UNIVERSITÀ DEGLI STUDI DI PALERMO
Dottorato di ricerca in Oncologia e Chirurgia Sperimentali
Dipartimento di Discipline Chirurgiche Oncologiche e Stomatologiche (Di.Chir.On.S.)

THE ROLE OF *HELICOBACTER PYLORI* AND EPSTEIN BARR VIRUS IN GASTRIC DISEASE: CORRELATIONS WITH P53 MUTATIONS, DNA METHYLATION AND MICROSATELLITE INSTABILITY

Doctoral Dissertation of:
Dr. Teresa Maria Assunta Fasciana

Tutor:
Prof. Anna Giammanco

The Chair of the Doctoral Program:
Prof. Antonio Russo

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Abstract

ABSTRACT

Gastric diseases can have an infectious etiology, in fact *Helicobacter pylori* (*H. pylori*) is considered the main risk factor and, more recently, also linked to infection by Epstein–Barr virus (EBV).

The outcome of the infection can be correlated on the relationship among environment, host and bacterial virulence factors.

These data showed that different factors should be evaluated in patients with gastric diseases to prevent and reduce the development of gastric cancer and Malt lymphomas.

SUMMARY

Introduction: Genetic predisposition, environmental factors, and infectious agents interact in the development of gastric diseases.

Helicobacter pylori and Epstein-Barr virus (EBV) infection has recently been shown to be associated with these diseases.

The aim of this study was to evaluate the relationship between *H. pylori* and EBV infection and correlations with, p53 mutations, DNA methylation and microsatellite instability and cytokines polymorphism in different gastric diseases in people living in Mediterranean area. The investigation was considered interesting because Sicily, for geographic localization constitute one of the most complex mixtures of different ethnic elements in Europe.

Methods: Gastric biopsy samples of 100 patients with different gastric diseases were used. *H. pylori* and EBV infection was diagnosed by the detection of the *ureaseA* and BAMHI-W respectively. The virulence factors, resistance to clarithromycin of *H. pylori*, polymorphism of IL-1RN, IL-10 and DNA methylation were detected by polymerase chain reaction.

Polymorphisms in the *IL-8*, and in the *IL-1β* 511 were detected by

PCR-restriction fragment length polymorphism (RFLP). Microsatellite instability (MSI) analysis was performed using the BAT26 primer set. Single-stranded conformational polymorphism (SSCP) was used for p53 mutation.

Results: Our result showed that the *H. pylori* and EBV infection is present respectively in 44% and in 37% of the population analyzed while coinfection was found in 26% of patients.

The polymorphism *s1m1i1* of *vacA* gene, was most frequent in Group Malt Lymphoma (p-value = 0.0169) in comparison to others, while the polymorphism *s2m2i2* was most frequent in Group normal gastric mucosa (p-value = 0.0359) and less frequent in Group gastric cancer (p-value = 0.0276). In other words the polymorphism *s1m1i1* was significantly associated to patients with Malt lymphoma, instead the polymorphism *s2m2i2* was significantly associated to patients with inactive chronic gastritis.

In addition, the presence of *cagA* gene was the most frequent in patients with gastric cancer (p-value = 0.0198), instead than the patients with inactive chronic gastritis where the positivity was less frequent (p-value = 0.0130).

A close correlation was found between the status of the *oipA*, *vacA* and *cagA* genes.

The analysis of the *23S rRNA* gene demonstrated that 32 (73%) patients were infected with *H. pylori*-susceptible strains and the remaining 12 (27%) with resistant strains.

Polymorphism of IL-β (-31), CT was more frequent in infected patients in contrast to non-infected patients (43.64% > 37.78%, p=0.039). For polymorphism IL-β (-511), CC was more frequent in non-infected patients than in infected patients (57.78>36.36, p=0.044). We observed that for polymorphism IL-1RN, type 1/2 was less frequent in patients with Malt lymphoma (p=0.0256). Similar results were obtained for polymorphism IL-8 (-251), type TA (p=0.0029).

Instead for polymorphism IL-10 (-1082), type AG and GA, were less and more frequent respectively, in patients with Gastric Cancer (p=0.0256 and p<0.0001 respectively). Finally in patients with normal

gastric mucosa, the polymorphism IL-10 (-819), type CC was less frequent (0.0047), instead type CT was more frequent in comparison to others (p=0.0085).

COX2 gene was more frequently methylated in infected patients in contrast to non-infected patients (30.91%>6.67%, p=0.0057).

Microsatellite stability was more frequent in non-infected patients in contrast to infected patients (95.56%>74.55%, p=0.01), vice versa microsatellite instability was more frequent in infected patients in contrast to non-infected patients (23.45%>4.44%, p=0.01).

There was a significant difference among groups for microsatellite stability (p=0.0121) and microsatellite instability (p= 0.0121) but with a significant level equal to 0.05. Nothing group had a percentages significant greater or less in comparison to others

Finally p53 mutation was observed in 26% of the cases, particularly no mutation was observed in patients with normal gastric mucosa, and our results showed that the exon 8 was mutated more frequent in patients with infection (*H. pylori* only, EBV only or both) in comparison to patients without infection (23.64% > 0.00%, p=0.0014), while exon 6 was more frequently mutated in patients with EBV infection (36.36%, p=0.0011), instead the exon 8 was more frequently mutated in patients with co-infected (42.31%, p<0.0001).

Conclusion: According to our analysis the gastric disease in Sicilian population has different pathways depending on the presence of the various factors included infectious agents such as *H. pylori* and EBV, and genetic factors of subject.

CHAPTER 1

Background

1.1 Introduction

Agreeing to WHO estimates for 2011, cancer now causes more deaths than all coronary heart disease or all stroke [1].

Discovering that microorganisms produce diseases was one of the main milestones for microbiology during the 19th century, and at the end of that century, microbiologists started looking for the origin of many diseases in these organisms, including cancer [2-3-4].

Gastric cancer is the fifth most common cancer and the third leading cause of cancer-related death globally, with an 952 000 new cases estimated and 723 000 deaths annually [5].

At today significant efforts has been focused on clarifying the pathology of gastric cancer [6]. In particular, the development of genome-wide analysis tools has enabled the detection of genetic and epigenetic alterations in gastric cancer; for example, aberrant DNA methylation in gene promoter regions is thought to play a crucial role in gastric carcinogenesis [7-8-9]. The etiological viewpoint is also essential for the study of gastric cancers, and two distinct pathogens, *Helicobacter pylori* (*H. pylori*) and Epstein-Barr virus (EBV), are known to participate in gastric carcinogenesis [10-11].

1.2 Role of *H. pylori* in gastric disease

After two decades of research, the role of *H. pylori* in certain types of gastric diseases is extensively accepted, comprising bacterium eradication as part of its cure [12]. Copious studies have been made to try and establish this

bacterium's specific mechanisms of interaction with humans, [13] virulence factors, [14] and to which secretion system its pathogenicity islands belong [15].

H. pylori is a spiral-shaped bacterium that grows in the mucus layer of the human stomach, causing inflammation know as gastritis [16]. Additional, it turns to ulcers, long-lasting anaemia , and growths in the stomach, which are more likely to get cancer [17-18-19-20]. *H. pylori* is mainly spread through contaminated water, food, saliva, or mouth to mouth contacts and possibly transmitted sexually via oral-genital contact [21]. Nearly, 50% of the global population is estimated to be infected by *H. pylori* [22], in which less than 2% develop gastric cancer [23]. The bacterium is thought to be first acquired during childhood in all nations[24] and mostly in developing countries. Moreover, the infection rate of children in developing countries is higher than that in the advanced countries, 80% compared to 10%, at the age of 20 years [25], while senior citizens in both types of countries have around 50% of infection at 60 years of age.

1.3 Principal *H. pylori* virulence factors

The most commonly virulence factors studied in *H. pylori* are encoded by the cytotoxin associated gene A (*cagA*), the vacuolating associated gene A (*vacA*) and *oipA* (outer inflammatory protein). *H. pylori* CagA is a high immunogenic protein with molecular weight of 120–140-kDa encoded by CagA gene located at the end of pathogenic island (*Cag PAI*)and is associated with cell injury and more severe clinical outcomes, such as gastric adenocarcinoma and duodenal ulcer[26].

Almost 60% of *H. pylori* strains isolated in Western countries are carrier of *cag PAI*, whereas approximately all of the East Asian isolates are *cag PAI*-positive.

H. pylori CagA is translocated into host cells following attachment of the bacteria to the cell via the cag PAI-encoded type IV secretion system[27].

Inside the host cell, CagA can be phosphorylated by either Abl kinase or Src family kinases on tyrosine residue at four distinct glutamate-proline-isoleucine-tyrosine-alanine (EPIYA) motifs present at the C-terminal region

of the protein, leading to morphological changes in the cell. Moreover, CagA can act directly in an unphosphorylated state to affect cellular tight junction, cellular polarity, cell proliferation and differentiation, cell scattering, induction of the inflammatory response and also cellular elongation[28].

Many investigations demonstrated that patients with antibodies against CagA protein showed higher rates of peptic ulcer disease and gastric cancer[29]. In addition, it has been demonstrated in vivo that *cagA* plays an important role in disease progression and development. Clinically, infection with the *cagA*-positive *H. pylori* strains has been associated with higher grades of gastric mucosal inflammation as well as severe atrophic gastritis and has been suggested to play an important role in the development of gastric carcinoma[30].

While there are at least 7 variable regions in the *vacA* gene; in the signal (*s*) region, of which one of two alleles can be present: *s1* or *s2*, in the middle (*m*) region, of which one of two alleles can be present; *m1* or *m2* and in intermediate region (*i1*, *i2*, and *i3*)[31]. These variable regions display different levels of toxicity to host cells, with *vacA s1/i1/m1* being most cytotoxic, followed by *s1/i1-2/m2*. The *s2/i2/m2* genotype has been found to induce little or no toxicity[32].

VacA assists *H. pylori* to colonize the stomach through exerting multiple effects on epithelial cells in the human host. VacA further crosses epithelial cell lineage and cause different effects on cells of immune system. It is suggested that *H. pylori* secretes VacA near the apical membrane of epithelial cells where it binds with the cell surface receptors and is internalized. After internalization, VacA exerts multifunctional effects on cellular organelles[33]. VacA plays a vital role in the survival of *H. pylori* by inducing a flow of ions and nutrients from the mucosa toward the stomach lumen which results into causing the alteration in the gastric epithelium cell integrity[34].

OipA is one of the outer membrane proteins with functions in adhesion.

