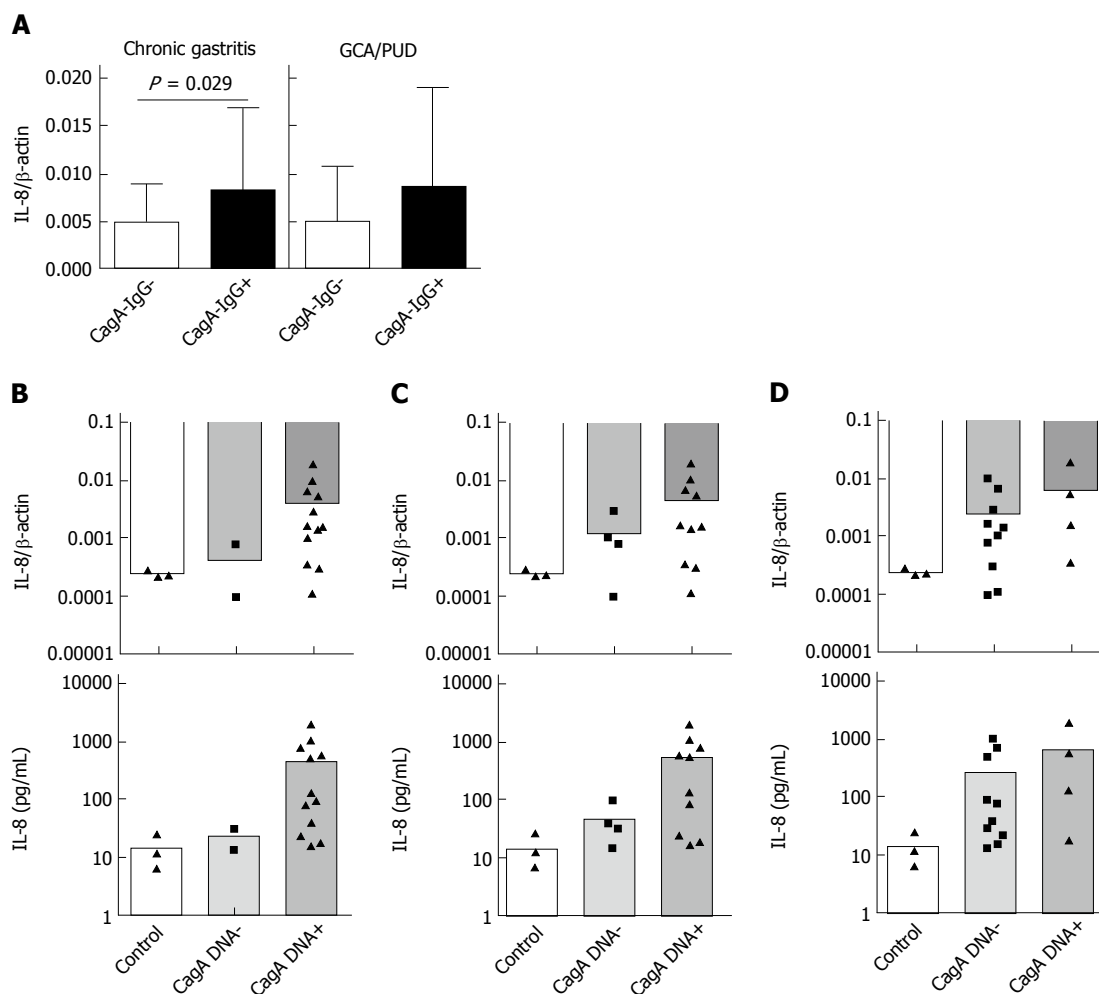


Figure 3 Inflammatory potential based on CagA-IgG in gastric mucosa *ex vivo* and *in vitro* using AGS cells. A: IL-8 mRNA expression was evaluated in antrum mucosa from patients with chronic gastritis [CG, AG, IM, without peptic ulcer disease (PUD) and gastric cancer (GC)] with ($n = 23$) and without CagA seropositivity ($n = 48$); B: IL-8 expression in antrum mucosa from patients with PUD and gastric cancer ($n = 4$ for CagA-IgG⁺ and $n = 6$ CagA-IgG⁻); C: IL-8 mRNA; D: IL-8 protein expression in supernatant are shown for subgroups dependent on *cagA*, *CagA* mRNA and anti-CagA-IgG status *in vitro* using co-culturing of *Helicobacter pylori* strains from patients with AGS cells.

vacA s1m1 polymorphism. This observation is further supported by data showing the dependency of apoptotic activity of *H. pylori* on *vacA*^[36]. This led us to believe that the immune response to *cagA* may be at least in part triggered by the effect of VacA on the gastric mucosa. Therefore, the amount of inflammation related to cell toxicity and apoptosis through VacA may influence the interaction of cellular CagA with the immune system and ultimately determine the immune response. The interaction between VacA and CagA has been in focus of several recent studies providing evidence for complex interaction and showing that VacA and CagA can counter-regulate or antagonize each other and affect the host-bacteria interaction^[37,38].

