Infection with Helicobacter Pylori Presenting the vacA s2m2 haplotype is Strongly Associated with Protection Against Gastric Cancer

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Abstract

Background: Infection with *Helicobacter pylori* is recognized as the main risk factor for gastric cancer (GC); the clinical outcome of this infection is variable and partially depends on the virulence of the infective strain. This study characterizes *H. pylori* virulence genes in patients with diverse gastric lesions, from preneoplasia to GC, from a South American region with high GC mortality rates.

Methods: We studied the virulence profiles of *H. pylori* strains to colonize the antrum of 318 patients with non-atrophic gastritis (NAG), 58 patients with preneoplastic lesions (PN), and 90 with GC from Ibagué, Colombia. The presence of *16S rDNA*, the *cagA* and *cagE* genes, and the *vacA* s_1, s_2, m_1, and m_2 alleles were determined by PCR.

Results: *H. pylori* infection was detected in 44% of all patients, 41.2% in NAG, 43.1% in PN and 54.4% of GC patients (p= 0.0813). *cagA* and *cagE* genes were significantly more frequent in and GC than in NAG (p= <.0001). The *vacA* s_1m_1 haplotype was significantly more frequent in PN (68%) and GC (65.3%) than in NAG (37.4%). The frequency of *vacA* s_2m_2 haplotype decreased significantly from NAG (42.7%) to PN (12%) and this to GC (4.1%). A total of 23 different genotypes were identified, with *cagA*+/*cagE*+/*vacA* s_1m_1 (84/205) as the more frequent in PN and GC and *cagA*-/*cagE*-/*vacA* s_2m_2 in NAG (49/205).

Conclusions: In the population studied, *vacA* s_2m_2 was identified as a significant marker for protection against PN and GC, and genotype *cagA*+/*cagE*+/*vacA* s_1m_1 as a marker for increased GC risk. We also found that patients with PN and GC had a higher frequency of *cagA*+/*cagE*+/*vacA* s_1m_1 *H. pylori* strains known to be aggressive.

Background

Gastric cancer (GC) is the third leading cause of cancer-related deaths worldwide. [1,2,3] In Colombia, it is the leading cause of cancer-related deaths in men and the fourth in women.[4,5] Globally, the GC incidence varies across different countries; in Latin America, an association between altitude and GC risk has been observed and Chile, Costa Rica, and Colombia are some of the countries with the highest mortality rates in the world.[4]

The bacteria *Helicobacter pylori* (*H. pylori*) is classified as a class I human carcinogen and recognized as the main risk factor for GC.[6,7] This bacteria promotes inflammation of the gastric mucosa that in some patients lead to premalignant lesions such as chronic atrophic gastritis, intestinal metaplasia, and dysplasia that precedes the appearance of GC.[6–13] Worldwide, there is no correlation between the prevalence of *H. pylori* infection and GC incidence.[14] This bacterium colonizes the gastric mucosa of 50% of the worldwide population and 80% in developing countries; still, only 1–3% of infected people develop premalignant gastric pathologies.[9,15,16]

In Colombia, whereas the prevalence of *H. pylori* infection varies widely between regions,[14,17–19] the mortality of GC as well as a higher prevalence of precancerous lesions are concentrated in the central part of the country that corresponds to the Andes Central Mountain Range, mainly in the Coffee axis

departments (Caldas, Risaralda, and Quindío), Norte de Santander, Boyacá, Huila, Cauca, Tolima, and Caquetá. Furthermore, the lowest GC mortality rates are reported in regions with low altitudes but a high prevalence of infection, such as the Guajira, Chocó, Córdoba, Putumayo, and Sucre departments.[12] Several theories have been raised regarding the association between altitude and GC, but they have not been well elucidated yet. It is likely that those mountain regions cluster host genetic, dietary, and environmental factors as well as bacterial genotypes that promote GC development.[8,11–13,20–25] Among the best-studied *H. pylori* virulence factors are cytotoxin-associated gene A (*cagA*) and vacuolating cytotoxin gene A (*vacA*). More virulent strains carry the *cagA* gene, which encodes the oncoprotein CagA, which induces a myriad of changes in the gastric mucosa: an increase of inflammation, the loss of epithelial polarity, the disruption of intercellular junctions, increases proliferation, reduces apoptosis, and eventually promotes carcinogenicity.[26–28] The *vacA* gene, which is virtually present in all *H. pylori* strains, encodes the VacA protein, which is responsible for cytoplasmic vacuoles and pores in the membrane of gastric epithelial cells and apoptosis.[29–31] Some *vacA* alleles are known to increase the risk of developing peptic ulcer disease and GC.[32–36] Infection with *H. pylori* strains that possess the *vacA* s_1m_1 genotype is associated with an increased risk of peptic ulceration and GC.[27,32, 37–39] However, little attention has been paid to evaluate the prognostic value of the *vacA* s_2m_2 haplotype.