Its functional status is regulated by slipped-strand mispairing that is based on the number of CT dinucleotide repeats in the 5′ region of the genes (switch ‘on’ as functional and switch ‘off’ as nonfunctional) [35] .

OipA was initially identified as a proinflammatory response-inducing protein based on the fact that *oipA* isogenic mutants reduced the induction of IL-8 from gastric epithelial cell lines [36].

Several studies revealed that OipA can induce inflammation and actin dynamics through the phosphorylation of multiple signaling pathways that usually interact with *cag PAI* (CagA)-related pathways. *H. pylori*-related inflammatory signaling related to gastric carcinogenesis is regulated by the activation of the phosphoinositide-3 kinase (PI3K)/Akt signaling pathway.

OipA-regulated IL-8 release through PI3K/Akt has recently been reported to be dependent on forkhead transcription factors of class O (FoxO) 1/3a inactivation [37].

The mechanisms of *H. pylori* pathogenesis by different virulence factors are showed in figure 1[38].

1.4 Role of EBV in gastric disease

Epstein-Barr virus (EBV) is a gamma DNA herpes virus that infects over 95% of the global population and remains an asymptomatic life-long infection[39]. B lymphocytes and epithelial cells are the major targets of EBV infection. As a common human tumor virus, EBV was shown to be associated with a vast number of human diseases, such as lymphomas, nasopharyngeal carcinoma (NPC), and gastric carcinoma.

The viral gene methylation might be a response of the host cell against foreign DNA; on the other hand, it might benefit EBV by allowing it to escape the immune response of the host[40]. Epigenetic changes caused by DNA methylation play the most striking role in the tumorigenesis.

DNA hypermethylation is the most important hallmark of EBV-associated gastric cancer that distinguishes it from other molecular subtypes of gastric cancer[41]. EBV-positive tumors display a distinctive promoter hypermethylation profile that mirrors their spectrum of mutations and gene expression[42]. Of particular interest, all EBV-positive tumors display CDKN2A but not MLH1 promoter hypermethylation, which represents the microsatellite instability (MSI) subtype of gastric cancer[43]. Strikingly, the prevalence of DNA hypermethylation in EBV-positive gastric cancer is showed in figure 2[44].

1.5 DNA methylation and Microsatellite instability in gastric diseases

Many studies have identified the transcriptional silencing by DNA methylation as a mechanism responsible for tumor suppressor inactivation. Methylation of promoter CpG islands leads to DNA structural changes and, consequentially, gene inactivation[45]. Several cancers, including gastric tumors, show methylation of multiple genes including *CDH1*, *DAPK*, *COX2*, *hMLH1* and *CDKN2A*[46].

Microsatellite instability (MSI) reflects an erroneous form of DNA replication in repetitive microsatellite sequences and has been considered a hallmark of mismatch repair gene inactivation. MSI has been associated with less aggressive tumor behavior and favorable prognosis in sporadic colorectal cancer[47]. MSI status has been determined by means of BAT26 mononucleotide repeats because this marker is quasi-monomorphic in normal DNA and has shown high sensitivity and specificity in the identification of MSI phenotype[48].

1.6 p53 mutation in gastric diseases

The mutation of the p53 gene is among the major alterations of the multi-step process of gastric carcinogenesis, while it has also been reported in pre-malignant lesions of the stomach, such as chronic gastritis, intestinal metaplasia and dyspepsia[49]. Kodama et (2007) al have suggested an accumulation of wild-type p53, especially in the *H pylori* infected mucosa probably due to the *H pylori*-induced DNA damage[50]. The present study shows the highest frequency of p53 mutation in the groups with *H pylori* infection, especially among the *cagA* positive cases, corroborating previous studies[51]. The high percentage of p53 mutation in EBV-associated and EBV-negative gastric carcinomas was observed by Lima et al (2008), demonstrating that the p53 mutation is a relevant alteration in gastric carcinogenesis independent of the infection [52].

1.7 Correlation between polymorphisms of the IL-8, IL1β, IL1RN and IL-10 genes and *H. pylori* infection.

The key pathophysiological event in *H. pylori* infection is the induction of an inflammatory response in the gastric mucosa, which is mediated and regulated by inflammatory cytokines produced by epithelial cells[53]. Polymorphisms in genes encoding cytokines such as interleukin (IL) IL-8, and IL-10 IL1β and IL1RN influence the cytokines' secretion levels and appear to contribute to the risk of developing gastroduodenal diseases[54-55].

Interleukin-8 is an proinflammatory cytokine that has an important role in the pathogenesis of *H. pylori*-induced diseases[56].

The high expression of IL-8 has been demonstrated in gastric mucosa infected with *H. pylori*. IL-8 causes chemotaxis and the activation of inflammatory cells in gastric mucosa infected with *H. pylori*[57].

The IL-8 gene, which is located on chromosome 4, exhibits a single nucleotide polymorphism (SNP TeA base transition) at 251 nt relative to the transcription start site. The A allele tends to be associated with increased IL-8 production and consequently with an amplified inflammatory response[58].

The IL-1 cluster IL-1β gene encoding IL-1β and the IL-1RN gene coding for the anti-inflammatory antagonist (IL-1ra) of IL-1 receptor are located on chromosome 2q14 and have a number of functionally relevant polymorphisms that could be correlated with the high or low production of IL-1β[59-60]. Substitution in the promoter region of the TATA box at the −511 position (CT; dbSNP: rs16944) and the −31 position (TC; dbSNP: rs1143627) has been observed. IL-1β is not only a determinant of a pro-inflammatory phenotype but also appears to be a major cofactor in maintaining and promoting *H. pylori* infection[61].

Was reported that the genotype IL-1β-511T+, IL-1β-31C+, and IL-1RN 2/2 are associated with an increased risk of developing gastric cancer[62].

Differently from the first two interleukins, IL-10 is an anti-inflammatory cytokine that downregulates cell-mediated immune responses and cytotoxic inflammatory responses. *H. pylori* can lead to IL-10 upregulation as a way to suppress an efficient immune response, which then favors infection and parasite survival[63]. The gene encoding human IL-10 is located on chromosome 1. Two SNPs that are associated with low IL-10 production

have been reported in the promoter region of this gene: a C and T base transition at position 819 and a C and A base transition at position 592.

The low IL-10 production is associated with increased gastric inflammation intensity and with an enhanced risk of gastric carcinoma in patients infected with *H. pylori*[64].

1.8 Resistance to clarithromycin in *H. pylori*

Proton-pump inhibitor (PPI), clarithromycin-based, triple therapy has been the recommended treatment for *H. pylori* eradication for the past 15 years[65].

Among the possible causes of failure, which are becoming more important every year, is the decrease in the number of peptic ulcer disease treated given that the eradication rate is always higher in peptic ulcer disease than in non-ulcer dyspepsia, and even more, antibiotic resistance to clarithromycin. Antimicrobial resistance in *H. pylori* is the consequence of mutations[66].

In the case of clarithromycin there are essentially three point mutations, which can occur at the two nucleotide positions 2142 (A2142G and A2142C) and 2143 (A2143G) in the peptidyl transferase loop of the *23S rRNA* gene; these mutations result in a conformational change leading to a decrease in binding of the drug. These mutations occur by chance, do not have an impact on bacterial fitness and can therefore remain for many generations[67].

CHAPTER 2

Patients and Methods

Study population

This study was performed on sample of 100 patients, 37% males and 63% females, with ages into range 20-87, mean 58.43 y.o. and standard deviation (SD) 16.44 y.o.

We included the patients in four groups:

• Group with normal gastric mucosa (Group NGM), composed of 36% males and 64% females, aged 22-71 years, mean 46.52 y.o. and standard deviation (SD) 14.74 y.o

• Group with active chronic gastritis (Group GCA) composed of 40% males and 60% females, aged 20-87 years, mean 46,64 y.o. and standard deviation (SD) 16.83 y.o

• Group with gastric cancer (Group GC), composed of 36% males and 64% females, aged 55-87 years, mean 70 y.o. and standard deviation (SD) 8.68 y.o

• Group with gastric MALT lymphoma (Group ML), composed of 36% males and 64% females, aged 57-80 years, mean 68,24 y.o. and standard deviation (SD) 6.24 y.o

Table 1 shows the clinical information of the patients.

Exclusion criteria for patient recruitment to the study were as follows: previous attempts to eradicate *H. pylori* and use of antibiotics or proton

pump inhibitor within the 2weeks prior to endoscopy. Each patient signed an informed-consent form before endoscopy.

DNA isolation from gastric biopsies, *H. pylori* and EBV DNA detection by PCR methodology.

The genomic DNA of the biopsies was extracted using a High Pure Template Preparation kit (Roche), in accordance with the manufacturer's instructions.

The extracted DNA was stored at -20 °C until use.

H. pylori infection was diagnosed by the detection of *ureaseA* gene by nested PCR, while the BAMHI-W fragment region of the EBV genome was used as the target to evaluate the presence of the virus, according to Di Carlo et al., (2011) and Giardina et al., (2008)[68-69].

Detection of *cagA*, typing of *vacA* and *oipA* and evaluation of point mutations in *23S rRNA* genes.

PCR was used to detect the presence of the *cagA* gene, the type of EPIYA motif, the typing of *vacA* alleles, and determine *oipA* status, according to Chiarini et al (2008)[70].

To establish percentage of clarithromycin resistance in the population examined, we determined the point mutations in the *H. pylori 23S rRNA* gene, according to Fasciana et al (2015)[71].

Genotyping of cytokine polymorphisms

Polymorphisms in the *IL-8*, and in the *IL-1β* 511 were detected by PCR-restriction fragment length polymorphism (RFLP).

To analyze the polymorphism at position -251 of the IL-8 gene, the primers used were 5'-TTCTAACACCTGCCACTCTAG-3' (forward) and 5'-CTGAAGCTCCACAATTTGGTG-3' (reverse).

The PCR products were digested with the restriction enzyme MfeI, and then visualized by electrophoresis on 3.5% agarose gel stained with ethidium bromide. The genotypes were coded as follows: T/T, a single band consisting of 108 bp; T/A, three bands consisting of 108 bp, 76 bp, and 32 bp; and A/A, two bands consisting of 76 bp and 32 bp.

