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TESI DI LAUREA

HER2-LOW IN GASTROESOPHAGEAL ADENOCARCINOMA: A REAL-WORLD PATHOLOGICAL PERSPECTIVE

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ABSTRACT

Introduction: Trastuzumab in combination with cytotoxic chemotherapy is the first-line therapy of choice in HER2-overexpressing metastatic gastroesophageal cancers. In the DESTINY-Gastric01 trial, a novel HER2-targeted antibody-drug conjugate Trastuzumab deruxtecan proved to be effective also in HER2-low disease, paving the way for novel therapeutic scenarios.

Materials and methods: we retrospectively selected a large series of 1109 formalin-fixed paraffin-embedded (FFPE) samples of gastroesophageal adenocarcinoma (n = 375 gastroesophageal junctional adenocarcinomas and 707 gastric adenocarcinomas; 502 surgical resection specimens and 607 biopsy specimens) analyzed by IHC for HER2 protein expression in seven Italian surgical pathology units from January 2018 to June 2022. We assessed the prevalence of HER2-low (i.e., HER2 1+ and HER2 2+ without amplification) and its correlation with clinical and histopathological features, other biomarkers' status, including MMR/MSI status, EBER and PD-L1 Combined Positive Score.

Results: out of 1089 assessable cases, 662 (60.8%) cases were HER2 0, 298 (27.4%) were HER2-low and 129 (11.8%) were HER2-high. Biopsy samples were enriched in HER2-low cases in comparison with surgical resection specimens (34.5% *versus* 18.9%; p<0.00001). The prevalence of HER2-low and HER2-high cases was higher in Lauren intestinal cases than in diffuse cases and lower in pure signet-ring cell carcinomas (SRC) (p=0.002). The distribution of HER2 0, HER2-low and HER2-high cases among the centers where the evaluation was performed was significantly different (p=0.007). HER2-low status did not correlate with localization, histotype and grading according to WHO, Ming classification, staging, other biomarkers' status and year of diagnosis.

Conclusions: in this work, we showed how the expansion of the HER2 spectrum might decrease reproducibility, especially in biopsy specimens, increasing inter-laboratory and interobserver variability. If controlled trials confirm the promising activity of novel anti-HER2 agents in HER2-low gastroesophageal cancers, a shift in the interpretation of HER2 status may need to be pursued, including a modification of existing HER2 assays and a well-defined characterization of HER2 expression beyond the current dichotomic HER2-positive and HER2-negative status.

INTRODUCTION

1.0 ESOPHAGEAL AND GASTRIC ADENOCARCINOMA

1.1 Esophageal adenocarcinoma

Esophageal adenocarcinoma is one of the most common types of malignant epithelial tumors of the esophagus. Its incidence it's different over the world, and there are improvements about the understanding of the reasons for this variance: environmental and dietary factors are some examples. According to the fifth-edition volume of the *WHO Classification of Tumors-Digestive System*, recent data demonstrate that adenocarcinoma of the esophagus and of the esophagogastric junction share many histological, biological and clinical features, so these two types of cancers can be discussed together. [1]

There are many different risk factors for esophageal adenocarcinoma, such as tobacco use, consuming hot beverages and foods, presence of gastro-esophageal reflux, obesity, while *Helicobacter pilory* infection has a protective role due to the reduced volume and acidity of gastric juice after infection.

Lastly, the condition called *Barrett's esophagus* represents a well-established precursor lesion for this type of tumor. It consists of the replacement of the squamous epithelium of the distal esophagus by metaplastic columnar epithelium, above the gastroesophageal junction. There are two types of metaplastic columnar epithelium: gastric type (with parietal and chief cells) and intestinal type (with goblet and Paneth cells). The latter characterizes *Barrett's esophagus.* [1].

1.1.1 Epidemiology

Esophageal cancer is the eighth most common tumor type and the sixth cause of cancer death all over the world, with over 600,000 cases and 540,000 deaths in 2020. There are two different subtypes of this tumor, that are esophageal adenocarcinoma and esophageal squamous cell carcinoma. The first one represents about 11% of all esophageal neoplasms, while the second one the 87%. Focusing on the esophageal adenocarcinoma, the areas with the highest incidence of it are North America, Western and Northern Europe, Australia and New Zealand. [2]

Furthermore, the incidence and the mortality rates linked to esophageal adenocarcinoma have been rising during the last four decades. [3]

The M:F ratio for this tumor is 4.4:1 and the average patient age with diagnosis of esophageal adenocarcinoma is in the seventh decade of life. [1]

1.2 Gastric adenocarcinoma

Gastric adenocarcinoma is a malignant epithelial neoplasm of the gastric mucosa, with glandular differentiation and it represents a heterogenous group of tumors regarding morphology, molecular, etiology and histogenesis features. [1][4]

Gastric/gastroesophageal junction adenocarcinoma represents more than 90% of gastric carcinomas and the most common histotype among gastric malignancies. [5]

Gastric carcinogenesis is process that consists of normal mucosa transformation into carcinoma, through different and progressive steps: chronic gastritis, mucosal atrophy, intestinal metaplasia, dysplasia and finally carcinoma. This sequence of events it's known as *Correa's cascade of multistep gastric carcinogenesis.*

There are different risk factors leading to this type of cancer, such as tobacco use, Epstein-Barr virus (EBV), dietary habits, genetic factors and *Helicobacter pylori* infection. The latter has an important role in carcinogenetic process, and it was classified as a type I carcinogen by the WHO in 1994. In particular, *H. pylori* virulence factors like CagA and VacA are associated to an increased risk of gastric cancer. [4]

According to Siewert's classification, gastric cardia adenocarcinoma can be included among esophagogastric junction cancers. For this reason, gastric adenocarcinoma and esophagus/esophagogastric junction adenocarcinoma can be discussed as one from a histological point of view.

Furthermore, the eighth edition of the Union for International Cancer Control (UICC) TNM classification and the American Joint Committee on Cancer (AJCC) state that any cancer involving esophagogastric junction whose epicenter is ≤ 2 cm nearby proximal stomach can be considered as one of the esophageal cancers. On the other side, tumors with an epicenter situated ≥ 2 cm into the proximal stomach are considered as gastric carcinomas, even if the esophagogastric junction is involved. [1]

1.2.1 Epidemiology

Gastric cancer is the third most common cause of cancer-related deaths worldwide and it also represents the fifth most common malignancy in both males and females, with over one million new cases in 2020 and 769,000 related deaths. Eastern Asia, Eastern Europe and South America represent the areas with the highest incidence of this tumor type, while Northern America and Northern Europe the lowest. In particular, gastric cancer is the most commonly diagnosed malignant neoplasm and the first cause of cancer-related deaths in different South-Central Asian countries. Additionally, the M:F ratio for gastric cancer is 2:1 and, during the last 50 years, there has been a decrease in distal gastric cancer incidence in Western countries. The reason is the lower prevalence of *Helicobacter pylori*

infections and the improvement of health and hygiene conditions. On the other side, there has been a rise of proximal gastric cancer (gastric cardia and gastroesophageal junction). This is probably caused by the growing incidence of gastroesophageal reflux disease and obesity.
However, despite of the decreasing incidence of this tumor type in some areas, an increasing number of gastric cancer cases is expected in the future, because of aging population. [2][5]

2.0 HISTOPATHOLOGICAL CLASSIFICATIONS

The most used histopathological classifications are Lauren's classification and World Health Organization (WHO) classification.

2.1 WHO 2019 CLASSIFICATION

2.1.1 Histotypes

According to the WHO classification, there are five main histopathological subtypes of gastric adenocarcinomas: tubular, papillary, poorly cohesive (including signet-ring cells), mucinous and mixed adenocarcinomas.

- **Tubular adenocarcinoma**: this is the most common subtype consisting of tubular, acinar and glandular structures of variable diameter. Neoplastic cells can be cuboidal, columnar or flattened by intraluminal mucin or cell debris. There are different levels of differentiations: for example, tumors with solid structures and barely recognizable tubules are classified as poorly differentiated tubular (solid) carcinoma, with a predilection for the cardia/esophagogastric region. [1] (**Fig.1**)
- **Papillary adenocarcinoma**: this is a rare subtype, histologically most commonly well differentiated. It consists of long finger-like processes with fibrovascular connective tissue cores, encircled by columnar or cuboidal cells. In some tumors it's possible to find also tubular structures (tubulopapillary). This type of gastric adenocarcinoma is associated with higher frequency of liver metastasis and lower survival. [1] (**Fig.1**)
- **Poorly cohesive adenocarcinoma (PCC):** isolated neoplastic cells or organized in aggregates without well-formed glands. This gastric adenocarcinoma type can be differentiated in signet-ring cell type or non-signet-ring cell type (PCC-NOS). The first one is characterized only by signet-ring cells, with a central droplet of cytoplasmatic mucin and an eccentrical nucleus, while the second one consists of a mix of different cell types: some cells resembling histiocytes or lymphocytes, others have an eosinophilic cytoplasm, others are pleomorphic with bizarre nuclei. These cells don't have classic signet-ring cell morphology. In addition, signet-ring cell carcinomas show lower sensitivity to chemotherapy.

