

# DETERMINATION OF STRAINS OF *Helicobacter pylori* AND OF POLYMORPHISM IN THE INTERLEUKIN-8 GENE IN PATIENTS WITH STOMACH CANCER

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**ABSTRACT – Context** - Gastric neoplasia is the second most common cause of death by cancer in the world and *H. pylori* is classified as a type I human carcinogen by the World Health Organization. However, despite the high prevalence of infection by *H. pylori* around the world, less than 3% of individuals carrying the bacteria develop gastric neoplasias. Such a fact indicates that evolution towards malignancy may be associated with bacterial factors in the host and the environment. **Objectives** – To investigate the association between polymorphism in the region promoting the IL-8 (-251) gene and the *H. pylori* genotype, based on the *vacA* alleles and the presence of the *cagA* gene, using clinical and histopathological data. **Methods** - In a prospective study, a total of 102 patients with stomach cancer and 103 healthy volunteers were analysed. Polymorphism in interleukin 8 (-251) was determined by the PCR-restriction fragment length polymorphism reaction and sequencing. PCR was used for genotyping the *vacA* alleles and the *cagA* in the bacterial strains PCR. Gastric biopsies were histologically assessed. **Results** - The *H. pylori* serology was positive for 101 (99%) of all patients analysed, and 98 (97%) of them were colonized by only one strain. In patients with mono-infection, 82 (84%) of the bacterial strains observed had the *s1b1m1* genotype. The *cagA* gene was detected in 74 (73%) of patients infected by *H. pylori*. The presence of the *cagA* gene was demonstrated as associated with the presence of the *s1b1m1* genotype of the *vacA* gene ( $P = 0.002$ ). As for polymorphism in the interleukin 8 (-251) gene we observed that the AA ( $P = 0.026$ ) and AT ( $P = 0.005$ ) genotypes were most frequent in the group of patients with gastric adenocarcinoma. By comparing the different types of isolated bacterial strains with the interleukin -8 (-251) and the histopathological data we observed that carriers of the A allele (AT and AA) infected by virulent strains (*m1s1 cagA+*) demonstrated a greater risk of presenting a degree of inflammation (OR = 24.75 CI 95% 2.29-267.20  $P = 0.004$ ) and increased neutrophilic activity (OR = 28.71 CI 95% 2.62-314  $P = 0.002$ ) in the gastric mucosa. **Conclusion** - Our results demonstrate that the interaction between polymorphism in the interleukin -8 (-251) gene, particularly with carriers of the A allele and the infecting type of *H. pylori* strain (*s1m1 cagA* positive) performs an important function in development of gastric adenocarcinoma.

**HEADINGS** - *Helicobacter pylori*. Stomach neoplasms. Polymorphism, genetic. Interleukin-8.

## INTRODUCTION

Infection by *H. pylori* is considered one of the most important factors in the pathogenesis of various gastrointestinal diseases, such as: chronic gastritis, atrophic gastritis, peptic ulcers, carcinoma and gastric lymphoma<sup>(14, 28)</sup>. However, the majority of infected patients remain asymptomatic carriers throughout their lives and only 20% may evolve towards a more serious gastrointestinal disease during their lives<sup>(14, 20)</sup>, with fewer than 3% being observed developing stomach cancer<sup>(10)</sup>. The great variability in clinical manifestations of *H. pylori* infection is associated to

several factors, including: bacterial virulence factors, environmental factors and genetic factors of hosts, or a combination of both<sup>(2, 8)</sup>.

Especially important among the bacterial virulence factors are cytotoxin *vacA* and *cagA*, which are associated with bacterial pathogenicity. Studies conducted in several countries have shown that *vacA*-type *s1m1* and *cagA*-positive *H. pylori* strains are associated with severe *H. pylori*-induced peptic ulcer disease<sup>(3, 11, 24)</sup>. Recent publications have also uncovered this association in Brazil<sup>(1, 7)</sup>.

However, bacterial virulence factors alone are not sufficient for determining clinical evolution of the

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infection, given that virulent strains are frequent in both patients with peptic ulcers and those with gastric carcinoma<sup>(18, 32)</sup>. Other factors in the host, especially those that regulate the immunological and inflammatory response, may also contribute to progression towards neoplasia<sup>(8, 9)</sup>.

