UNIVERSITÀ DEGLI STUDI DI PADOVA

CORSO DI LAUREA MAGISTRALE IN MEDICINA E CHIRURGIA

DIPARTIMENTO DI MEDICINA DIMED

U.O.C di Anatomia Patologica

Direttore: Ch.mo Prof. Angelo Paolo Dei Tos

Tesi di Laurea Magistrale

ASSOCIATON BETWEEN EPSTEIN-BARR VIRUS, H. PYLORI INFECTION AND TUMOUR INFILTRATING LYMPHOCYTES IN A SERIES OF PERUVIAN GASTRIC ADENOCARCINOMAS

Relatore:

Ch.mo Prof. Matteo Fassan

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1. ABSTRACT

Background. Helicobacter pylori (HP) and Epstein-Barr virus (EBV) infections have been recognized as possible triggering factors for gastric cancer (GC). Their involvement in the process of gastric carcinogenesis has been extensively confirmed by numerous studies based on different pathogenetic mechanisms. Recent publications focusing on epigenetic analyses and infection-related histopathological features associated with the two infectious agents, suggest they may serve as predictive markers for future immune-modulating therapies. Tumour-infiltrating lymphocytes (TILs) have also been identified as predictive biomarkers for immunotherapy in several types of malignant tumours including gastric adenocarcinoma.

Aim of the study. The purpose of this study is to analyse the association of TILs levels with HP and EBV infection in gastric cancer.

Materials and methods. A total of 98 resected GC Peruvian cases were included in the study. Analysis for evaluation of TIL levels was performed by a pathologist using hematoxylin and eosin (H&E) staining. The assessment of CD8 and CD3 positive T-lymphocyte density (by immunohistochemical staining) was instead calculated using software. HP detection in the samples was performed by quantitative PCR (qPCR). In Situ Hybridization (ISH) technique was used for the detection of EBV infection, while gene expression was assessed by qPCR. Methylome analysis of EBV-related genes was performed by the Illumina Infinium MethylationEPIC BeadChip and the methylation status of the genes was detected by PCR.

Results. Regarding TIL levels assessed in the samples, the median was 30%. The percentage of EBV+ detected by ISH was 24.1% while the EBV+ percentage calculated by qPCR technique was 41.8%. EBV-related gene methylation was 70% and HP+ percentage was 58.2%. The following parameters were associated with longer survival: younger age (p=0.024), early stages (p=0.001), HP+ (p=0.036) and low CD8 density (p=0.046).

Higher TIL level was more frequently observed in intestinal subtype ($p<0.001$), grade-2 ($p<0.001$), EBV PCR+ ($p=0.001$) and methylation of EBV-related genes ($p=0.007$). Higher TIL and EBV positive cases share 8 genes with similarly methylated status in the metabolomic analysis. Higher CD8 density was associated with EBV PCR+ ($p=0.012$) and HP- ($p=0.005$).

Conclusions. HP infection and lower CD8 T-lymphocyte density predict longer survival. Higher levels of TILs in gastric cancer are associated with EBV+ and methylation status of EBV-related genes. Conversely, lower CD8 T-lymphocyte infiltration is associated with HP+ in GC.

RIASSUNTO

Premesse. Le infezioni da Helicobacter pylori (HP) e da virus di Epstein-Barr (EBV) sono state identificate come possibili fattori scatenanti il cancro gastrico (GC). Il loro coinvolgimento nel processo di carcinogenesi gastrica è stato ampiamente confermato da numerosi studi sui differenti meccanismi patogenetici. Recenti pubblicazioni incentrate sulle analisi epigenetiche e sulle caratteristiche istopatologiche correlate alle infezioni associate ai due agenti infettivi, suggeriscono che potrebbero fungere da marcatori predittivi per le future terapie immunomodulanti. Anche i linfociti infiltranti il tumore (TIL) sono stati identificati come possibili biomarcatori predittivi per l'immunoterapia in diversi tipi di tumori maligni compreso il carcinoma gastrico.

Scopo dello studio. Lo scopo di questo studio è di indagare l'associazione dei livelli di TIL con l'infezione da HP ed EBV nel cancro gastrico.

Materiali e metodi. Sono stati inclusi nello studio un totale di 98 campioni di GC resecati da 98 pazienti peruviani. L'analisi per la valutazione dei livelli di TIL è stata eseguita da un patologo utilizzando la colorazione istologica con ematossilina-eosina (H&E). La valutazione della densità dei linfociti T CD8 e CD3 positivi (mediante colorazione immunoistochimica) è stata invece calcolata con un software. La rilevazione dell'HP nei campioni è stata eseguita mediante PCR quantitativa (qPCR). Per la rilevazione dello stato d'infezione da EBV è stata utilizzata la tecnica di ibridazione in situ (ISH), mentre l'espressione genica è stata valutata mediante qPCR. Lo stato di metilazione dei geni correlati a EBV è stato rilevato mediante PCR mentre l'analisi del metiloma è stata eseguita utilizzando il kit di Illumina Infinium MethylationEPIC BeadChip.

Risultati. Per quanto riguarda i livelli di TIL valutati nei campioni, la mediana era del 30%. La percentuale di EBV+ rilevata mediante ISH è stata del 24,1%, mentre la percentuale di EBV+ calcolata mediante tecnica qPCR è stata del 41,8%. La metilazione genica legata all'EBV era del 70% e la

percentuale di HP+ del 58,2%. I seguenti parametri sono stati associati a una sopravvivenza più lunga: età più giovane (p=0,024), stadi precoci (p=0,001), HP+ (p=0,036) e bassa densità di CD8 (p=0,046). Un livello più elevato di TIL è stato osservato più frequentemente nel sottotipo intestinale (p<0,001), nel grado di stadiazione 2 (p<0,001), nella EBV PCR+ (p= 0,001) e nella metilazione dei geni correlati all'EBV (p=0,007). I casi con TIL più elevati e quelli positivi all'EBV condividono 8 geni con uno stato di metilazione simile nell'analisi metabolomica. Una maggiore densità di CD8 è stata associata a EBV PCR+ (p=0,012) e HP- (p=0,005).

Conclusioni. L'infezione da HP e una minore densità di linfociti T CD8 predicono una sopravvivenza più lunga. Livelli più elevati di TIL nel carcinoma gastrico sono associati a EBV+ e allo stato di metilazione dei geni correlati a EBV. Al contrario, una minore infiltrazione di linfociti T CD8 è associata a HP+ nel GC.

2. INTRODUCTION

2.1. PATHOLOGY OF GASTRIC CANCER (GC)

Gastric cancer (GC) is a malignant epithelial tumour of the gastric mucosa with glandular differentiation. Gastric cancers are a histologically heterogeneous group of neoplasms that arise in epidemiological and molecular contexts characteristic of this type of neoplasm [1].

Clinically, gastric cancer may be asymptomatic in the early stages while symptoms such as dysphagia, asthenia, weight loss and vomiting may appear in the advanced stages of the disease. As for the main hereditary syndromes affecting the stomach, these are: hereditary diffuse gastric cancer, gastric adenocarcinoma with proximal polyposis of the stomach and familial gastric cancer [2].

Gastric adenocarcinoma accounts for approximately 95% of all malignant stomach cancers [2,3]. Thus, most gastric cancers are adenocarcinomas, which may arise from the glands of the most superficial, or mucosal, layer of the stomach. Therefore, unless otherwise stated, in this thesis, when referring to gastric cancer (GC), we will mainly refer to adenocarcinomas.

However, there are other cancers that arise from the stomach, including lymphomas of the mucosa-associated lymphoid tissue, which originate from the lymphoid tissue of the stomach, and leiomyosarcomas, which originate from the muscles surrounding the mucosa [5].

The stomach is divided into several anatomical sub-areas, including the heart, fundus, body, pylorus and antrum. These areas are distinguished by histological differences, anatomical demarcations, or both. A distinction that will be emphasised in this thesis is that between adenocarcinomas arising from the cardia (cardia gastric adenocarcinoma) and those arising in other parts of the stomach (non-cardia gastric adenocarcinoma), as they have different epidemiological patterns and causes.

The etiology of stomach cancer is multifactorial; several risk factors are involved in the development of this neoplasm, including genetic factors. Among the risk factors, Helicobacter pylori plays a significant role.

Numerous studies and trials have confirmed the hypothesis that gastric adenocarcinoma develops through a multi-stage process that begins with chronic gastritis triggered mainly by Helicobacter pylori and progresses through atrophy, intestinal metaplasia and dysplasia (intraepithelial neoplasia) to carcinoma.

Various genetic factors are counted among the risk factors. Loss of E-cadherin expression, due to a mutation in the cadherin 1 (*CDH1*) gene, is the primary carcinogenic event in hereditary diffuse gastric cancer. Proximal gastric adenocarcinomas, on the other hand, probably arise from gastro-esophageal reflux or Helicobacter pylori gastritis [1].

2.2. CLASSIFICATION

There are several classifications of gastric adenocarcinomas: (a) clinical-pathological: it divides gastric carcinomas into early GC and advanced GC; (b) histological: it divides GCs into intestinal or diffuse, this classification considers growth patterns (expansive or infiltrative) and certain histo-types that identify different tumour forms; (c) molecular [4]. The histological classifications according to Lauren's and according to WHO will be briefly analysed later. It is crucial, when classifying GC, to understand how far the carcinoma infiltrates the mucosa. In the past, Kodama's 1983 Japanese endoscopic classification of gastric cancer growth patterns was used, but this classification is now outdated.

If the tumour remains confined to the lamina propria, before the muscolaris mucosae the likelihood of spread is very low, on the contrary if it invades the submucosa the spread is more frequent [3].

Gastric adenocarcinomas can be also classified according to macroscopic appearance into: (a) protruding: the tumour is polypoid or fungiform; (b) penetrating: the tumour is ulcerated; (c) superficial diffuse: the tumour extends along the mucosa or superficially infiltrates the stomach wall; (d) linitis plastica: the tumour infiltrates the stomach wall with an associated fibrous reaction resulting in a rigid leathery stomach; (e) miscellaneous: the tumour has at least one or more than 2 of the features of the other types.

Prognosis is better in protruding tumours than in infiltrating tumours because protruding tumours become symptomatic earlier [3]. Another classification instead stratifies GC adenocarcinomas into two main histological types: diffuse and intestinal [4]. These two types of gastric adenocarcinomas look different under the microscope, differ in sex ratio, age at diagnosis and other epidemiological aspects that characterise and differentiate them from each other.

Laurén's histological classification

The intra- and inter-tumour heterogeneity of gastric tumours is considerable, giving rise to numerous classifications describing their appearance. Among the most widely used are: Laurén's and the World Health Organization (WHO) classification [5]. According to Lauren's classification, gastric adenocarcinoma is classified into two main histological types:

- the intestinal type which accounts for 55% of cases, is typically found in elderly patients and men, and is formed by more or less differentiated glands with mucin production;
- the diffuse type, in 25% of cases, in young patients and mainly women, has a diffuse growth with typical signet ring cells (Table 1).

Diffuse carcinomas are poorly differentiated and consist of solitary or poorly cohesive tumour cells in the absence of gland formation. In contrast, intestinal carcinomas are mostly moderately diffuse and form glandular structures reminiscent of colorectal adenocarcinomas, which explains the name of the subtype [6].

Since its establishment in 1965, Laurén's classification of gastric adenocarcinoma has been the most widely used and most studied of all gastric adenocarcinoma classification systems. Laurén divided the histology of gastric carcinoma into the two groups listed above, namely the intestinal type and the diffuse type; however, the indeterminate type was later included to describe a histology not common to the two described above [3,4].

Signet's ring cell carcinoma results from this classification, included in the diffuse type. Most studies have shown that the intestinal type is the most common of the gastric neoplasms, followed by the diffuse type and finally the indeterminate type [5-7]. The intestinal type has also been shown to be associated with intestinal metaplasia of the gastric mucosa and there is a clear correlation with the presence of Helicobacter pylori. In some studies, the incidence of the diffuse type has been found to be higher in younger and female patients [8], this evidence may suggest that there are distinct tumour development pathways for intestinal adenocarcinoma and diffuse type of stomach cancer.