The 2019 WHO Classification of digestive system puts stress on the importance of distinguishing the two different types of poorly cohesive adenocarcinoma, this because the non-signet ring cell type presents poorer prognosis compared to pure signet ring cell carcinomas. [1][4] (**Fig.1**)

- **Mucinous adenocarcinoma:** this tumor type is determined by extracellular mucin pools that accounts for > 50% of the tumor area. There are two main growth patterns: the first one consists of recognizable glandular structures or tubules lined by columnar epithelium with interstitial mucin, while the second one has chains or single tumor cells (like signet-ring cells) surrounded by mucin. [1] (**Fig. 2**)
- **Mixed adenocarcinoma**: it shows two or more different histological elements: glandular (tubular/papillary) and signet-ring cell/poorly cohesive. These two components can be combined, adjacent, or separated.
  Patients with this type of adenocarcinoma have a poor prognosis than those with only one histological component. [1] (**Fig. 2**)

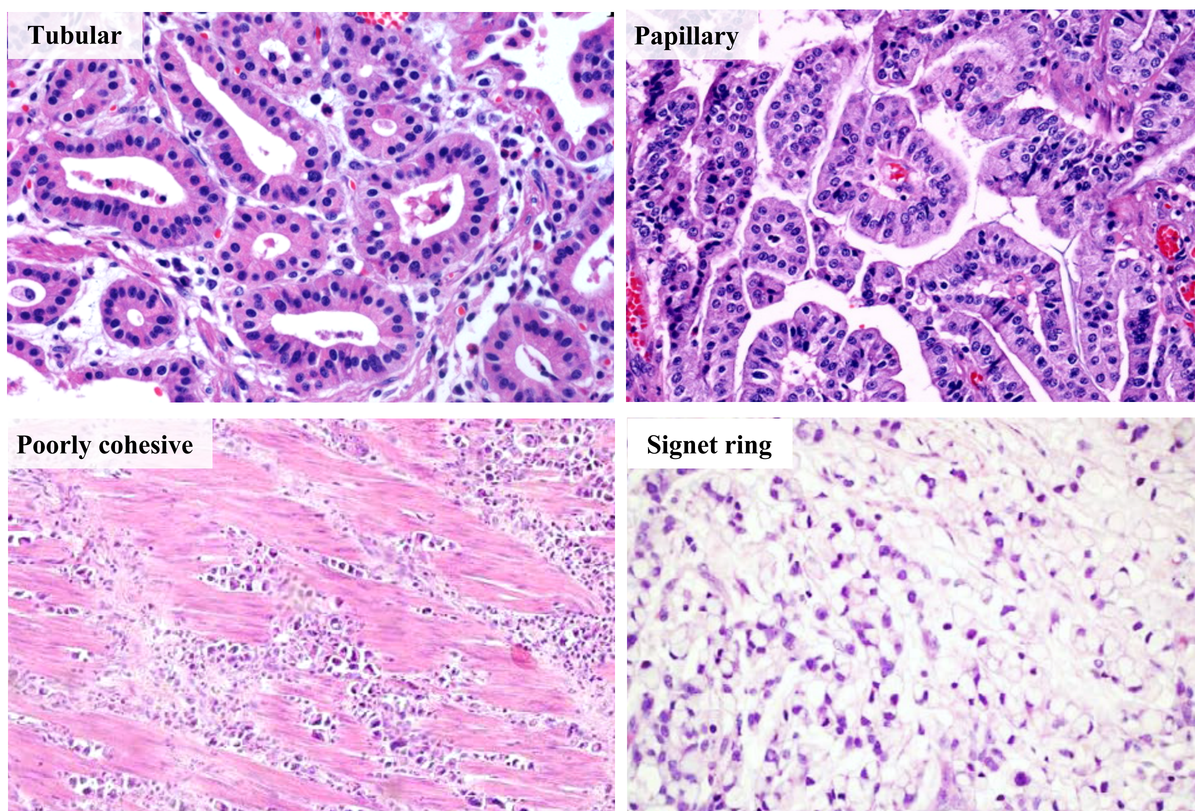

Figure 1: tubular, papillary, poorly cohesive and signet ring types. (Grillo F, et al. – Pathologica 2020)

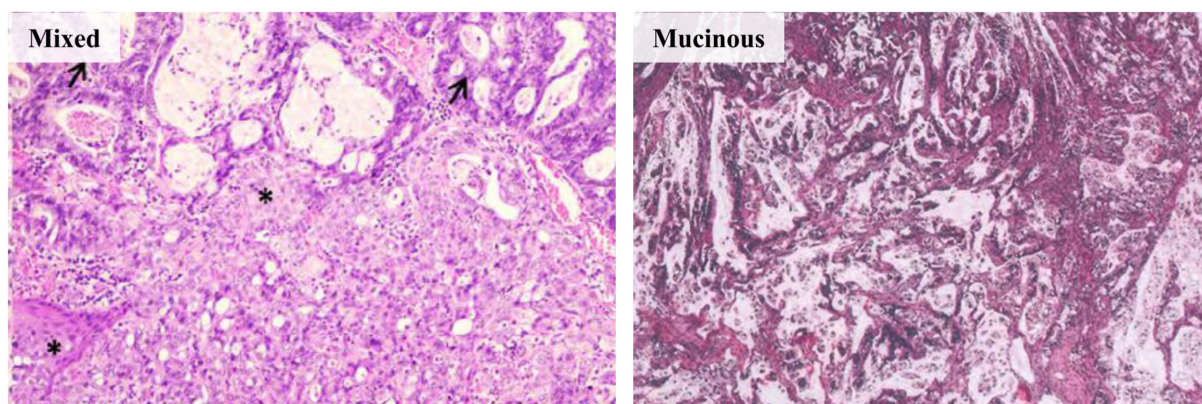

Figure 2: mixed type (Grillo F, et al. – Pathologica 2020) and mucinous type (WHO Classification of Tumours-Digestive system Tumours 2019)

2.1.2 Grading

It applies to tubular and papillary adenocarcinomas, not to the other gastric adenocarcinoma subtypes. Well-differentiated adenocarcinomas show well-formed glands, while poorly differentiated adenocarcinomas have poorly formed gland and present solid areas or individual cells.

There are two levels of grading: low-grade (well or moderately differentiated) and high-grade (poorly differentiated). [1]

2.2 LAUREN'S CLASSIFICATION

This classification recognizes two main histological types, diffuse type and intestinal type. Intestinal type presents glands or papillae, while diffuse type shows an infiltrative growth pattern without cellular cohesion. If a tumor presents both of these aspects, it's called mixed carcinoma, while solid poorly differentiated or undifferentiated tumors that don't belong to these subtypes are included in indeterminate category. .[4]

Lauren's classification is commonly used by clinicians and surgeons, because it differentiates subtypes based on epidemiologic settings, clinicopathologic profiles and biological behaviors. Indeed, there are some important differences between different categories. For example, intestinal type is usually associated with Helicobacter pylori and Correa's cascade, whereas diffuse type shows E-cadherin expression loss. .[4]

In addition, intestinal type gastric adenocarcinomas tend to metastasize haematogenously, while poorly cohesive phenotype of diffuse types through peritoneal surface.

Finally, mixed gastric adenocarcinoma is characterized by a poorer prognosis compared to diffuse or intestinal types.[4]

2.3 MING’S CLASSIFICATION

According to this classification, there are two main types of gastric carcinoma: expansive and infiltrative. The first one is characterized by tumor nodules due to carcinoma growth en masse and its expansion, the second one presents cells that invade individually and widely. There are different levels of cell maturation between these two categories, but glands are more common in expanding type. [6]

An important difference between expanding and infiltrative types is the histogenetic origin. In fact, intestinal metaplasia has a significant role in expanding carcinoma type development, but not in the infiltrative type. Other differences can be found in sex and age of patients, survival rate and epidemiological distribution. [6]

So, Ming’s classification allows specialists to analyze some aspects of gastric cancer.

3.0 PREDICTIVE BIOMARKERS

Studying predictive biomarkers is a fundamental step to improve gastroesophageal adenocarcinoma therapy, that can be personalized for each patient based on his molecular profile.

Some important predictive biomarkers involved in this type of cancer are HER2, microsatellite instability, positivity to Epstein-Barr virus and PD-L1.

An important consideration is that, according to the study of The Cancer Genome Atlas (TCGA) in 2017, esophageal and gastric adenocarcinomas have molecular similarities, so they can be considered as a single entity for clinical trials of therapies. [5]

3.1 HER2 (Fig. 3)

The HER2 receptor (human epidermal growth factor receptor - 2) is a transmembrane glycoprotein that belongs to epidermal growth factor receptor family (EGFR). This family regulates epithelial cells differentiation, growth and survival and it includes HER1 (ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4), where ErbB represents a group of proteins with four receptor tyrosine kinases.

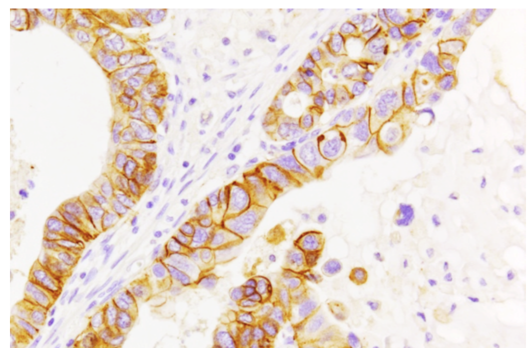

Figure 3: HER2 expression

HER proteins have in common structural elements, such as transmembrane domain, extracellular ligand-binding domain and intracellular protein tyrosine kinase. HER2 is different because it doesn’t bind any ligand.

HER2 can be activated by heterodimerization, that involves ligand-activated EGFR or HER3,

or by homodimerization. The latter is frequent when there is a high concentration of HER2, as it's possible in cancers. [7]

Some studies showed a possible involvement of HER2 in gastroesophageal junction cancer but, while the predictive role of HER2 has been demonstrated, its role as a prognostic factor in gastric/gastroesophageal carcinoma isn't clear. Anyway, recent reports prove that HER2 overexpressed is linked to a poor prognostic factor for survival and an increased disease recurrence. [8]

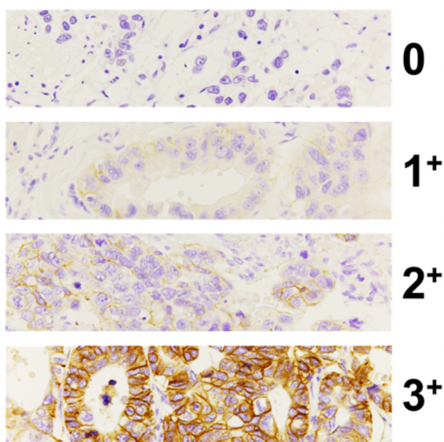

Figure 4: HER2 different scores

HER2 overexpression is associated to intestinal histotype and proximal gastric adenocarcinoma, and it is related to 30% of gastroesophageal junction's cancers.