Genetic polymorphisms, particularly those occurring in the region that promotes the genes that codify inflammatory cytotoxins have been associated to an increase in the synthesis of those interleukins and have emerged as important determinants of susceptibility to cancer<sup>(12, 25)</sup>.

*H. pylori* indirectly stimulates activation of a cascade of cytotoxins responsible for development of the inflammatory process. Infection by *H. pylori*, induces the gastric epithelial cells to secrete interleukins with chemotactic properties for neutrophils and mononuclear cells such as interleukin (IL)-8, IL-6 and IL-1, leading to a proliferative response, with a dense infiltrate of neutrophils and plasmatic cells in the gastric mucosa, leading to active chronic gastritis<sup>(17)</sup>.

Some studies have described the gene that codifies IL-8 as having the polymorphism of an A/T base pair in the promoting region (-251) that is associated with an increase in synthesis of that interleukin by gastric epithelial cells<sup>(29, 33)</sup>. It is thus associated with an elevated risk of developing stomach cancer<sup>(25, 29, 33)</sup>.

In Belém, PA, Brazil, a high rate of prevalence of infection by the bacteria has been described in adult patients with gastric disturbances<sup>(22, 23)</sup>. Furthermore, the Northern Region of Brazil has a high frequency of gastric pathologies such as peptic ulcers and gastric adenocarcinoma<sup>(5)</sup>.

## METHODS

### Patients and control sample

Peripheral blood and gastric fragment samples were collected from 102 patients with gastric carcinoma from the state of Pará, at the Endoscopy Service of the Ophir Loyola University Hospital, during the period of September 2007 to September 2008. During endoscopy, four biopsy fragments were taken from the stomach of each patient. Biopsies of the cancer lesion and the adjacent area (perilesion) were obtained from every patient for histological analysis, and two antrum specimens were also analysed using molecular methods. None of the patients had received antimicrobial drugs, H2-receptor antagonists, acid pump inhibitors, nonsteroidal anti-inflammatory drugs, or any medication for at least 60 days prior to endoscopy.

For the control sample peripheral blood samples were collected from 103 patients lacking clinical or metabolic diseases and asymptomatic for gastrointestinal disturbances, who were thus not submitted to endoscopic exams. All the individuals included (patients and control) were from the same socioeconomic level and had similar cultural habits, and all were natives of Pará state with the same ethnic background, approximately 50% Portuguese, 40% Amerindian, 10% African<sup>(5)</sup>. The study was approved by the Ethics Committee at Núcleo de Medicina Tropical, Belém,

PA (Protocol 036/2007 – CEP/NMT). All patients gave their informed consent to participate in the experiment.

### Detection of infection by *H. pylori*

In the control group, presence of the specific *H. pylori* IgG and *cagA* antibodies was studied in serum samples. To detect specific *H. pylori* IgG antibodies the commercial HIK anti-*H. pylori* EIA kit was used (Monobind, Inc, USA), and the Helicobacter P-120 EIA commercial kit, from VIVA Diagnostica, Hürth, Germany was employed for researching anti-*cagA* antibodies, both being utilized according to the manufacturers' technical descriptions.

### DNA isolation

Total DNA was extracted from frozen gastric biopsy specimens using the following procedure: 10 µl of proteinase K and 300 µl of lysis buffer (200 mM Tris-HCl, 25 mM EDTA, 300 mM NaCl, 1.2 % sodium dodecyl sulfate) were added to the biopsy specimens. The mixture was incubated at 55°C for 12h. The lysate was extracted with an equal volume of phenol-chloroform, precipitated with isopropanol, and washed with 70% ethanol. The pellet was dried and suspended in 200 µl of sterile distilled water. DNA extracts were stored at -20°C.

### PCR amplification and detection of amplified DNA products

One set of primers (p1 and p2)<sup>(16)</sup> that amplifies a gene fragment of 298 bp present in all strains of *H. pylori* was used to detect bacterial DNA. Only positive samples were used for further study.