WHO histological classification

Published in 2010, the World Health Organization (WHO) classification appears to be the most detailed of all patho-histological classification systems. It is noteworthy that the WHO classification includes not only adenocarcinoma of the stomach, but also all other types of gastric cancer of lower frequency, with an exhaustive list [30]. The type of gastric adenocarcinoma is divided into several subgroups, including papillary, tubular, mucinous and mixed carcinoma, which can be compared to the indeterminate type of Laurén's classification (Table 1).

Gastric adenocarcinoma of the poorly cohesive type includes Signet ring cell carcinoma. All other classified gastric adenocarcinomas may be classified as uncommon due to their low clinical significance. Interestingly, in the WHO classification, the most common type of gastric cancer is tubular adenocarcinoma, followed by papillary and mucinous types second in frequency. Signet ring cell carcinoma accounts for a frequency of about 10% of gastric cancers and is characterised by the presence of Signet ring cells in more than 50% of the tumour tissue [31-32].

Table 1. *Laurén and World Health Organization classification.*

Laurén classification	World Health Organization classification
Intestinal type	Papillary adenocarcinoma Tubular adenocarcinoma Mucinous adenocarcinoma
Diffuse type	Signet-ring cell carcinoma and other poorly cohesive carcinomas
Indeterminate type	Mixed carcinoma Adenosquamous Carcinoma Squamous cell carcinoma Hepatoid adenocarcinoma Carcinoma with lymphoid stroma Choriocarcinoma Carcinosarcoma Parietal cell carcinoma Malignant rhabdoid tumour Mucoepidermoid carcinoma Paneth cell carcinoma Undifferentiated carcinoma Mixed adeno-neuroendocrine carcinoma Endodermal sinus tumour Embryonal carcinoma Pure gastric yolk sac tumour Oncocyte adenocarcinoma

2.3. MOLECULAR CHARACTERIZATION OF GASTRIC ADENO CARCINOMA

Histologically based classification systems for gastric cancer have little clinical utility, making the development of analysis of its molecular and clinical characteristics, which has been complicated by histological and etiological heterogeneity because this could guide patient therapy an urgent priority.

The objectives of the study of The Cancer Genome Atlas (TCGA) were to develop a robust molecular classification of gastric cancer and to identify dysregulated pathways and candidate drivers of distinct classes of gastric cancer for testing targeted therapies [43].

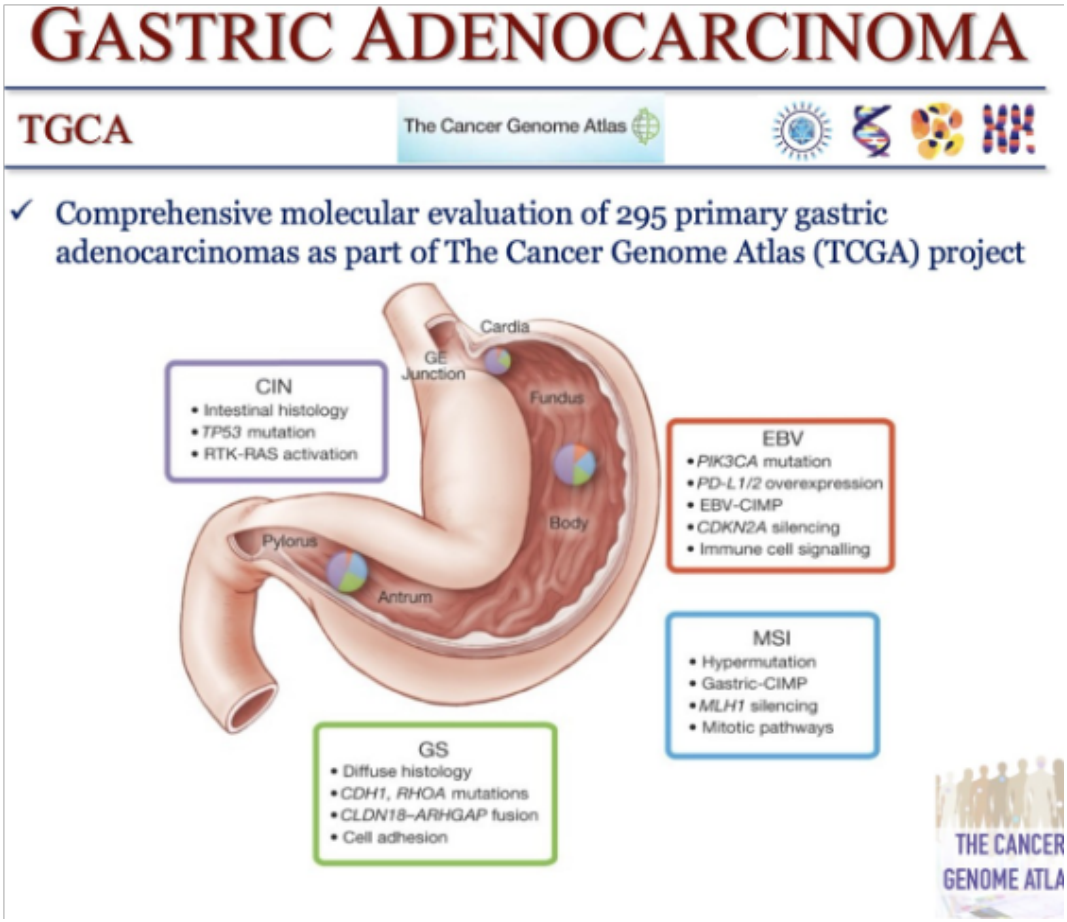


Figure 1. *The Cancer Genome Atlas [43].*

Molecular classification of gastric adenocarcinoma

In recent years, there has been a growing interest in a molecular-based classification of gastric carcinomas [44]. The Cancer Genome Atlas decided to perform a comprehensive mutational analysis of the tumour tissues of 295 patients with gastric adenocarcinomas, prior to treatment (chemotherapy or radiotherapy), and identified four main groups based on gene expression patterns:

- EBV-associated GC: have specific related mutations and have an over-expression of PD-L1-2 responding well to immunotherapy (Table 2).

Table 2. *EBV-Associated Gastric Carcinoma.*

Clinical	• 5-10% of all gastric adenocarcinomas • Predominantly men (2:1), mean age 65 years • Cardia/ Fundus/ Body >> Antrum
Histology	• 80% of gastric cancers with lymphoid are EBV positive
Molecular	• High levels of DNA methylation • PIK3CA mutations 80% • CDKN2A silencing • Amplification of PDL-1 and overexpression by IHC
Prognosis	• Improved prognosis • Potential role for PDL-1 immunotherapy

- MMR-deficient GC: have a high TMB (tumour mutational burden) making them respond well to immunotherapy. This subgroup comprised 20% of the samples and was defined as microsatellite unstable tumours. These tumours had elevated mutation rates, with affected genes that included those commonly observed in other cancers a, such as *PIK3CA, ERBB3,*

ERBB2 (HER2) and *EGFR*. Is present MLH1/PMS2/MSH2/MSH6 protein deficiency (Table 3).

Table 3. *MMR-deficient Gastric Carcinoma.*

Clinical	• 10-200% of all gastric adenocarcinomas • Women > men; older age (median 72 years) • Antrum >> Body/Fundus or Cardia
Histology	• Intestinal type (tubular, mucinous, papillary) • Lymphoid stroma • May be associated with Helicobacter pylori infection
Molecular	• Most are sporadic due to loss of MLH1/PMS2 from MLH1 promoter hypermethylation • Mutations in *PIKC3A* 42%, *ERBB3* 26%
Prognosis	• Improved prognosis (most studies) • Role for PD-1 blockade immunotherapy

- CIN (chromosomal instability): this third subgroup, comprised 50% of the samples, represented chromosomally unstable tumours. These tumours exhibited marked aneuploidies, amplification of receptor tyrosine kinases, VEGFA and cell-cycle mediators (*CCNE1, CCND1* and *CDK6*), and p53 mutations. These tumours presented more frequently in the gastro-esophageal junction/cardia) [44-47].

- Genomically stable GC: the remaining 20% of the samples were classed as genomically stable because they lacked the features of the other three tumour subtypes, it is characterised by few mutations and a stable genome. These tumours were predominantly of the diffuse histological Laurén's variant and thus with a very aggressive clinical disease phenotype (Table 4) [44-47].

Table 4. *Genomically stable Gastric Carcinoma.*

Clinical	• 20% of all gastric adenocarcinomas • Diagnosed at an earlier age (median 59 years) • Occurs in all sites of the stomach
Histology	• Poorly cohesive gastric carcinoma (diffuse type) including those with signet ring cell differentiation
Molecular	• *CDH1* (E-cadherin) mutation, including patients' hereditary diffuse gastric cancer (HDGC) • *RHOA* mutations • CLDN18-ARHGAP fusion
Prognosis	• Poor prognosis with limited response to chemotherapy compared to other subtypes

2.4. EPIDEMIOLOGY

Gastric cancer in the World

Gastric cancer (GC) is a global health problem, diagnosing more than 1 million people worldwide each year. GC remains the third leading cause of cancer death and is the fourth most common type of cancer, despite declining incidence and mortality worldwide over the past five decades [6].
According to estimates from the GLOBOCAN project of the International Agency for Research on Cancer (IARC) [2], in 2018 there were 1,033,701 new cases of gastric cancer (representing 5.7% of all diagnosed cancer cases) and 782,685 deaths related to gastric cancer worldwide. [7]
Therefore in 2018, gastric cancer was the fifth most commonly diagnosed type of cancer and, again with reference to the same year, it is estimated to be responsible for 8.2% of all cancer deaths, accounting for 1 in 12 deaths and making it the third most common cause of cancer-related death after lung and colorectal cancers, whose percentages are 18.4% and 9.2%, respectively [8,9].

Incidence, mortality and geographical variability

The incidence of GC varies up to tenfold depending on geographic region, suggesting that environmental or genetic factors influence clinico-pathological features and carcinogenesis.
Gastric cancer is uniformly rare in adults younger than 50 years of age in all populations and countries. GC incidence rates increase progressively with age and reach a plateau between the ages of 55 and 80. On average, men are more susceptible than women, as GC incidence rates are two to three times higher in men than in women [7-8].
This suggests that the incidence of GC differs by sex, but it is important to emphasise how it is influenced by geographical variability [9-10]. The incidence thus shows enormous geographical diversity: it is observed that more than 50% of new cases occur in developing countries. Areas with the highest likelihood of GC development include regions such as Central and

South America, Eastern Europe and East Asia (China and Japan) [12]. Low-risk regions include Australia and New Zealand, South Asia, North and East Africa and North America. In Europe, the ratio varies between 10-30% [13]. Gastric cancer (GC) is a multifactorial disease for which alternative prevention, considering a proper diet, early diagnosis and follow-up with adequate treatment, leads to a reduction of recorded episodes especially in the young population [16,17]. Actually, GC is rather rare among the young and has a low prevalence in the young population, under 45 years of age, where no more than 10% of patients are affected by the development of the disease [18-20].

In recent years, there has been an increase in the five-year survival rate, which is probably due to early diagnosis using the endoscopic examination method, which allows early diagnosis and resection of the tumour [13].

