Genetic polymorphisms in the cag pathogenicity island of Helicobacter pylori and risk of stomach cancer and high-grade premalignant gastric lesions

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Abstract

Helicobacter pylori (Hp) infects the stomach of about half of the human population and is strongly associated with the risk of gastric cancer (GC) and its premalignant precursors. The cag pathogenicity island (cagPAI) is a region of the Hp genome encoding for key molecular machinery involved in the infection process. Following a sequencing study, we selected 50 genetic polymorphisms located in seven cagPAI genes and tested their associations with the risk of advanced gastric premalignant lesions and GC in 1220 subjects from various Latin American populations showing the whole spectrum of phenotypes from gastritis to GC. We found that three polymorphisms of cagA are associated with the risk of advanced gastric premalignant lesions (incomplete intestinal metaplasia [i.e., Type 2 and 3] or dysplasia), and that six polymorphisms located in cagA, cagL and cagI were associated with risk of GC. When corrected for multiple testing none of the associations were statistically significant.

Abbreviations: ASR, age-standardized incidence rate; AUC, area under the curve; cagPAI, cag pathogenicity island; CG, chronic gastritis; CI, confidence interval; CLR, conditional logistic regression; GC, gastric cancer; Hp, Helicobacter pylori; IL-8, interleukin 8; IM, intestinal metaplasia; MAF, minor allele frequency; OR, odds ratio; RSCU, relative synonymous codon usage; SIFT, Sorting Intolerant From Tolerant; T4SS, type IV secretion system.

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However, scores built by integrating the individual polymorphisms were significantly associated with the risk of advanced gastric premalignant lesions and GC. These results have the potential of establishing markers for risk stratification in the general population, in view of targeting Hp eradication to high-risk population groups.

**KEYWORDS**

gastric cancer, genetic polymorphisms, *Helicobacter pylori*, pathogenicity island, premalignant gastric lesions

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**1 | INTRODUCTION**

Gastric cancer (GC) is an important public health problem worldwide, as it affects over 1 million people annually, with nearly 800,000 cases dying of the disease in 2018 (https://gco.iarc.fr/today/). Its incidence varies widely among countries, with East Asia and Latin America as high incidence areas (age-standardized incidence rates [ASR] of 22.4 and 8.7, respectively), whereas incidence has been declining steadily in Europe and North America, with current ASR of 8.1 and 6.9, respectively.1

The natural history of GC is well known. Acute stomach inflammation can become chronic and lead to atrophic gastritis, which can in turn progress to intestinal metaplasia (IM) and dysplasia, before eventually becoming cancer.2

The most important established risk factor for GC is an infection by *Helicobacter pylori* (Hp). Hp is considered a Class 1 carcinogen by the International Agency for Research on Cancer.3 The association of Hp with GC risk is strong, with odds ratio (OR) in the order of six for noncardia GC.4 Hp infection has also been reported to be associated with increased risk of premalignant gastric lesions, as well as gastric ulcer.5 Hp infection is one of the most prevalent infections in the human population. It is estimated that about half of the world population carries Hp,6 with infection rates in adults ranging from values in the order of 25% in North America to over 60% in Latin America and the Caribbean.7,8 However, most infected people have only mild (usually in the form of gastritis) or no symptoms at all, and only a small fraction of infected individuals will develop serious sequelae such as advanced premalignant gastric lesions, GC or gastric ulcer.2,5

Not all Hp strains carry the same risk of such advanced endpoints. Hp has a very high rate of genetic variability.9 In particular, the cagA pathogenicity island (cagPAI) is a key virulence factor and is crucial in the pathogenesis of Hp-associated diseases.10 The cagPAI stretches over 40 kb in the Hp genome and encodes for 31 genes that form a type IV secretion system (T4SS) similar to molecular machinery used by other bacteria and consisting of a molecular syringe that injects CagA and other bacterial molecules into cells of the host gastric mucosa.11 This in turn triggers a cascade of inflammatory events involving production of interleukin 8, inflammation and morphological changes ultimately leading to advanced premalignant gastric lesions and GC.12 We hypothesized that genetic variability in cagPAI genes at the level of polymorphisms in individual bases might also show association with risk for advanced premalignant gastric lesions and GC. To this end, we performed a first study on a small number of samples from Mexico and Venezuela.9 Although we identified some polymorphisms associated with GC risk, the study was limited by the small sample size and the lack of samples of subjects with intermediate-risk like IM. We, therefore, launched a new study doing whole-genome sequencing for a total of 75 samples consisting of 37 subjects with chronic gastritis (CG), 21 with IM and 16 with GC cases from Mexico, Colombia and a reference strain. We identified nonsynonymous cagPAI variants that were associated with the risk of IM/GC combined or either lesion.13 The preliminary results of the sequencing study, however, need replication and validation with a much larger sample size, which we accomplished with the present work.