Amplification of *vacA* signal sequences and middle regions was performed by PCR with oligonucleotide primers described by Atherton et al.<sup>(3)</sup>. The strains were initially characterized as being either type s1 or s2 and as either type m1 or m2. All s1 strain alleles were further characterized as either variant s1a or s1b. The previously described F1 and B1 primers were used to detect *cagA*<sup>(31)</sup>.

All PCR mixtures were prepared in a volume of 25 µl containing 0.5 nM of each primer; 1X PCR buffer; 1.5 nM MgCl<sub>2</sub>; sterilized water, 0.2 nM deoxynucleoside; 1.25u Taq DNA polymerase, and 2 µl DNA sample. The mixtures were placed in a thermal cyclor.

PCR amplification was performed under the following conditions: initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing and extension for 1 min, and final extension at 72°C for 10 min. Annealing temperatures were set at 58°C for primers VA3-F/VA3-R, VA4-F/VA4-R, F1/B1, and at 63°C for SS1-F, SS3-F, SS2-F/VA1-R. Negative and positive controls were used in all reactions. PCR products were visualized by electrophoresis in 2% agarose gel, stained with ethidium bromide, and examined under UV illumination.

### Detection of polymorphism in the IL-8 gene

Polymorphisms of the IL-8 (-251) gene were characterized using the PCR-restriction fragment length polymorphism (PCR-

RFLP) method. The volume for the PCR was 25 µL, containing 0.5 mM of each primer, 1 X PCR buffer, 1.5 mM of MgCl<sub>2</sub>, 0.2 mM of each nitrogenated base, 1.25U of Taq DNA polymerase, 50 ng of DNA and sterile water. The forward and reverse primers for PCR were: 5'-TTCTAACACCTGCCACTCTAG-3' and 5'-CTGAAGCTCCACAATTTGGTG-3', respectively.

The PCR products were digested with *MfeI*, overnight at 37°C and separated by electrophoresis in 2% agarose gel stained with ethidium bromide. The homozygote genotype for allele T (T/T) generated a product of 108pb. The A/A genotype generates a product with two bands (76 and 32pb). The heterozygous genotype (A/T) presented three bands (108, 76 and 32pb). Later on, patients carrying the A/T allele were confirmed through DNA sequencing.

PCR products were submitted to direct sequencing in both directions using reagents from the Big Dye Terminator kit (version 3.1, Applied Biosystems, USA) and analysed in the ABI 3730 automatic sequencer (Applied Biosystems, USA). The sequences obtained were edited using the SEQUENCER programme, and aligned with the BIOEDIT programme.

### Histological evaluation

The biopsy specimens were fixed in 10% buffered formalin solution, embedded in paraffin, cut into sequential 0.4-µm sections, and stained with hematoxylin and eosin. Histopathological parameters were graded from 0-3 using the criteria described in the updated Sydney classification system<sup>(13)</sup> for analysing chronic inflammation, polymorphonuclear activity and intestinal metaplasia.

### Statistical analysis

The Hardy-Weinberg equilibrium of the IL-8 gene allele was assessed using chi-square tests. Haplotype frequencies were estimated using Arlequin software<sup>(27)</sup>.

The chi-square tests and G test were utilised for comparing the variables for sex, age, *cagA* status, *H. pylori* and genotype frequencies between patients and control. The risks of carriers of the different alleles developing adenogastric carcinoma were calculated with the odds ratio. The data were analysed using Bioestat version 5.0 software<sup>(4)</sup>. Differences were considered statistically significant for *P* values less than 0.05.

## RESULTS

### Epidemiological data

Table 1 shows comparisons between the frequency of sexes, age group and infection by *H. pylori* between the two groups studied. The patients presented an average age of 58 years, with ages ranging from 20 to 90 years. As for the control group, it presented an average age equal to 36 years, with ages ranging from 18 to 81 years. Patients with adenogastric carcinoma presented higher ages than those of the control group.

Based on the serological research carried out on the two groups, we observed a greater frequency in the presence of IgG anti-*H. pylori* and anti-*cagA* antibodies in patients with gastric adenocarcinoma than in the control group (Table 1).