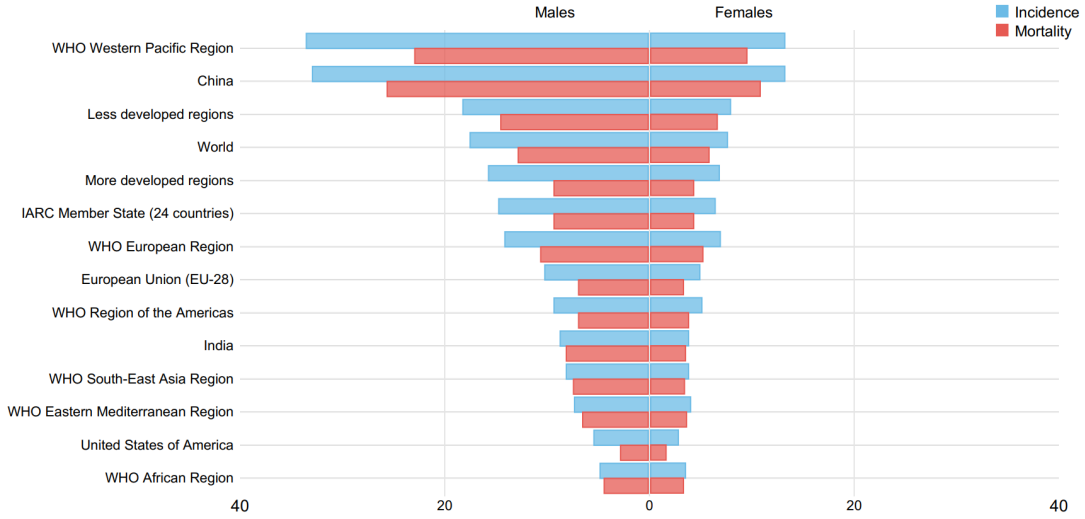


***Figure 2**. Incidence and mortality of gastric cancer in the World].*

Gastric cancer in Peru

The highest incidence rates of GC occur in East Asia and South America. More than two-thirds of cases globally occurred in low- and middle-income countries, according to GLOBOCAN 2018 [14].

In Peru, GC is among the second in males and the third most frequent malignancy in females. Furthermore, it is important to consider that in this country, GC is the third most common cancer and causes the highest absolute number of cancer deaths [14,15].

A figure that characterises the situation in the Andean country of Peru concerns the prevalence of two agents considered carcinogenic for gastric cancer: Helicobacter pylori (HP) and Epstein Barr virus (EBV).

Helicobacter pylori infection in the gastric mucosa has been associated with low socioeconomic status and its prevalence in adults living in Latin American developing countries is between 70% and 80% [20,21].

The HP infection rate is over 60% in the Peruvian population. Epstein-Barr virus infection is more frequent in gastric cancer than in chronic gastritis.

Recent studies suggest that co-infection with HP and EBV may have a synergistic carcinogenic effect and is more frequently found in GC than in chronic gastritis [22,23].

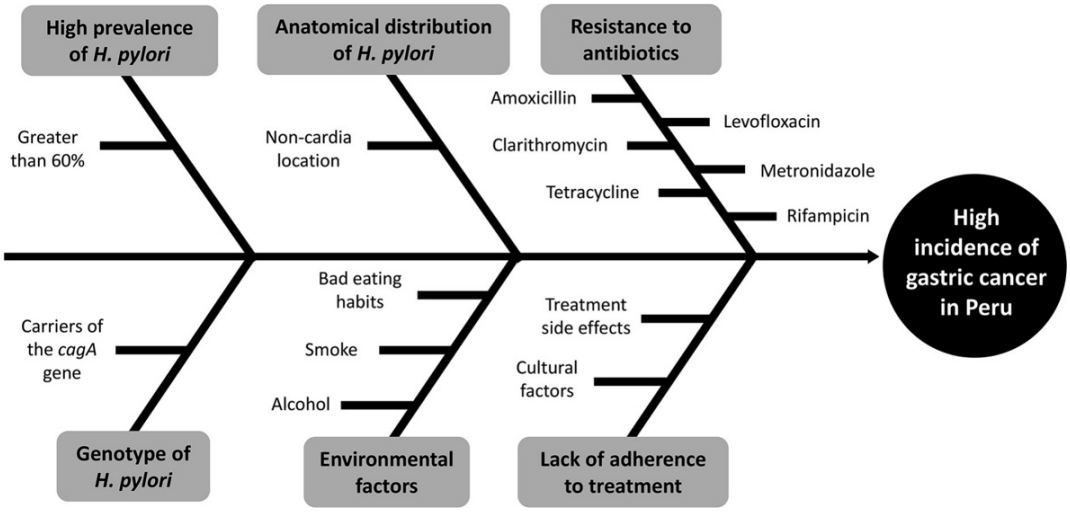


Figure 3. *Factors affecting the incidence of gastric cancer in Peru [24].*

Risk factors

Gastric cancer is a multifactorial disease in that both environmental and genetic factors play a role in its etiology. Some of these risk factors, such as age and gender, are not modifiable, while others, such as smoking and Helicobacter pylori infection, potentially are [24]. Several factors have been studied to have a significant impact on the increased risk of developing GC, such as family history, diet, alcohol consumption, smoking, Helicobacter pylori and Epstein-Barr virus (EBV) infections [30].

Risk factors for cancers arising from the cardia and non-cardia regions of the stomach may be different and are distinguished below (Table 5). Common risk factors for cardia-GC and non-cardia GC include advanced age, male sex, tobacco smoking, radiation and family history. It has been observed that taking aspirin and statins can prevent both cancers.

Factors associated only with cardia-GC, but not with non cardia-GC, include obesity and gastroesophageal reflux disease (GERD). In contrast, exclusive risk factors for non-cardia gastric cancer include HP infection (at least in Western countries), Epstein-Barr virus, low socioeconomic status and likely dietary factors such as low fruit and vegetable consumption and high intake of salty, processed or smoked foods [24].

Chronic HP infection is the leading cause of gastric cancer and accounts for approximately 89% of distal gastric cancer cases worldwide [25,26]. The prevalence of HP infection in adults exceeds 50% in many industrialised countries, however, there is substantial regional variation observed in the distribution of its prevalence. It has been shown that the prevalence of HP infection is higher in Central and South America and parts of Asia and Eastern Europe than in North America, Australia and Western Europe [27]. Up to 10% of gastric cancers can be attributed to less common causes, such as EBV infection, autoimmune gastritis and Menetrier's disease [28,29].

Table 5. *Some prominent risk factors for gastric Cardia and non-Cardia GC [24].*

RISK FACTORS FOR GASTRIC CANCER	
CARDIA	**NON CARDIA**
Age	Age
Male sex	Male sex
Tobacco smoking	Tobacco smoking
Race	Race
Family history	Family history
Low physical activity	Low physical activity
Fiber intake	Fiber intake
Radiation	Radiation
—	Helicobacter pylori
—	Epstein–Barr virus
—	Low socioeconomic status
—	High intake of salty and smoked food
—	Low consumption of fruits and vegetables
Obesity	—
GERD	—

2.5. HELICOBACTER PYLORI (HP) INFECTION

Pathobiology of Helicobacter pylori-induced gastric cancer

Helicobacter pylori (HP) is a Gram-negative bacterium that has been classified as a class I carcinogen for GC development by the World Health Organization since 1994 [33,34]. The genomes of HP are heterogeneous and for this reason encode different virulence factors that differ in the role they play in influencing the clinical outcome of infection.

The damaging mechanisms of HP are:

- the vacuolysing toxin VacA, encoded by the gene of the same name *(vacA)*, which enables the bacterium to obtain nourishment from cells by forming vacuoles in them; the toxin also causes direct damage to the gastric epithelium and stimulates an acute inflammatory process;
- the CagA protein, encoded by the cytotoxin A *(cagA)* associated gene that is introduced into the gastric cell. CagA acts by inducing changes in cell structure, promoting cell proliferation and transformation: this is a mechanism underlying infection-related gastric carcinogenesis [43]. In addition, this protein activates signaling pathways that induce cellular changes as well as proinflammatory cytokine production [44,45].

The proinflammatory potential caused by the cagA-positive and vacA allelic variants of HP may explain the association of HP with severe atrophic gastritis, peptic ulcer and gastric adenocarcinoma [41,42].

Numerous epidemiological studies have shown that HP infection is one of the risk factors for the development of GC. The effect of H. pylori on the process of carcinogenesis has been described by two main mechanisms: (a) an inflammatory reaction caused by HP infection that indirectly affects the gastric mucosa and (b) the result of the direct action of the HP pathogen causing changes to gastric epithelial cells at the epigenetic level [35]. Thus, different virulence factors of H. pylori, such as CagA or VacA, are known to increase the risk of GC development [36]. HP with cagA and vacA correlates

with an increased risk of developing both intense tissue responses (related to the inflammatory response) and premalignant and malignant lesions in the distal stomach [37].

Furthermore, Helicobacter pylori infection compromises the gastric tissue microenvironment, promoting epithelial-mesenchymal transition (EMT) and further progression of gastric carcinogenesis induced by chronic inflammation due to persistent infection caused by the infection [38,39].

Previous studies have revealed for the first time that the stomach microbiota influences the response to cancer immunotherapies and that therefore HP serology could be a powerful tool to tailor cancer immunotherapy treatment. It has also been suggested that the presence of HP infection in GC is associated with a favourable prognosis in relation to lymphocyte density, as will be discussed in this thesis, and that an HP+ GC is responsive to immune checkpoint inhibitors and that this improves prognosis [26, 27].

Prevalence of Helicobacter pylori infection in Peruvian patients with gastric cancer

Certain types of Helicobacter pylori, particularly those positive for the cytotoxin A-associated virulence factor *(cagA)* gene, are more likely to cause GC [48-50]. HP is estimated to cause 65-80% of all cases of gastric cancer, or an estimated 660,000 new cases per year [51, 52].

The association between HP infection in the gastric mucosa and low socioeconomic status has been highlighted. The prevalence of HP in adults living in Latin American countries, including Peru, is between 70% and 80% [40]. In contrast, the prevalence in developed countries is estimated to be around 50% of the total population [46, 47].

The extent of the association between Helicobacter pylori and the incidence of gastric cancer is not entirely certain: it is unclear whether the incidence is subject to underestimation because HP infection and circulating antibody response may be lost with tumour development and therefore, retrospective studies are subject to bias due to the classification of cases as HP negative when they had been infected with HP in the past [51].

Retrospective case-control studies are however limited by the fact that HP infection is necessarily assessed after cancer development, as antibodies may have been lost.

HP does not colonise areas of cancer, or intestinal metaplasia or atrophy and there is evidence that with the development of advanced gastric disease the organism can be eliminated from the stomach over time [53]. With the loss of infection therefore, the level of circulating anti-HP antibodies decreases, and patients with GC may be seronegative to HP even if they have been infected in the past [54].

Assuming an average prevalence of HP of 35% in developed countries and 85% in developing countries, an Odds Ratio of 5.9 (the measure of association between the exposure and the outcome of HP-associated GC) suggests that approximately 65%-80%, respectively, of non-cardia GCs are attributable to HP infection and thus potentially preventable through infection control. However, HP infection does not appear to increase the risk of cardia-GC. In fact, it should be noted that, at least in Western countries, HP is an important risk factor only for non-cardia GC, but not for cardia GC as previously described [51].

It should be noted that a further reason for the underestimation of HP prevalence in GC cases is related to the method of detection of HP infection. In epidemiological studies, Helicobacter pylori infection is usually detected by means of an enzyme-linked immunosorbent assay (ELISA). However, the infection may disappear spontaneously from the mucosa during the progression of atrophy and could lead to a substantial under-detection of the infection and an underestimation of its effect on the risk of gastric cancer (GC). More recent studies using the Western blot test have found a higher relative risk. Indeed, it is known that antibodies detected by Western blot persist longer after the loss of infection [55].

With the Western blot test, almost all non-cardia GCs were classified as Helicobacter pylori positive, and the OR was more than three times higher than that assessed by ELISA, supporting the hypothesis that HP infection is a necessary condition for non-cardia GCs [56,57].

The decline in HP prevalence, which is probably due to improved hygiene conditions and extensive use of antibiotics, may be one of the main reasons for the rapid decline in the incidence of non-cardia GC [59]. Epidemiological studies have shown a significant association between the prevalence of HP infection and contamination of water sources, as the prevalent route of infection in Peru is probably fecal-oral via contaminated water [59].