Immunohistochemistry is used to analyze HER2 status. There are different scores, like score 0, 1+, 2+ and 3+. When there is a tumor classified with a score 2+, it's better to execute FISH (fluorescent in situ hybridization). A tumor is considered HER2-positive when its score is 3+ or 2+ with FISH-detected HER2 amplification. [5] (**Fig. 4**)

According to a ToGA (Trastuzumab for Gastric Cancer) trial, in patients who present advanced HER2-positive gastric/gastroesophageal junction cancers, the combination of Trastuzumab and fluoropyrimidine plus cisplatin chemotherapy improves overall survival. [5].

However, there is an important consideration to do: HER2 expression can be heterogenous within neoplasms, with a significant impact on Trastuzumab efficacy. Indeed, a heterogeneous expression of HER2 is related to shorter progression-free survival after Trastuzumab-containing first-line chemotherapies. For this reason, it's necessary to acquire a representative tumoral material performing at least 6 endoscopic biopsy specimens.

Trastuzumab efficacy can also be affected by acquired resistance, a phenomenon that can be explained by HER2 expression heterogeneity: on the one hand, Trastuzumab-containing chemotherapies eradicates HER2-positive neoplastic cells but, on the other hand, it also gives proliferative advantage to HER2-negative malignant cells. In order to solve the acquired resistance problem, new HER2-targeted molecules and combinations have been developed, like Trastuzumab-deruxtecan (T-Dxd). This antibody-drug conjugate affects both HER2 positive cells and HER2 negative neoplastic clones and it consists of a Trastuzumab molecule connected to a cytotoxic topoisomerase I inhibitor by a cleavable linker. [5]

According to dr. Kohei Shitara's study (National Cancer Center Hospital East in Kashiwa, Japan), Trastuzumab-deruxtecan showed benefits across patients with HER2 positive and HER2-low

advanced gastric cancer (HER2-low is a new classification that includes tumors with HER2 score 1+ or 2+ and FISH negative).
The randomized phase II study, called DESTINY-Gastric01, included HER2 positive advanced gastric cancer patients in a primary cohort, with 125 randomized patients who received the drug and 55 patients represented controls. For HER2-low cancers, a single-arm exploratory cohort for T-Dxd treatment was created. Patients with HER2-positive gastric/gastroesophageal junction cancers treated with T-Dxd showed benefits, improving objective response rate and overall survivor (from 8.1 to 12.5 months after treatment). At the same time, the study demonstrated that T-Dxd was effective also in HER2-low patients. [9]

3.2 MISMATCH REPAIR DEFICIENCY (MMRd)/MICROSATELLITE INSTABILITY (MSI)

MSI gastric carcinomas present loss of mismatch repair (MMR) proteins' functions, that is typically caused by mutational inactivation or epigenetic silencing of DNA mismatch repair genes. This leads to microsatellite instability.
This tumor subtype generally arises in older patient's gastric antrum, but it's possible to find it also in Lynch syndrome and non-polyposis colorectal cancer syndrome. [5]
Most of MSI gastric carcinomas are characterized by an intestinal-type morphology, a lower rate of nodal metastasis and a more favorable prognosis. [5]
Microsatellite status is studied using molecular assays or immunohistochemical analysis of MMR proteins' expression, such as MLH1, PMS2, MSH2 and MSH6.
Moreover, Pembrolizumab is used as second-line therapy against MSI gastric carcinomas.[5]

3.3 EBER

The percentage of gastric tumors that result positive for Epstein-Barr virus (EBV) is about 9% of gastric/gastroesophageal adenocarcinomas. This tumor type, that is characterized by CpC island methylation (CIMP) and high levels of DNA promoter hypermethylation, has a positive prognosis and it's usually situated in the gastric body or fundus. [5]
The most used method to detect EBV is called EBV-encoded RNA (EBER) in situ hybridization (ISH). In addition to false-negative EBER-ISH results caused by RNA degradation, there may be also false-positive results due to cross-reactivity, poorly fixed tissues and non-specific staining. [10]
As regards molecular alterations, in EBV positive gastric cancers there are different mutations, in particular: *PIK3CA (80%), ARID1A (55%), BCOR (23%),* together with *CDKN2A* promoter hypermethylation (100%) and overexpression of Programmed Death Ligands 1 and 2 (PD-L1 and PD-L2). [5]

Kim and colleagues' study demonstrates a relevant response to Pembrolizumab in patients with EBV-positive gastric tumors. [5]

3.4 PD-L1

PD-L1 (Programmed Death Ligand 1) can be analyzed in particular in locally advanced, recurrent or metastatic gastric cancers, in order to identify patients who can be treated with PD-1 (Programmed Death protein) inhibitors. [11]

While PD-1, a checkpoint protein localized on the surface of T cells, has an important role in the inhibition of immune system, PD-L1 is a transmembrane glycoprotein situated on the surface of malignant tumor cells, and it binds to PD-1. When tumor cells recognize PD-1 proteins, they react producing more PD-L1 proteins. In this way, PD-1/PD-L1 link is possible, and it causes T cells apoptosis. This binding is responsible for the decrease of T cell-mediate immune surveillance and, consequently, for tumor evasion. [12]

PD-1/PD-L1 aces is considered a fundamental therapeutic target in cancer immunotherapy thanks to the use of PD-1 and PD-L1 inhibitors. Immunohistochemistry (IHC) assays can be used to detect tumors expressing PD-L1 positive.

Monoclonal antibodies used in therapy against this tumor subtype are Avelumab, Atezolizumab and Durvalumab. [5]

AIM OF THE THESIS

Gastric and gastroesophageal junction cancers are among the leading causes of cancer-related deaths worldwide. If Trastuzumab combined to cytotoxic chemotherapy is consider as the first-line therapy for HER-overexpressing metastatic gastroesophageal cancers, the new antibody-drug conjugated Trastuzumab-Deruxtecan has proved to be effective also in HER2-low disease, paving the way for novel therapeutic scenarios.

In this real-world study, the prevalence of HER2-low and its correlation with clinical, histopathological and molecular features, including MMR/MSI status, EBER and PD-L1 combined positive score, are analyzed. While HER2 overexpression has been largely investigated, few studies have provided data on the prevalence of HER2 1+ and not amplified HER2 2+ cases (classified as HER2-low).

MATERIALS AND METHODS

1.0 STUDY DESING

We retrospectively selected a total of 1109 formalin-fixed paraffin-embedded (FFPE) samples of gastroesophageal adenocarcinoma (n = 375 GEJ adenocarcinomas and 707 gastric adenocarcinomas; 502 surgical resection specimens and 607 biopsy specimens) analyzed by IHC for HER2 protein expression from January 2018 to June 2022. The cases were selected from the Surgical Pathology Units of Padua University Hospital (Padua, Italy), Ospedale Policlinico San Martino IRCCS (Genoa, Italy), Fondazione IRCCS Casa Sollievo della Sofferenza (San Giovanni Rotondo, Italy), "Città della Salute e della Scienza" Turin University (Turin, Italy), Santa Chiara Hospital (Trento, Italy), Fondazione IRCCS Policlinico San Matteo (Pavia, Italy), and Santa Maria della Misericordia University Hospital (Udine, Itay). Original slides were jointly re-evaluated, in order to assess the number of biopsy fragments, histotype and grading according the WHO 2019 criteria [1], Lauren and Ming classes, poorly cohesive carcinoma class according to the European Chapter of International Gastric Cancer Association (IGCA). Information regarding staging, neoadjuvant therapy and biomarkers' status (HER2, PD-L1, MMR/MSI status, EBER) was collected from the pathology reports.

2.0 HISTOLOGICAL SAMPLE PREPARATION

Tissue microscopic analysis is possible thanks to a process made of different steps and called "tissue processing", that begins with tissue fixation and finishes with section cutting on the microtome. Classic procedure is explained below.

1. Obtaining fresh specimens

Fresh tissue specimens can be removed from various sources. After the dissection, the tissue has to be fixed as soon as possible to avoid deterioration, which is a natural consequence of tissue removal from its setting.

2. Fixation

The specimen is put in a liquid fixative that slowly penetrates the tissue and preserves it, causing chemical and physical changes. The most common liquid fixative used is formalin (formaldehyde solution), with a penetration rate of 0.8 mm/h.

Specimens remain in liquid fixative for long enough to allow it to penetrate every part of the tissue and to permit chemical reaction of fixation. Usually, this time is between 6 and 24 hours and it depends on sample size.
Purpose of fixation is to arrest tissue degradation.

3. Tissue sampling

After fixation, it's necessary to select specific areas of tissue samples for examination. This step is made by pathologist, who describes the specimen macroscopically and selects the most representative parts of it. These parts are placed in labeled cassettes reporting patient's name, surname, and his histological identification number. When the specimen is put inside them, the surface that pathologist wants to analyze faces the bottom of the cassettes.
While cassettes are put in the processor, the remaining part of the tissue previously sampled is generally placed in vacuum bags.