In the Tolima Department of Colombia, a central mountainous region with high GC mortality rates, *H. pylori* infection prevalence varies between 59% and 66% but the correlation between the bacterial genotypes and gastric lesions has not been properly studied.[32,40–42]

Methods

In this study, we characterized *H. pylori* virulence genes in patients with NAG, PN, or GC from Tolima, with a particular interest in the study of *vacA* s_2m_2 haplotype.

Patients and sampling

A total of 466 patients with gastric pathologies were recruited during the period 2010–2019 in Ibagué, including 90 patients with GC who had undergone upper gastrectomy at the Federico Lleras Acosta Hospital and 376 patients who had undergone upper gastrointestinal endoscopy as part of the dyspepsia study at the Javeriano Medical Center. A gastric biopsy from the pyloric antrum region was taken from each patient, placed in 70% alcohol solution, and stored at -20°C until studied. Biopsies from antrum and tumor lesions were placed in paraformaldehyde for histology studies. All patients were informed about the study and if willing to participate they were asked to sign an informed consent letter. The research protocol used in the study was approved by the University of Tolima Ethics Committee and adhered to the Helsinki Declaration.

Histopathological examinations

Biopsies in paraformaldehyde were embedded in paraffin for histopathological diagnosis. Antral and tumor sections were stained with hematoxylin and eosin and evaluated independently by three surgical pathologists to establish the diagnosis in each case. Patients were assigned to one of the three groups: 1.

chronic non-atrophic gastritis (NAG); 2. PN, which included patients with chronic and atrophic gastritis, intestinal metaplasia, and dysplasia; and 3. GC.

Molecular identification of H. pylori

DNA was extracted from the gastric antrum biopsy specimens with the DNeasy Blood and Tissue Kit (QIAGEN, USA) following the manufacturer's instructions. DNA was quantified using a Nanodrop ND™ 1000 UV-Vis spectrophotometer of Thermo Scientific and quality was evaluated by using the ratio of absorbance at 260 and 280 nm (A260/A280). To test for the presence of *H. pylori*, a 537-bp fragment of the Sub unit 16 of ribosomal DNA (*16Ss rDNA*) gene was amplified using polymerase chain reaction (PCR) with the primers ACT-1 and ACT-2, as previously described (ref). DNA extracted from the *H. pylori* NCTC 11638 strain (donated by the National Institute of Cancerology, Bogotá, Colombia) was used such as positive control. Ultrapure water instead of DNA was used such as negative control. The final volume of 25 µl contained 9.5 µl of ultrapure water, 1 µl of each primer, 12.5 µl of the BIOLINE MyTaq™ Extract from the PCR kit, and 1.5 µl of DNA. The thermocycling program was previously described by López et al.[40]

Amplification and typing of the cagA, cagE, and vacA genes

To determine the genotype of *H. pylori* virulence genes the samples were subjected to PCR for the *cagA* and *cagE* genes and the signal regions (s_1 and s_2 alleles) and mid-region (m_1 and m_2 alleles) of *vacA* using primers and conditions previously described.[33, 40,43] As a control for one possible inhibition of the reaction, a fragment from the human β-globin gene was amplified. All PCRs were performed in a Bio-Rad Dual-Touch 1000 thermocycler. The amplified products were visualized on a Thermo Fisher™ ultraviolet light transilluminator on 1.5% agarose gels at 100 volts for 60 minutes using ethidium bromide (0.4%). All primers used in this study are listed in Supplementary table 1, Additional File 1.