2.6. EPSTEIN-BARR VIRUS (EBV)

EBV-associated gastric cancer

Apart from HP infection, the second factor associated with GC development is the Epstein–Barr virus (EBV). EBV is a ubiquitous infectious factor: the EBV genome subsists in the tumor cells and transforming EBV proteins are expressed by the tumour cells themselves [60]. EBV-associated carcinoma accounts for 5-20% of gastric carcinomas, is more frequent in males and has predominantly proximal gastric localization (ossicular mucosa). Histologically it is a poorly differentiated neoplasm, and it is characterised by significant inflammation, which in this case is not due to the presence of a large number of mutations, but rather to the massive release of cytokines by immune cells in response to EBV infection. Thus, a major lymphocyte infiltrate develops and, as an associated phenomenon, activation of the PD1-PDL1 axis that inhibits the recognition of tumour cells by T lymphocytes. Immunotherapy therefore acts by inhibiting this axis through binding to PDL1 present on tumour cells [106,107] (Fig. 4).

An IHC (immunohistochemistry) examination shows the presence of the viral Ag EBER (small non-coding RNA) localised in the nucleus of human cells infected with EBV in tumour cells. It is important to note that EBER is not found in chronic gastritis or metaplastic atrophy (earlier steps in the Correa cascade) (Fig. 5). This means that the infection may be the primum movens, which induces the development of the neoplasm, with a mechanism that does not necessarily involve passing through intermediate

histological phenotypes. EBV infection occurring in a subject with a definite lesion may accelerate tumour progression [108,109] (Fig. 4.2).

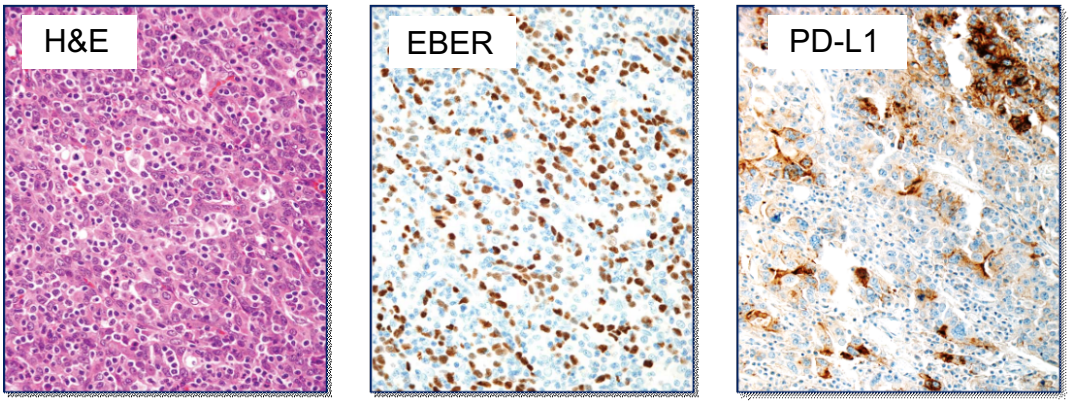


***Figure 4**. EBV-associated gastric adenocarcinomas.*

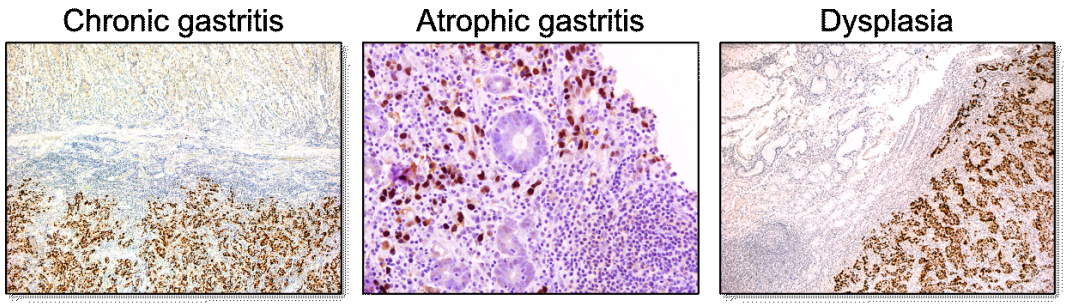


***Fig 4.2** EBER in the multistep cascade.*

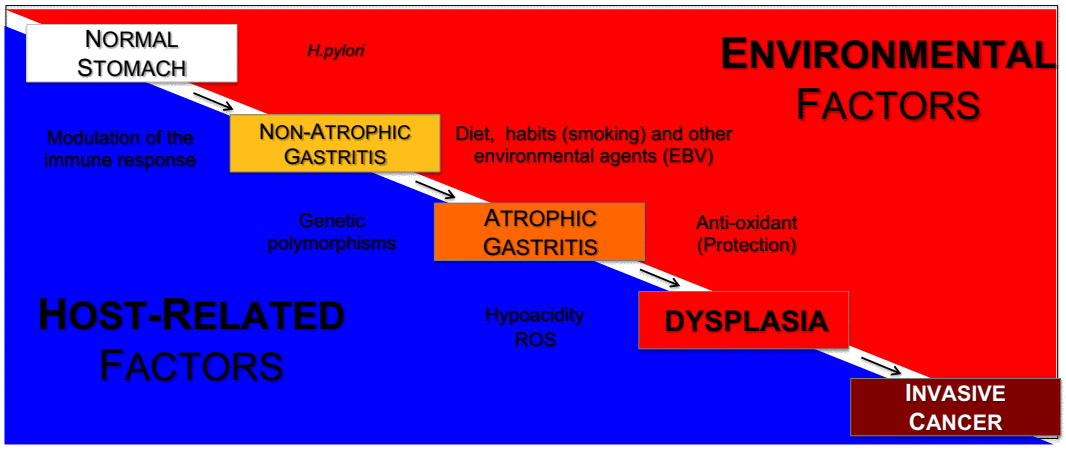


***Figure 5.** The gastric precancerous cascade: Correa's cascade.*

Epstein-Barr virus (EBV) is uniformly present in gastric adenocarcinoma subtype with extensive lymphocyte infiltration [64,65].

EBV infection of B lymphocytes produces a proliferative stimulus for the cells that results in: (a) hyperplasia and expansion of infected B cells with concomitant expansion of reactive T cells; (b) production of antibodies against host and virus proteins due to polyclonal activation of B cells.

The immune response also involves an expansion of CD8+ T cells with an inversion of the CD8/ CD4 ratio. The acute phase is followed by a latency phase of the virus within memory B cells. In this phase, certain viral proteins including LMP-1 and -2, EBNA and EBERs are expressed.

Immunity plays a key role in controlling the proliferation of EBV-infected B cells; when this control fails, the uncontrolled proliferative stimulus will be an element in a multifactorial process leading to neoplastic transformation [63]. EBV causes a strong human leukocyte antigen (HLA) class I–restricted, antigen-specific CD8+ cytotoxic T-lymphocyte (CTL) response in infected individuals [66]. This response is believed to play an important role in controlling the virus during both primary infection and in the long-term carrier state.

Recent studies on the immunophenotypic characterisation of EBV-associated gastric carcinoma have shown that (a) lymphocytes infiltrating EBV+ tumour nests are predominantly CD8+ T cells, many of which express perforin; (b) 4 times more CD8+ T cells infiltrate EBV+ cases than EBV- cases (c) the marker index for Ki-67, a proliferation-associated antigen, in CD8+ cells is 4 times higher in EBV+ cases than in EBV- cases; (d) there is evidence of close contact between carcinomas and CD8+ cells; (e) all EBV+ and EBV- gastric carcinoma cells express MHC class I, whereas markers of immunological activation, such as MHC class II, ICAM-1 and Fas/Apo-1 expression, are more evident in EBV+ cases [67].

Extensive lymphocyte infiltration is a consistent feature of EBV+ gastric carcinoma and may be associated with the generally favorable prognosis of EBV+ gastric carcinomas [68-71].

T-cells may infiltrate, attracted by cytokines such as IL-1 secreted by gastric cancer cells, or may expand clonally in response to certain antigens presented by the carcinoma tissue [72].

EBV is associated with CpG dinucleotide hypermethylated pattern and longer survival [23, 24], more specifically, the EBV-associated gastric adenocarcinoma shows global CpG island methylation of the promoter region of various cancer-related genes [73,74]. Epigenetic alterations, such as methylation of the CpG islands of the promoter region of various cancer-associated genes, are now considered to be one of the main mechanisms of development and progression of gastric carcinoma [75,76]. In the study of EBV-associated gastric carcinoma, it was found that global DNA methylation and subsequent gene silencing typically occur in this specific type of gastric carcinoma [77-80]. Both p16 and E-cadherin expression were decreased or abnormal in association with promoter methylation of each corresponding gene in EBV-associated gastric carcinoma, but this correlation is still equivocal in EBV-negative gastric adenocarcinoma [77,78,79]. Since EBV-associated GC shows distinct clinicopathological features, such as male predominance, preferential presence of the gastric body and a diffuse histology, it is reasonable to assume that high or low CpG island methylation status is closely associated with some clinic - pathological features characteristic of EBV-negative gastric carcinoma [78-81]. Recent studies indicate that EBV presence could also predict efficacy of checkpoint immune inhibitors in GC [82].

Prevalence of Epstein-Barr virus in Peruvian patients with gastric cancer

About 20% of all cancer cases have a virus as an etiological factor and in this regard, Epstein-Barr virus (EBV) is associated with several types of malignancies, including gastric cancer [85].

Among all types of gastric cancer, adenocarcinomas account for about 95% of cases [9] and about 10% of these are associated with EBV [87]. Thus, about 10% of GCs have been described as EBV-positive, but there is

insufficient evidence for a distinct etiological role of EBV in the development of GCs [61]. EBV-positive gastric carcinomas differ according to patient characteristics such as gender, age or anatomical subsite and geographical location [62]. EBV-positive cancers comprise about 9% of all cancers classified by The Cancer Genome Atlas (TCGA) network, but the different prevalence according to geographical region should be noted: in Asia the prevalence is about 15% while in Europe it is around 5%[6].

EBV-associated gastric cancer affects more men and, globally, ranks first in terms of virus-associated deaths. The number of EBV-related deaths in GC is exponentially proportional to age, especially after the age of 60 years [86]. EBV can reach the stomach through saliva as a free viral particle in virus-infected B lymphocytes and in infected oropharyngeal epithelial cells [88]. The stomach is generally classified into two topographical sub-sites, the cardia (upper part of the stomach) and the non-cardia (lower part of the stomach) [9]. EBV loses infectivity more easily once it reaches the stomach, which would explain the higher prevalence of EBV-associated gastric cancers in the upper part of the organ, i.e. the cardia-GC subtype [89].

Association between coinfection of Helicobacter pylori and Epstein-Barr Virus with gastric cancer

The association of HP and EBV co-infection with GC could be explained by the preclinical finding (based on the infectious mechanisms of the two pathogens) of a synergy of both pathogens to induce carcinogenesis. The dual infection with HP and EBV could act synergistically to increase IL-17 expression and maintain an inflammatory state that increasingly damages the gastric mucosa [90-95]. The results of some studies indicate that HP infection and its virulent strains are frequent and widespread in Peruvian regions, these studies conducted on the Peruvian population showed that co-infection of HP and EBV could have a synergistic carcinogenic effect and that this combined effect would be more frequent in GC than in chronic gastritis (CG) [83,84].

The persistence of a Helicobacter pylori and Epstein-Barr virus (EBV) package promotes aggressive GC, and the molecular mechanisms underlying HP- and EBV-mediated GC aggressiveness are not well characterised.