4. Embedding

Embedding is divided into two main parts: imbibition and pouring of paraffine.
Imbibition with paraffine is carried out using automatic processors and different solutions and chemical reagents. It starts with dehydration, that consists of successive immersions of the specimen in a series of alcohol solutions with increasing concentration. This step is necessary because melted paraffine wax is hydrophobic, so most of the water inside a specimen must be removed and replaced by alcohol (which is miscible with water). The sequence of increasing concentrations avoids distortion of the tissue and, generally, it includes 70% alcohol, 95% alcohol and finally 100% alcohol. Now, tissue is water-free and contains alcohol, which is unfortunately immiscible with melted paraffine wax. To overcome this problem, an intermediated solvent miscible with both alcohol and paraffine is used. This agent, typically xylene, substitutes alcohol in the tissue and then it will be displaced by paraffine wax during the successive step. This process is named "clearing" and the intermediated solvent is called "clearing agent". The latter has another important role in tissue processing: it removes a consistent part of fat from the specimen, otherwise wax infiltration would be impeded.
Then, melted paraffine wax can infiltrate the tissue, at a temperature of 60°C. This wax gives tissue physical properties to be subsequently sectioned during the following step.

Pouring of paraffine is the second main part of embedding and, during it, the specimen is transformed into a block of paraffine that will be cut on the microtome. This process is carried out using an "embedding centre", which is composed of:

- Heated paraffine wax container
- Melted paraffine dispenser
- Heated cassettes containers
- A cold plate (about -20°C)
- Heated working area (about 60°).

Cassettes are put inside heated containers to keep them warm. Then, the laboratory technician takes one cassette, opens it and selects one embedding mold basing on the specimen seizure. Once some paraffine wax has been put inside the mold using the melted paraffine dispenser, the tissue sample is switched from cassette to embedding mold. It's important to maintain orientation of the specimen previously established by the pathologist, because it will represent the "plane of section" of the tissue. Now, specimen is made to adhere to the bottom of the mold using pestles. After that, the cassette is placed on top of the embedding mold, more paraffine is added and the mold is finally placed on the cold plate to solidify.

When solidification is completed, the paraffine block can be removed from the mold and it is ready for the cutting step.

5. Cutting

The aim of this step is to obtain thin slices of the specimen (2-3 microns of thickness) in order to analyze them in the microscope. Rotary microtome is the most used and it is composed of these main parts:

- Knife holder base, that can be moved to or away from the block
- Knife holder, which includes blade clamp to hold the blade
- Block holder, where the block is placed. In a rotary microtome, this part slides up and down during rotation of the advancement handwheel, while the blade stands still.
- Advancement handwheel, that rotates in one direction moving the block up and down and closer to the knife.
- Water baths thermostatically controlled at 40-45°C, where slices are put after sectioning

Cutting is divided into two main parts: trimming and sectioning.

Trimming consists of cutting block to eliminate excess of paraffine. In this way, the surface of the specimen representing the plane of section is completely and clearly visible. During this step, block is placed in the block holder and then it is moved to the knife. When blade starts to cut paraffine, laboratory technician keeps rotating the advancement handwheel till all specimen surface is visible. To trim, section thickness is set at 15-20 microns.

After this procedure, block is overheated, so it is put on a cold plate to obtain better and thinner slices.

The second step is sectioning. The procedure is the same as trimming, but section thickness is different: here, it is set at 2-3 microns.
After obtaining specimen slices, they are picked using a little brush and then placed on the surface of water bath. The latter is thermostatically controlled because heated water avoid formation of folds.
Finally, sample sections are caught with polarized or unpolarized slides, basing on stain that will be made.
Slides with specimen sections are placed in an incubator to be dried. Before staining step, sections need to be dewaxed and rehydrated. To get this, slides are immersed firstly in xylene, to remove paraffine wax from the sections, and then in a series of alcohol solutions with decreasing concentration. Now, it's possible to proceed with staining step.
When sections are stained, slides have to be mounted. Tissue needs to be dehydrated, so slides are washed in a series of alcohols with increasing concentration and then there is a passage in xylene. The latter is fundamental because of its affinity with the mounting medium, commonly Canada balsam. After balsam has been put on tissue sections, a cover slip is placed on the slide which is finally left to dry.

3.0 IMMUNOHISTOCHEMISTRY (IHC)

Immunohistochemistry is a method used to detect protein markers in tissues. While hematoxylin and eosin staining is not specific, IHC marks precise proteins basing on antigen-antibody complexes.
Antigens are proteins located within or on the surface of a cell. Pathologist searches for the presence of antigens in the tissue to make a better diagnosis and, consequently, decides which treatment is the most suitable for that tumor type. [13]

Polyclonal and Monoclonal antibodies

A primary antibody binds to the target antigen. This antibody can be polyclonal or monoclonal: the first one has affinity for different parts (epitopes) of an antigen and it consists of a mix of different antibodies molecules, while the second type that binds to one single epitope and it's a preparation of antibodies chemically identical.
Monoclonal antibodies are obtained by hybridomas: an antigen is inoculated into a mammal (generally mouse) activating its immune response. Therefore, B cells begin to produce antibodies against this antigen and then they are isolated. B cells from the spleen of the animal are fused with immortal myeloma cells, creating a new cell line (hybridoma). The latter has a particular feature: it can produce antibodies with a high reproductivity typical of myeloma cells.

Then, from the heterogenous population of cells the hybridoma is able to produce, the type of antibody I want to use is successively selected, and it is left to proliferate on petri dishes, obtaining equal single epitope antibodies.
Polyclonal antibodies are cheaper and more sensitive because they bind many epitopes, but this can lead to cross-reactivity to non-target antigens. On the other hand, monoclonal antibodies are more expensive, more specific, and less sensitive. [14]

Antigen retrieval and blocking proteins
Antigen retrieval and blocking proteins are fundamental in IHC procedure.
Antigen retrieval consists of antigenic sites exposition, to allow antigen-antibody binding. This process is necessary because formaldehyde fixation causes protein cross-linking (methylene bridges), which masks epitopes. Antigen retrieval breaks these methylene bridges, exposing antigenic sites. This step is achieved by two possible methods: heat-induced or proteolytic-induced epitope retrieval.
Blocking proteins prevents non-specific antibody binding and reduces false positive results. In this step, it's possible to use proteins like bovine serum albumin (BSA) or casein. When avidin-biotin detection system is used, endogenous biotin has to be blocked because it is present in many tissues.[15]

Detection systems
Fluorochromes and conjugated enzymes can reveal primary antibody-antigen binding. If antibody is labeled with a fluorochrome, the site of reaction between antibody and antigen is detected by fluorescence (immunofluorescence). On the other hand, if an enzyme is used as a marker, it will produce a colored precipitate when a proper substrate in present (immunohistochemistry).
Immunofluorescence is less stable over time than immunohistochemistry, which is more sensitive and more precise.
Commonly used enzymes in immunohistochemistry are *alkaline phosphatase (ALP), β-galactosidase* and *horseradish peroxidase (HRP).* [16]
There are different immunohistochemical methods to detect antigens, such as direct, indirect and methods with immune or avidin-biotin complex.

- **Direct method:** it is the easiest method because it consists of an antibody specifically directed against an antigen. This specific antibody is linked to an enzyme and then a substrate is added producing a stained precipitate, that allows to visualize the antigen. [16]
- **Indirect method:** the antigen is bound to a non-conjugated primary antibody. After, this complex reacts with a secondary enzyme-linked antibody, that binds the Fc part of the primary antibody. In particular, the two antibodies originate from different animal species. [16]

- **Method with immune complex:** there are three important elements in this method: a primary antibody directed against the antigen, a secondary bridging antibody and the immune complex, composed of two IgG molecules linked to enzyme molecules (alkaline phosphatase β-galactosidase or horseradish peroxidase). Primary antibody binds to the antigen, then the bridging antibody binds to Fc portion of the primary antibody and finally the immune complex (which is from the same species as the primary antibody) is added, attaching itself to the secondary antibody. These immune complexes don't precipitate in solution and the enzyme is visualized with substrate-chromogen reaction. A great vantage of this immunohistochemical method is that it's possible to obtain a good stain even though part of antigen was destroyed by processing and fixation processes. [16]
- **Method with avidin-biotin complex:** this is a recent method, based on the capacity of avidin to link four vitamin biotin molecules. As the previous method, there are three components: a primary antibody directed against the antigen, a secondary biotin-linked antibody (which can bind to the primary antibody) and a complex of enzyme-linked biotin and avidin. Biotin attached to the secondary antibody can connect itself to this complex thanks to free avidin's binding sites. Enzyme is visualized using the proper chromogen. The great affinity between avidin and biotin makes this method particularly sensitive. [16]

Substrates and chromogens

An enzyme is a catalyst that works on a substrate to accelerate its conversion in product, by the formation of an intermediate enzyme-substrate complex.

A single enzyme molecule can transform more substrate molecules in product and this gradual amplification increase IHC sensitivity.

Based on the enzymes used, there are different chromogens that can act as substrates, functioning as electron donors in the reaction. At the end, a final and coloured product is created, and it will detect the antigen through its precipitation in the reaction point.

Immunohistochemical techniques need the precipitation of the terminal, stained product in the site where it has been created. For this reason, chromogens that form soluble terminal products aren't used for these techniques.

An example of substrate for horseradish peroxidase (HRP) enzyme is *3-3'-diaminobenzidine (DAB)*, a derivate of benzene. It produces an intense, alcohol-resistant, brown stain. HRP catalyzes reaction between the substrate and H_2O_2. While DAB oxidizes creating the final product of the reaction (that is a stained molecule), the hydrogen peroxide reduces itself becoming water. [16]

Counterstain

Counterstain is used to visualize better antibody localization and its relationship with cellular structures within the tissue. In IHC, the most popular counterstain used is hematoxylin, which stains nuclei blue. [15]

What's important is to choose a counterstain appropriate to the substrate used. If the chosen chromogen produces terminal products that are alcohol and organic solvents insoluble (like DAB), it's possible to use alcohol-based dyes to counterstain. Whereas, if these final products are alcohol and organic solvents soluble, it's not possible to use alcohol to decolorize.[16]

For this study…

IHC stains were performed using the Bond Polymer Refine Detection Kit (Leica Biosystems, Newcastle upon Tyne, UK) on BOND-MAX automated IHC stainer (Leica Biosystems) and the UltraView DAB Detection Kit on Ventana Benchmark Ultra automated IHC staining system (Roche Diagnostics, Basel, Switzerland).