Whether a host will develop an immune response to an infection may be influenced by multiple factors. The seroprevalence may typically change during the course of infection, however, it is only partially true for *H. pylori* infection that shows relatively similar pattern during the life-time starting with early infection to death. We have previously shown that anti-CagA-IgG

seropositivity was similar in different age groups (above or below 30 years) from *H. pylori* positive subjects^[22]. Recently, the similar serological pattern was shown in children, where anti-CagA-IgG was positive in 32% despite the very young age^[33]. Based on this observation, we speculate that the initial infection with *H. pylori* and according very first contact to CagA may determine the serological status of the host, which will then remain stable through the whole life until *H. pylori* treatment, disappearance or death. In this regard, the host factors and especially genetic predisposition may play the very important role. Certain host factors such as genetic polymorphism (exp. HLA) have previously been suggested to be associated with susceptibility or resistance to *H. pylori* infection^[39]. Furthermore, nonfunctional TLR1 SNP 602S/S has been associated with a reduced risk of *H. pylori*-induced gastritis^[40]. Also, a genome-wide association study identified an association between TLR1 and *H. pylori* seroprevalence that could potentially explain the variation^[41]. However, TLR1 is not the only candidate gene, and IL1-beta

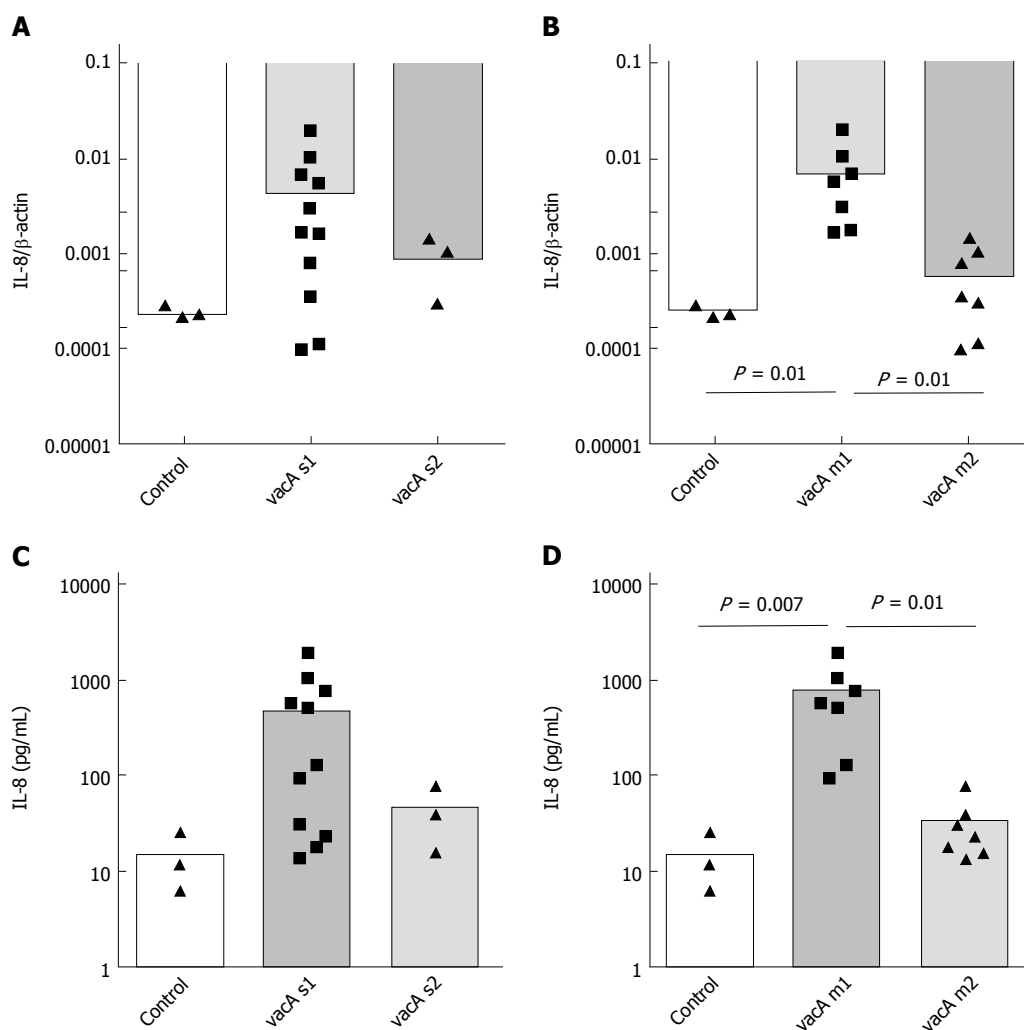


Figure 4 Inflammatory potential of *Helicobacter pylori* strains in relation to *vacA* polymorphism. *Helicobacter pylori* strains from patients were co-cultivated with AGS cell and A-B: IL-8 mRNA; C-D: IL-8 expression in supernatant were measured using qPCR and ELISA.

should be also considered as a potential determinant^[42].

From the clinical perspective, our data support significant association of *H. pylori* CagA seropositivity and corpus predominant gastritis, atrophic alterations in gastric mucosa. However, the absence of anti-CagA-IgG does not preclude the infection of individual with the more virulent CagA positive *H. pylori* strain. Based on the current data, the knowledge of individual anti-CagA-IgG status does not allow any specific prognostic clinically-relevant management in support of existing recommendation^[43,44].

One of the limitations of our study is that due to the low number of patients, we were unable to suitably address the host related genetic factors. In the present study, we focused on the systemic anti-CagA-IgG production and the locally produced IgA response may be an interesting target for evaluation. Even though we could correlate CagA-IgG data from two different tests, there still may be some difference related to different techniques^[25]. Nevertheless, we observed the best specificity with the ELISA kit while immunoblot although had slightly higher sensitivity, it was also

associated with high number of false positive results (data not shown). Furthermore, even though the *H. pylori* CagA-IgG positive and negative groups were well balanced, the higher number of subjects were female and potential gender specificity cannot be fully excluded.