Through a study of the molecular mechanism involved in HP- and EBV-driven gastric epithelial cell proliferation, it was shown that co-infection is significantly more advantageous for pathogens, as it creates a favourable microenvironment for increased pathogen-associated gene expression. EBV latent genes (ebna1 and ebna3c) are more highly expressed in co-infection than in solitary EBV infection. The HP-associated genes 16S rRNA, *cagA* and *vacA* are also highly expressed during co-infection compared to HP alone [9]. Furthermore, EBV and HP together create a microenvironment that can induce cell transformation and oncogenesis through dysregulation of cell cycle regulatory genes, GC markers, oncosopressor genes and anti-apoptotic genes in infected gastric epithelial cells through gankyrin [19]. Increased expression of gankyrin, which is a small oncoprotein, modulates several cell signaling pathways, leading to the initiation of the mechanism of oncogenesis. Gankyrin shows an expression pattern similar to that of ebna3c both at the level of transcription and expressed proteins, suggesting a possible correlation. These results suggest a new insight into the interaction between the two oncogenic agents (HP and EBV) that would lead to increased carcinogenic activity in gastric epithelial cells through overexpression of gankyrin. EBV and HP would thus mediate an increased expression of gankyrin, which is able to dysregulate the expression of genes associated with the development of the neoplastic process, or of tumour suppressor genes, or those in response to DNA damage or pro-apoptotic genes [137].

2.7. TUMOUR-INFILTRATING LYMPHOCYTES (TILs) AND ITS PROGNOSTIC VALUE FOR GASTRIC CANCER

The tumour microenvironment (TME) is the internal environment of malignant tumour progression, host anti-tumour immune response and

normal tissue destruction; all these processes occur in the TME [96]. Therefore, the TME is emerging in importance as a crucial factor in understanding the relationship between the immune system and tumour [97, 98]. Tumour-infiltrating lymphocytes (TILs) are an important component of the TME and represent the action exerted by the host's anti-tumour immune response [99-100]. Several studies in gastric cancer have suggested that TILs and their components may guide the selection of patients who are candidates for immunotherapy and checkpoint blockade therapy [101, 102]. This suggests that the evaluation of TILs in daily pathological diagnosis is becoming increasingly important. In particular, quantitative analysis of immune gene expression has shown a high correlation with TILs [103], suggesting that the assessment of TILs may be a viable, less expensive and readily available alternative to genome-wide analysis methods [104].

For instance, a higher level of tumour-infiltrating lymphocytes (TILs) has been shown to correlate with longer survival in certain tumour types, including gastrointestinal and breast cancer [105], which are characterised by a greater response to checkpoint inhibitors.

Evaluation of the immune response to cancer is becoming increasingly important, as this response has been shown to have significant prognostic implications. The study of this immune response is gaining increasing recognition, becoming a subject of study along with immunotherapies that are being evaluated and implemented in different tumour types [107].

T-lymphocyte-mediated adaptive immunity is believed to play a key role in antitumour immunity. Recent results show that high densities of immune cells related to adaptive immunity, i.e. total T lymphocytes, both cytotoxic T cells and memory cells, are associated with increased survival, which would indicate that adaptive immunity plays a key role in preventing tumour progression [131].

In contrast, studies have not found a positive impact on patient survival from tumour-infiltrating B cells; in fact, previous studies would have concluded that B lymphocytes and humoral immunity are instead associated with tumour progression.

From the studies performed, it is hypothesised that the prognostic role of TILs is mainly due to a decrease in metastatic potential and that therefore TIL density is related to the presence of lymph node metastases but not to the depth of tumour invasion [132,133].

This hypothesis is supported by the following evidence: (a) firstly, clones with metastatic potential usually contain higher amounts of aberrantly expressed proteins, including those contributing to metastasis, which act as tumour-associated antigens that are more easily recognised and destroyed by in situ immune reactions and thus by lymphocytes; (b) secondly, a high density of TILs is indicative of a healthy immune system, whereby immune reactions occurring in the lymph nodes may exert an adequate function against tumour cells that have converged in the lymph nodes; (c) thirdly, the tumour burden of metastatic foci in the lymph nodes is smaller in volume than that of primary foci and thus metastatic foci are more likely to be completely destroyed by the immune reaction [134].

Methods of evaluating TIL levels

Methodologies for assessing TILs have been diverse and only a few studies have evaluated the relationship between TIL levels and clinicopathological features of tumours [97-100]. Recently, the International Immuno-Oncology Biomarkers Working Group (IBWG) has proposed a standardised methodology to assess TILs in different malignancies, including gastrointestinal malignancies [114]. Furthermore, recent reports have described that the density of immune cell subpopulations belonging to TILs, such as T lymphocytes and activated macrophages, could be sensitive to epigenetic associations [105,106]. To perform the study of this thesis, a comprehensive analysis method was developed to investigate the relationship between TIL level based on IBWG methodology [97] and EBV infection assessed by ISH, qPCR and methylation of EBV-related genes in a cohort of Peruvian patients with resected GC. Furthermore, the density of immune cell subpopulations (CD3- and CD8-positive lymphocytes) was

calculated and their specific relationships with the mentioned infections and tumour characteristics were assessed [114].

2.8. DNA METHYLAYION AS A MOLECULAR BIOMARKER IN GASTRIC CANCER

DNA methylation is a process that induces gene silencing and heterochromatin formation and is involved in the regulation of gene expression, genomic imprinting, X-chromosome inactivation and silencing of centromeric regions. This mechanism is also involved in the host defence mechanism, silencing DNA of exogenous origin and preserving the integrity of the genome from the action of transposons and retroviruses [135].

There are three ways in which DNA methylation can contribute to neoplastic development: (a) hypomethylation of proto-oncogenes that are activated into oncogenes, (b) ipermethylation of oncosopressor genes resulting in loss of function and (c) directed mutagenesis.

Furthermore, DNA methylation patterns can alter the expression of cancer-associated genes, which is why aberrant DNA methylation has proven to be a promising biomarker for early cancer diagnosis [136].

2.9. IMMUNOTHERAPY IN GASTRIC CANCER

The immune system, due to the central tolerance processes it undergoes, cannot recognise cellular elements and self-structures. However, when a tumour with many genetic mutations is present, new antigens and neo-epitopes are formed. These constitute an alarm bell for the immune system as it recognises these cells as non-self and thus tries to attack the neoplasm. We distinguish two basic phases of this immune attack revolving around the T cell:

- Lymph node phase in which the APCs after phagocytosing cell debris or other material derived from the tumour cell, process and present this tumour antigen on the MHC class 2. These APCs then migrate to the lymph node where they must activate the T cell (with the antigen specific TCR) allowing its clonal expansion (activation);

- These T lymphocytes then, at the peripheral level, can recognise the neoplasm and destroy it directly by exploiting the mechanisms of cell-mediated immunity [116,117].

Thus, for the immune system to function, both activation at the lymph node level of the T lymphocyte with the dendritic cell and direct contact of the T lymphocyte with the tumour cell are required. However, for both of these steps there are negative modulation signals called immunological checkpoints. At the lymph node level, between the APC and the T lymphocyte there is the CTLA-4 molecule (Cytotoxic T-Lymphocyte Antigen 4) expressed by the T lymphocyte, which dampens the activation signal provided by the APC via the B7 molecule (which initially bound CD28 on the T lymphocyte providing an activating signal) [118]. At the peripheral level, the PD-1/PD-L1 axis prevails as an inhibitory signal with PD-1 (Programmed cell death protein 1) expressed on the T lymphocyte and its binding partner on the tumour cell. This is always a natural intrinsic (protective) blocking mechanism, since if a complete attack on the tumour were to occur, the patient would still risk death. A neoplasm gains an evolutionary advantage when the expression of PD-L1 significantly increases, enabling it to evade immune control [119].

The immunotherapeutic drugs currently used in clinical practice are monoclonal antibodies inhibiting these immunological checkpoints. These drugs have the potential to reactivate the anti-tumour immune response [116-120]. The study of PDL1 expression makes it possible to consider introducing immunotherapy into the therapeutic protocols of gastric adenocarcinomas. PD-L1 is a receptor up-regulated by tumour cells as an escape from the immune system. The first evidence of PDL1 expression in gastric carcinoma came from the KEYNOTE 012 study (Clinicaltrials.gov identifier NCT01848834) in 2016. This trial evaluated the use and efficacy of Pembrolizumab in the context of metastatic stage PDL1-positive gastric and gastroesophageal junction adenocarcinomas [121]. In a subsequent trial (KEYNOTE 059) it was evaluated that PDL1 overexpression was found

in 57% of patients with gastric carcinoma and that they responded to immunotherapy [120-123]. In 2017, the FDA (Food and Drug Administration) approved the use of Pembrolizumab in the context of patients with metastatic gastric cancer who had not responded to other therapies [124].

Today, there are anti-PD-1 (Pembrolizumab and Nivolumab) and anti-PD-L1 (Atezolizumab and Durvalumab) drugs available, which act on the T lymphocyte and the neoplastic cell respectively, removing the activating blockade on the T cell, which is thus free to destroy this cell. The step at the lymph node level can also be modulated using anti-CTLA-4 drugs (Ipilimumab) that allow greater clonal expansion of anti-tumour T-cells and thus greater T-cell infiltration into the neoplasm and subsequent destruction of the neoplastic cells [125,126]. Research in the field of immunotherapy is aimed at identifying markers predictive of response to therapy. The most important biomarker is the number of mutations: a Tumour Mutational Burden (TMB) is linked to a greater response to these drugs. This is because with increased mutations comes increased neoantigen load and increased T-cell activation. These tumours, by virtue of their high neoantigen load, are recognised by the T cells leading to a high inflammatory infiltrate initially only in the periphery, at the level of the invasion front, but subsequently with the blockage of the PD1/PD-L1 axis there is infiltration by T lymphocytes inside the neoplastic mass leading to its destruction. In fact, the neoplastic cells sense the attack by the T lymphocytes via IFN-γ signalling and in response, they intensify PD-L1 expression (Fig. 6). In the absence of drugs inhibiting this binding, T lymphocytes are prevented from performing their cytotoxic action. In the figure below (Fig. 7), a comparison is shown between a tumour with microsatellite instability (MSI), with many lymphocytes positive for both PD1 and PD-L1 at the front of the neoplasm, and a tumour with microsatellite stability (MSS), which on histological observation appears 'silent' [127-130].