TABLE 1. Epidemiological characteristics of the control group and patients

Demographic data	Control n = 103	Stomach cancer n = 102	P
Age (yr)			
<50	72	20	<0.001
>50	31	82	
Sex			
Male	59	65	0.423
Female	44	37	
IgG-anti <i>H. pylori</i>			
Positive	61	101	<0.001
Negative	42	1	
IgG anti <i>cagA</i> (HP+)			
Positive	34	74	0.033
Negative	27	27	

Test: control vs stomach cancer

### Genotyping of *H. pylori vacA* and *cagA* genes

In all patients with gastric adenocarcinoma who were positive on serologic IgG *H. pylori* specific, it was possible to isolate DNA from *H. pylori* in gastric biopsies. In characterizing the *vacA* alleles of bacterial strains we observed that all of the isolated strains presented the *vacA* gene. Of all patients analysed, 97% (98/101) were colonized by only one strain, which contained only one *vacA* genotype (*s1/m1* or *s1/m2* or *s2/m2*) and 3% (3/101) presented infection by more than one strain, with detection of more than one *vacA* genotype (*s1/m1* and *s2/m2*).

Among patients with mono-infection, 88% (86/98) contained the *m1* allele and 12% (12/98) the *m2* allele. With regard to the *vacA* gene signal sequence region we observed that 96% (94/98) presented the *s1* allele, of those 96% (90/94) were of the *s1b* variable and 4% (4/94) of the *s1a* variable. The *s2* allele was observed in 4% (4/98) of the patients.

Three combinations of the different alleles in the signal and median sequence region were identified (*s1/m1*, *s1/m2*, *s2/m2*). In patients with mono-infection 4% (4/98) of the bacterial strains observed had the *s1a/m1* genotype; 84% (82/98) the *s1b/m1* genotype, 8% (8/98) the *s1b/m2* genotype and 4% (4/98) possessed the *s2/m2* genotype.

As for the *cagA* gene, it was detected in 73% (74/101) of patients infected by *H. pylori*. The presence of the *cagA* gene was demonstrated as associated with the presence of the *s1b/m1* genotype of the *vacA* gene (Table 2).

In the three patients who presented infection by multiple strains, we observed the following genotypes: *s1a/m1-s2/m2 cagA+*, *s1b/m1-s2/m2 cagA+* and *s1a/m1-s2/m2 cagA-* (Table 2).

### Polymorphism in the IL-8 gene

Table 3 demonstrates the distribution of the IL-8 -251 genotypes. In both the control group (*P* = 0.811) and patients with adenocarcinoma (*P* = 0.791) the polymorphism studied was in the Hardy-Weinberg equilibrium. Comparing the

TABLE 2. Genotyping of *H. pylori* strains isolated from patients with gastric adenocarcinoma

<i>vacA</i> Genotype	Genotype <i>cagA</i>		Total (%)
	Positive (%)	Negative (%)	
<b>Monoinfection</b>			
<i>s1a-m1</i>	1	3	4
<i>s1b-m1</i>	68	14	82
<i>s1b-m2</i>	3	5	8
<i>s2-m2</i>	-	4	4
<b>Multiple infection</b>			
<i>s1a-m1/ s2-m2</i>	1	1	2
<i>s1b-m1/ s2-m2</i>	1	-	1
<b>Total</b>	<b>74</b>	<b>27</b>	<b>101</b>

Monoinfection: *vacA* x *cagA*  $G_{williams} = 18.9493, P = 0.0020$

TABLE 3. Frequency of the IL-8 (-251) genotype in the control group and in patients with gastric adenocarcinoma

Genotypes	Control	Patients	OR (IC 95%)	P-value
T/T	42	21	-	
A/T	42	56	2.66 (1.37 - 5.15)	0.005
A/A	19	25	2.63 (1.17 - 5.82)	0.026
Carriers of A	61	81	2.65 (1.42 - 4.93)	0.002

frequencies of genotypes for the IL-8 (-251) gene, we observed that the patients with gastric adenocarcinoma differed from those in the control group, presenting an increase in the frequency of AA and AT genotypes (Table 3).