In summary, we show that seropositivity for CagA in subjects with *H. pylori* infection is positive in one third of *H. pylori* infected European population despite the presence of CagA positive strain. The immune response to CagA was associated with various bacterial factors and most importantly with *H. pylori vacA* gene polymorphisms. Our data support a crucial role of bacterial and probably host-related factors that co-determine the complex interaction with *H. pylori* and define the immunologic and clinical phenotypes of the infection.

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COMMENTS

Background

Helicobacter pylori (*H. pylori*) -related peptic ulcer disease and gastric adenocarcinoma are commonly associated with *cagA*⁺ *H. pylori* strains. However, seropositivity against CagA varies among different studies with positivity below 50% in multiple studies from Europe.

Research frontiers

Infection with *H. pylori* induces strong and sustained inflammation in mucosa and triggers immune positivity against *H. pylori*. Although similar immune response to CagA is expected for *H. pylori cagA*⁺ strains, the positivity is substantially lower. The mechanism responsible for the seropositivity to CagA is not sufficiently understood. This knowledge may be helpful to identify the factors responsible for the differences in clinical phenotype of *H. pylori* infection. Furthermore, it may also facilitate the preventive and treatment strategies.

Innovations and breakthroughs

In this well-characterized cohort of patients, we demonstrated a low anti-CagA-IgG positivity in *H. pylori* infected patients, which was independent to the high rate of *H. pylori cagA* positive strains. Immune response to *H. pylori* CagA was strongly associated with atrophic gastritis, increased mucosal inflammation and IL-8 expression. Most importantly, we observed a strong association of anti-CagA positivity to *H. pylori vacA* s and m polymorphisms, which also correlated with the inflammatory potential *in vitro* in AGS cell lines. Altogether, our data suggest that *H. pylori vacA* polymorphism may determine the immune response to CagA through modulation of mucosal inflammation.

Applications

These data strengthens the role of *H. pylori vacA* polymorphisms and immune response to CagA and in *H. pylori* infection. Whether *H. pylori vacA* may become a clinical tool for risk stratification of *H. pylori*-related diseases needs further evaluation.

Peer-review

The results in this manuscript have demonstrated that seropositivity for CagA in subjects with CagA positive *H. pylori* status is present in one third of *H. pylori* infected European population. The immune response to CagA is associated with various bacterial factors and most importantly with *vacA* gene polymorphisms. The data supported that both bacterial and host-related factors determined the complex interaction of *H. pylori* with the immunologic system and clinical phenotypes of the infection.

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