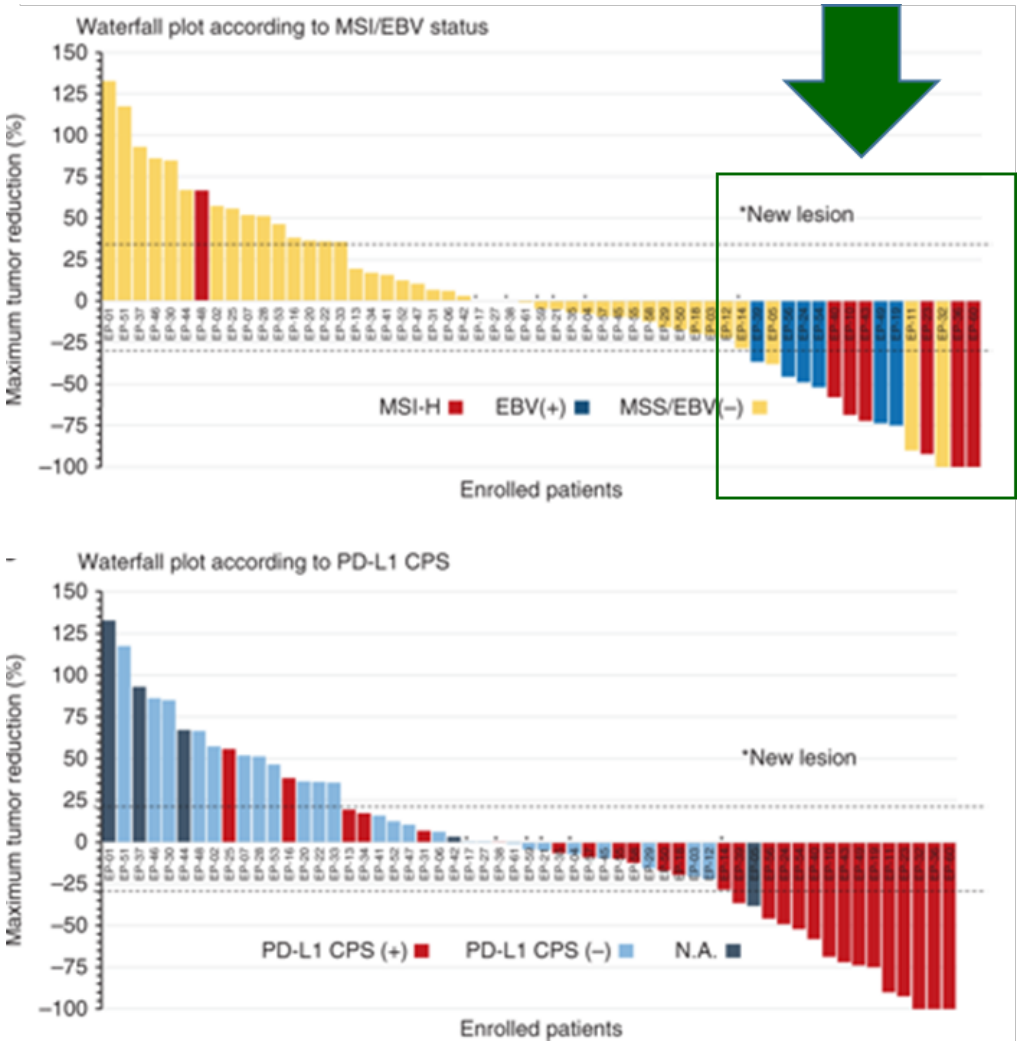


***Figure 6**. Comprehensive molecular characterization of clinical responses to PD-1 inhibition in metastatic gastric cancer.*

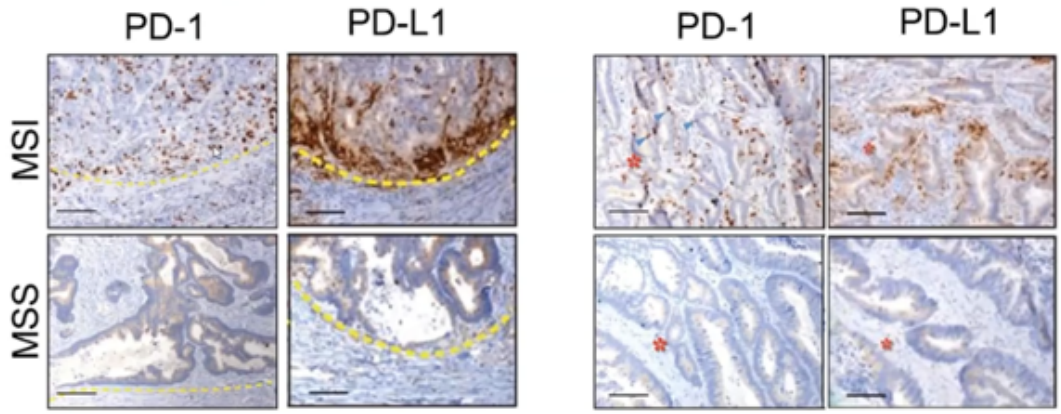


***Figure 7**. Histological comparison between a microsatellite instable (MSI) tumour with many lymphocytes positive for both PD1 and PD-L1, and a microsatellite stable (MSS) tumour.*

3. AIM OF THE STUDY

The aim of this study is to investigate the association between Epstein-Barr virus (EBV) and Helicobacter pylori (HP) infection with Tumour-infiltrating lymphocyte (TIL) levels in gastric cancer (GC). EBV and HP infections have been extensively recognised as triggers of gastric cancer (GC). Analyses of the correlation between the infectious agent and the different level of TILs suggest new perspectives for the identification of predictive markers for immune-modulating therapies, as suggested by recent publications.

Tumour-infiltrating lymphocytes (TILs) have also been identified as a predictive biomarker for immunotherapy in different malignancies.

Notably, in this study, were investigated: (a) the clinicopathological features of the samples analysed related to CD3 and CD8 positive T lymphocytes density; (b) the association between tumour-infiltrating lymphocyte (TIL) levels and clinicopathological features of gastric tissues; (c) the association between TIL levels and the methylation in genes related to EBV; (d) the association between TILs and the pathological features of different infectious agents. Consequent objectives of the study are to demonstrate the association between clinicopathological tissue characteristics and the different duration of survival of GC patients.

The finding that epigenetic regulation can mediate the immune detection capacity of gastric tumours has the ultimate goal of planning further research to lead to the identification of new biomarkers of response to immunotherapy.

4. MATERIALS AND METHODS

Subjects

Gastric cancers that were histologically diagnosed and underwent surgery at the Instituto Nacional de Enfermedades Neoplasicas (INEN) in a period from 2015 to 2021 were included in the study.

Patients included in the study were asked to read and sign the informed consent. The research project was presented and approved by the research and ethics committee whose reference is Protocol Number #050-2015-CIE/INEN. All included cases were given an ISH test for EBV along with/or an assessment of the level of methylation of EBV-related genes [9].

The case selection criterion involved the selection of samples that had the highest gene count of EBV infection-associated genes detected by PCR technique. Cases selected from those with the highest gene counts were previously selected with counts up to 166210.5 copies/µL [11].

Tumour specimens

Tumour samples were collected and stored until use at the Institute's Biobank at a storage temperature of -80°C. Similarly, FFPE (Formalin-Fixed Paraffin-Embedded) samples were stored in the pathology department archive. Tissue microarrays (TMAs) were constructed from tumour cores approximately 6.0 mm in diameter, taken from the invasive areas of each sample from the FFPE blocks of the selected samples. Finally, series of sections of approximately 4µm were prepared to be used for immunohistochemical staining (IHC) [16].

Immunohistochemistry

The sample sections were rehydrated in phosphate-buffered saline (PBS) and antigen retrieval was performed by soaking them in 0.1% trypsin solution in PBS at 37 °C for 5-10 min. Alternatively, antigen was recovered by heating the sections in microwave for 5 min, repeating 4 times (total 20 min) in buffer solution.

Further treatment of the sections required that they be treated with normal goat serum or normal horse serum concentrated at 10% in PBS, a treatment that should last 45 minutes. The antihuman primary antibodies used for IHC were CD8 (clone C8/144B, IS623, Dako) and CD3 (IS503, Dako, Glostrup, Denmark).

Finally, the sample sections were further incubated in alkaline phosphatase-streptavidin solution (Vector Laboratories, Burlingame, CA; 1:1000 dilution) for a duration of 30 minutes at room temperature. At this stage, reaction with the Fast-Red Substrate System (Dakopatts) or Dako® Fuchsin + Substrate-Chromogen took place. Background staining was performed with Mayer's hematoxylin solution, after which the sections were dehydrated using ascending alcohols to xylene and mounted on slides.

Measurement of TILs

For the measurement of the TIL level, several steps were followed: first FFPE tissue samples were taken and stained by means of hematoxylin-eosin (H&E) staining, resulting in stained slides.

As previously mentioned in the introduction of the thesis, the IBWG method was used to evaluate TILs in the stromal compartment, a method that was performed by a pathologist (Fig. 8). In this way, it was possible to assess the density of T lymphocytes [6].

The different densities of CD3 and CD8 T-lymphocytes were calculated by the VisionPharm software: the number of staining-positive cells was counted in relation to the total number of cells in five high-resolution fields located in the stromal compartment; this procedure was always performed under the supervision of a pathologist (Fig. 9). The immunostained slides were digitally scanned with an Olympus BX63 scanner (Olympus, Tokyo, Japan) at 20x magnification. The digital images obtained were visualised with the Visiopharm Integrator System software version 6.6.1.2572 (Visiopharm, Hørsholm, Denmark).

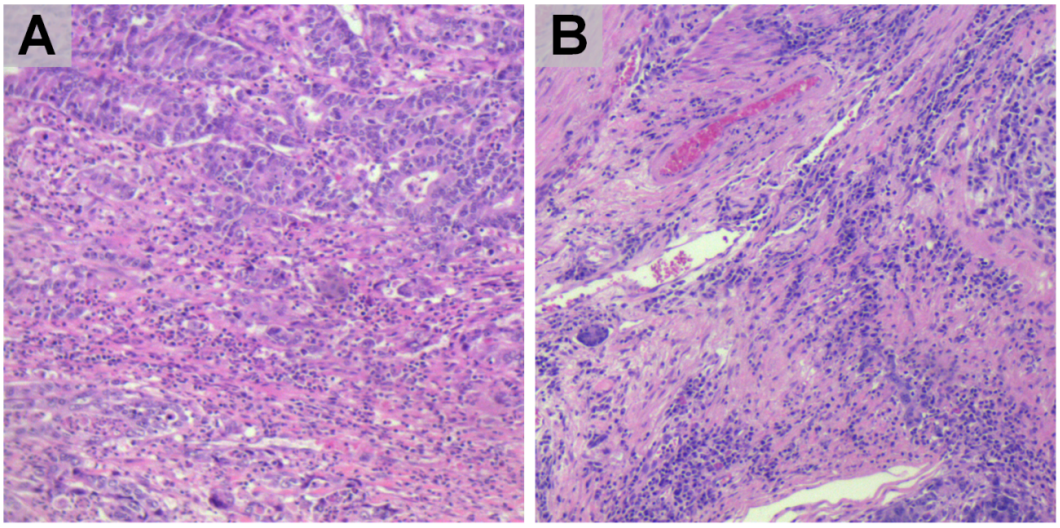


***Figure 8**. H&E staining of gastric cancer: representative slides of stromal compartment with high (A,B) level of Tumor Infiltrating Lymphocytes. (Magnification: ×200).*

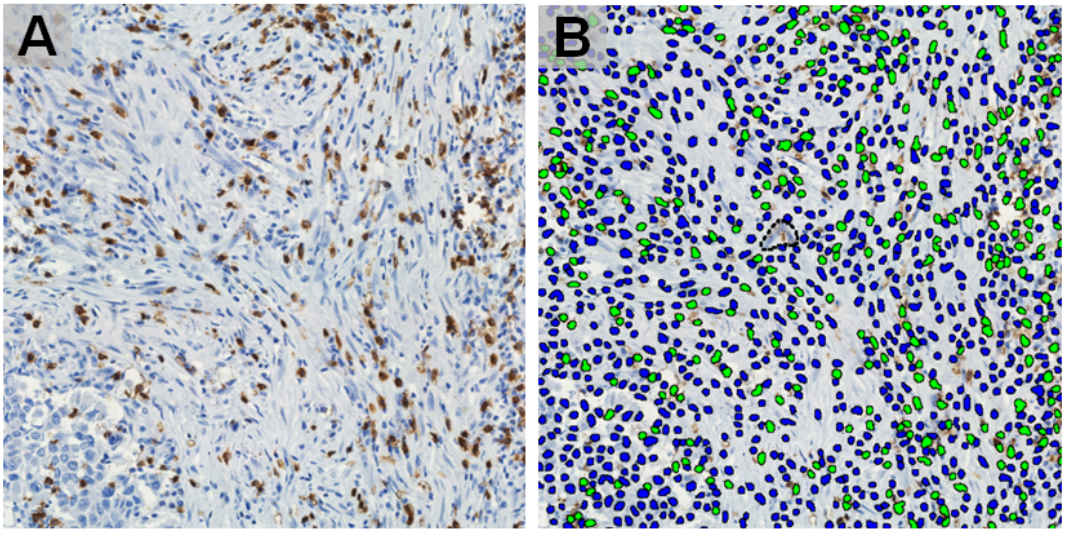


***Figure 9**. Identification of T cells CD8 in immunohistochemistry staining images (A) by machine learning-based image processing (B) showing positive (green) and negative (blue) cells. (Magnification: ×400).*

EBV ISH

Fluorescein-labelled oligonucleotide probes were used to perform chromogenic ISH for EBV-encoded RNA (EBER). The probe was enzymatically digested (ISH protease 3, Ventana) while an iViewBlue detection kit (Ventana) using the BenchMark ULTRA staining system was used for detection.