### Effects of the IL-8(-251) polymorphism, *cagA* status and *vacA* gene polymorphism on histological degree of gastritis in noncancerous gastric mucosa adjacent to cancer

By simultaneously comparing the different types of isolated bacterial strains with the IL-8 (-251) and the histopathological data we observed that carriers of the A allele (AT and AA) infected by virulent strains (*mIs1 cagA+*) demonstrated a greater risk of presenting a degree of inflammation (OR = 24.75 IC 95% 2.29-267.20  $P = 0.004$ ) and increased neutrophilic activity (OR = 28.71 IC 95% 2.62-314  $P = 0.002$ ) in the gastric mucosa (Table 4). In that analysis we excluded patients who presented infection with multiple strains (1 AA and 2 TT) and the one patient who did not present infection by *H. pylori* had the IL-8 (-251) A/T genotype.

TABLE 4. Combined effect of IL-8 (-251) polymorphism and bacterial virulence factors (*vacA* e *cagA*) on alteration of the gastric mucosa

IL-8	Strains <i>H. pylori</i>	GI		OR (95% IC)	P	NA		OR (95% IC)	P
		1	2 and 3			1	2 and 3		
TT	NV	3	1	-	-	3	1	-	-
TT	V	6	9	4.5 (0.5-3.04)	0.49	5	10	6.03 (0.26-45.02)	0.73
Carrier A	NV	1	4	12.07 (0.69-3.63)	0.33	1	4	12.07 (0.3-1.4)	0.57
Carrier A	V	8	66	24.75 (2.29-267.20)	0.01	7	67	28.71 (2.62-314)	0.002

GI = Degree of inflammation (1 = low; 2 and 3 = moderate to high), NA = Neutrophilic activity (1 = low; 2 and 3 = moderate to high)

NV = non-virulent strain (*s2m2 cagA* negative e *s1m2 cagA* negative)

V = virulent strains (*s1m1 cagA+*, *s1m2 cagA+*, *s1m1 cagA* negative)

## DISCUSSION

This study was developed in order to increase knowledge regarding factors such as the type of infecting bacterial strain and polymorphism in the IL-8 (-251) gene in gastric carcinogenesis in Pará state.

In comparing the group of patients with gastric adenocarcinoma and the control group we observed that the patients presented greater age on average. Several studies have also described an average age of more than 52 years for development of stomach cancer, considering that it is a multifactor process resulting from exposure to endogenous and exogenous factors. Furthermore, an infection by *H. pylori* determines the occurrence of a chronic inflammation of the gastric mucosa that can persist for decades, in a multiple-stage process that is usually sequential and confers an increased risk for developing stomach cancer over a period of many years<sup>(17, 28)</sup>.

In this study the *vacA* alleles and the presence of the *cagA* gene were characterized in patients with stomach cancer. It was observed that the predominant genotype in the isolated *H. pylori* strains was *s1b/m1/cagA* positive. Other studies carried out in Brazil have also observed that association<sup>(7, 22, 30)</sup>.

Colonization of the gastric mucosa by *s1m1cagA* positive strains of *H. pylori* is associated with a more intense inflammatory response and greater levels of damage to DNA in epithelial cells. Inadequate repair of DNA lesions in cells may lead to mutations and genomic instability, constituting the initial stage of the carcinogenesis process<sup>(26, 30)</sup>.

Besides the presence of the virulent strain, factors in the host, such as polymorphism in the IL-8 gene have also been studied as a possible factor that influences development of stomach cancer<sup>(25)</sup>. IL-8 is a potent chemotactic factor and an activator of polymorphonuclear leukocytes and macrophages, contributing to an inflammatory response<sup>(15)</sup>. The neutrophils induced by IL-8 synthesis activate radicals such as nitrous oxide that present a mutagenic potential, and thus may cause mutations in the gastric epithelial cells<sup>(15, 17)</sup>.

Some studies have demonstrated that patients infected by bacterial strains that possess a *cag* pathogenicity island present high levels of IL-8 in the stomach tissue, and thus a more intense local inflammatory response<sup>(6, 19)</sup>.

Furthermore, polymorphism in the IL-8 (-251) has been associated to the increased expression of IL-8<sup>(21)</sup>. Several papers have demonstrated an association between IL-8

(-251) polymorphism and an increased risk of developing gastroduodenal diseases<sup>(21, 25, 30)</sup>.