Statistical methods

The chi-square (X^2) independence tests were performed to evaluate differences between the epidemiological variables, the presence of *H. pylori* infection, and the bacterial genotypes, and the gastric pathology of the patients. A two-tailed p-value (α) < 0.05 was considered statistically significant. The data were processed with R software version 3.6.1.

Results

A total of 466 patients with different gastric pathologies were included in this study. There were 285 women (61.2%) and 181 men (38.8%), with an average age of 52.1 ± 16 years. A 2:1 ratio of women to men was observed in patients with premalignant pathologies, while in patients with GC, this proportion was reversed (2:1 ratio of men to women) (Figure 1).

The histological analysis showed NAG in 318 (68.2%), PN in 58 (12.5%) and GC in 90 (19.3%) patients. Of the patients with GC, 40 (44.4%) had intestinal-type GC (GCI), 25 (27.8%) had diffuse-type GC (GCD) and 25 (27.8%) had mixed-type GC (GCM). Among the 40 GCI cases 27 (67.5%) were men; of the 25 GCD cases 17 (68%) were men and of the 25 GCM cases 18 (72%) were men.

The molecular diagnosis tests showed that 44% (205/466) of the patients were infected with *H. pylori*, 59.5% (122/205) were women and 40.5% (83/205) were men. The *H. pylori* prevalence was lower than 50% in most gastric histological lesions, except in GC (54.4%). The infection status was not associated with the sex, age, or gastric pathology of the patients. The molecular diagnosis results are presented in Table 1.

Table 1
Molecular detection of *H. pylori* in 466 antral gastric tissue.

Variable	Population n† (%)	*H. pylori* detection‡		p-value
		Negative	Positive	
Sex				
Female	285 (61.2)	163 (57.2)	122 (42.8)	0.5839
Male	181 (38.8)	98 (54.1)	83 (45.9)	
Age				
< 50 years	187 (40.1)	90 (48.1)	97 (51.9)	<.0001*
≥ 50 years	279 (59.9)	171 (61.3)	108 (38.7)	
Pathology§				
NAG	318 (68.2)	187 (58.8)	131 (41.2)	0.0813
PN	58 (12.5)	33 (56.9)	25 (43.1)	
GC	90 (19.3)	41 (45.6)	49 (54.4)	

†n (%): Number and percent in each category

‡Molecular detection of H. pylori was performed from partial amplification of *16S rDNA* gene

§ The gastric pathologies of patients were classified in three categories: non-atrophic gastritis (NAG), preneoplastic lesions (PN) and gastric cancer (GC)

*p-value <0.05

In all *H. pylori*-positive biopsies, the *cagA, cagE,* and *vacA* genes were amplified in 59.5%, 66.3%, and 94.6% of cases, respectively (Table 2). Strains with the *cagA* and *cagE* genes were significantly more frequent in PN and GC than in NAG (p-value 0.004 and <0.0001, respectively), and OR values showed that the presence of these genes increased around 10 times the risk for GC. As the severity of gastric pathology increased, the proportion of strains with *cagA* and *cagE* also increased (Figure 2). These genes were present in almost 90% of *H. pylori* strains in GC, regardless of the subtype of cancer (intestinal or diffuse).

PCR amplification of the signal regions (s_1 and s_2 alleles) and mid-regions (m_1 and m_2 alleles) of *vacA* showed different allelic combinations (Supplementary table 2, Additional File 1) with a predominance of s_1m_1 (47.8%) and s_2m_2 (29.8%). The s_1m_1 genotype was significantly more frequent in patients with PN lesions (68%, p-value 0.001) or GC (65.3%, p <0.0001) than in patients with NAG (37.4%), and OR values indicated a risk for GC of 18 times higher when s_1m_1 is present. Besides, the s_2m_2 haplotype was frequent in NAG (42.7%) but drastically decreased in PN (12%,) with an OR value of 0.1544 and was almost absent in GC cases (4.1%) with an OR value as low as 0.0547 (Figure 2 and Table 2).