Evaluation of the EBV gene and HP gene expression

EBV gene expression was detected by searching the *BNRF1* gene region. In contrast, for the search of HP-associated genes, the presence of the colonising genes *hspA* and *UreA* in frozen DNA samples was assessed. These genes were targeted by quantitative polymerase chain reaction (qPCR) in the LightCycler 96 Instrument thermal cycler (Roche, Mannheim, Germany). EBV or HP positivity of the sample was confirmed when ≥10 copies/μL were detected in the sample [9].

Evaluation of methylation in genes related to EBV

Genomic DNA was isolated from frozen GC samples using the classical method of using either phenol/chloroform or isoamylalcohol and proteinase K. The EpiTect Bisulphite kit (Qiagen, Germany) was used for bisulphite treatment as previously described using the Mastercycler nexus gradient thermocycler (Eppendorf, Germany).

In contrast, the methylation status analysis of the six gene promoters *RASSF1, CDKN2A, MGMT, GSTP1, HOXA10* and *TP73* was performed in the LightCycler® 96 Instrument thermal cycler (ROCHE®). The results obtained were interpreted with the aid of the LightCycler® 96 System Version 2.0 software [15].

Primer sequences were forward:

- for *CDNK2A*: 5' TGGAGTTTTCGGTTGATTGGTT 3' and reverse: 5' AACAACGCCCGCACCTCCT 3';
- for *HOXA10:* forward: 5' A TCGGAAGTGCGTTATTTCGTG 3' and reverse:5' TTCCGTCTCTCGACTCGAAACT 3';

- for *TP73:* forward: 5' GGGTCGGGTAGTTCGTTTTG 3' and reverse: 5' CGATTTCGCTACGTCCCCT 3';
- for *RASSF1A:* forward: 5' ATTGAGTTGCGGGAGTTGGT 3' and reverse:5' ACACGCTCCAACC GAATACG 3';
- for *GSTP1*: forward: 5' GTCGGCGTCGTGATTTAGTATTG 3' and reverse: 5' AAACT ACGACGACGAAACTCCAA 3';
- for *MGMT:* forward: 5' GCGTTTCGACGTTCGTAGGT 3' and reverse: 5' CACTCTTCCGAAAACGAAACG 3'.

The Illumina Infinium MethylationEPIC BeadChip was used to perform DNA methylation profiling. This BeadChip Kit presents over 850,000 CpGs in enhancer regions, promoters and CpG islands, gene bodies, according to the manufacturer's instructions. These results are contained in FFPE tumour tissues from a subset of 24 GC cases in the Molecular Genomics Core laboratory, USC Norris Comprehensive Cancer Center (Los Angeles, CA, USA). The methylation values for the individual CpG sites were obtained as β-values. A β value was generated for each CpG locus reflecting a measure of the percentage of methylated (associated β value = 1) and unmethylated (associated β value = 0) probes.
The calculation of β values for each probe are continuous variables that are obtained by dividing the intensity of the methylated probe by the combined intensity of the methylated and unmethylated probes.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 21. At the genome-wide level, DNA methylation was analysed, which allowed the identification of differentially methylated regions (DMRs) in relation to both TILs levels (with a cutoff of 30%) and ISH status of EBER (positive or negative sample) using the Illumina Infinium MethylationEPIC BeadChip kit. The different comparisons of the variables that defined the different categories were carried out with the chi-square test or Fisher's exact test, depending on the assessment of the case. OS was defined as the elapsed

time from the date of surgery until death from any cause or last life information. Relative to this, survival rates were estimated using the Kaplan-Meier method. All tests were two-sided, and differences were considered significant when $p<0.05$.

5. RESULTS

Clinicopathological features

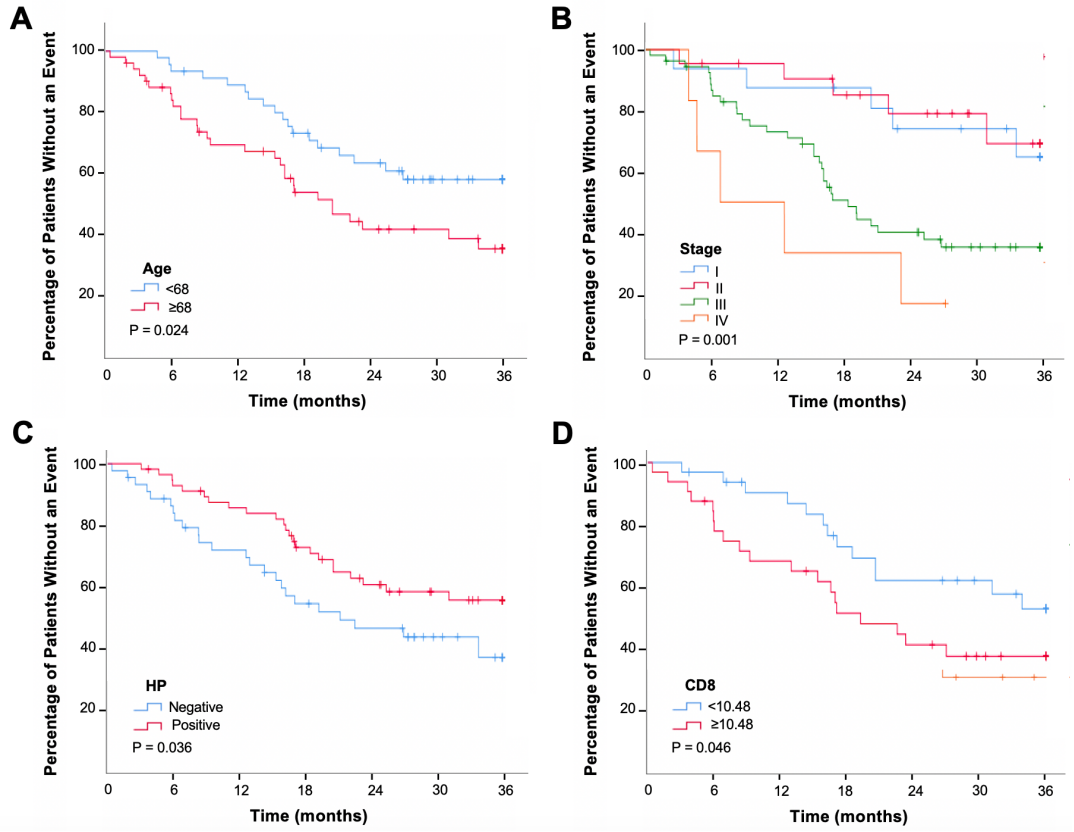
In the analysis of the entire series of 98 cases included in the study, the median age was 68 years and 41.8% were women. Most of the GC cases analysed had a low or undifferentiated grade (52%), 58.2% had intestinal histology, 65.3% of patients had lymphovascular and perineural invasion (58.2% of cases).

The most common stages analysed were III-IV (61.2% of the total). Most patients have been involved of the antrum of the stomach (55.1%).

HP was detected in 56.1% of the samples. With respect to TIL: the mean in the samples was 30% and the median densities of CD3 and CD8 T lymphocytes were 21.07% and 10.48%, respectively.

Factors associated with shorter survival were older age (p=0.024), the presence of lymphovascular invasion (LVI)(p=0.007), advanced disease stage (p=0.001), absence of HP (p=0.036) and higher CD8 density (p=0.046) (Fig. 10). The level of TILs was not associated with survival during the study (p=0.594) (Fig.10).

***Figure 10**. Kaplan-Meier curve for overall survival by age (A), stage (B), HP (Helicobacter Pylori) status (C) and CD8 positive T cell density (D).*



Determination of EBV status through ISH, qPCR and methylation

Epstein-Barr virus status was positive by EBER-ISH in 24.1% of the 79 cases evaluated and positive by qPCR in 41.8% of the total 98 cases. Analyses showed no relationship between a detection performed with EBV-ISH and one performed with qPCR (kappa index=0.012, p=0.864). Furthermore, no significant association was established between survival and EBV-ISH (p=0.345) or qPCR (p=0.809).

An up-regulated methylation status in at least one gene was found in 70% of the 78 cases evaluated (Table 6). Methylation of *RASSF1, CDKN2A, MGMT, GSTP1, HOXA10* and TP73 was found in 37.2%, 48.7%, 34.6%, 11.5%, 15.4% and 10.3%, respectively. A significant association was also found between methylation status and the presence of EBV infection assessed by qPCR (kappa index=0.328, p=0.003) or ISH (kappa index=0.158, p=0.025). Methylome analysis by Infinium MethylationEPIC BeadChip in 24 tumour samples revealed that the DMR cutoff >30% with p-value <0.05 relative to EBER-ISH status identified 88 DMR-related genes (from 116 DMRs).

Table 6. *Clinicopathological features.*

P value < 0.05
*1: Well-differentiated; 2: Moderately differentiated; 3-4: Poorly differentiated and Undifferentiated.
** at least one gene.

Features	n=98	%	OS at 3y (%)	p value
Age (Median)				**0.024**
<68	46	46.9	60.9	
≥68	52	53.1	44.2	
Gender				0.788
Male	57	58.2	52.6	
Female	41	41.8	51.2	
Lauren subtype				0.507
Intestinal	57	58.2	49.1	
Diffuse	28	28.6	53.6	
Mixed	13	13.3	61.5	
Histological grade*				0.301
1	14	14.3	64.3	
2	33	33.7	45.5	
3-4	51	52.0	52.9	
LVI				**0.007**
No	34	34.7	67.6	
Yes	64	65.3	43.8	
PNI				0.167
No	41	41.8	53.7	
Yes	57	58.2	50.9	
Location				0.183
Antrum	54	55.1	55.6	
No-Antrum	44	44.9	47.7	
Resection				0.055
Subtotal	57	58.2	57.9	

Features	n=98	%	OS at 3y (%)	p value
Total	41	41.8	43.9	
Pathological stage				**0.001**
I	16	16.3	68.8	
II	22	22.4	77.3	
III	54	55.1	40.7	
IV	6	6.1	16.7	
HP PCR				0.036
Negative	43	43.9	44.2	
Positive	55	56.1	58.2	
TIL stromal (Median)				0.594
<30	48	49.0	52.1	
≥30	50	51.0	52.0	
CD3 density (Median) (n=71)				0.241
<21.07	35	49.3	54.3	
≥21.07	36	50.7	44.4	
CD8 density (Median) (n=64)				**0.046**
<10.48	32	50.0	59.4	
≥10.48	32	50.0	40.6	
CD8/CD3 ratio (n=63)				0.413
Low	31	49.2	51.6	
High	32	50.8	46.9	
EBER-ISH (n=79)				0.345
Negative	60	75.9	50.0	
Positive	19	24.1	42.1	
EBV (qPCR) (n=98)				0.809
Negative	57	58.2	54.4	
Positive	41	41.8	48.8	

Methylated EBV-related genes (n=78)**				0.494
No	23	29.5	47.8	
Yes	55	70.5	52.7	

P value < 0.05

*1: Well-differentiated; 2: Moderately differentiated; 3-4: Poorly differentiated and Undifferentiated.

** at least one gene.

Association between TILs and clinicopathological features in gastric tissues

Analyses of the association between TILs levels and HP and EBV showed that: (a) a higher level of TILs was associated with intestinal histological subtype (p<0.001) and well or moderately differentiated grade (p<0.001); (b) CD3 lymphocyte density was not associated with HP (p=0.531) nor with other variables (p>0.01); (c) a higher density of CD8 lymphocytes was associated with the absence of HP infection.