**2 | MATERIALS AND METHODS**

**2.1 | Study population**

We used samples from multiple studies to represent a wide range of Latin American populations from high- and low-risk of GC, and of the whole spectrum of lesions from gastritis to GC. Details concerning eligibility, recruitment, data and sample collection, endoscopic examination and pathological diagnoses of each study have been published elsewhere.5,9,14-20 Subjects were at least 30 years old, and recruited
from four countries in Latin America, with contrasting risk for GC. Venezuela (Tachira province) with high-risk, Colombia with five cities representing both high- and low-risk areas, and two countries with lower risk, Mexico (two hospitals in Mexico City) and Paraguay (two hospitals in Asunción City) in a varied period from the early 1990s to early 2000s; however, in each site recruitment time spanned no more than 3 years and all disease groups were collected within the same time period. All study subjects signed an informed consent and ethical clearance was obtained from the committee of each recruitment as well as the coordinating center.

Eligible subjects were those who were confirmed positive for \textit{cagA} gene in biopsy samples. From the parent studies, we were able to locate 2114 eligible samples, of sufficient quality from either biopsies or DNA extracted from biopsies (1397 from Venezuela, 325 from Colombia, 262 from Mexico and 130 from Paraguay).

### 2.2 | Sample preparation

The DNA was extracted from frozen tissues of biopsy samples using QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

In cases where biopsy DNA was not available and \textit{Hp} culture was positive (n = 75 from Mexico and n = 2 from Colombia), DNA of cultured strains was purified using the guanidine thiocyanate-EDTA-Sarkosyl method\textsuperscript{21} in Mexico, and with a Pure-Link Genomic DNA Mini Kit (Life Technologies, Carlsbad, California) according to the manufacturer’s instructions in Colombia. A whole-genome amplification was carried out on samples with low-levels of DNA (37% of the total), using the Genomiphi V2 DNA Amplification Kit (GE Healthcare Life Sciences, Marlborough, Massachusetts) according to manufacturer’s instructions.

### 2.3 | Polymorphism selection

We aimed to study selected genes of the pathogenicity island cagPAI, namely \textit{cagA}, \textit{cagC}, \textit{cagE}, \textit{cagI}, \textit{cagL}, \textit{cagX}, \textit{cagYc} and \textit{cagY}. They were chosen because their functions have been well characterized in the T4SS, including \textit{CagA},\textsuperscript{22} \textit{CagE}\textsuperscript{23} and \textit{CagY},\textsuperscript{24} or because they are present extracellularly (ie, as pilus or bacterial surface-related proteins), suggesting possible interactions with host cells, like \textit{CagC},\textsuperscript{23}\textsuperscript{25} \textit{CagI},\textsuperscript{26} \textit{CagL},\textsuperscript{27} \textit{CagX}\textsuperscript{28} and \textit{CagY}.\textsuperscript{29} Variants on these genes were identified by a whole-genome sequencing study performed on 74 \textit{Hp} strains isolated from patients from Mexico and Colombia, 37 CG, 21 with IM and 16 with GC.\textsuperscript{13} We selected candidate polymorphisms from SNPs with at least 7.5% frequency in the 74 sequenced strains (ie, observed in at least five strains), then applied the following criteria: for non-synonymous variants, OR of \( \geq 1.5 \) or \( \leq 0.67 \) for GC + IM compared to CG, and OR \( \geq 3.0 \) or OR \( \leq 0.33 \) for synonymous variants. In addition to these candidate variants, all variants present at the same codon, regardless of their overall frequencies, were included for assay development to ascertain the frequency of candidate variants at a given codon.

To test for samples that are \textit{Hp} positive but cagPAI negative, we added two polymorphisms in housekeeping genes mapping outside of the cagPAI (\textit{atpA}\text{185} and \textit{ureI}\text{60}; ie, samples that would give no amplification for markers within the cagPAI but show signals for the two markers in housekeeping genes).