In our study, we observed a greater frequency in the AT and AA allele genotypes among patients with adenocarcinoma than in the control group, with the presence of allele A being associated with the risk of developing gastric adenocarcinoma. Regarding the histopathological data, we observed that patients with genotypes AA and AT presented higher levels of inflammation and neutrophilic activity than carriers of genotype TT.

The presence of the A allele in the -251 position of the IL-8 gene was associated with an increase in the risk of stomach

cancer in Japanese, Korean, Chinese and Iranian populations<sup>(21, 25, 29, 33)</sup>. Additionally, some studies have demonstrated that patients carrying the A allele present higher production of IL-8, leading to alteration in the quality and intensity of inflammatory responses produced by the host after exposure to *H. pylori*<sup>(29, 33)</sup>.

Our data indicate that the interaction between polymorphism in the IL-8 (-251) gene, particularly with carriers of the A allele and the infecting type of *H. pylori* strain (*s1m1 cagA* positive) performs an important function in development of gastric adenocarcinoma.

Vinagre RMD, Corvelo TCO, Arnaud VC, Leite ACK, Barile KAS, Martins LC. Determinação das cepas do *Helicobacter pylori* e do polimorfismo do gene da interleucina-8 em pacientes com câncer gástrico. Arq Gastroenterol. 2011;48(1):46-51.

**RESUMO – Contexto** - A neoplasia gástrica é a segunda causa mais comum de morte por câncer no mundo e o *H. pylori* é classificado como carcinógeno humano tipo I pela Organização Mundial de Saúde. Entretanto, apesar da elevada prevalência da infecção pelo *H. pylori* em todo mundo, menos de 3% de indivíduos portadores dessa bactéria desenvolvem neoplasias gástricas. Tal fato indica que a evolução para malignização possa estar associada a fatores bacterianos, do hospedeiro e do ambiente. **Objetivos** - Investigou-se a associação do polimorfismo da região promotora do gene IL-8 (-251) e do genótipo do *H. pylori*, baseado nos alelos *vacA* e na presença do gene *cagA*, com a clínica e os dados histopatológicos. **Métodos** - Em estudo prospectivo, 102 pacientes com câncer gástrico e 103 voluntários saudáveis foram analisados. O polimorfismo da IL-8 (-251) foi determinado pela reação de PCR-RFLP e sequenciamento. Para genotipagem dos alelos *vacA* e do gene *cagA* das cepas bacterianas foi utilizada a PCR. As biopsias gástricas foram avaliadas histologicamente. **Resultados** - A sorologia para o *H. pylori* foi positiva em 101 (99%) de todos os pacientes analisados, e 98 (97%) deles foram colonizados por apenas uma cepa bacteriana. Em pacientes com monoinfecção, 82 (84%) das cepas bacterianas observadas apresentavam o genótipo *s1bml1*. O gene *cagA* foi detectado em 74 (73%) dos pacientes infectados pelo *H. pylori*. A presença do gene *cagA* demonstrou estar associada com a presença do genótipo *s1bml1* do gene *vacA* ( $P = 0,002$ ). Quanto ao polimorfismo do gene da IL-8 (-251), observou-se que os genótipos AA ( $P = 0,026$ ) e AT ( $P = 0,005$ ) foram mais frequentes no grupo de pacientes com adenocarcinoma gástrico. Comparando os diferentes tipos de cepas bacterianas isoladas, com o polimorfismo do gene da IL-8-251 e dados histopatológicos, observou-se que, portadores do alelo A (AT e AA) infectados por cepas virulentas (*m1s1 cagA+*), demonstraram risco aumentado de apresentar maior grau de inflamação (OR = 24,75 IC 95% 2,29-267,20  $P = 0,004$ ) e aumento da atividade neutrofílica (OR = 28,71 IC 95% 2,62-314  $P = 0,002$ ) na mucosa gástrica. **Conclusão** - Os resultados demonstram que a interação entre o polimorfismo do gene da IL-8, particularmente em portadores do alelo A, e o tipo de cepa infectante do *H. pylori* (*s1m1 cagA* positiva) desempenha importante função no desenvolvimento do câncer gástrico.

**DESCRIPTORIOS** - *Helicobacter pylori*. Neoplasias gástricas. Polimorfismo, genético. Interleucina-8.

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