Table 2
Status of *cagA, cagE*, and v*acA* genes in patients infected with *H. pylori.*

Gene or haplotype	Population no.† (%)	Gastric pathology§			NAG vs PN		NAG vs GC	
		NACG (%)	PN (%)	GC (%)	p-value	OR	p-value	OR
cagA								
Negative	83 (40.5)	72 (55.0)	6 (24.0)	5 (10.2)	0.00455	3.8644 (1.45-10.29)	<0.0001*	10.739 (4.01-28.81)
Positive	122 (59.5)	59 (45.0)	19 (76.0)	44 (89.8)				
Total	205	131	25	49				
cagE								
Negative	69 (33.7)	60 (45.8)	5 (20.0)	4 (8.2)	0.016489	3.3803 (1.19-9.54)	<0.0001*	9.507 (3.23-27.96)
Positive	136 (66.3)	71 (54.2)	20 (80.0)	45 (91.8)				
Total	205	131	25	49				
vac A								
s_1m_1	98 (47.8)	49 (37.4)	17 (68.0)	32 (65.3)	0.001644	6.4762 (1.79-23.43)	<0.0001*	18.2857 (4.16-80.26)
s_2m_2	61 (29.7)	56 (42.7)	3 (12.0)	2 (4.1)		0.1544 (0.04-0.55)		0.0547 (0.0125-0.24)
Total	159	105	20	34				

†no. (%): number and percent in each category

§ The gastric pathologies of patients were classified into three categories: non-atrophic gastritis (NAG), preneoplastic lesions (PN), and gastric cancer (GC)

*p-value <0.05

A total of 23 different genotypes and one case of coinfection were identified in the infected patients (Supplementary Figure 1, Additional File 1), with a predominance of the *cagA*+/*cagE*+/*vacA* s_1m_1 (41%) and

cagA-/c*agE*-/*vacA s2m2* (24%) genotypes. Patients with NAG presented the highest variation in genotypes (21 different genotypes), followed by patients with PN (10 genotypes), patients with GCI (9 genotypes), and finally, patients with GCD and GCM (5 genotypes each); there seems to be a selection of genotypes as the disease progress. In patients with NAG, the genotypes *cagA*-/*cagE*-/*vacA s2m2* and *cagA*+/*cagE*+/*vacA* s_1m_1 were the most frequent (35.1 and 31.3%, respectively) while in patients with PN and GC, the *cagA*+/*cagE*+/*vacA* s_1m_1 genotype was the most prevalent (60 and 57.1%, respectively). The *cagA*-/*cagE*-/*vacA* s_2m_2 genotype was present in only one patient with GC resulting in an OR value of 0.066 as compared with the NAG group (Supplementary Figure 2, Additional File 1, Supplementary table 3, Additional File 1).

Discussion

GC is a multifactorial disease associated with genetic, environmental, and infectious factors, with *H. pylori* infection as the more important risk factor. GC associated with *H. pylori* infection is the result of long-term chronic inflammation in the gastric mucosa that when unregulated may lead to tissue damage, which may progress to atrophic gastritis, intestinal metaplasia, dysplasia, and eventually GC.[44,45] Bacterial genotypes play an important role in clinical outcomes, particularly when they are associated with an increased inflammatory response; however, this association may vary between and within different populations.[46]

In Colombia, the prevalence of *H. pylori* infection and the risk for GC vary among the different departments.[10, 58, 60] The association between gastric lesions and *H. pylori* virulence factors have been evaluated in Colombian departments with a similar prevalence of infection but contrasting GC risk and higher frequencies of *cagA*-positive and *vacA* s_1m_1 genotypes were found in populations with a higher risk of GC than in low-risk areas.[47–49] In Ibagué, located in the Andean mountain region with high GC mortality rates,[12,13,50,51] only studies with small samples size have been carried out, analyzing *cagA* and the *s* and *m* regions of *vacA*.[40–42, 52] In the present study we aimed to do a more comprehensive analysis of genotypes in virulence genes in larger groups of patients.

In our studied population, the most frequent type of GC was GCI (44.4%), similar to previous works,[55–58] while the frequency of GCM (27.8%) was higher than that reported in other countries. [70,71] Of note, 20% of the GC patients had an average age of diagnosis of 42 years, which shows the need for the implementation of early detection programs to identify and treat the disease.