### 2.4 | Genotyping

We submitted a total of 122 assays for development at Kbiosciences-LGC (Hoddesdon, UK). Triallelic or multi-allelic polymorphisms (N = 20) were designed as multiple biallelic assays. For example, two assays were designed for each triallelic polymorphism, each distinguishing the major allele and one of the minor alleles. Complex polymorphisms spanning two or more bases (ie, with two or more polymorphic sites within the same codon resulting in amino acid changes) were broken down into multiple biallelic assays.

Given the very high genetic variability of \textit{Hp}, not all selected polymorphisms were suitable for the development of genotyping assays. A total of 29 assays failed either at the level of bioinformatic testing, or at validation based on genotyping of up to 96 samples of \textit{Hp} from cultured strains. Eventually, we obtained 93 validated genotyping assays.

Genotyping was performed at the German Cancer Research Center (DKFZ) in Heidelberg, Germany, in 384-well plates, using KASP technology as recommended by the manufacturer. In addition to the samples, negative controls and duplicated samples (5%) used for quality control purposes were put on each plate and genotyped under the same conditions as the other samples. The personnel performing the genotyping was blind to the diagnosis status. PCR plates were read on a Viia 7 Real-Time PCR System and genotypes were called with the QuantStudio software (ThermoFisher Applied Biosystems, Waltham, Massachusetts).

### 2.5 | Quality control and data preprocess

We verified genotype concordance between duplicated samples. This led to the exclusion of 18 samples that had more than two discordant calls. The concordance rate of the duplicates in the remaining samples was 99.25%. We further removed 24 unintended duplicate samples. In addition, samples with a call rate of less than 50% were discarded for subsequent analysis. Finally, we also removed 46 subjects with duodenal ulcer, leaving a total number of 1220 samples for analysis. We checked that allelic frequencies of all polymorphisms were in general agreement with those observed in the sequencing analysis reported in our previous work.\textsuperscript{13}

We removed the following assays from the analysis: two polymorphisms with very low minor allele frequency, eight with missing data for more than 33% of the samples; and 52 assays that were individual components of multi-allelic assays, representing 18 polymorphisms, and were replaced by consensus genotypes for a given codon, as well as three assays from multi-allelic polymorphisms, for which the
assay for the other allele could not be developed. The data preprocess step left 52 polymorphisms available for multiple imputations.

2.6 | Statistical and bioinformatic analysis

2.6.1 | Multiple imputation

Multiple imputation imputes incomplete multivariate data by chained equations using fully conditional specification implemented by the MICE R package as described by Van Buuren. Briefly, each variable has its own imputation model. Built-in imputation models are provided based on types of data such as continuous, unordered and ordered categorical data. Each missing value was imputed multiple times. A total number of 30 imputed datasets were created, each with five iterations.

2.6.2 | Univariate association analysis

The outcome of interests was cancer vs noncancer or high-grade vs low-grade premalignant lesions. One by one screening of each marker was performed with conditional logistic regression (CLR) stratified on country and adjusted for baseline covariates, such as age, sex, education status, smoking status, length of refrigerator use and grain intake levels, separately for the two endpoints. R package survival was used on each imputed data set for the CLR model and summarized with R package MICE.

2.6.3 | Multivariable association analysis

Markers that were significant (P < .05) from the adjusted univariate CLR model were fit simultaneously into multivariable CLR model. Sensitivity of the multivariable model was also evaluated with additional CLR model were fit simultaneously into multivariable CLR model. Sensitivity was scaled to a proportion by dividing it with the total number of genetic markers in the multiple CLR model. This proportion was then directly used as the predicted risk probability, together with the true label of the subject’s outcome, to produce the AUC. An AUC value of larger than 0.5 reflects the performance of the score method as a classifier.

The functional effect of nonsynonymous polymorphisms was evaluated with the use of the Sorting Intolerant From Tolerant (SIFT) algorithm (https://sift.bii.a-star.edu.sg). The impact of polymorphisms on codon usage bias was evaluated according to Lafay et al.

3 | RESULTS

The final dataset used for statistical analysis consisted of 1220 samples (Table 1), and included 73.2% of subjects with low-grade premalignant lesions, 14.5% of subjects with high-grade premalignant lesions (consisting of IM Grade 2 and 3, and dysplasia) and 12.3% of subjects with GC.