Four-μm-thick FFPE sections were incubated with the primary antibodies MSH6 (clone EP49; Agilent, Santa Clara, CA; dilution 1:25), PSM2 (EP51; Agilent), MLH1 (clone ES05; Agilent), MSH2 (FE11; Agilent), PATHWAY HER2/ neu (4B5; Ventana Medical Systems, Roche Diagnostics), and PD-L1 (22C3; Dako and SP142; Ventana Medical Systems, Roche Diagnostics).

For the evaluation of HER2, the four-tier score was adopted; equivocal cases were analyzed by HER2 fluorescent in situ hybridization (FISH) to test for gene amplification. HER2 1+ and not-amplified 2+ cases were reclassified as HER2-low, while HER2 amplified 2+ and 3+ cases were classified as HER2-high[17].

Deficient mismatch repair (MMRd) status was assessed by testing MSH2, MSH6, MLH1 and PSM2, and samples were defined as MMRd when one or both proteins resulted negative[18].

PD-L1 was evaluated by using the Combined Positive Score (CPS). Thresholds of 1, 5, and 10 were used for the analysis[19].

Regarding EBER, the Bond ready-to-use ISH EBER Probe was used in a Leica Bond-Max automation system according to the manufacturer's instructions (Leica Biosystems) to detect EBV infection.

4.0 FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH)

FISH is a procedure that detects specific DNA sequences location in chromosomes in metaphase o interphase cells, using fluorescent probes.

DNA double helix is significantly stable, due to hydrogen bonds between bases. When these bonds are broken, they are able to re-form in favourable conditions. This feature is the basis of molecular

hybridization, a process where DNA or RNA sequence, attached to a fluorescent reporter molecule, works as a probe to identify or quantify its complementary sequence. [20]

Principles of FISH

Firstly, probe is labelled directly or indirectly (**Fig. 5**): in the direct method, the probe is attached to a fluorochrome (such as rhodamine), while in the indirect one a hapten (like biotin) binds to the probe. Successively, labelled probe and target DNA are denatured with heat or chemicals, to form new bonds between the probe and the target. In this way, the probe specifically hybridizes to its complementary sequence on the chromosome. In the direct method, there's a colored signal on the hybridization site that can be visualized using fluorescence microscopy, whereas the indirect method bases on immunohistochemistry and antigen-antibody binding, that is visualized with a colored histochemical reaction visible with light microscopy or fluorochromes with ultraviolet light. [21]

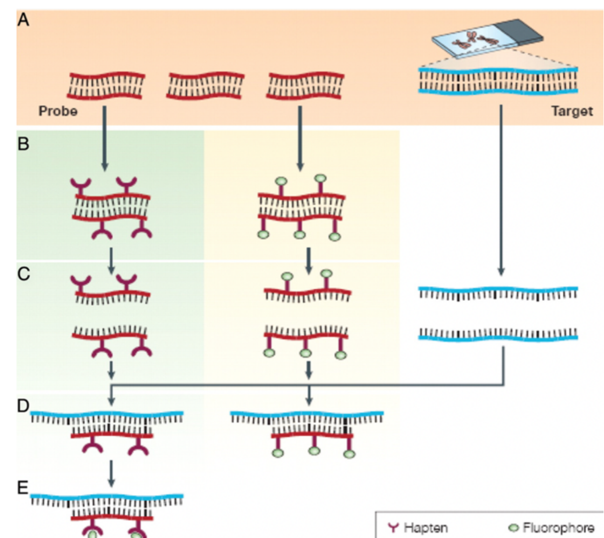

Figure 5: The principles of fluorescence *in situ* hybridization. (**A**) The basic elements are a DNA probe and a target sequence. (**B**) DNA probe is labelled indirectly with a hapten (left panel) or directly labelled with a fluorochrome (right panel). (**C**) The labelled probe and the target DNA are denatured (**D**) They are then combined, which allows the annealing of complementary DNA sequences. (**E**) If the probe has been labelled indirectly, an extra step is required for visualization of the non-fluorescent hapten. Finally, the signals are evaluated by fluorescence microscopy. *Nature Reviews Genetics 6, 784 (2005)*

Direct method is faster than the indirect one, which is characterized by better signal amplification thanks to the use of several layers of antibodies. [20]

Probes

There are three main types of probes: whole-chromosome painting probes, repetitive sequence probes and locus-specific probes.

- **Whole-chromosome painting probes:** complex DNA probes derived from a single type of chromosome that has been microdissected, amplified and labelled to highlight the whole chromosome homogenously. This probe type is called in this way because of the hybridization of fluorescently labelled chromosome-specific probes to cells. Individual chromosomes and chromosomal aberrations are visualized, in particular during metaphase. [21]
- **Repetitive sequence probes:** they bind to precise chromosomal regions that contain short sequences, that are present in many copies. [21]

- **Locus-specific probes:** generally genomic clones, they vary in size based on the nature of cloning vector (such as plasmids or BAC vector). This type is helpful to visualize structural rearrangements, like chromosomal translocations, inversions or deletions. [21]

Applications

FISH had a primary role in mapping genes, in particular in the Human Genome Project (HGP). Nowadays, this procedure is largely used in clinical diagnosis. FISH makes possible to visualize chromosomal abnormalities (such as deletions, duplications and translocations) and to search for multiple sites simultaneously, when hybridization probes have been marked with different fluorophores. All these applications are fundamental, because identifying genes involved in cancer development provides targets for directed therapy. [21]

Furthermore, FISH is the gold standard to detect HER-2 status, like in breast cancer and gastroesophageal adenocarcinoma. Indeed, when HER2 has a score of 2+, it's necessary performing FISH because tumor with HER2 score 2+ with FISH-detected are considered HER2-positive, while those with FISH-negative are not. Consequently, the result impacts on better therapy choices.

5.0 STATISTICAL ANALYSIS

Chi-square, Fisher's exact tests and Kruskall wallis ANOVA were used, where appropriate. p values < 0.05 were considered statistically significant.

RESULTS

Clinicopathological findings

Overall, the mean age of the patients was 69.46±12.25 years (median 71; range 19–96). The male-to-female ratio was 2.04. Out of 1109 samples analyzed, 502 (45.3%) were surgical resection specimens and 607 (54.7%) were biopsies; 1073 (96.8%) were primary tumors, and 36 (3.2%) were metastasis. Overall, our case series included 375 (33.6%) GEJ adenocarcinomas and 707 (63.8%) gastric adenocarcinomas. In 27 (24.3%) metastatic samples, whether the primary tumor was gastric or gastroesophageal was unknown. For 87 GEJ adenocarcinomas the data on the distance from the gastroesophageal junction was available. Among these 87 cases, 22 (25.3%) were Siewert 1, 52 (59.8%) were Siewert 2, and 13 (14.9%) were Siewert 3. Among the gastric adenocarcinomas, 201 (28.4%) were localized in the corpus/fundus, 467 (66.1%) in the antrum/angulus and 39 (5.5%) cases in the antrum/corpus.

With regard to the histotype according to WHO 2019 classification, 546 (49.2)% of cases were tubular, 35 (3.2%) were papillary, 245 (22.1%) were poorly cohesive, 222 (20.0%) were of mixed histotype, 30 (2.7%) were mucinous, 15 (1.5%) were carcinomas with lymphoid stroma, 11 (1.0%) cases were of a rare histotype (i.e., 6 were adenosquamous, 3 were undifferentiated, one was a mixed neuroendocrine-non-neuroendocrine neoplasm (MiNeN), one was mucoepidermoid) and in 5 (0.5%) cases the histotype was not assessable.

When investigating the distribution across Lauren's classes, 597 (53.8%) of cases were intestinal, 254 (22.9%) were diffuse, 225 (20.3%) were mixed, 27 (2.5%) were indeterminate and in 5 (0.5%) cases the histotype according to Lauren's was not assessable.

With regard to Ming classification, 190 (17.1%) of cases were expansive, 575 (51.8%) were infiltrative, and 344 (31.1%) were not assessable.

As to the grading, only tubular, papillary and mixed (tubular component) cases were graded. Out of 803 cases, 413 (51.4%) were high-grade, 268 (33.4%) were low-grade and 122 (15.2%) were not assessable, due to the biopsy being not representative of the tumor or due to therapy artifacts.

With regard to the tumor extent, the surgical specimens of our cases series were distributed as follows: 52 (10.4%) were pT1, 56 (11.2%) were pT2, 223 (44.4%) were pT3, 150 (29.9%) were pT4, and 21 (4.1%) were pTx.

When investigating lymph node involvement, 146 (29.1%) of the surgical specimens were pN0, 88 (17.5%) were pN1, 95 (18.9%) were pN2, 141 (28.1%) were pN3, and 32 (6.4%) were pNx.

HER2-low: prevalence and clinicopathological associations (Table 1)

Overall, HER2 expression was scored as follows: 662 (59.7%) cases were HER2 0, 201 (18.1%) cases were HER2 1+, 97 (8.8%) cases were not amplified HER2 2+, 35 (3.2%) cases were amplified HER

2+, and 94 (8.4%) cases were HER2 3+. In 20 (1.8%) cases scored as HER2 2+, the result of the FISH was either indeterminate or not available and will be excluded from further evaluations. Out of 1089 assessable cases, 662 (60.8%) cases were HER2 0, 298 (27.4%) were HER2-low and 129 (11.8%) were HER2-high.