For the RFLP analysis of the IL-1β -511 polymorphism, the region containing the polymorphic site was amplified according to Rad et al (2003)[72]. PCR conditions were as follows: 95°C for 5 min, then 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and finally 72°C for 5 min. The products were digested with 10 U of AvaI at 37°C for 3 h. Fragments were analyzed by electrophoresis on a 3% agarose gel and stained with ethidium bromide. This yielded products of 190 and 114 bp (allele 1) and 304 bp (allele 2). The IL-1RN exon 2 polymorphism was analyzed according to Rad et al (2003)[72]. Conditions used were as follows: 95°C for 5 min, then 35 cycles of 95°C for 30 s, 50° C for 30 s, 72°C for 30 s, and finally 72°C for 5 min. The PCR products were analyzed by electrophoresis on a 2%agarose gel stained with ethidium bromide. Allele 1 (4 repeats) was 410 bp, allele 2 (2 repeats) was 240 bp, allele 3 (5 repeats) was 500 bp, allele 4 (3 repeats) was 325 bp, and allele 5 (6 repeats) was 595 bp in length according to Forte et al (2008)[73].

Amplification Refractory Mutation System (ARMS-PCR) was used to type, 819 C/T and 1082G/A IL-10 SNP, as described by Crivello et al (2006)[74].

Valuation of P53 mutation and single-stranded conformation polymorphism (SSCP) analysis.

Primers P1 and P2 (table 2) were used in a standard PCR to amplify the 2.9-kb fragment of the genomic DNA which contains exons 5 to 9 of the p53 gene[75]. The amplified fragments were purified from a 1% agarose gel after electrophoresis. For the SSCP analyses, PCR was performed as described by Effert et al (1996)[76]. Distinct primer pairs (table 2) were used to amplify exons 5 to 8 of the p53 gene in separate PCRs for 30 cycles at 94, 55, and 72°C. Five microliter of the PCR product was used, and SSCP analysis was performed as described by Effert et al (1996)[76].

Bisulfite modification and methylation-specific PCR (MSP)

DNA from tissues was subjected to treatment with sodium bisulfite as described by Herman at al. The modified DNA was amplified with primers specific for either the methylated or unmethylated sequences of *hMLH1*,

COX2, DAPK, CDKN2A and *CDH1* (table 3). PCR was individually performed as described by Ferrasi et al (2010)[77].

Microsatellite instability analysis (MSI)

MSI analysis was performed using the BAT26 primer set (5'-TGACTACTTTTGACTTCAGCC-3', sense and 5'-AACCATTCAACATTTTTAACCC-3', antisense). Sense primer was labeled with 6-FAM. PCR was performed in a final volume of 25 μL containing 1 × PCR buffer, 3.0 mmol/L MgCl2, 0.2 μmol/L dNTPs, 0.4 μmol/L of each primer, 2 U of Platinum Taq DNA Polymerase (Invitrogen) and 50 ng of DNA. The thermal conditions were 94°C/5 min followed by 40 cycles (94°C/1 min, 50°C/1 min and 72°C/1 min) and a final extension at 72°C/7 min. The dye-labeled PCR products were analyzed with ABI PRISM 3130 Genetic Analyzer using Genescan 3.7 software (Applied Biosystems) according to Hoang JM et al (1997)[48].

Histology analysis

The diagnosis of gastroduodenal disease was based on endoscopic and histological examination and it was established according to the Sidney System classification[78].

Statistical analysis

All data were analyzed with Matlab statistical toolbox version 2008 (MathWorks, Natick, MA, USA) for 32 bit Windows. Data are presented as number and percentage for categorical variables and continuous data are expressed as mean ± standard deviation (SD), unless otherwise specified. The χ^2 test and Fisher's exact test were performed to evaluate significant differences of proportions or percentages between two groups. Particularly Fisher's exact test was used where the χ^2 test was not appropriate. The multicomparison chi-square test was used to define significant differences among groups, if chi-square test was positive (p-value <0.05) then residual analysis with Z-test was performed to localized the highest or lowest significant presence. For continuous data, we performed the Student's T-test for unpaired data to evaluate significant differences between two groups,

instead to evaluate significant differences among three o more groups, we performed the multicomparison ANOVA test. If ANOVA test was positive (p-value <0.05), pairwise comparisons were performed with Scheffé's test.

Particularly we considered in this study five groups of patients:

•Group NIG with no infection patients only, composed by 44.44% males and 55.56% females, with ages into range 22-87, mean 57.67 y.o. and standard deviation (SD) 17.82 y.o

•Group IG with infection patients only (only *H. pylori* (Hp) or only EBV or both), composed by 30.91% males and 69.09% females, with ages into range 20-87, mean 59.07 y.o. and standard deviation (SD) 15.16 y.o

•Group EBV with EBV infection patients only, composed by 45.45% males and 54.55% females, with ages into range 25-77, mean 61.09 y.o. and standard deviation (SD) 13.65 y.o

•Group Hp with Hp infection patients only, composed by 27.78% (5/18) males and 72.22% (13/18) females, with ages into range 31-81, mean 58.53 y.o. and standard deviation (SD) 14.81 y.o

•Group EBV-Hp with both EBV and Hp infection patients, composed by 26.92% males and 73.08% females, with ages into range 20-87, mean 58.58 y.o. and standard deviation (SD) 15.91 y.o.

CHAPTER 3

Results

Our result showed that the *H. pylori* and EBV infection is present respectively in 44% and in 37% of the population analyzed while coinfection was found in 26% of patients.

The single percentages, founded in correlation to different gastric diseases, are reported in the graphic 1.

In each group of patients analysed, the rate of coinfection founded is showed in graphic 2.

The presence of *H. pylori* was found in 12 males [27% (12/44)] while the females had a sharply higher prevalence [72% (32/44)]. The same percentage was found in the group of patients EBV infected.

In finally the coinfection was found in 7 males [27% (7/26)] and in 19 females [73% (19/26)].

About the characterization of *H. pylori* strains, the polymorphism of *vacA* gene, the presence of *cagA* gene and the status of *oipA* gene, evaluated directly on gastric biopsies, is reported in table 4.

Particularly, the polymorphism *s1m1i1* of *vacA* gene, was more frequent in Group ML (p-value = 0.0169) in comparison to the others, while the polymorphism *s2m2i2* was more frequent in Group NGM (p-value = 0.0359) and less frequent in Group GC (p-value = 0.0276). In other words the polymorphism *s1m1i1* was significantly associated to patients with ML, instead the polymorphism *s2m2i2* was significantly associated to patients with GCA.

In addition, the presence of *cagA* gene was more frequent in patients with GC (p-value = 0.0198), instead than the patients with NGM where the positivity was less frequent (p-value = 0.0130).

About the distribution of the EPIYA motif, we found the motif ABC present in 96% of the cases, while in 4% cases the motif ABCC was present, as showed in graphic 3.

A strong association between the presence of mosaic s1i1m1 and *cagA* positivity and between mosaic *s2i2m2* and *cagA* negativity was found.

Similarly the *H. pylori* with *oipA* gene ON, was more frequent in patients with GC (p-value = 0.0198), while less frequent (p-value = 0.0130) in patients with NGM.

The data are showed in table 5.

The distribution of *vacA* mosaicism with respect to the *cagA* gene is shown in table 6.

About the number of CT repeats, the most prevalent in the 'ON' frame status had six CT repeats, as found to be the case in other studies[79]. ON status of *oipA* gene was observed more frequently in the GC group. Among out-frame status 'OFF variants, the most prevalent was the patterns with eight CT repeats as reported in table 7.

A close correlation was found between the status of the *oipA*, *vacA* and *cagA* genes and, indeed, the 'ON' status occurred in all the *cagA*-positive strains, associated with *s1i1m1* mosaicism.

The analysis of the *23S rRNA* gene demonstrated that 32 (73%) patients were infected with *H. pylori*-susceptible strains and the remaining 12 (27%) with resistant strains. The predominant point mutation observed among the 12 *H. pylori* resistant strains was, in 10 cases (83 %), A2143G and A2142G in 2 cases (17%), as showed in graphic 4.

The distribution of strains *H. pylori*-susceptible and resistant, among gender and age of patients enrolled, is showed in table 8, while in table 9 is reported the association between resistance or susceptibility to clarithromycin and *vacA*, *cagA* or *oipA* status.

The quantification of EBV DNA, conducted by real time PCR quantitative, showed in 97% of cases (36/37) the presence of a low charge of viral DNA, while in 3% of the cases (1/37) was present a charge of 10^3 of viral DNA.

Table 10 shows, age, sex gender, disease, all polymorphisms of IL analyzed, methylation status and microsatellite instability between patients without

infection and patients with infection, considering EBV only, Hp only or both.

From Table 10, it resulted that for polymorphism IL-β (-31), CT was more frequent in IG in contrast to NIG (43.64% > 37.78%, p=0.039). For polymorphism IL-β (-511), CC was more frequent in NIG than IG (57.78>36.36, p=0.044). Particularly for methylation status, *COX2* was more frequent in IG in contrast to NIG (30.91%>6.67%, p=0.0057). Finally for microsatellite instability, MSS was more frequent in NIG in contrast to IG (95.56%>74.55%, p=0.01), vice versa MSI was more frequent in IG in contrast to NIG (23.45%>4.44%, p=0.01).

In Table 11 we showed, age, sex gender, disease and all polymorphisms of IL analyzed, methylation status and microsatellite instability among patients without infection (NIG), patients with Hp infection only, patients with EBV infection only and patients with Hp and EBV simultaneously.

From Table 11, we showed that for polymorphism IL-1RN, type 1/1, there was a significant difference among groups (p= 0.046) but with a significant level equal to 0.05, no group had percentages significant greater or less in comparison to others. For methylation status, type *COX2* there was a significant difference among groups (p<0.0001), particularly, the HP-EBV group was the most frequent for patients with COX2 presence (p=0.0105), and analogous for *CDKN2A* (p=0.0310). Finally for microsatellite instability there was a significant difference among groups of MSS (p=0.0121) and MSI (p= 0.0121) but with a significant level equal to 0.05, no group had percentages significant greater or less in comparison to others

Table 12 shows all polymorphisms of IL analyzed, methylation status and microsatellite instability, presented in this study, considering patient's groups differentiated for disease type: Normal Gastric Mucosa, Active Chronic Gastritis, Malt lymphoma and Gastric Cancer.

From Table 12, we observed that for polymorphism IL-1RN, type ½, was less frequent in patients with ML (p=0.0256), analogous for polymorphism IL-8 (-251), type TA (p=0.0029)

Instead for polymorphism IL-10 (-1082), the types AG and GA, were less and more frequent respectively in patients with GC (p=0.0256 and p<0.0001 respectively). Finally in patients with normal gastric mucosa, the

polymorphism IL-10 (-819), type CC was less frequent (0.0047), instead type CT was more frequent in comparison to others (p=0.0085).