Fifty polymorphisms located in caga (29 polymorphisms), cagC (2), cagE (5), cagI (4), cagL (8), and cagX (2) were retained in the final statistical analysis. We performed two sets of analyses. In the first, we compared subjects who had low-grade premalignant lesions (n = 893) with subjects who had high-grade premalignant lesions (n = 177). Four polymorphisms (cagA1283, cagA2551, cagA3490_3491 and cagX31_32) showed associations with increased risk of high-grade lesions. After multivariable association analysis, only cagA1283, cagA2551 and cagA3490_3491 remained significant. Next, we combined the three polymorphisms into a score, whereby the number of risk-increasing alleles at any of the three polymorphisms is added up for each study subject; the score can thus have any value between 0 and 3 (Figure S1). The score was also associated with the risk of high-grade lesions, with an OR = 1.99 for each additional risk allele, and a strong statistical significance (P = 2.56 × 10^-6). The AUC of the high-risk lesion score was 0.64 (95% CI 0.60-0.69). Relaxing the threshold for inclusion in the score to P < .1 or P < .2 resulted in larger numbers of polymorphisms in the scores, but not to significantly better AUC (data not shown). Results of the polymorphisms showing significant associations at P < .05 and included in the score are shown in Table 2, whereas results for all polymorphisms are reported in Table S1.

In the second analysis, we compared subjects who did not have GC (n = 1070) with subjects with GC (n = 150). Three polymorphisms (cagA2419, cagA3435 and cagL400) were associated with increased risk of GC and three (cagA1576, cagL1007 and cagL184) were associated with decreased risk. Some pair-wise combinations of the six polymorphisms showed weak but significant correlation (cagL184 and cagL400: r² = .28, P = 2.20 × 10^-35, cagL1007 and cagL184: r² = .19, P = 1.82 × 10^-19, cagL1007 and cagL400: r² = .09, P = 2.28 × 10^-8).

Like for the previous analysis we built a score using the risk-increasing alleles of all six polymorphisms (ie, we considered the reference allele instead of the variant allele for cagA1576, cagL1007 and cagL184). The GC score has values between 0 and 6 (Figure S2). Each additional risk allele in the score gives an OR = 2.42, and this result is strongly significant (P = 5.41 × 10^-8). The AUC of the cancer score was 0.65 (95% CI 0.61-0.70). In this case too, using different
thresholds for inclusion in the score (P < .1 or P < .2) did not lead to significantly higher AUC (data not shown). Results of the polymorphisms showing significant associations at P < .05 and of the score are shown in Table 3 and the results of all polymorphisms are reported in Table S1.

There were no significant interactions between the six polymorphisms associated with GC risk (15 pair-wise interaction tests, one at a time) or the three polymorphisms associated with risk of high-grade lesions (three pair-wise interaction tests, one at a time; data not shown).

The two polymorphisms in housekeeping genes (atpA185 and ureI60) did not show any association with the risk of either high-grade lesions or GC (data not shown).

GC patients were also subdivided according to the histological classification of their tumors (intestinal vs diffuse). We then performed an exploratory case-case analysis for the six polymorphisms associated with GC risk to see if they could be associated with the risk of one specific histology. None of the polymorphisms showed significantly different frequencies between two histology types (intestinal vs diffuse), with P-values ranging from .33 to .87 (Table S3).

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**TABLE 1** Study population

| Diagnosis                        | Mexico | Paraguay | Colombia | Venezuela | Total |
|----------------------------------|--------|----------|----------|-----------|-------|
| Chronic gastritis                | 84     | 35       | 50       | 271       | 440   |
| Atrophic gastritis/IM1           | 22     | 18       | 102      | 311       | 453   |
| Total low-grade                  | 106    | 53       | 152      | 582       | 893   |
| IM2 + 3/dysplasia                | 14     | 12       | 40       | 111       | 177   |
| Total noncancer                  | 120    | 65       | 192      | 693       | 1070  |
| Cancer                           | 41     | 16       | 15       | 78        | 150   |
| Total                            | 161    | 81       | 207      | 771       | 1220  |
| Male                             | 39.1%  | 55.6%    | 50.2%    | 53.4%     | 51.5% |
| Median age (25%-75%)             | 51 (41-64) | 51 (40-62) | 52 (42-61) | 47 (40-55) | 48 (40-58) |