Out of the 501 surgical resection specimens in our case series, 357 (71.3%) were HER2 0, 63 (12.6%) were HER2 1+, 32 (6.3%) were not amplified HER2 2+, 14 (2.8%) were amplified HER2 2+, 35 (7.0%) were HER2 3+; 95 (18.9%) were HER2-low, 49 (9.8%) were HER2 high. Out of the 588 biopsies, 305 (51.9%) were HER2 0, 138 (23.4%) were HER2 1+, 65 (11.1%) were not amplified HER2 2+, 21 (3.6%) were amplified HER 2+, 59 (10.0%) were HER2 3+; 203 (34.5%) were HER2-low and 80 (13.6%) were HER2-high. Overall, a higher prevalence of HER2-low was found among biopsy specimens ($p<0.00001$) (**Figure 6**). The mean number of biopsy fragments of adenocarcinoma per sample was: 4.53 ± 2.32 for HER2 0 cases, 4.73 ± 2.30 for HER2-low cases, and 4.22 ± 1.92 for HER2-high cases. When using 6 biopsies as a cut-off value, no difference in the distribution of HER2 0, low and high was found between cases with <6 biopsy fragments and cases with ≥6 biopsy fragments.

Out of 1055 primary tumor samples, 642 (60.9%) were HER2 0, 289 (27.4%) were HER2-low and 124 (11.7%) were HER2-high. Out of 34 metastatic samples, 20 (58.8%) were HER2 0, 9 (26.5%) were HER2-low, and 5 (9.5%) were HER2-high.

Out of 368 GEJ adenocarcinomas, 226 (61.4%) were HER2 0, 91 (24.7%) were HER2-low and 32 (13.9%) were HER2-high. Among the 22 Siewert 1 cases, 15 (68.2%) were HER2 0, 6 (27.3%) were HER2-low, and 1 (45.5%) was HER2-high. Among the 52 Siewert 2 cases, 28 (53.8%) were HER2 0, 15 (28.8%) were HER2-low, and 9 (17.4%) were HER2-high. Among the 13 Siewert 3 cases, 6 (46.2%) were HER2 0, 6 (46.2%) were HER2-low, and 1 (7.6%) was HER2-high.

Out of 696 gastric adenocarcinomas, 423 (60.8%) were HER2 0, 199 (28.6%) were HER2-low and 74 (10.6%) were HER2-high. Out of 199 gastric cases from the corpus/fundus, 120 (60.3%) were HER2 0, 61 (30.7%) were HER2-low and 18 (9.0%) were HER2-high. Out of 458 gastric cases from the antrum/angulus, 271 (59.2%) were HER2 0, 133 (29.0%) were HER2-low and 54 (11.8%) were HER2-high. Out of 39 gastric cases from the antrum/corpus, 32 (82.0%) were HER2 0, 5 (12.8%) were HER2-low and 2 (5.2%) were HER2-high (**Figure 6**).

Regarding the histotype according to the WHO 2019 classification, among the 534 tubular adenocarcinomas 304 (56.9%) were HER2 0, 156 (29.2%) were HER2-low and 74 (13.9%) were HER2-high. Out of the 34 papillary adenocarcinomas, 19 (55.9%) were HER2 0, 10 (29.4%) were HER2-low and 5 (14.7%) were HER2-high. Out of the 243 poorly cohesive carcinomas, 162 (66.7%) were HER2 0, 57 (23.5%) were HER2-low and 24 (9.8%) were HER2-high. Out of the 218 mixed adenocarcinomas, 141 (64.7%) were HER2 0, 55 (25.5%) were HER2-low and 22 (10.1%) were

HER2-high. Out of the 30 mucinous adenocarcinomas, 21 (70.0%) were HER2 0, 8 (26.7%) were HER2-low and 1 (3.3%) was HER2-high. Out of the 15 carcinomas with lymphoid stroma, 7 (46.7%) were HER2 0, and 6 (40.0%) were HER2-high.

When investigating the classification proposed by the European Chapter of the IGCA[22], among 73 PC-NOS cases, 40 (54.8%) were HER2 0, 21 (28.8%) were HER2-low and 12 (16.4%) were HER2-high. Out of the 124 PC-NOS/SRC, 82 (66.1%) were HER2 0, 32 (25.8%) were HER2-low, and 10 (8.1%) were HER2-high. Out of 41 SRC, 37 (90.3%) were HER2 0, 3 (7.3%) were HER2-low, and 1 (2.4%) was HER2-high. The lower prevalence of HER2-high cases in PC-NOS/SRC and of HER2-low and HER2-high cases appeared to be statistically significant (p=0.00224).

Regarding Lauren classification, among 585 intestinal cases, 334 (57.1%) were HER2 0, 171 (29.2%) were HER2-low and 80 (13.7%) were HER2-high. Out of the 252 diffuse cases, 168 (66.7%) were HER2 0, 60 (23.8%) were HER2-low and 24 (22.9%) were HER2-high. Out of the 221 mixed cases, 143 (64.7%) were HER2 0, 56 (25.3%) were HER2-low and 22 (10.0%) were HER2-high. Out of the 27 indeterminate cases, 13 (48.1%) were HER2 0, 11 (40.7%) were HER2-low and 3 (11.1%) were HER2-high.

Regarding Ming classification, among 190 expansive cases, 119 (62.6%) were HER2 0, 50 (26.3%) were HER2-low and 21 (11.1%) were HER2-high. Out of 564 infiltrative cases, 358 (63.5%) were HER2 0, 141 (25.0%) were HER2-low and 65 (14.0%) were HER2-high.

Out of the 410 high-grade cases, 248 (60.4%) were HER2 0, 117 (28.6%) were HER2-low and 45 (11.0%) were HER2-high. Out of the 258 low-grade cases, 150 (58.1%) were HER2 0, 72 (119.1%) were HER2-low and 36 (13.9%) were HER2-high.

Regarding tumor extent of the surgical specimens, among the 52 surgical specimens classified as pT1, 44 (84.6%) were HER2 0, 3 (5.8%) were HER2-low, and 5 (9.6%) were HER2-high. Out of the 56 surgical specimens classified as pT2, 36 (64.3%) were HER2 0, 13 (23.2%) were HER2-low, and 7 (12.5%) were HER2-high. Out of the 56 surgical specimens classified as pT2, 36 (64.3%) were HER2 0, 13 (23.2%) were HER2-low, and 7 (12.5%) were HER2-high. Out of the 223 surgical specimens classified as pT3, 154 (69.1%) were HER2 0, 45 (20.1%) were HER2-low, and 24 (10.8%) were HER2-high. Out of the 149 surgical specimens classified as pT4, 107 (71.8%) were HER2 0, 32 (21.4%) were HER2-low, and 10 (6.8%) were HER2-high. Out of the 21 surgical specimens classified as pTx, 16 (76.2%) were HER2 0, 3 (14.3%) were HER2-low, and 2 (9.5%) were HER2-high.

Regarding lymph node involvement, among the 146 surgical specimens classified as pN0, 110 (75.3%) were HER2 0, 26 (17.8%) were HER2-low, and 10 (6.9%) were HER2-high. Out of the 88 surgical specimens classified as pN1, 61 (69.4%) were HER2 0, 21 (19.3%) were HER2-low, and 10 (11.3%) were HER2-high. Out of the 95 surgical specimens classified as pN2, 62 (65.3%) were HER2 0, 20 (21.0%) were HER2-low, and 13 (13.7%) were HER2-high. Out of the 101 surgical specimens

classified as pN3, 101 (72.1%) were HER2 0, 27 (20.3%) were HER2-low, and 12 (8.6%) were HER2-high.

Out of 177 pre-treated samples, 116 (65.6%) were HER2 0, 46 (25.9%) were HER2-low and 15 (8.5%) were HER2-high. Out of 912 non-pre-treated samples, 546 (59.9%) were HER2 0, 252 (27.6%) were HER2-low and 912 were HER2-high.

Associations between HER2-low and other biomarkers' status (Table 2)

Overall, 248/1089 (22.8%) cases were investigated for PD-L1 expression; 45 (18.1%) cases were CPS<1, 77 (31.1%) were 1≤CPS<10, 126 (50.1%) were CPS≥10. Out of the 45 cases with CPS<1, 31 (68.9%) were HER2 0, 10 (22.2%) were HER2-low, and 4 (8.9%) were HER2-high. Out of the 77 cases with 1≤CPS<10, 54 (70.2%) were HER2 0, 17 (22.1%) were HER2-low, and 6 (7.8%) were HER2-high. Out of the 126 cases with CPS≥10, 73 (57.9%) were HER2 0, 34 (27.0%) were HER2-low, and 19 (15.1%) were HER2-high (**Figure 6**).

EBER expression was investigated in 223/1089 (20.5%) cases; 213 (95.5%) were EBER negative and 10 (4.5%) were EBER positive. Out of the 213 EBER negative cases, 122 (57.3%) were HER2 0, 65 (30.5%) were HER2-low, and 26 (12.2%) were HER2-high. Out of 10 EBER negative cases, 5 (50.0%) were HER2 0, 3 (30.0%) were HER2-low and 2 (20.0%) were HER2-high.

MMR/MSI status was investigated in 545/1089 (50.0%) cases; 484 (88.8%) were MMRp and 61 (11.2%) were MMRd/MSI. Among the MMRd cases, 56/61 (91.8%) had MLH1/PMS2 loss, 3/61 (4.8%) had MSH2/MSH6 loss, 1/61 (1.6%) had MLH1/MSH6 loss and 1/61 (1.6%) had PMS2 loss. Out of 484 MMRp cases, 281 (58.1%) were HER2 0, 141 (29.1%) were HER2-low, 62 (12.8%) were HER2-high. Out of 61 MMRd cases, 37 (60.6%) were HER2 0, 19 (30.0%) were HER2-low, 5 (8.2%) were HER2-high. Out of 56 cases with MLH1/PMS2 loss, 33 (59.0%) were HER2 0, 18 (32.1%) were HER2-low, 5 (8.9%) were HER2-high. Out of 3 cases with MSH2/MSH6 loss, 2 (66.7%) were HER2 0 and 1 (33.3%) was HER2-low. The cases with MLH1/MSH6 loss and PMS2 loss were both HER2 0.