The genotyping of virulence genes showed that in our population the frequency of the *cagA* gene was lower (59.5%) than that reported in previous investigations in Colombian mountain regions [33,48,64,65] and other Latin American countries, with 67 to 80% reported in patients with premalignant lesions. [34,66–68] The presence of the *cagE* gene has been suggested as a better marker than the *cagA* gene about the integrity of the Cag Pathogenicity Island (*cag PAI*), and it has been observed with a frequency greater than 80% in patients with gastrointestinal diseases in other countries.[46,69] In the present study, 66.3% of infected patients were positive for *cagE*, which was lower than in previous reports in Colombian strains.[33,54] However, the frequency of these genes was significantly higher in patients with severe lesions, and *cagA*

was present in 76% of PN and 89.8% in GC, whereas *cagE* was in 80% of PN and 91.8% in GC. These results are in agreement with previous studies in Colombia [33,48] and confirm the value of using these genes as markers for PN and GC.

Analysis of the *vacA* gene alleles showed that in the group of patients with PN and GC, the most frequent combination was s_1m_1, whereas the s_2m_2 genotype was rare; in contrast, in patients with NAG, the most frequent combination was s_2m_2 (Table 2). These results are consistent with those of previous reports, where the s_1m_1 genotype has been widely associated with the presence of chronic inflammation and the development of severe gastric pathologies.[70] while the *vacA* s_2m_2 nontoxic strains are more frequent in patients with non-ulcer dyspepsia and mild gastritis.[35,71–74]

The distributions of the tested genes strengthen the observed association of the genes with the disease; thus, the percentage of strains harboring the *cagA* and *cagE* genes and the *vacA* s_1m_1 haplotype increased as the severity of the gastric pathology increased (Fig. 2). A similar trend was previously reported by Cittely et al.[48] in Colombian patients from Bogotá and in patients with gastroduodenal disorders in other countries.[36] However, little attention has been paid to the value of *vacA* s_2m_2 as a potential marker of disease risk; our results show a strongly significant protective factor of s_2m_2 for PN (OR 0.1544) and even stronger for GC (OR 0.0547) where this *vacA* haplotype is almost absent. Thus, carrying strains with the *vacA* s_2m_2 haplotype significantly reduces the risk for gastric cancer and to determine vacA alleles there is no need to isolate and culture *H. pylori*, the test can be done in DNA from biopsies. These make PCR amplification of *vacA* alleles a useful and accessible biomarker to assess the risk for gastric cancer.

In this study, we analyzed genotype profiles using results from all genes and alleles studied and found as many as 23 genotypes; two of them commonly reported and another 21 different combinations that are less commonly found (Supplementary Fig. 1, Additional File 1). We aimed to see if building a genotype would improve the value of these tests as markers for disease risk. The most frequent genotypes were *cagA+/cagE+/vacA* s_1m_1 (41%) and *cagA-/cagE-/vacA* s_2m_2 (23.9%). A positive association was found between the severity of these lesions and the *cagA+/cagE+/vacA* s_1m_1 *H. pylori* genotype, while the *cagA-/cagE-/vacA* s_2m_2 genotype was rare in patients with PN and GC and was more frequent in patients with NAG, which supports the relationship between histological features and *H. pylori* genotypes reported in previous studies. [35,71,74,75]

Conclusions

In conclusion, the present study shows that the *cagA*+/*cagE*+/*vacA* s_1m_1 genotype is a marker for increased GC risk, whereas *cagA-/cagE-/vacA* s_2m_2 is a marker for decreased risk. Of importance, our results suggest that the determination of *vacA* alleles is useful enough to evaluate the risk for disease and the presence of s_2m_2 is a strong marker for protection against gastric cancer.

Abbreviations

16s rDNA= Subunit 16 of ribosomal DNA

cag PAI= cag pathogenicity island

cagA= cytotoxin-associated gene A

cagE= cytotoxin-associated gene E

GC= gastric cancer

GCD= diffuse-type gastric cancer

GCI= intestinal-type gastric cancer

GCM= mixed-type gastric cancer

H. pylori= *Helicobacter pylori*

PCR= polymerase chain reaction

NAG= non-atrophic gastritis

PN= preneoplasia

vacA= vacuolating cytotoxin gene A

X^2= The chi-square.

Declarations

Ethics approval and consent to participate.

All subjects included in the study provided his/her written informed consent for study participation. Similarly, the study protocol was developed in concordance with The Declaration of Helsinki (subsection of ethics approval and consent to participate).