**TABLE 2** Associations between polymorphisms in Hp cagPAI genes and risk of high-grade premalignant gastric lesions (P < .05)

| Polymorphism | Major allele DNA | Minor allele DNA | Codon number | Major allele amino acid | Minor allele amino acid | Low-grade Frequencya | High-grade frequencya | ORb | 95% CI | P   |
|--------------|------------------|------------------|--------------|-------------------------|-------------------------|----------------------|----------------------|-----|-------|-----|
| cagA1283     | A                | C                | 428          | Asn                     | Thr                     | 29.9%                | 41.5%                | 1.69 | 1.08-2.64 | .021 |
| cagA2551     | G                | A                | 851          | Ala                     | Lys                     | 10.1%                | 19.9%                | 2.29 | 1.41-3.71 | .001 |
| cagA3490_3491| wild mut         | 1162             | Cys          | Ser                     | 11.7%                   | 23.8%                | 2.16                 | 1.32-3.54 | .002 |
| Scorec       |                  |                  |              |                         |                         |                      |                      | 1.99 | 1.50-2.63 | 2.56 × 10⁻⁶ |

Note: cagX31_32 showed association with risk of high-grade premalignant gastric lesions (P < .05) as well (Table S2), but the association was not significant after multivariable analysis, therefore it was not included in the score.

Abbreviation: cagPAI, cag pathogenicity island; HP, Helicobacter pylori; OR, odd ratio.

**Note:** Minor allele frequencies in subjects with low-grade and high-grade premalignant lesions, respectively.

**Note:** Odds ratios, stratified by country and adjusted for age, sex, education status, smoking status, length of refrigerator use and grain intake levels.

**Note:** Score composed of the three above polymorphisms. Minor alleles of each marker contributed to the score equally. The score ranges from 0 to 3. The OR is referred to the risk of each additional risk allele.
We reported in Figure S3 the positions within the respective genes of the polymorphisms showing associations with the risk of either high-grade premalignant lesions or GC.

All results showed here were generated with data after multiple imputation. Analyses performed using raw data before multiple imputation gave essentially the same results (data not shown).

Study subjects can be subdivided according to their geographic area of origin between regions at low risk of GC, including Mexico, Paraguay and coastal areas of Colombia and regions at high-risk, including Venezuela and mountain areas of Colombia. We compared frequencies of polymorphisms of subjects with low-grade premalignant lesions between low risk (n = 683 subjects) and high-risk regions (n = 210). None of the three polymorphisms associated with the risk of high-grade premalignant lesions or the six polymorphisms associated with the risk of GC showed significant differences between the regions (data not shown).

None of the associations we reported for the individual polymorphisms are significant if multiple testing is taken into account (with a Bonferroni-corrected threshold of 0.05/53 polymorphisms [50 polymorphisms, three of which are triallelic] × 2 sets of analyses [low-grade vs high-grade and noncancer vs cancer]) = 0.00047). Evaluation of the results with a false discovery rate also shows that none of the individual associations are significant (data not shown).

None of the nonsynonymous variants associated with risk of either high-grade lesions or GC was predicted by SIFT to have a relevant functional effect, with the exception of cagL184, where the polymorphism we found to be associated with risk of GC causes an amino acid substitution, with the major allele coding for glutamine and the minor allele coding for lysine. This replacement is predicted to be not tolerated and possibly resulting in disruption of the protein function.

We also analyzed the relative codon usage according to Lafay et al. Both alleles of polymorphism cagA3435, which is associated with the risk of GC, code for aspartic acid. However, the major allele has a relative synonymous codon usage (RSCU) of 0.54, while the minor allele has a value of 1.46. The RSCU is the observed frequency of a codon, divided by the frequency expected if all possible codons for that amino acid were used equally. Thus, RSCU = 1 indicates a lack of bias, RSCU < 1 shows that a codon is underrepresented and RSCU > 1 that a codon is overrepresented with respect to the expected.