HER2 evaluation in matched samples

Overall, 64 paired samples (i.e., biopsy and surgical resection specimen of the same patient) were included in the analysis. Out of 64 paired biopsies and surgical specimens, 30 (46.9%) had a concordant HER2 score, and 34 (53.1%) pairs had discordant HER2 scores. All the 30 concordant samples were labeled as HER2 0. The 17 pairs of discordant samples were labeled as follows: HER2 1+ (biopsy) and HER2 0 (surgical specimen) in 11 (64.7%) pairs, amplified HER2 2+ (biopsy) and HER2 3+ in 2 pairs (11.8%), not amplified HER2 2+ (biopsy) and HER2 0 (surgical specimen) in 2

pairs (11.8%); HER2 1+ (biopsy) and not amplified HER2 2+ (surgical specimen) in 1 (5.9%) pair and HER2 1+ (biopsy) and HER2 3+ (surgical specimen) in 1 (5.9%) pair.

Among the 15 patients with concordant HER2 scores, 10 (66.7%) did not receive neoadjuvant therapy, and 5 (33.3%) received neoadjuvant therapy between the endoscopic sampling and surgical resection. Among the 17 patients with discordant HER2 scores, 10 (58.9%) did not receive neoadjuvant therapy, and 7 (41.2%) received neoadjuvant therapy between the endoscopic sampling and surgical resection.

HER2 low prevalence according to the year of evaluation and center where the evaluation was performed (Table 3).

The 1089 samples included in the studies were selected from the surgical pathology units of seven centers dating back to 2018. The prevalence of HER-low cases was: 47/174 (27.0%) in 2018, 70/277 (25.3%) in 2019, 59/206 (28.7%) in 2020, 93/315 (29.5%) in 2021, 29/117 (24.7%) in 2022. No statistically significant difference in the distribution of HER2 0, HER2-low and HER2-high cases was found over the years.

The prevalence of HER-low cases within the various centers was: 94/402 (23.4%) in Center 1, 87/302 (28.9%) in Center 2, 53/147 (36.1%) in Center 3, 21/110 (19.1%) in Center 4, 28/69 (40.6%) in Center 5, 7/35 (20.0%) in Center 7, 8/24 (33.3%) in Center 8. The distribution of HER2 0, HER2-low and HER2-high cases was found to be statistically different (p=0.007). Of note, when applying positive/negative scoring system to our cohort, the p-value was not significant (p=0.3).

The prevalence of HER-low cases in biopsy samples within the various centers was: 72/197 (36.5%) in Center 1, 44/167 (26.3%) in Center 2, 45/104 (43.2%) in Center 3, 4/28 (14.3%) in Center 4, 27/59 (45.8%) in Center 5, 4/12 (33.3%) in Center 7, 7/21 (33.3%) in Center 8.

The prevalence of HER-low cases in surgical resection specimens within the various centers was: 22/205 (10.7%) in Center 1, 43/135 (17.0%) in Center 2, 8/43 (18.6%) in Center 3, 17/82 (20.7%) in Center 4, 1/10 (10.0%) in Center 5, 4/12 (33.3%) in Center 7, 0/14 (0.0%) in Center 8.

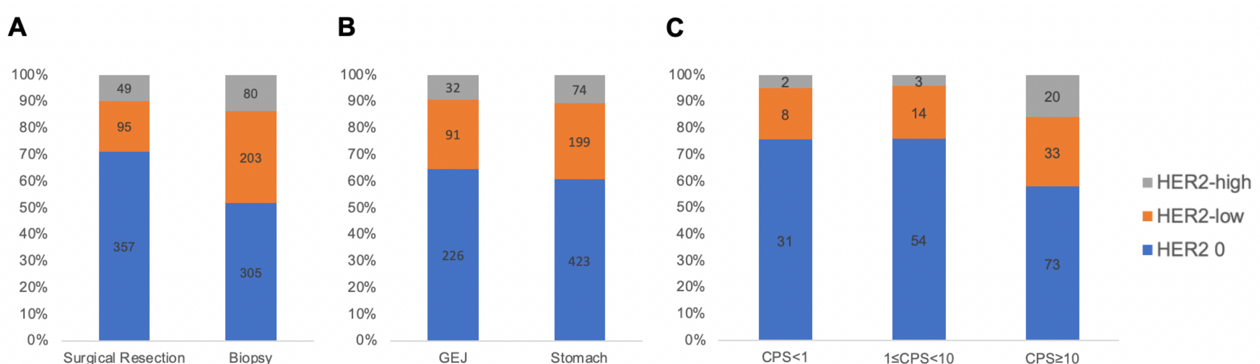

Figure 6: distribution of HER2 0, HER2-low and HER2-high gastroesophageal adenocarcinomas according to to (A) type of specimen, (B) tumor location and (C) PD-L1 expression by CPS.

DISCUSSION AND CONCLUSIONS

Trastuzumab deruxtecan (T-DXd) is a novel HER2-targeted antibody-drug conjugate containing an anti-HER2 antibody and a cytotoxic topoisomerase I inhibitor[23]. In the randomized phase II study (DESTINY-Gastric01) patients with HER2-positive advanced G/GEJ cancer treated with T-DXd achieved prolonged progression-free and overall survival, compared with those treated with chemotherapy of physician's choice (irinotecan or paclitaxel). Patients with HER2 1+ and not amplified (i.e., ISH-negative) HER2 2+ were included in a single-arm exploratory cohort. With an overall response rate for each of this group was 26.3% and 9.5%, respectively, T-DXd proved to be effective, even if to a lower degree, in patients with HER2-low disease[24].

Based on the four-tiered HER2 scoring system, HER2 status can be defined as positive or negative in order to identify those who might benefit from Trastuzumab. However, the promising results of the DESTINY-Gastric01 trials identify the new molecular subgroup of HER2-low G/GEJ adenocarcinomas.

This is the first study to investigate the clinico-pathologic and molecular features of HER2-low G/GEJ cancers in the real-world setting of a large multi-Institutional series. While HER2 overexpression has been largely investigated, few studies have provided data on the prevalence of HER2 1+ and not amplified HER2 2+ cases.

Cappellesso[25] and colleagues investigated HER2 status in 1040 G/GEJ adenocarcinomas using tissue microarrays (TMAs), reporting a prevalence of HER2-low cases of 19.9%. In two smaller cohorts the prevalence was 18.5%[17] and 12.9%[26]. In this context, i) the enrichment in either biopsy samples or surgical specimens, ii) the use of TMAs *versus* whole slides sections, iii) interobserver variability and the iv) IHC assay and clone may be accountable for the variability of HER2-low prevalence among the different studies.

In locally advanced unresectable and metastatic G/GEJ patients, the evaluation of HER2 status is based on endoscopic biopsy specimens. Due to the high levels of HER2 expression heterogeneity[8], international guidelines recommend more than 6 samples to be taken. According to our results, when compared to the surgical specimens the biopsy samples were enriched in HER2-low cases, (34.5% *versus* 18.9%; $p<0.00001$) and there was no difference in the fraction of HER2-low between cases with <6 biopsy fragments and cases with ≥6 biopsy fragments. This might be attributed to the fact that in the biopsy membranous staining is evaluated in a minimum of 5 cohesive cells while in surgical specimens it is evaluated in ≥10% of the neoplastic cells. Furthermore, while previous works evaluated the concordance of HER2 status between biopsy and surgical specimens using a positive/negative scoring system, the introduction of a three-tiered scoring system (*i.e.,* 0/low/high) in the diagnostic algorithm may cause higher discordance rates between biopsies and surgical samples due to the heterogeneous nature of HER2 expression. In fact, when applying a positive/negative

scoring system to our 32 pairs of matched biopsy and surgical resection, the discordance rate is 3.1%, and when applying a 0/low/high scoring system, the discordance rate rises to 43.4%. Another point to consider is that pre-analytical issues such as hyperfixation and cold ischemia are more common in biopsies and may lead to unreliable HER2 evaluation[27], [28]. This warrants some caution in relying on HER2 IHC/ISH of endoscopic biopsy specimens alone to identify HER2-low patients for targeted treatment regimens.

The interobserver agreement in the assessment of HER2-low expression has been investigated in invasive breast cancer. Previous studies have reported the lowest agreement rate to be between HER2 0 *versus* HER 1+ cases. HER2 0 versus HER2 3+ cases were found to have the highest agreement[29], [30]. In our study, we found a statistically significant distribution ($p=0.007$) of HER2 0, HER2-low and HER2-high cases among the seven centers where the evaluation was performed. The difference remained significant even when considering biopsy or surgically specimens alone. However, when applying a positive/negative scoring system to the same cohort, the difference in the distribution of HER2-positive and HER2-negative cases was not statistically significant. These data suggest that a three-tiered scoring system might result in lower inter-laboratory agreement and lower reproducibility of HER2 IHC assay.

No significant differences emerged concerning patients' age, sex, gastric *versus* gastroesophageal localization, Siewert class, and corpus fundus *versus* antrum/angulus. Furthermore, no statistically significant association between HER2-low status and WHO 2019 histotype, Lauren classification, Ming classification, and grading. was found. However, with a similar trend to that of HER2-high, the fraction of HER2-low cases is higher in tubular and papillary adenocarcinomas and in Lauren intestinal adenocarcinomas. We also demonstrated that HER2-low status can be found in rare histotypes, including carcinoma with lymphoid stroma, adenosquamous and undifferentiated carcinoma. When applying the IGCA classification to poorly cohesive carcinomas, we found a significantly lower prevalence of HER2-low and HER2-high among "pure" signet ring cell carcinomas. Due to the rarity of this histotype, scarce molecular data are available in the literature and a systematic evaluation of HER2 expression has not been performed yet. However, as for colorectal cancer, it can be hypothesized that the peculiar morphology and the aggressive clinical course might be associated with a specific molecular profile. On this matter, a study by Woo and colleagues suggested that non-specific staining in the marginated cytoplasm might undermine HER2 evaluation.