This study was approved by the bioethics committees of Tolima University with ethical permission number 013 on 23 November 2006; Federico Lleras Acosta Hospital with ethical permission number 18 on 14 February 2010; and the Javeriano Medical Center with ethical permission number 02 on 31 July 2018.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

None declared. The authors declare that they have no competing interests related to the subject matter or materials discussed in this article.

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Authors' contributions

AGT: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing – original draft, writing – review & editing

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All authors read and approved the final manuscript.

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Figures

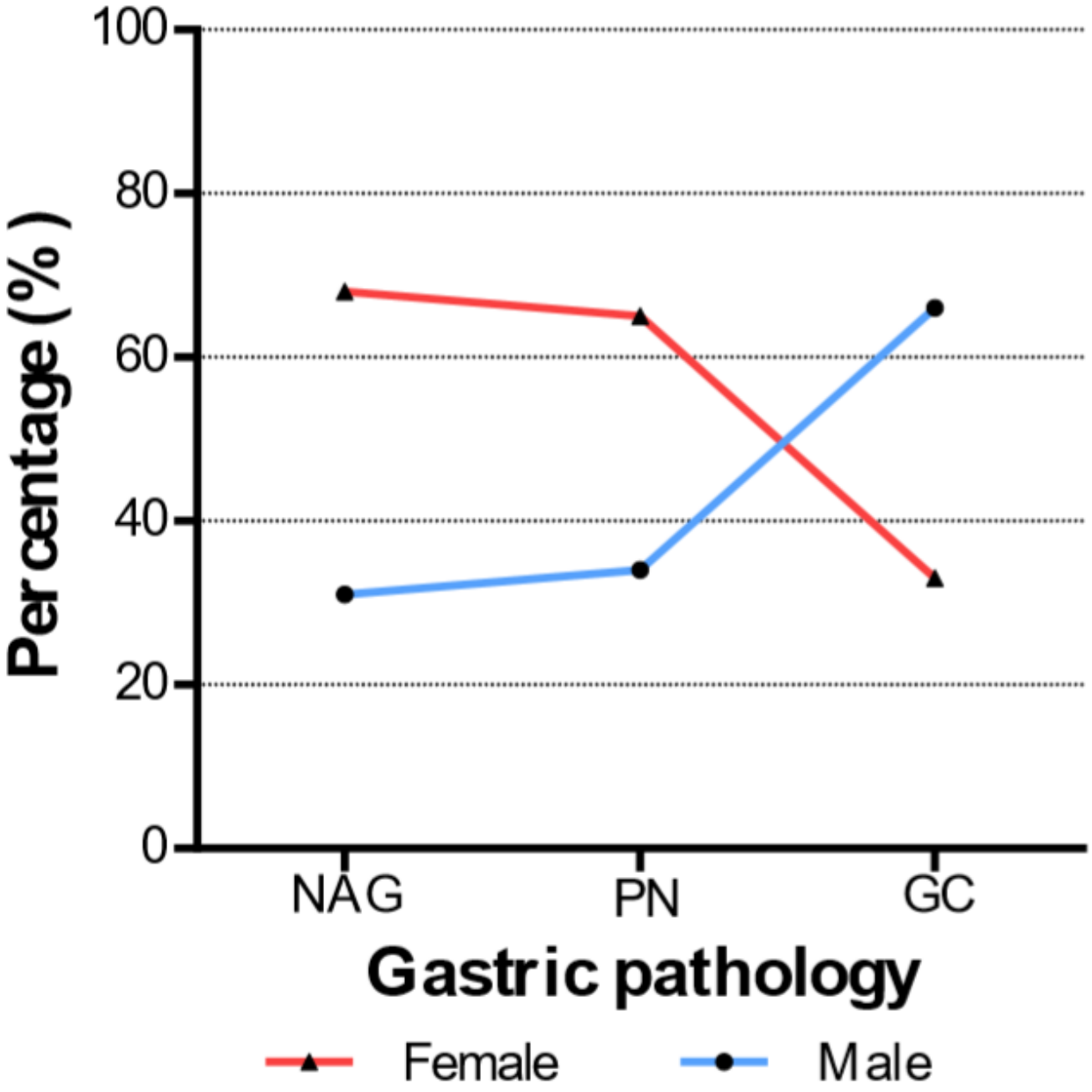

Figure 1

Distribution of sample according to gastric pathology and sex (n=466).