| Polymorphism | Major allele DNA | Minor allele DNA | Codon number | Major allele amino acid | Minor allele amino acid | Noncancer frequency | Cancer frequency | ORb | 95% CI | P |
|--------------|-----------------|-----------------|--------------|-------------------------|-------------------------|--------------------|-----------------|-----|--------|---|
| cagA1576     | G               | A               | 526          | Ala                     | Thr                     | 8.1%               | 2.2%            | 0.22 | 0.06-0.78 | .020 |
| cagA2419     | A               | G               | 807          | Arg                     | Thr                     | 13.3%              | 18.8%           | 1.83 | 1.07-3.13 | .028 |
| cagA3435     | C               | T               | 1143         | Asp                     | Asp                     | 6.0%               | 10.7%           | 2.44 | 1.16-5.16 | .019 |
| cagI1007     | C               | T               | 336          | Ala                     | Val                     | 29.4%              | 24.2%           | 0.59 | 0.36-0.97 | .039 |
| cagL184^c    | G               | C               | 62           | Glu                     | Lys                     | 11.2%              | 6.1%            | 0.46 | 0.22-1.00 | .049 |
| cagL400      | A               | G               | 134          | Ile                     | Val                     | 21.2%              | 30.8%           | 1.95 | 1.08-3.54 | .28 |
| Score^d      |                |                 |              |                         |                         | 2.42               | 1.77-3.30       | 5.41 × 10^-8 |

Abbreviation: cagPAI, cag pathogenicity island.

aMinor allele frequencies in subjects with and without gastric cancer, respectively.
bOdds ratios, stratified by country and adjusted for age, sex, education status, smoking status, length of refrigerator use and grain intake levels.
ccagL184 has a third allele, which is not significantly associated with risk of gastric cancer.
dScore composed of the six above polymorphisms. Risk alleles of each marker contributed to the score equally. The score ranges from 0 to 6. The OR is referred to the risk of each additional risk allele.

4 | DISCUSSION

We studied the possible associations between polymorphisms in key genes of the Hp cagPAI and risk of high-grade premalignant gastric lesions and of GC in several Latin American populations. Our study is the first to examine the associations of HP cagPAI sequence variants with gastric pathology in unselected gastric biopsy specimens, using high throughput genotyping assays. To date, Hp genotyping on biopsy specimens has been almost limited to CagA EPIYA motif patterns. There are more recent studies based on sequencing technology, reporting associations between specific Hp genetic variants and clinical phenotypes. However, these sequencing studies have been performed on a limited number of samples and with Hp strains isolated from the patients. It is important to note that Hp culture from clinical specimens is not always successful. Even if Hp is detectable by PCR or by histology, culture often fails, indicating potential serious bias in the studies based only on cultured strains. In addition, such strain-based studies often lack covariate information relevant to GC, which is very relevant considering that GC is a multifactorial disease.

In our previous study with Hp from patients with CG, IM or GC from Mexico and Colombia, a large number of genetic polymorphisms were found in the cagPAI genes studied. In the present work, we decided to analyze polymorphisms with increased possibility of being functionally relevant and study their possible associations with risk for high-grade premalignant gastric lesions and for GC. We started from a large pool of candidate polymorphisms, but reduced it...
to 50 polymorphisms, representing a fraction of our initial list. Development of genotyping assays was one of the major difficulties we faced in this project. The very high-genetic diversity of Hp, particularly in the cagPAI, makes it prohibitive to develop genotyping assays for many polymorphisms. As a result, we lost many candidate SNP located in hypervariable regions, leaving more SNP located in rather conserved regions, which may in fact be relevant for gene function.

To ensure a sufficient level of quality we discarded samples with a call rate lower than 50%, resulting in a loss of over 40% of the initial samples. Consequently, to cope with the high rate of missing data in the remaining dataset we chose to apply multiple imputations. We compared the results obtained from data before and after multiple imputation and we did not observe notable differences, which suggests that multiple imputation performed reliably and that our final results are robust.

We found that three polymorphisms in cagA were associated with risk of high-grade premalignant gastric lesions and six polymorphisms in cagA, cagI and cagL were independently associated with risk of GC.

Once cagA is internalized into gastric epithelial cells it interacts with a myriad of intracellular targets including kinases or proteins of the cytoskeleton. Two of the positions we found associated with the disease are located in Domain II (cagA1283 associated with high-grade preneoplasia and cagA1576 with GC) which is a region important for the interaction of cagA with the inner left of the cytoplasmic membrane. Another position is located in Domain III (cagA2419, associated with GC risk) that may affect the intramolecular N-terminal/C-terminal interaction important for the recruitment of PAR1,88 which in turn may lead to the activation of the Ras-ERK MAPK pathway.