Finally in table 13 we report, age, sex gender, disease and all polymorphism of IL analyzed, methylation status and microsatellite instability, among patients with Hp infection only, patients with EBV infection only and patients with Hp and EBV simultaneously.

From table 13, we observed that for polymorphism IL-1RN type: 1/1 and 2/2 there were significant differences among groups (p=0.0395 and p= 0.0499, respectively) but at significant level equal to 0.05, no significant more or less frequent among groups were individuated. In this case can only observe more patients with 1/1 polymorphism (but no significant) in EBV group, analogous there were more patients with 2/2 (but no significant) in Hp group.

The p53 mutation was observed in 26% of the cases, in particular no mutation was observed in patients with normal gastric mucosa.

The results about the p53 mutation and its correlation with different gastric diseases and different infections are showed in table 14.

From table 14, it resulted that the exon 8 was mutated most frequently in patients with infection (Hp only, EBV only or both) in comparison to patients without infection (23.64% > 0.00%, p=0.0014).

In table 15 we showed, age, sex gender, disease and P53 mutation considered in this study, among patients without infection (NIG), patients with Hp infection only, patients with EBV infection only and patients with Hp and EBV simultaneously.

From table 15, we showed that the exon 6 was mutated more frequently in patients with EBV infection (36.36%, p=0.0011), instead the exon 8 was more frequent in patients co-infected (42.31%, p<0.0001).

From table 16, we observed that only for P53 mutation there were significant differences among patients with NGM, GCA, ML and GC, with exon 8 mutated, but at significant level of 0.05,

Finally p53 mutation was observed in 57% of the patients infected with *cagA* positive *H. pylori*.

CHAPTER 4

Discussion

H. pylori was included among the carcinogen agent as a Class 1 Carcinogen in 1994[80]. Patients with chronic *H. pylori* infection have an physiological and morphological changes within the gastric environment and the risk for neoplastic transformation. Recently, it was establish that EBV is also linked to the development of a quota of gastric carcinoma. The relationship between EBV infection and gastric cancer has been demonstrated by strong pieces of evidence such as the monoclonality of the viral genome and its presence in almost all tumour cells[81].

Moreover there is evidence to suggest that genetic factors can be contribute to the manifestation of functional gastrointestinal disorders. For example polymorphisms in genes that code cytokines influence cytokine secretion levels and appear to contribute to the risk of gastric diseases[82].

In addition infection with *H. pylori* and EBV can lead to the aberrant expression of p53 protein through the induction of host DNA damage, the methylation of multiple genes including *CDH1, DAPK, COX2, hMLH1* and *CDKN2A* can lead to DNA structural changes and gene inactivation.

Finally MSI has been considered a hallmark of mismatch repair gene inactivation[83-84-85].

In our study, the positivity rate of *H. pylori* in patients with gastric cancer, was higher than that in patients with normal gastric mucosa, active gastritis chronic and Malt lymphoma, 48%, 44%, 40% and 44% respectively.

While, EBV was detected in 52% of the patients with gastric cancer, in patients with normal gastric mucosa, active gastritis chronic and Malt lymphoma was more less, 20%, 40% and 36% respectively. Anyhow the

rate of *H. pylori* in all patients analyzed was 45,83% and the rate of EBV was 38,54%.

In patients with gastric cancer the rate of coinfection was higher than that in patients with normal gastric mucosa, active chronic gastritis and Malt lymphoma 56%, 33%,53% and 42% respectively.

In any case the prevalence of *H. pylori* and EBV infection, founded in this study, is in agreement with reported results from various world regions.

The *cagA* and *vacA* genes are commonly used as markers to characterize *H. pylori* virulence, and several epidemiological studies have reported geographical variations in the circulation of the virulence factors pertaining to microorganism. In Sicily the *cagA* gene is present in 52,27 % of strains, in 97 % of the strains it is associated with the ABC EPIYA motif and in most it is associated with the presence of the *s1i1m1 vacA* allele.

In this study, the rate of *H. pylori* resistance to clarithromycin founded is high, occurring in 27% of the cases and the most frequent point mutation, in the peptidyltransferase loop region of the *23S rRNA* gene was A2143G in 83 % of resistant strains. These data confirm those reported by Fasciana et al (2015) in a previous study[71].

Of interest, *H. pylori* resistance to clarithromycin has been found to be more frequently observed in female (57%) than in male patients (13%). A possible explanation for this finding may be a wider use of antibiotics for curing infections, with a greater prevalence in females, with, for example, urinary tract infections[86].

Despite the known importance of *H. pylori* and EBV in the gastric cancer aetiology, few studies have focused on the interrelationship of these two agents in gastric cancer cases. Thus, we investigated the presence of both *H. pylori* and EBV, in parallel with the status of DNA methylation, microsatellite instability the different polymorphisms of the IL-8, IL1β, IL1RN and IL-10 and the mutation of tumour suppressor p53.

About the DNA methylation we demonstrate that 88% of gastric cancer presents methylation for about one in five of the genes analyzed. COX2 was the gene found to be most commonly hypermethylated (32%) followed by CDH1 (20%), DAPK (12%), CDKN2A (12%) and hMLH1 (12%) reporting the same distribution showed by Ferrasi et al (2010)[77].

The same rate was observed in patients with Malt lymphoma while in patients with active chronic gastritis and normal gastric mucosa a lower rate of methylation was observed.

Moreover statistical correlation was found between co-infection and DNA methylation specially with COX2 and CDKN2A genes.

In the present study, the detection rate of MSI in gastric cancer and in Malt lymphoma was 28% and 24% respectively, while a less rate was detected in patients with normal gastric mucosa and active chronic gastritis, 8% and 4% respectively.

Among the gastric cancer patients in our case, the rate of MSI was similar those reported by Sheng et all.

The main reason for the inconsistent detection rate of MSI is the low number of patients involved in the study.

In addition MSI was observed in 30,77% of patients with coinfection, in 27,78% of patients with only *H. pylori* infection and in 9,09% of patients with only EBV infection.

MSI detection in gastric diseases can be used to predict the risk of gastric cancer, and those showing MSI need to be followed up regularly for early diagnosis. MSI may be another molecular mechanism involved in the multi-step process during coinfection and during *H. pylori* infection.

Therefore MSI can serve as a sensitive indicator of genetic instability in gastric cancer.

Probably due the low sample size, we did not find a correlation between polymorphisms in the IL-8, IL1β, IL1RN and IL-10 genes and coinfection.

The only significant statistic data were obtained between presence of coinfection and 1/1 polymorphisms of IL1RN.

Finally the present study shows the highest frequency of p53 mutation in the groups with co-infection, and in particular the exon 8 presents high rate of mutation.

A strong correlation was found between the presence of *cagA* gene and p53 mutation.

In conclusion, despite a limitation of this study was the limited number of patients who were evaluated, our results showed that the tumorigenesis is a multistep process that involve various factors to investigate these molecular

events will allow for a better understanding of the gastric pathogenesis and will lead to the translation of these insights into the clinical arena.

CHAPTER 5

Tables and Figures

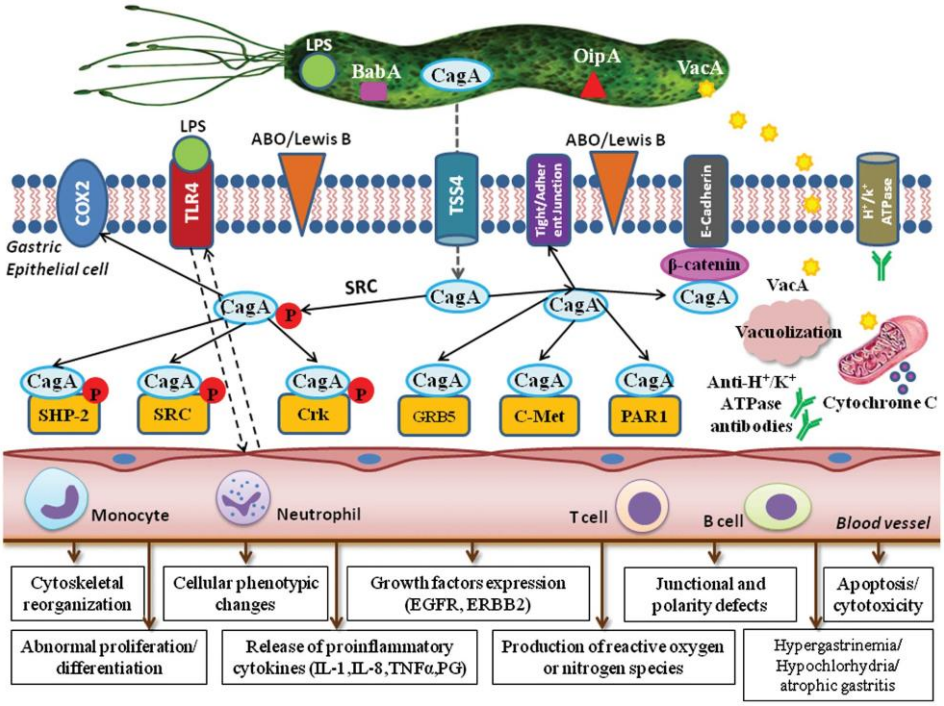


Figure 1. Several virulence factors, such as CagA and VacA, encoded by *H. pylori* genes, interacting with gastric epithelial cells and the immune system[38].

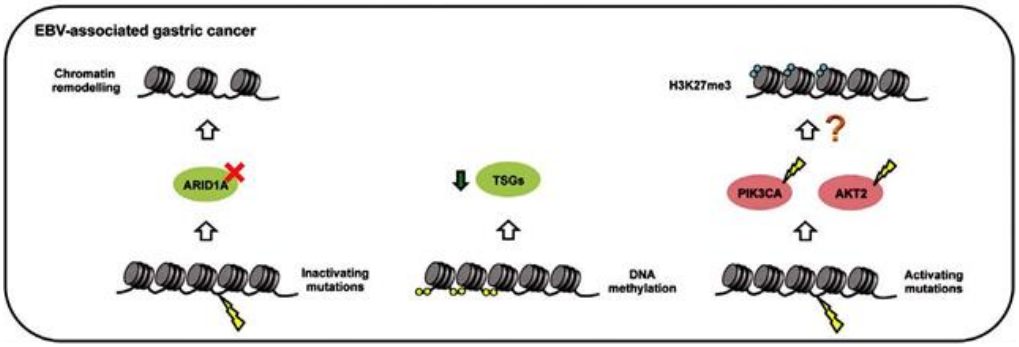


Figure 2. The role of EBV in gastric cancer[44].