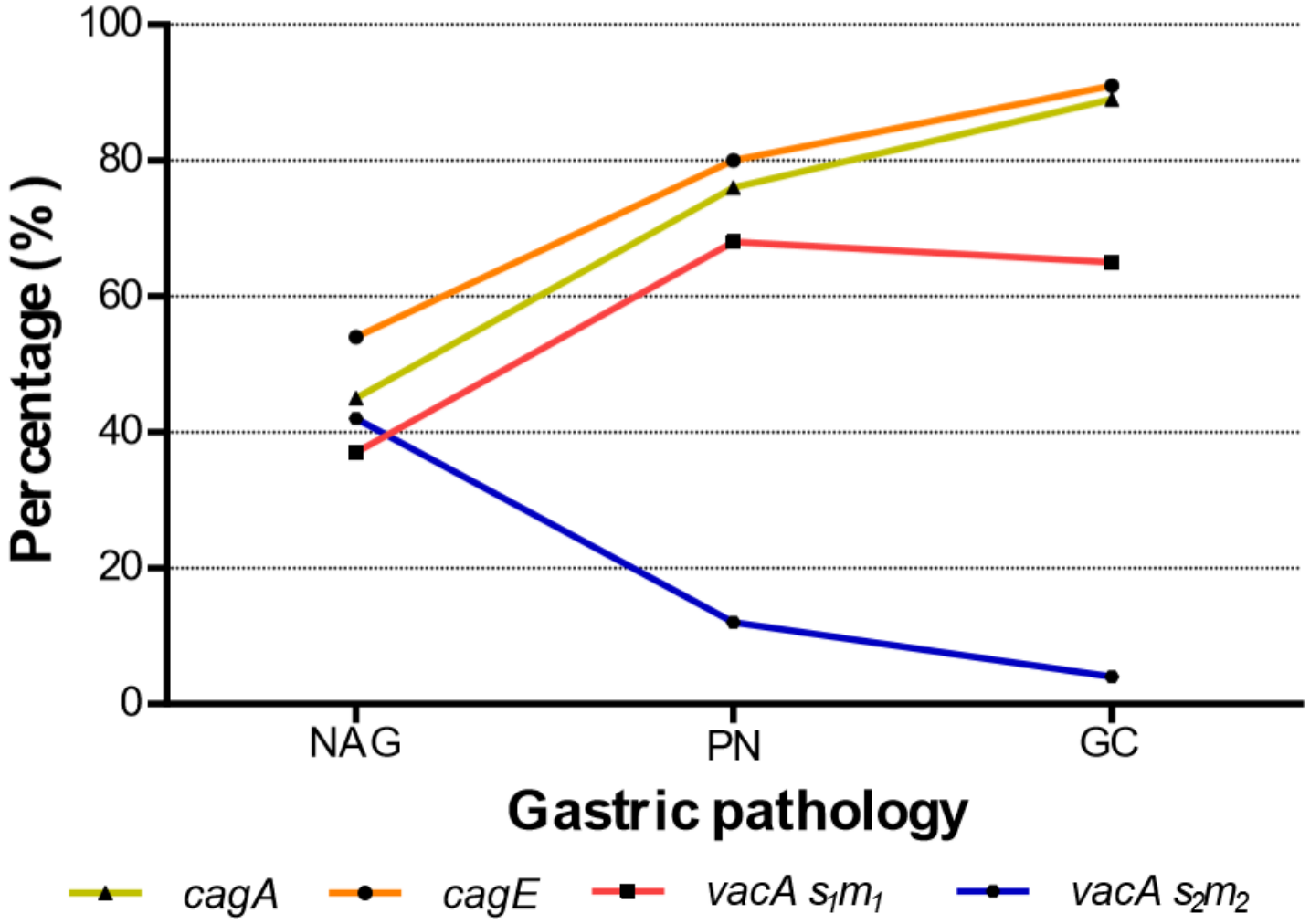

Figure 2

Frequency of H. pylori strains with the genescagA, cagE, and s1m1 ands2m2 vacA haplotypes in infected patients according to their clinical group.

Supplementary Files

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