Until recently, the main focus of CagL variants has been hypervariable-amino acid residues 58 to 62,29 located upstream of the RGD motif that is crucial for host integrin binding,40 and the cagL184 polymorphism found in our study resides at this region (residue 62). In accordance with this, a meta-analysis of worldwide strains39 found an overall positive association between polymorphisms in the 58 to 62 region and GC. Yeh et al showed that polymorphisms at residues 58 and 59 induce a corpus shift of gastric integrin $\alpha_5\beta_1$,33 although an in vitro study in AGS cells that tested various combinations of amino acids at residues 58 and 59 did not find functional differences.41 We genotyped cagL172 (codon 58) and did not find any significant association. However, our analysis with SIFT predicts that the amino acid substitution caused by cagL184 at codon 62 is not tolerated and likely to disrupt the function of the protein. As noted by Tafreshi et al,42 it is possible that variation at residues 58 and 59 might work in concert with variation at residues 60 to 62 in influencing the structural integrity of CagL and therefore its function.43 More recent sequencing studies have identified a number of variants in this gene. The other cagl variant associated with GC in our study, on residue 134, has been reported with variable results in other studies in Latin America,34,36,37 although this variant has not been detected in East Asian strains.33,35 Thus, although the data from these Western strains are inconclusive in terms of its association with GC, this cagl variant is likely to represent a Western strain-specific marker. Furthermore, residue 134 is located within a structurally important region of cagl containing a disulfide bond that bridges helix 5 to the C-terminus of the short $\alpha_4$ helix.52,43

One study that examined sequence variants of cagl, in addition to those of cagl, of East Asian strains, did not detect the cagl1007 variant at residue 336 that showed the association with GC in our study.35

When we took into account multiple testing, none of the associations of the individual polymorphisms remained significant; however, scores generated by the combinations of the individually associated polymorphisms were associated with the risk of high-grade premalignant gastric lesions and of GC, respectively, with strong statistical significances. Taken together, these results suggest that individual variants, if confirmed in further studies, could be useful to gain insight about molecular mechanisms of gastric carcinogenesis, but not likely to be of much use for risk stratification in the population. On the other hand, scores could be useful for risk stratification in the general population, in view of targeting Hp eradication in population groups at particularly high-risk of developing high-grade premalignant lesions or GC. The AUC we obtained for both scores were promising, although clearly not at the level where it can be envisaged to use these scores as predictive tools in the general population. However, it is likely that additional studies of genetic variants located in other Hp genes will uncover further risk-associated polymorphisms. Future versions of the scores generated with the larger number of risk variants will have better predictive power.

Exploratory analyses of our previous discovery work13 found 19 polymorphisms showing associations with the risk of IM and/or GC (P < .05). Six of them could be studied here, however, none of them showed significant associations with either IM or GC risk. Major reasons for these differences may include the much smaller sample size of our previous analysis (74 samples in total), the inclusion of samples from additional different countries in the current study and differences between culturable and unculturable strains. Additional minor factors may include the fact that diagnoses were grouped in different ways, with the high-grade premalignant gastric lesions consisting of only IM cases in Rizzato et al, and of IM2 + IM3 + dysplasia here and with or without adjustment of covariates.

The main strengths of our work are the inclusion of unculturable Hp strains and a large sample size, even taking into account the loss of many subjects due to the low quality of the samples. Sample size remains relatively large when considering the different subgroups of subjects, with 177 subjects with high-grade premalignant gastric lesions and 150 GC cases.

Limitations include the fact that we studied only Latin American populations and therefore findings are not necessarily generalizable to other populations, considering the known large differences between Hp strains of different populations.44,45 On the other hand, Latin America is one of the areas with the highest Hp infection rates and with the highest GC mortality rates.1 Hispanic populations also represent large minorities in the USA and other countries. Additionally, this work did not include large structural variations. However, we studied EPIYA and CM motifs in our sequencing study and we did not observe any association with risk of IM and/or GC.53 Moreover, structural
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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA ACCESSIBILITY

The primary data for this work will be made available to researchers who submit a reasonable request to the corresponding author, conditional to approval by all the collaborators. Data will be stripped from all information allowing identification of study participants. The data are not publicly available due to privacy or ethical restrictions.

ETHICS STATEMENT

All study subjects signed an informed consent and ethical clearance was obtained from the committee of each recruitment as well as of the coordinating center.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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