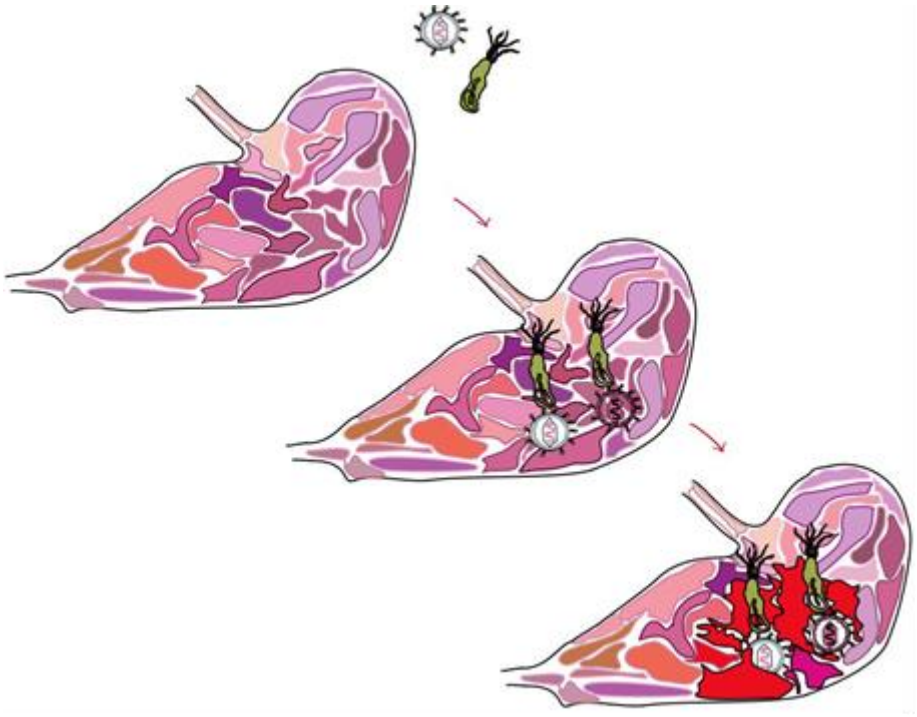


Figure 3. *H. pylori* and EBV coinfection in gastric diseases[44].

Table 1. Age, gender and symptoms of patients enrolled.

Parameters	**Sample data**
Age	58.27 ± 16.26
Gender	
Male	37% (37/96)
Female	63% (63/96)
Symptoms	
NGM	25.00% (25/100)
GCA	25.00% (25/100)
ML	25.00% (25/100)
GC	25.00% (25/100)

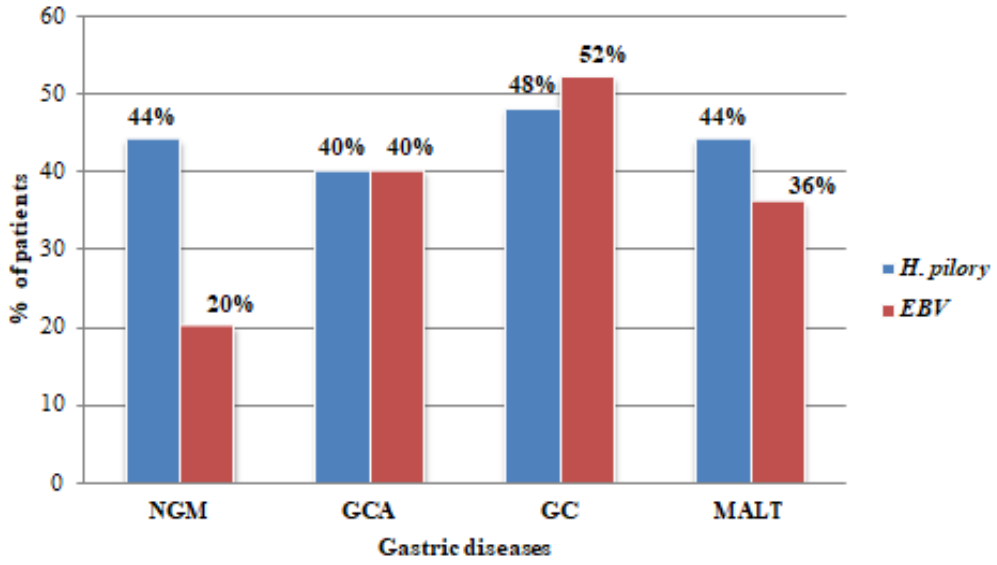
NGM = normal gastric mucosa; GCA = active chronic gastritis; GC = gastric cancer; ML = MALT lymphoma;

Table 2. Sequences of oligonucleotides used for evaluation of p53 mutation

Primer	Sequence	Fragment length
P1	GACGGAATTCGTCCCAAGCAATGGATGAT	2.9 kb
P2	GTCAGTCGACCTTAGTACCTGAAGGGTGA	
P3	TTCCTCTTCCTGCAGTACT	209 bp
P4	AGCTGCTCACCATCGCTAT	
P5	GGCCTCTGATTCCTCACTGA	170 bp
P6	GCCACTGACAACCACCCTTA	
P7	TGTTGTCTCCTAGGTTGGCT	139 bp
P8	CAAGTGGCTCCTGACCTGGA	
P9	CCTATCCTGAGTAGTGGTAA	164 bp
P10	TCCTGCTTGCTTACCTCGCT	
P11	GACCTGGTCCTCTGACTGCT	320 bp
P12	ACGGCCAGGCATTGAAGTCT	

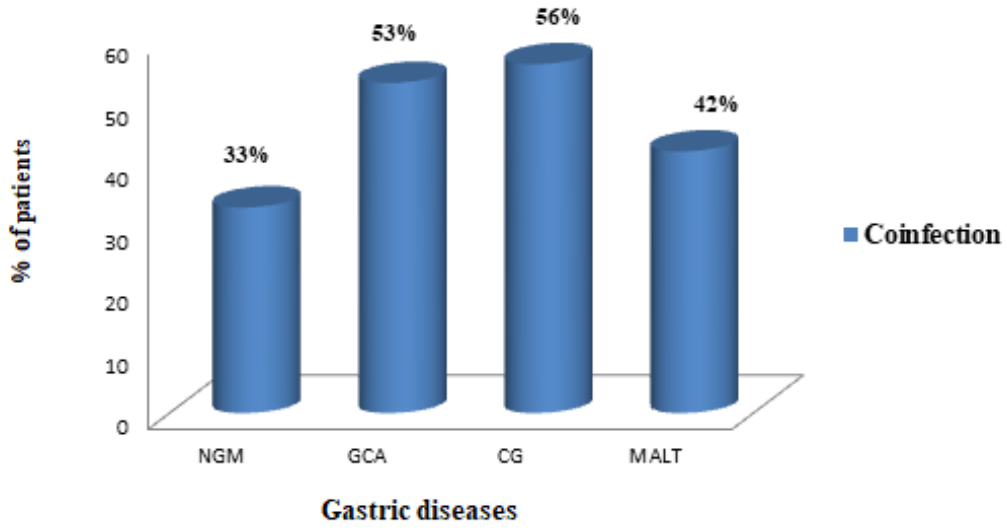
Table 3. Primer Sequences and PCR conditions for methylation-specific PCR (MSP) analysis.

Gene	Primer (5'-3') forward	Primer (5'-3') reverse	Size (bp)
COX2	M TTAGATACGGCGGCGGCGGC	TCTTTACCCGAACGCTTCCG	161
	U ATAGATTAGATATGGTGGTGGTGGT	CACAATCTTTACCCAAACACTTCCA	171
DAPK	M GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	98
	U GGAGGATAGTTGGATTGAGTTAATGTT	CAAATCCCTCCCAAACACCAA	116
CDH1	M TTAGGTTAGAGGGTTATCGCGT	TAACTAAAAATTCACCTACCGAC	115
	U TAATTTTAGGTTAGAGGGTTATTGT	CACAACCAATCAACAACACA	97
hMLH1	M TATATCGTTCGTAGTATTCGTGT	TCCGACCCGAATAAACCCAA	153
	U TTTTGATGTAGATGTTTTATTAGGGTTGT	ACCACCTCATCATAACTACCCACA	124
CDKN2A	M TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150
	U TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151



NGM = Normal gastric mucosa; GCA = active chronic gastritis; GC = gastric cancer; ML = Malt lymphoma

Graphic 1. Distribution of *H. pilory* and EBV infection in different gastric diseases.

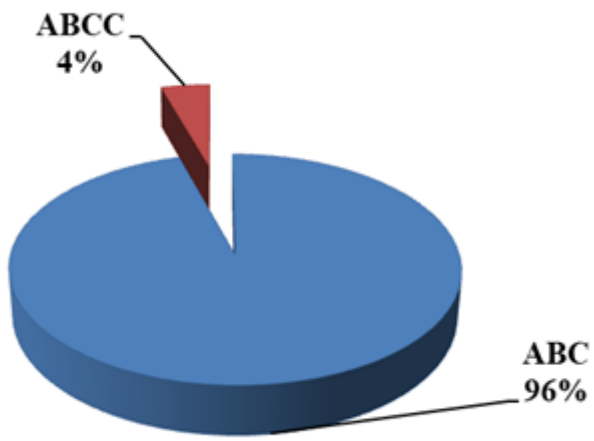


NGM = Normal gastric mucosa; GCA = active chronic gastritis; GC = gastric cancer; ML = Malt lymphoma

Graphic 2. Distribution of coinfection in different gastric diseases.

Table 4. Polymorphism of *vacA* gene, presence of *cagA* gene and status of *oipA* gene.

Parameters	**Sample data**
Polymorphism of Gene *vacA*	
s1m1-m2i1	2.27% (1/44)
s1m2i2	4.55% (2/44)
s1m2i1	6.82% (3/44)
s1m1i1	40.91% (18/44)
s2m2i2	45.45% (20/44)
Gene *cagA* (†)	
Negative	47.73% (21/44)
Positive	52.27% (23/44)
Gene *oipA* (†)	
OFF	47.73% (21/44)
ON	52.27% (23/44)



Graphic 3. Percentage of EPIYA motif.

Table 5. Characterization of *H. pilory* virulence factors and their distribution in different gastric diseases.