Table 7. *Evaluation of relationship among Tumor-infiltrating-lymphocytes and clinicopathological features.*

Features	TIL <30% (n=48)	TIL ≥30% (n=50)	p	CD8 <10.48	CD8 ≥10.48	p
Age (Median)			0.070			0.8
<68	27 (58.7)	19 (41.3)		14	13	
≥68	21 (40.4)	31 (59.6)		18	19	
Gender			0.973			0.313
Male	28 (49.1)	29 (50.9)		16	20	
Female	20 (48.8)	21 (51.2)		16	12	
Lauren subtype			**<0.001**			0.955
Intestinal	17 (29.8)	40 (70.2)		21	20	
Diffuse	24 (85.7)	4 (14.3)		7	8	
Mixed	7 (53.8)	6 (46.2)		4	4	
Histological grade*			**<0.001**			0.165
1	8 (57.1)	6 (42.9)		8	3	
2	6 (18.2)	27 (81.8)		13	12	
3-4	34 (66.7)	17 (33.3)		11	17	

Features	TIL <30% (n=48)	TIL ≥30% (n=50)	p	CD8 <10.48	CD8 ≥10.48	p
LVI			0.065			0.171
No	21 (61.8)	13 (38.2)		12	7	
Yes	27 (42.2)	37 (57.8)		20	25	
PNI			0.658			0.611
No	19 (46.3)	22 (53.7)		14	12	
Yes	29 (50.9)	28 (49.1)		18	20	
Location			0.149			0.611
Antrum	30 (55.6)	24 (44.4)		20	18	
No-antrum	18 (40.9)	26 (59.1)		12	14	
Pathological stage			0.086			0.095
I	11 (68.8)	5 (31.3)		6	4	
II	8 (36.4)	14 (63.6)		11	5	
III	28 (51.9)	26 (48.1)		15	20	
IV	1 (16.7)	5 (83.3)		0	3	
H. pylori (PCR)			0.213			**0.005**
Negative	18 (41.9)	25 (58.1)		8 (29.6)	19 (70.4)	
Positive	30 (54.5)	25 (45.5)		24 (64.9)	13 (35.1)	
EBER-ISH			0.098			0.077
Negative	32 (53.3)	28 (46.7)		19	18	
Positive	6 (31.6)	13 (68.4)		3	10	
EBV (qPCR)			**0.001**			**0.012**
Negative	28 (68.3)	13 (31.7)		20	10	
Positive	20 (35.1)	37 (64.9)		12	22	
Methylated EBV- related genes			**0.007**			0.174

Features	TIL <30% (n=48)	TIL ≥30% (n=50)	p	CD8 <10.48	CD8 ≥10.48	p
No	16 (69.6)	7 (30.4)		10	5	
Yes	20 (36.4)	35 (63.6)		16	19	
RASSF1***			**0.011**			**0.048**
Negative	28 (57.1)	21 (42.9)		20	12	
Positive	8 (27.6)	21 (72.4)		6	12	
CDKN2A* (p16)			0.484			**0.025**
Negative	20 (50.0)	20 (50.0)		18	9	
Positive	16 (42.1)	22 (57.9)		8	15	
MGMT***			0.797			0.488
Negative	23 (45.1)	28 (54.9)		16	17	
Positive	13 (48.1)	14 (51.9)		10	7	
GSTP1***			0.126			0.571
Negative	34 (49.3)	35 (50.7)		24	21	
Positive	2 (22.2)	7 (77.8)		2	3	
HOXA10***			0.735			0.517
Negative	31 (47.0)	35 (53.0)		21	21	
Positive	5 (41.7)	7 (58.3)		5	3	
TP73***			0.205			0.063
Negative	34 (48.6)	36 (51.4)		26	21	
Positive	2 (25.0)	6 (75.0)		0	3	

*1: Well-differentiated; 2: Moderately differentiated; 3-4: Poorly differentiated and Undifferentiated;
** at least one gene, *** Methylated status

Association between TILs and EBER-ISH status, Epstein Bar gene expression or methylation signature

Higher TIL level and higher CD8 lymphocyte density were associated with EBV positivity confirmed by qPCR (p=0.001 and p=0.012, respectively). The trend with EBV positive status was shown with the ISH test (p-values p=0.098 and 0.077 respectively) (Table 6).

In addition, a clear association between a higher TIL level and methylation of at least one of the six EBV-related genes (p-value of 0.007) and also the association between elevated TILs and methylation of the *RASSF1A* oncosopressor gene (p=0.011) was shown. In addition, the methylation status of the *RASSF1A* gene (p=0.048) in question, together with the methylation status of *CDKN2A* (p=0.025), were associated with a higher density of CD8+ cells (Table 7). CD3 cell density, on the other hand, was not associated with EBV status by ISH technique (p=0.124) nor with other assessments of EBV status (p-value greater than 0.05).

Another important result concerns the methylome analysis using the Infinium MethylationEPIC BeadChip kit. Thirty-two genes related to differentially methylated regions (from 146 DMRs) were identified that were associated with a high density of TILs at >30%. This methylome analysis had a DMR cutoff >30% with a p-value <0.05.

Finally, eight genes were shown to be shared by both the state with TIL density >30% and the EBV+ state obtained by the EBER-ISH method. The genes are:

- *SORCS3:* lower, Sortilin related VPS10 domain containing receptor 3.
- *TBC1D14*: lower, TBC1domain family member 14.
- *TMEM260*: lower, transmembrane protein 260.
- *TNNT3*: lower, troponin T3, fast skeletal type.
- *HEATR4:* major, HEAT repeat containing 4.
- *RGMA:* major BMP repulsive guidance molecule co-receptor a.
- *MTRNR2L1:* minor, MT-RNR2like 1.
- *SH2D4A:* minor, SH2 domain containing 4A.

6. DISCUSSION

This study showed a considerable association between TIL levels and EBV+ status leading to the conclusion that higher TIL levels are significantly associated with EBV positivity. In more detail: it was observed that a higher density of CD8 T lymphocytes and thus higher levels of TILs were associated with both EBV positivity and the methylation status of EBV-related genes [138]. EBV-associated CG showed global methylation of CpG islands in the promoter region of cancer-related genes. These epigenetic alterations, such as methylation at the level of the CpG islands in the promoter region of the genes, were extensively described in our study. In particular, the methylation pattern in GC with EBV+ was extensively described in our results, underlining the evidence that DNA methylation and the resulting gene silencing are characteristic features of this type of gastric cancer.

Another piece of evidence we considered important in analysing the association between TILs and EBV-associated GC was the finding that the elevated TIL level was also significantly associated with the methylation status of at least one of the 6 gastric cancer-associated genes we evaluated in the study. This suggests that the process that causes gene methylation would be a possible means of mediating the body's immune activity against EBV-positive tumours [139].

The explanation for this possible direct correlation between elevated TILs, methylation of tumour genes and mediation of the immune response is suggested by the evidence that the methylation status of the 8 genes analysed in this study is similarly dysregulated in other tumour types with elevated levels of TILs. Thus, this methylation status of 8 genes is not regulated in the same way as other tumours with elevated TILs and in GCs that were EBV-ISH positive by genome-wide DNA methylation status analyses. As reported in the results, the *SORCS3* gene is altered in its methylation status in the samples analysed and has been described by

previous studies as a gene related to gastrointestinal tumour progression [139,140].

Recent studies have uncovered a significant correlation between epigenetic modifications (particularly alterations in the methylation status of genes) and the immune system's ability to detect gastric cancer; this epigenetic regulation could therefore mediate the immune detection capability of GCs [141-144]. Long interspersed nuclear element-1 (LINE-1) comprises about 17% of human genomic DNA, has a high density of CpG sites in its untranslated region that are hypermethylated in normal cells. This finding suggests that the methylation level of LINE-1 could be used as a marker of methylated genomic DNA content. Furthermore, it has been shown that in many human epithelial malignancies the hypo-methylated state of LINE-1 is associated with a poorer prognosis, even in GC patients, thus the tumour hypomethylation state of LINE-1 is associated with a poorer clinical outcome not only in GC, but also in other epithelial malignancies.

The interesting hypothesis that was considered in reaching the conclusions of this study was that the level of methylation of GC-associated genes would appear to be influenced by the density of TILs. This hypothesis is based on the established evidence that TILs have the function of protecting the host from the aggressive behavior of tumour cells, evidence that confirms better clinical outcomes in patients with GC with a higher density of TILs. In addition, other evidence to point out is that the methylation level of LINE-1 is higher in TILs than in tumour cells, so it is reasonable to assume that GCs with a higher density of TILs show higher genomic methylation values once tumour tissues are analysed (tissues with a mixture of tumour and non-tumour cells such as TILs).

However, the high level of LINE-1 methylation found in tumour tissues with a high density of TILs may not be entirely due to the high density of TILs. Indeed, in EBV-positive gastric carcinomas it has been shown that a high level of DNA methylation is due to the intrinsic molecular characteristics of this molecular subtype of GCs. Thus EBV-associated GCs exhibit high

levels of methylation regardless of TIL density, but in this study the clear association with the presence of a high density of TILs was highlighted.
Secondly, a possible explanation for this correlation between TILs and hyper methylation for GCs that do not exhibit the molecular characteristics typical of an EBV-associated GC is that the high density of TILs leads to increased levels of DNA methyltransferases and, consequently, to higher levels of genomic DNA methylation in tumour cells [142].
This hypothesis that the association between EBV infection and elevated TILs (and higher CD8 lymphocyte density) is mediated by a methylated DNA pattern needs further research but it is suggestive that this finding could lead to the identification of new biomarkers of response for GC immunotherapy:
Another significant association found by the study of the series of 98 Peruvian patients is that between a higher density of CD8+ lymphocytes and a worse prognosis, thus a lower survival of patients. This effect could be explained by the relationship between CD8-positive levels and the level of PD-L1- and FOXP3-positive pro-tumour T-lymphocytes [10-13,20]. In this case, the PD-1/PD-L1 axis prevails as an inhibitory signal with PD-1 (Programmed cell death protein 1) expressed on the T lymphocyte and its ligand, PDL-1, expressed on the tumour cell. The neoplasm gains an evolutionary advantage when PD-L1 expression increases significantly, enabling it to evade immune control. PD-L1 is a receptor up-regulated by tumour cells to evade the immune system and this would explain the worse prognosis associated with samples with a higher density of CD8+ lymphocytes. Immunotherapeutic drugs currently used in clinical practice are monoclonal antibodies that inhibit these immunological checkpoints. These drugs have the potential to reactivate the anti-tumour immune response [30–53,143].
A second association with longer survival in the series of GCs studied is that with HP infection. This relationship between HP+ and better prognosis had already been shown in other studies [144].
Linking to what has been discussed above, the prognostic feature of low CD8+ lymphocyte density associated with HP infection explains the more

favourable prognosis of HP+ tumours suggesting that a more active immune system with GC is the most plausible explanation for this longer survival.

Recent published studies have shown that there is a reduced GC response to immunotherapy in patients with HP infection. This evidence could be related to the results obtained during the analysis of this thesis whereby HP-positive GC samples showed reduced tumour infiltration by CD8+ T lymphocytes [138,142].

One limitation of this study to consider is the small size of the case series analysed. For this reason, it is reasonable to consider the need for further validation from other, larger studies.

Tissue microarrays (TMAs) were used so the volume of samples analysed was rather small, however the selection of the area of invasive tumour for assessment was carefully selected by a pathologist. Most of the samples obtained in the real world are similarly small in volume, as obtaining the samples by gastroscopy does not ensure a bigger analysable volume. This would give considerable accuracy value to the choice of this study.

7. CONCLUSIONS

In conclusion, upon cross-sectional analysis of possible associations between EBV, HP and TILs levels in the GC, we found that:

- higher levels of TILs in GC are associated with EBV infection status and this correlation would appear to be mediated by the methylation status characteristic of EBV-associated genes.
- In contrast, HP infection is associated with a longer survival of GC patients, this favourable prognosis could be explained by the lower infiltration of CD8 T lymphocytes.

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