No statistically significant association was found between HER2 expression pattern and MMRd/MSI, EBER, and PD-L1 (CPS≥1 and CPS≥10). However, PD-L1 CPS≥10 cases were enriched in HER2-low and HER2-high. KEYNOTE-811 phase III clinical trial investigated whether the addition of pembrolizumab to chemotherapy and trastuzumab in HER2-overexpressing cases[31]. The results of

this landmark study demonstrated improved efficacy of the triple therapy compared with chemotherapy and trastuzumab double therapy, with a higher objective response rate in PD-L1-expressing tumors (CPS≥1). In KEYNOTE-811, 84.1% of participants had a PD-L1 CPS of ≥1. In our series, 86.2% (25 /29) of HER2-high cases were PD-L1 CPS ≥1, and 65.5% (19/29) were PD-L1 CPS of ≥10; while 83.6% (51/61) of HER2-high cases were PD-L1 CPS ≥1 and 55.7% were PD-L1 CPS ≥10.

As for breast cancer, HER2-low expressing gastroesophageal adenocarcinoma is emerging as a novel distinct entity, possibly challenging the current diagnostic-therapeutic process, shifting from a binary to a 3-tiered scoring system. In this work we showed how the introduction of this new entity might decrease reproducibility, especially in biopsy specimens, increasing inter-laboratory and interobserver variability. Many opportunities to refine the assessment of HER2-low gastroesphageal cancers exist. Future perspectives include: a modification of existing HER2 assays to increase reproducibility, the delivery of specific training for gastrointestinal pathologists, the incorporation of digital quantitative analysis in the workflow and the introduction of complementary biomarkers.

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APPENDIX (TABLES)

Table 1. Clinico-pathologic features according to HER2 status

	HER2 0	HER2-low	HER2-high	Total	p-value
Type of Specimen					
Surgical Resection	357 (71.3%)	95 (18.9%)	49 (9.8%)	501 (100%)	p<0.00001
Biopsy	305 (51.9%)	203 (34.5%)	80 (13.6%)	588 (100%)	
Number of biopsy fragments					
Mean ± SD	4.53 ± 2.32	4.73 ± 2.30	4.22 ± 1.92	4.56 ± 2.27	NS
Type of sample					
Primary tumor	642 (60.9%)	289 (27.4%)	124 (11.7%)	1055 (100%)	NS
Metastasis	20 (58.8%)	9 (26.5%)	5 (14.7%)	34 (100%)	
Site of primary tumor					
GEJ	226 (61.4%)	91 (24.7%)	32 (13.9%)	368 (100%)	NS
Stomach	423 (60.8%)	199 (28.6%)	74 (10.6%)	696 (100%)	
- **corpus/fundus**	120 (60.3%)	61 (30.7%)	18 (9.0%)	199 (100%)	NS
- **antrum/angulus**	271 (59.2%)	133 (29.0%)	54 (11.8%)	458 (100%)	
- **antrum/corpus**	32 (82.0%)	5 (12.8%)	2 (5.2%)	39 (100%)	
na	13 (52.0%)	8 (32.0%)	4 (16.0%)	25 (100%)	
Histotype					
Tubular	304 (56.9%)	156 (29.2%)	74 (13.9%)	534 (100%)	
Papillary	19 (55.9%)	10 (29.4%)	5 (14.7%)	34 (100%)	
Poorly cohesive	162 (66.7%)	57 (23.5%)	24 (9.8%)	243 (100%)	
- **PC-NOS**	40 (54.8%)	21 (28.8%)	12 (16.4%)	73 (100%)	p=0.002
- **PC-NOS/SRC**	82 (66.1%)	32 (25.8%)	10 (8.1%)	124 (100%)	
- **SRC**	37 (90.3%)	3 (7.3%)	1 (2.4%)	41 (100%)	
- **na**	3 (60.0%)	1 (20.0%)	1 (20.0%)	5 (100%)	
Mixed	141 (64.7%)	55 (25.5%)	22 (10.1%)	218 (100%)	NS
Mucinous	21 (70.0%)	8 (26.7%)	1 (3.3%)	30 (100%)	
Carcinoma with lymphoid stroma	7 (46.7%)	6 (40.0%)	2 (13.3%)	15 (100%)	
Other	5 (45.5%)	5 (45.5%)	1 (9.1%)	11 (100%)	
na	3 (75.0%)	1 (25.0%)	0 (0.0%)	4 (100.0%)	
Lauren Classification					
Intestinal	334 (57.1%)	171 (29.2%)	80 (13.7%)	585 (100%)	NS
Diffuse	168 (66.7%)	60 (23.8%)	24 (9.5%)	252 (100%)	
Mixed	143 (64.7%)	56 (25.3%)	22 (10.0%)	221 (100%)	
Indeterminate	13 (48.1%)	11 (40.7%)	3 (11.1%)	27 (100%)	
na	3 (75.0%)	1 (25.0%)	0 (0.0%)	4 (100.0%)	
Ming Classification					
Expansive	119 (62.6%)	50 (26.3%)	21 (11.1%)	190 (100%)	NS
Infiltrative	358 (63.5%)	141 (25.0%)	65 (14.0%)	564 (100%)	
na	184 (54.8%)	108 (32.2%)	43 (12.8%)	335 (100%)	
Grading					

High-grade	248 (60.4%)	117 (28.6%)	45 (11.0%)	410 (100%)	NS
Low-grade	150 (58.1%)	72 (28.0%%)	36 (13.9%)	258 (100%)	
na	66 (55.9%)	32 (27.1%)	20 (17%)	118 (100%)	
Staging of surgical specimens					
pT					
pTX	16 (76.2%)	3 (14.3%)	2 (9.5%)	21 (100%)	NS
pT0	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
pT1	44 (84.6%)	3 (5.8%)	5 (9.6%)	52 (100%)	
pT2	36 (64.3%)	13 (23.2%)	7 (12.5%)	56 (100%)	
pT3	154 (69.1%)	45 (20.1%)	24 (10.8%)	223 (100%)	
pT4	107 (71.8%)	32 (21.4%)	10 (6.8%)	149 (100%)	
pN					
pNX	23 (71.8%)	5 (15.7%)	4 (12.5%)	32 (100%)	NS
pN0	110 (75.3%)	26 (17.8%)	10 (6.9%)	146 (100%)	
pN1	61 (69.4%)	21 (19.3%)	10 (11.3%)	88 (100%)	
pN2	62 (65.3%)	20 (21.0%)	13 (13.7%)	95 (100%)	
pN3	101 (72.1%)	27 (20.3%)	12 (8.6%)	140 (100%)	
Neoadjuvant therapy					
Yes	116 (65.6%)	46 (25.9%)	15 (8.5%)	177 (100%)	NS
No	546 (59.9%)	252 (27.6%)	114 (12.5%)	912 (100%)	

Abbreviations: na: not assessable; SD: standard deviation; GEJ: gastroesophageal junction.

Table 2. Association between HER2 status and the other biomarkers'

	HER2 0	**HER2-low**	**HER2-high**	**Total**	**p-value**
PD-L1 (CPS)					
CPS<1	31 (68.9%)	10 (22.2%)	4 (8.9%)	45 (100%)	NS
1≤CPS<10	54 (70.2%)	17 (22.1%)	6 (7.8%)	77 (100%)	
CPS≥10	73 (57.9%)	34 (27.0%)	19 (15.1%)	126 (100%)	
EBER					
negative	122 (57.3%)	65 (30.5%)	26 (12.2%)	213 (100%)	NS
positive	5 (50.0%)	3 (30.0%)	2 (20.0%)	10 (100%)	
MMR/MSI status					
MMRp,MSS,MMRp/MSS	281 (58.1%)	141 (29.1%)	62 (12.8%)	484 (100%)	NS
MMRd,MSI,MMRd/MSI	37 (60.6%)	19 (31.2%)	5 (8.2%)	61 (100%)	
- **MLH1/PMS2 loss**	33 (59.0%)	18 (32.1%)	5 (8.9%)	56 (100%)	NS
- **MSH2/MSH6 loss**	2 (66.7%)	1 (33.3%)	0 (0.0%)	3 (100%)	
- **MLH1/MSH6 loss**	1 (100%)	0 (0.0%)	0 (0.0%)	1 (100%)	
- **PMS2 loss**	1 (100%)	0 (0.0%)	0 (0.0%)	1 (100%)	

Abbreviations: CPS: combined positive score; MMRp: mismatch repair proficient; MMRd: mismatch repair deficient; MSI: microsatellite instability.

Table 3. Distribution of her 2 0, HER2-low and HER2-high according to year of evaluation and center where the evaluation was performed.

	HER2 0	HER2-low	HER2-high	Total	p-value
Year					
2018	104 (59.8%)	47 (27.0%)	23 (13.2%)	174 (100%)	NS
2019	172 (62.1%)	70 (25.3%)	35 (12.6%)	277 (100%)	
2020	121 (58.7%)	59 (28.7%)	26 (12.6%)	206 (100%)	
2021	189 (60.0%)	93 (29.5%)	33 (10.5%)	315 (100%)	
2022	76 (65.0%)	29 (24.7%)	12 (10.3%)	117 (100%)	
Center					
Center 1	263 (65.4%)	94 (23.4%)	45 (11.2%)	402 (100%)	p=0.007
Center 2	180 (59.6%)	87 (28.9%)	35 (11.6%)	302 (100%)	
Center 3	74 (50.3%)	53 (36.1%)	20 (13.6%)	147 (100%)	
Center 4	75 (68.2%)	21 (19.1%)	14 (12.7%)	110 (100%)	
Center 5	37 (53.6%)	28 (40.6%)	4 (5.8%)	69 (100%)	
Center 6	20 (57.1%)	7 (20.0%)	8 (22.9%)	35 (100%)	
Center 7	13 (54.2%)	8 (33.3%)	3 (12.5%)	24 (100%)	