Parameters	**Group NGM**	**Group GCA**	**Group GC**	**Group ML**	**Multivariate and Univariate analysis**
Polymorphism *vacA*					
s1m1i1	9.09% (1/11)	10.00% (1/10)	58.33% (7/12)	81.82% (9/11)	p < 0.0001 (C) * ML, p=0.0169 (Z) **
s1m2i1	0.00% (0/11)	10.00% (1/10)	16.67% (2/12)	0.00% (0/11)	p = 0.308 (C)
s1m2i2	9.09% (1/11)	0.00% (0/10)	8.33% (1/12)	0.00% (0/11)	p = 0.589 (C)
s2m2i2	81.82% (9/11)	80.00% (8/10)	8.33% (1/12)	18.18% (2/11)	p < 0.0001 (C) * NGM, p=0.0359 (Z) ** KG, p=0.0276 (Z) ***
s1m1-m2i1	0.00% (0/11)	0.00% (0/10)	8.33% (1/12)	0.00% (0/11)	p = 0.435 (C)
Gene *cagA* (†)					
Negative	90.91% (10/11)	80.00% (8/10)	8.33% (1/12)	18.18% (2/11)	p < 0.0001 (C) * NGM, p=0.0130 (Z) ** KG, p=0.0198 (Z) ***
Positive	9.09% (1/11)	20.00% (2/10)	91.67% (11/12)	81.82% (9/11)	p < 0.0001 (C) * KG, p=0.0198 (Z) ** NGM, p=0.0130 (Z) ***
Gene *oipA* (†)					
Off	90.91% (10/11)	80.00% (8/10)	8.33% (1/12)	18.18% (2/11)	p < 0.0001 (C) * NGM, p=0.0130 (Z) ** KG, p=0.0198 (Z) ***
On	9.09% (1/11)	20.00% (2/10)	91.67% (11/12)	81.82% (9/11)	p < 0.0001 (C) * KG, p=0.0198 (Z) ** NGM, p=0.0130 (Z) ***

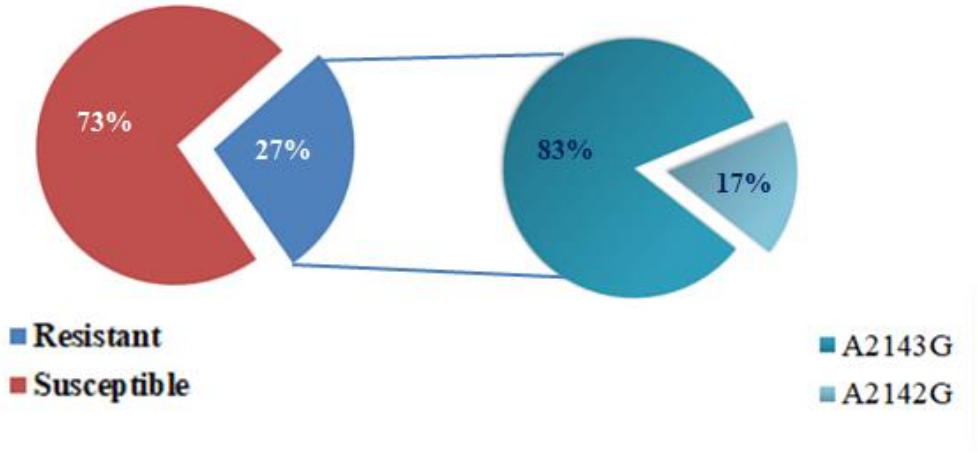
* = significant test; ** = significant most frequent; *** = significant less frequent; C = multicomparison chi-square test; Z= Z-test ; HP = *Helicobacter pylori*; NGM = normal gastric mucosa; GCA = active chronic gastritis; GC = gastric cancer; ML = Malt lymphoma; † = cases where gene *vacA* was present; p=p-value

Table 6. Distribution of *vacA* mosaicism with respect to the *cagA* gene

vacA mosaic	cagA-positive (23 cases)	cagA-negative (21 cases)
s1i1m1	19	0
s1i1m2	2	0
s1i2m2	1	1
s2i2m2	0	20
s1i1m1-m2	1	0

Table 7. *oipA* CT repeat patterns

Sequence of signal peptide-encoding region of *oipA*	**N° CT**	**N° of strains**
OFF Status		
ATGAAAAAAGCTCTCTTA……...CTCTCTCTCTCTCTCTCTCGTT	9	5
ATGAAAAAAGCCCTCTTACTAACTCTCTCTCTCTCTCTCTCGTTT	8	7
ATGAAAAAAGCTCTCTTGCTAACTCTCTCTCTCTCTCTCTCTCGTT	10	2
ATGAAAAAAGCTCTTTTA……CTCTCTCTCTCTCGTT	6	3
ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCTCTCTCGTT	7	2
ATGAAAAAGCTCTCTTA……CTCTCTCTCTCTCTCGTTCTGG	7	1
ATGAAAAAAGCCCTCTTA……..CTCTCTCTTTCTCTCGTTTT	4+2	1
Total		**21**
ON Status		
ATGAAAAAAGCTCTCTTACTAATTCTCTCTCTCTCTCGTT	5	4
ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCTCTCGTT	6	15
ATGAAAAAAGCCCTCTTA……..CTCTCTCTCTCTCTCTCTCTCGT	11	2
ATGAAAAAAGCTCTCTTACTAACT CTCTCTCTCTCTCTCTCGTT	9	1
ATGAAAAAAGCTCTTTTACTCTCT CTCTCTCTCTCGTT	8	1
Total		**23**



Graphic 4. Percentage of resistance to clarithromycin in *H. pylori* evaluated directly from biopsies sample and point mutation in *23 rRNA* gene.

Table 8. Distribution of strains *H. pylori*-susceptible and resistant correlated with gender and age of patients

Variable	*n°*	Resistance to clarithromycin	%
Gender			
Male	37	5	13%
Female	63	7	57%
Age (years)			
≤ 50	29	3	10%
>50	71	9	12%

Table 9. Correlation between resistance and polymorphism of *vacA* gene, presence of *cagA* gene and status of *oipA* gene.

Variable	*n°*	Resistance to clarithromycin	%
vacA			
s1i1m1	19	7	36
s1i1m2	2	1	50
s1i2m2	2	1	50
s2i2m2	20	3	15
s1m1-m2	1	0	0
cagA			
Negative	21	9	42
Positive	23	3	13
oipA			
On	23	8	34
Off	21	4	19

Table 10. Age, sex gender, disease, polymorphism of IL genes, methylation status and microsatellite instability among patients enrolled.

Parameters	**NIG**	**IG**	**NIG vs. IG**
Age	57.67 ± 17.82	59.07 ± 15.16	0.672 (T)
Gender			
Male	44.44% (20/45)	30.91% (17/55)	0.235 (CY)
Female	55.56% (25/45)	69.09% (38/55)	0.235 (CY)
Disease			
NGM	28.89% (13/45)	20.0% (11/55)	0.424 (CY)
GCA	26.67% (12/45)	23.64% (13/55)	0.908 (CY)
GC	20.0% (9/45)	29.09% (16/55)	0.417 (CY)
ML	24.44% (11/45)	25.45% (14/55)	0.908 (CY)
Polymorphism IL-1RN			
1/1	64.44% (29/45)	52.73% (29/55)	0.328 (CY)
1/2	15.56% (7/45)	25.45% (14/55)	0.324 (F)
2/2	15.56% (7/45)	20.0% (11/55)	0.611 (F)
1/3	4.44% (2/45)	0.0% (0/55)	0.20 (F)
4/4	0.00% (0/45)	1.82% (1/55)	1.00 (F)
Polymorphism IL-10 (-1082)			
AA	53.33% (24/45)	45.45% (25/55)	0.560 (CY)
AG	22.22% (10/45)	20.0% (11/55)	0.980 (CY)
GA	4.44% (2/45)	10.91% (6/55)	0.415 (CY)
GG	20.0% (9/45)	23.64% (13/55)	0.846 (CY)
Polymorphism IL-10 (-819)			
CC	64.44% (29/45)	56.36% (31/55)	0.538 (CY)
CT	31.11% (14/45)	38.18% (21/55)	0.598 (CY)
TT	4.44% (2/45)	5.45% (3/55)	1.00 (F)
Polymorphism IL-8 (-251)			
AA	35.56% (16/45)	25.45% (14/55)	0.380 (CY)
TA	35.56% (16/45)	47.27% (26/55)	0.328 (CY)
TT	28.89% (13/45)	27.27% (15/55)	0.964 (CY)
Polymorphism IL-β (-31)			

CC	15.56% (7/45)	20.0% (11/55)	0.754 (CY)
CT	37.78% (17/45)	43.64% (24/55)	**0.039 * (CY)**
TT	46.67% (21/45)	36.36% (20/55)	0.402 (CY)
Polymorphism IL-β (-511)			
CC	57.78% (26/45)	36.36% (20/55)	**0.044* (F)**
CT	28.89% (13/45)	47.27% (26/55)	0.068 (F)
TT	13.33% (6/45)	16.36% (9/55)	0.781 (F)
Methylation status			
CDH1	6.67% (3/45)	18.18% (10/55)	0.135 (F)
DAPK	6.67% (3/45)	9.09% (5/55)	0.727 (F)
COX2	6.67% (3/45)	30.91% (17/55)	**0.0057* (CY)**
hMLH1	4.44% (2/45)	14.55% (8/55)	0.178 (F)
CDKN2A	4.44% (2/45)	12.73% (7/55)	0.180 (F)
Microsatellite			
MSS	95.56% (43/45)	74.55% (41/55)	**0.01* (CY)**
MSI	4.44% (2/45)	25.45% (14/55)	**0.01* (CY)**

* = significant test; T = Student's T test for unpaired data; CY = chi-square test with Yates correction; F = Fisher's exact test ; NGM = Normal gastric mucosa; GCA = active chronic gastritis; GC = gastric cancer; ML = Malt lymphoma; p=p-value; NI = no infected group; IG=patients infected by EBV only, by HP only or both, MSS= microsatellite stability, MSI= microsatellite instability.

Table 11. Age, sex gender, disease, polymorphism of IL genes, methylation status and microsatellite instability among patients correlated with infections.

Parameters	**NIG**	**Group HP**	**Group EBV**	**Group HP-EBV**	**Multivariate and Univariate analysis**
Age	57.67 ± 17.82	58.53 ± 14.81	61.09 ± 13.65	58.58 ± 15.91	p=0.300 (A)
Gender					
Male	44.44% (20/45)	27.78% (5/18)	45.45% (5/11)	26.92% (7/26)	p=0.362 (C)
Female	54.56% (25/45)	72.22% (13/18)	54.55% (6/11)	73.08% (19/26)	p=0.362 (C)
Disease					
NGM	28.89% (13/45)	38.89% (7/18)	9.09% (1/11)	15.38% (4/26)	p=0.173 (C)
GCA	26.67% (12/45)	16.67% (3/18)	27.27% (3/11)	26.92% (7/26)	p=0.846 (C)
KG	20.0% (9/45)	16.67% (3/18)	36.36% (4/11)	34.62% (9/26)	p=0.347 (C)
ML	24.44% (11/45)	27.78% (5/18)	27.27% (3/11)	23.08% (6/26)	p=0.983 (C)
Polymorphism IL-1RN					
1/1	64.44% (29/45)	33.33% (6/18)	81.82% (9/11)	53.85% (14/26)	**p=0.046*# (C)**
1/2	15.56% (7/45)	27.78% (5/18)	9.09% (1/11)	30.77% (8/26)	p=0.291 (C)
2/2	15.56% (7/45)	38.89% (7/18)	9.09% (1/11)	11.54% (3/26)	p=0.078 (C)
1/3	4.44% (2/45)	0.00% (0/18)	0.00% (0/11)	0.00% (0/26)	p=0.476 (C)
4/4	0.00% (0/45)	0.00% (0/18)	0.00% (0/11)	3.85% (1/26)	p=0.411 (C)
Polymorphism IL-10 (-1082)					
AA	53.33% (24/45)	38.89% (7/18)	45.45% (5/11)	50.0% (13/26)	p=0.767 (C)
AG	22.22% (10/45)	22.22% (4/18)	27.27% (3/11)	15.38% (4/26)	p=0.845 (C)
GA	4.44% (2/45)	16.67% (3/18)	9.09% (1/11)	7.69% (2/26)	p=0.452 (C)
GG	20.0% (9/45)	22.22% (4/18)	18.18% (2/11)	26.92% (7/26)	
Polymorphism IL-10 (-819)					
CC	64.44% (29/45)	66.67% (12/18)	36.36% (4/11)	57.69% (15/26)	p=0.904 (C)
CT	31.11% (14/45)	33.33% (6/18)	63.64% (7/11)	30.77% (8/26)	p=0.213 (C)
TT	4.44% (2/45)	0.00% (0/18)	0.00% (0/11)	11.54% (3/26)	p=0.273 (C)
Polymorphism IL-8 (-251)					
AA	35.56% (16/45)	27.78% (5/18)	27.27% (3/11)	23.08% (6/26)	p=0.721 (C)
TA	35.56% (16/45)	44.44% (8/18)	45.45% (5/11)	50.0% (13/26)	p=0.671 (C)
TT	28.89% (13/45)	27.78% (5/18)	27.27% (3/11)	26.92% (7/26)	p=0.998 (C)

Polymorphism IL-β (-31)					
CC	15.56% (7/45)	16.67% (3/18)	45.45% (5/11)	11.54% (3/26)	p=0.088 (C)
CT	37.78% (17/45)	50.0% (9/18)	18.18% (2/11)	50.0% (13/26)	p=0.258 (C)
TT	46.67% (21/45)	33.33% (6/18)	36.36% (4/11)	38.46% (10/26)	p=0.753 (C)
Polymorphism IL-β (-511)					
CC	57.78% (26/45)	33.33% (6/18)	27.27% (3/11)	42.31% (11/26)	p=0.147 (C)
CT	28.89% (13/45)	38.89% (7/18)	54.55% (6/11)	50.0% (13/26)	p=0.224 (C)
TT	13.33% (6/45)	27.78% (5/18)	18.18% (2/11)	7.69% (2/26)	p=0.311 (C)
Methylation status					
CDH1	6.67% (3/45)	16.67% (3/18)	18.18% (2/11)	19.23% (5/26)	p=0.397 (C)
DAPK	6.67% (3/45)	16.67% (3/18)	0.00% (0/11)	7.69% (2/26)	p=0.406 (C)
COX2	6.67% (3/45)	16.67% (3/18)	36.36% (4/11)	46.15% (12/26)	**p<0.0001* (C) Group HP-EBV **, p=0.0105 (Z)**
hMLH1	4.44% (2/45)	11.11% (2/18)	18.18% (2/11)	15.38% (4/26)	p=0.358 (C)
CDKN2A	4.44% (2/45)	5.56% (1/18)	0.00% (0/11)	23.08% (6/26)	**p=0.0324* (C) Group HP-EBV **, p=0.0310 (Z)**
Microsatellite					
MSS	95.56% (43/45)	72.22% (13/18)	90.91% (10/11)	69.23% (18/26)	**p=0.0121*# (C)**
MSI	4.44% (2/45)	27.78% (5/18)	9.09% (1/11)	30.77% (8/26)	**p=0.0121*# (C)**

* = significant test; ** = significant most frequent; *** = significant less frequent; # = no localized significant modality with significant level < 0.05; A= one way ANOVA test; Sh = post hoc Scheffé's test for pairwise comparison; C = multicomparison chi-square test; Z= Z-test; NGM = Normal gastric mucosa; GCA = active chronic gastritis; KG = gastric cancer; ML = Malt lymphoma; p=p-value; NI = no infected group. MSS= microsatellite stability, MSI= microsatellite instability.

Table 12. Polymorphism of IL genes, methylation status and microsatellite instability considering different diseases.

Parameters	Normal gastric mucosa	Active chronic gastritis	Malt lymphoma	Gastric cancer	Multivariate and Univariate analysis
Polymorphism IL-1RN					
1/1	52.0% (13/25)	64.0% (16/25)	72.0% (18/25)	44.0% (11/25)	0.190 (C)
1/2	28.0% (7/25)	24.0% (6/25)	0.0% (0/25)	32.0% (8/25)	**p=0.0251* (C) Malt lymphoma ***, p=0.0256 (Z)**
2/2	20.0% (5/25)	8.0% (2/25)	28.0% (7/25)	16.0% (4/25)	0.318 (C)
1/3	0.0% (0/25)	4.0% (1/25)	0.0% (0/25)	4.0% (1/25)	0.564 (C)
4/4	0.0% (0/25)	0.0% (0/25)	0.0% (0/25)	4.0% (1/25)	0.387 (C)
Polymorphism IL-10 (-1082)					
AA	44.0% (11/25)	48.0% (12/25)	52.0% (13/25)	52.0% (13/25)	0.932 (C)
AG	32.0% (8/25)	32.0% (8/25)	20.0% (5/25)	0.0% (0/25)	**p=0.0161* (C) Gastric cancer ***, p=0.0256 (Z)**
GA	0.0% (0/25)	0.0% (0/25)	0.0% (0/25)	32.0% (8/25)	**p<0.0001* (C) Gastric cancer **, p<0.0001 (Z)**
GG	24.0% (6/25)	20.0% (5/25)	28.0% (7/25)	16.0% (4/25)	0.761 (C)
Polymorphism IL-10 (-819)					
CC	28.0% (7/25)	52.0% (13/25)	80.0% (20/25)	80.0% (20/25)	**p<0.0001* (C) Normal gastric mucosa***, p=0.0047 (Z)**
CT	64.0% (16/25)	40.0% (10/25)	20.0% (5/25)	16.0% (4/25)	**p=0.0012* (C) Normal gastric mucosa**, p=0.0085 (Z)**
TT	8.0% (2/25)	8.0% (2/25)	0.0% (0/25)	4.0% (1/25)	0.510 (C)
Polymorphism IL-8 (-251)					
AA	24.0% (6/25)	16.0% (4/25)	44.0% (11/25)	36.0% (9/25)	0.137 (C)
TA	48.0% (12/25)	56.0% (14/25)	8.0% (2/25)	56.0% (14/25)	**p=0.001* (C) Malt lymphoma ***, p=0.0029 (Z)**
TT	28.0% (7/25)	28.0% (7/25)	48.0% (12/25)	8.0% (2/25)	**p=0.0193*# (C)**
Polymorphism IL-β (-31)					
CC	16.0% (4/25)	20.0% (5/25)	16.0% (4/25)	20.0% (5/25)	p=0.965 (C)
CT	56.0% (14/25)	40.0% (10/25)	36.0% (9/25)	32.0% (8/25)	p=0.330 (C)
TT	28.0% (7/25)	40.0% (10/25)	48.0% (12/25)	48.0% (12/25)	p=0.429 (C)
Polymorphism IL-β (-511)					
CC	32.0%	48.0%	56.0%	48.0%	p=0.383 (C)

	(8/25)	(12/25)	(14/25)	(12/25)	
CT	52.0% (13/25)	36.0% (9/25)	32.0% (8/25)	36.0% (9/25)	p=0.479 (C)
TT	16.0% (4/25)	16.0% (4/25)	12.0% (3/25)	16.0% (4/25)	p=0.972 (C)
Methylation status					
CDH1	8.0% (2/25)	8.0% (2/25)	20.0% (5/25)	20.0% (5/25)	p=0.393 (C)
DAPK	8.0% (2/25)	4.0% (1/25)	12.0% (3/25)	12.0% (3/25)	p=0.719 (C)
COX2	12.0% (3/25)	16.0% (4/25)	28.0% (7/25)	32.0% (8/25)	p=0.266 (C)
hMLH1	4.0% (1/25)	8.0% (2/25)	16.0% (4/25)	12.0% (3/25)	p=0.528 (C)
CDKN2A	4.0% (1/25)	8.0% (2/25)	12.0% (3/25)	12.0% (3/25)	p=0.719 (C)
Microsatellite					
MSS	92.0% (23/25)	96.0% (24/25)	72.0% (18/25)	76.0% (19/25)	p=0.052 (C)
MSI	8.0% (2/25)	4.0% (1/25)	28.0% (7/25)	24.0% (6/25)	p=0.052 (C)

* = significant test; ** = significant most frequent; *** = significant less frequent; # = no localized significant modality with significant level < 0.05; C = multicomparison chi-square test; Z= Z-test; NGM = Normal gastric mucosa; GCA = active chronic gastritis; KG = gastric cancer; ML = Malt lymphoma; p=p-value. MSS= microsatellite stability, MSI= microsatellite instability.

Table 13. Polymorphism of IL genes, methylation status and microsatellite instability considering patients with infection.

Parameters	**Group HP**	**Group EBV**	**Group HP-EBV**	**Multivariate and Univariate analysis**
Age	58.53 ± 14.81	61.09 ± 13.65	58.58 ± 15.91	p=0.891 (A)
Gender				
Male	27.78% (5/18)	45.45% (5/11)	26.92% (7/26)	p=0505 (C)
Female	72.22% (13/18)	54.55% (6/11)	73.08% (19/26)	p=0505 (C)
Disease				
NGM	38.89% (7/18)	9.09% (1/11)	15.38% (4/26)	p=0.093 (C)
GCA	16.67% (3/18)	27.27% (3/11)	26.92% (7/26)	p=0.698 (C)
KG	16.67% (3/18)	36.36% (4/11)	34.62% (9/26)	p=0.365 (C)
ML	27.78% (5/18)	27.27% (3/11)	23.08% (6/26)	p=0.929 (C)
Polymorphism IL-1RN				
1/1	33.33% (6/18)	81.82% (9/11)	53.85% (14/26)	**p=0.0395*# (C)**
1/2	27.78% (5/18)	9.09% (1/11)	30.77% (8/26)	p=0.370 (C)
2/2	38.89% (7/18)	9.09% (1/11)	11.54% (3/26)	**p=0.0499*# (C)**
1/3	0.00% (0/18)	0.00% (0/11)	0.00% (0/26)	p=1.00 (C)
4/4	0.00% (0/18)	0.00% (0/11)	3.85% (1/26)	p=0.567 (C)
Polymorphism IL-10 (-1082)				
AA	38.89% (7/18)	45.45% (5/11)	50.0% (13/26)	p=0.767 (C)
AG	22.22% (4/18)	27.27% (3/11)	15.38% (4/26)	p=0.682 (C)
GA	16.67% (3/18)	9.09% (1/11)	7.69% (2/26)	p=0.629 (C)
GG	22.22% (4/18)	18.18% (2/11)	26.92% (7/26)	
Polymorphism IL-10 (-819)				
CC	66.67% (12/18)	36.36% (4/11)	57.69% (15/26)	p=0.837 (C)
CT	33.33% (6/18)	63.64% (7/11)	30.77% (8/26)	p=0.149 (C)
TT	0.00% (0/18)	0.00% (0/11)	11.54% (3/26)	p=0.170 (C)
Polymorphism IL-8 (-251)				
AA	27.78% (5/18)	27.27% (3/11)	23.08% (6/26)	p=0.929 (C)
TA	44.44% (8/18)	45.45% (5/11)	50.0% (13/26)	p=0.928 (C)
TT	27.78% (5/18)	27.27% (3/11)	26.92% (7/26)	p=0.998 (C)

Polymorphism IL-β (-31)				
CC	16.67% (3/18)	45.45% (5/11)	11.54% (3/26)	p=0.057 (C)
CT	50.0% (9/18)	18.18% (2/11)	50.0% (13/26)	p=0.164 (C)
TT	33.33% (6/18)	36.36% (4/11)	38.46% (10/26)	p=0.941 (C)
Polymorphism IL-β (-511)				
CC	33.33% (6/18)	27.27% (3/11)	42.31% (11/26)	p=0.650 (C)
CT	38.89% (7/18)	54.55% (6/11)	50.0% (13/26)	p=0.664 (C)
TT	27.78% (5/18)	18.18% (2/11)	7.69% (2/26)	p=0.205 (C)
Methylation status				
CDH1	16.67% (3/18)	18.18% (2/11)	19.23% (5/26)	p=0.977 (C)
DAPK	16.67% (3/18)	0.00% (0/11)	7.69% (2/26)	p=0.299 (C)
COX2	16.67% (3/18)	36.36% (4/11)	46.15% (12/26)	p=0.128 (C)
hMLH1	11.11% (2/18)	18.18% (2/11)	15.38% (4/26)	p=0.860 (C)
CDKN2A	5.56% (1/18)	0.00% (0/11)	23.08% (6/26)	p=0.084 (C)
Microsatellite				
MSS	72.22% (13/18)	90.91% (10/11)	69.23% (18/26)	p=0.370 (C)
MSI	27.78% (5/18)	9.09% (1/11)	30.77% (8/26)	p=0.370 (C)

* = significant test; ** = significant most frequent; *** = significant less frequent; # = no localized significant modality with significant level < 0.05; A= one way ANOVA test; Sh = post hoc Scheffé's test for pairwise comparison; C = multicomparison chi-square test; Z= Z-test; NGM = Normal gastric mucosa; GCA = active chronic gastritis; KG = gastric cancer; ML = Malt lymphoma; p=p-value. MSS= microsatellite stability, MSI= microsatellite instability.

Table 14. Age, sex gender, disease, and P53 mutation, in patients without infection and patients with infection.

Parameters	**NIG**	**IG**	**NIG vs. IG**
Age	57.67 ± 17.82	59.07 ± 15.16	0.672 (T)
Gender			
Male	44.44% (20/45)	30.91% (17/55)	0.235 (CY)
Female	55.56% (25/45)	69.09% (38/55)	0.235 (CY)
Disease			
NGM	28.89% (13/45)	20.0% (11/55)	0.424 (CY)
GCA	26.67% (12/45)	23.64% (13/55)	0.908 (CY)
KG	20.0% (9/45)	29.09% (16/55)	0.417 (CY)
ML	24.44% (11/45)	25.45% (14/55)	0.908 (CY)
P53 mutation			
Exon 5	2.22% (1/45)	1.82% (1/55)	0.566 (CY)
Exon 6	4.44% (2/45)	10.91% (6/55)	0.415 (CY)
Exon 7	0.00% (0/45)	1.82% (1/55)	0.920 (CY)
Exon 8	0.00% (0/45)	23.64% (13/55)	0.0014 * (CY)
Exon 9	0.00% (0/45)	3.64% (2/55)	0.566 (CY)

* = significant test; T = Student's T test for unpaired data; CY = chi-square test with Yates correction; F = Fisher's exact test ; NGM = Normal gastric mucosa; GCA = active chronic gastritis; KG = gastric cancer; ML = Malt lymphoma; p=p-value; NI = no infected group; IG=patients infected by EBV only, by HP only or both

Table 15. Characteristics and statistical analysis among groups: NIG, HP, EBV and HP-EBV and p53 mutations.

Parameters	**NIG**	**Group HP**	**Group EBV**	**Group HP-EBV**	**Multivariate and Univariate analysis**
Age	57.67 ± 17.82	58.53 ± 14.81	61.09 ± 13.65	58.58 ± 15.91	p=0.300 (A)
Gender					
Male	44.44% (20/45)	27.78% (5/18)	45.45% (5/11)	26.92% (7/26)	p=0.362 (C)
Female	54.56% (25/45)	72.22% (13/18)	54.55% (6/11)	73.08% (19/26)	p=0.362 (C)
Disease					
NGM	28.89% (13/45)	38.89% (7/18)	9.09% (1/11)	15.38% (4/26)	p=0.173 (C)
GCA	26.67% (12/45)	16.67% (3/18)	27.27% (3/11)	26.92% (7/26)	p=0.846 (C)
KG	20.0% (9/45)	16.67% (3/18)	36.36% (4/11)	34.62% (9/26)	p=0.347 (C)
ML	24.44% (11/45)	27.78% (5/18)	27.27% (3/11)	23.08% (6/26)	p=0.983 (C)
P53 mutation					
Exon 5	2.22% (1/45)	0.00% (0/18)	0.00% (0/11)	3.85% (1/26)	0.788 (C)
Exon 6	4.44% (2/45)	5.56% (1/18)	36.36% (4/11)	3.85% (1/26)	0.0036 * (C) EBV group **, p=0.0011 (Z)
Exon 7	0.00% (0/45)	0.00% (0/18)	0.00% (0/11)	3.85% (1/26)	0.411 (C)
Exon 8	0.00% (0/45)	5.56% (1/18)	9.09% (1/11)	42.31% (11/26)	<0.0001* (C) Group HP-EBV **, p<0.0001 (Z)
Exon 9	0.00% (0/45)	0.00% (0/18)	0.00% (0/11)	7.69% (2/26)	0.121 (C)

* = significant test; ** = significant most frequent; *** = significant less frequent; # = no localized significant modality with significant level < 0.05; A= one way ANOVA test; Sh = post hoc Scheffé's test for pairwise comparison; C = multicomparison chi-square test; Z= Z-test; NGM = Normal gastric mucosa; GCA = active chronic gastritis; KG = gastric cancer; ML = Malt lymphoma; p=p-value; NI = no infected group

Table 16. Correlation between gastric diseases and p53 mutations.

Parameters	Normal gastric mucosa	Active chronic gastritis	Malt lymphoma	Gastric cancer	Multivariate and Univariate analysis
P53 mutation					
Exon 5	0.0% (0/25)	4.0% (1/25)	0.0% (0/25)	4.0% (1/25)	0.564 (C)
Exon 6	0.0% (0/25)	4.0% (1/25)	12.0% (3/25)	16.0% (4/25)	0.143 (C)
Exon 7	0.0% (0/25)	0.0% (0/25)	0.0% (0/25)	4.0% (1/25)	0.387 (C)
Exon 8	0.0% (0/25)	8.0% (2/25)	24.0% (6/25)	20.0% (5/25)	0.045* (C)
Exon 9	0.0% (0/25)	0.0% (0/25)	4.0% (1/25)	4.0% (1/25)	0.564 (C)

* = significant test; ** = significant most frequent; *** = significant less frequent; # = no localized significant modality with significant level < 0.05; C = multicomparison chi-square test; NGM = Normal gastric mucosa; GCA = active chronic gastritis; KG = gastric cancer; ML = Malt lymphoma; p=p-value

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9. Scientific products

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Helicobacter pylori and Epstein-Barr virus infection in gastric diseases: Correlation with IL-10 and IL1RN polymorphism.
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F. Lorusso, M. P. Caleca, C. Bellavia,D. Pistoia, S. Gallina, R. Speciale, F. Dispenza, **T. Fasciana**, G. Capra.
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Teresa Fasciana, Nicola Serra, Giuseppina Capra, Chiara Mascarella, Maria Rosa. Simonte, Sara Cannella, Miriam Sciortino, Letizia Scola, Dario Lipari, Anna Giammanco.
In patients with Helicobacter pylori and Epstein-Barr virus coinfection: there is a correlation with IL-10 and IL1RN polymorphism?
46° Congresso SIM (Palermo 26-29 Settembre)

Teresa Fasciana, Giuseppina Capra, Chiara Mascarella, Anna Giammanco.
44° Congresso SIM (Pisa 25-28 Settembre)
Coinfection of Helicobacter pylori and Epstein-Barr virus in patients with gastric diseases in Southern Italy.
Selected as best poster in the section "Interaction host-microorganisms"

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