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Tesi di Laurea

PATHOLOGIC LANDSCAPE OF HER2-LOW IN GASTROESOPHAGEAL ADENOCARCINOMA: REAL-WORLD DATA

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ABSTRACT

Background. Human epidermal growth factor receptor 2 (HER2) is the first molecular biomarker which has been exploited for advanced gastroesophageal cancers' targeted therapy. On the basis of the results from the ToGA trial, the anti-HER2 monoclonal antibody trastuzumab combined with chemotherapy is routinely used as the first-line therapy for HER2-positive advanced gastroesophageal cancers. The DESTINY-Gastric01 trial showed that a novel HER2-targeted antibody-drug conjugate, Trastuzumab-deruxtecan, in addition of being valuable in overcoming the problem of HER2 intra-tumour heterogeneity, has proved to be effective also in those cancers which have a low HER2-expression rate (HER2-low disease, defined as HER2 immunohistochemistry 1+ or 2+ without gene amplification confirmed by in situ hybridization), thus paving the way for novel therapeutic scenarios.

Aim of the study. The purpose of this study was to evaluate the prevalence of HER2-low expression in a large real-world and multi-institutional series of cases of gastroesophageal adenocarcinomas. In addition to the prevalence analysis, the study also aimed to evaluate the correlation between this low expression rate with a series of clinical and histopathological features of gastroesophageal cancers.

Materials and methods. We retrospectively evaluated a total of 1.210 formalin-fixed paraffin-embedded samples of gastroesophageal adenocarcinomas which were analyzed by immunohistochemistry for HER2 protein expression in eight Italian surgical pathology units in the period between January 2018 and June 2022. We assessed the prevalence of HER2-low (*i.e.*, HER2 1+ and HER2 2+ without amplification) in the cohort of available samples. Each specimen was also evaluated and categorized considering different features, such as the type of specimen (surgical or biopsy), number of biopsy fragments, tumor localization, histotype according to the WHO 2019 criteria, grading, histopathological characteristics according to Lauren and Ming classification systems. It was considered also the year and the center where the specimen was collected.

Information regarding staging, neoadjuvant therapy and other biomarkers' status (PD-L1, dMMR/MSI status, EBER) was also collected from the pathology reports.

Results. HER2 status could be assessed in 1.189/1.210 cases. Among the 1.189 assessable cases, 710 (59,7%) were HER2 0, 217 (18,3%) were HER2 1+, 120 (10,1%) were not amplified HER2 2+, 41 (3,4%) were amplified HER2 2+, and 101 (8,5%) were HER2 3+. The prevalence of HER2-low was 28,3% (95% CI 25,8 to 31,0%) overall, and was higher in biopsy specimens (34,9%, 95% CI 31,2 to 38,8%) rather than in surgical resection specimens (21,0%, 95% CI 17,7 to 24,6%) ($p<0,0001$). Moreover, HER2-low prevalence ranged from 19,1 to 40,6% among centers ($p=0,0005$), and from 26,6 to 40,6% according to the clone of the antibody used for the immunohistochemical staining ($p=0,01$). It was pointed out also that HER2-low prevalence was lower in pure signet-ring carcinomas ($p=0,07$).

Conclusion. In the light of the promising activity of trastuzumab-deruxtecan in advanced HER2-low expressing gastroesophageal cancers and in the wake of the knowledge available on HER2-low breast cancers, the new "HER2-low" category in gastroesophageal cancers may be the starting point toward a reconsideration of the world of HER2 expressing cancers. However, this work shows how the expansion of the HER2 spectrum might raise problems in reproducibility, especially in biopsy specimens, decreasing inter-laboratory and inter-observer concordance. This may make it necessary to better define the characterization of HER2-low category.

RIASSUNTO

Presupposti dello studio. Il recettore 2 del fattore di crescita epidermico umano (HER2) è il primo marcatore biomolecolare che è stato sfruttato per la terapia mirata dei tumori gastroesofagei avanzati. Sulla base dei risultati dello studio ToGA, l'anticorpo monoclonale anti-HER2 trastuzumab in combinazione con la chemioterapia viene utilizzato di routine come terapia di prima linea per i tumori gastroesofagei avanzati HER2-positivi. Lo studio DESTINY-Gastric01 ha dimostrato che un nuovo anticorpo coniugato mirato a HER2, trastuzumab-deruxtecan, oltre a essere valido per superare il problema dell'eterogeneità intra-tumorale di HER2, si è dimostrato efficace anche in quei tumori che hanno un basso tasso di espressione di HER2 (malattia HER2-low, definita dal fatto di avere una valutazione immunoistochimica giudicata HER2 1+ o 2+ senza amplificazione genica confermata dall'ibridazione in situ), aprendo così la strada a nuovi scenari terapeutici.

Scopo dello studio. Lo scopo di questo studio era quello di valutare la prevalenza della bassa espressione di HER2 in un'ampia serie di casi di adenocarcinomi gastroesofagei, provenienti da più centri di patologia italiani. Oltre all'analisi della prevalenza, lo studio mirava anche a valutare la correlazione tra questo tasso di bassa espressione con una serie di caratteristiche cliniche e istopatologiche dei tumori gastroesofagei.

Pazienti e metodi. Abbiamo valutato retrospettivamente un totale di 1.210 campioni di adenocarcinomi gastroesofagei fissati in formalina e inclusi in paraffina, analizzati mediante immunoistochimica per l'espressione della proteina HER2 in otto unità di patologia chirurgica italiane nel periodo compreso tra gennaio 2018 e giugno 2022. Abbiamo valutato la prevalenza di HER2-low (definito come HER2 1+ o HER2 2+ senza amplificazione) nella coorte di campioni disponibili. Ogni campione è stato inoltre valutato e categorizzato considerando diverse caratteristiche, come il tipo di campione (chirurgico o bioptico), il numero di frammenti bioptici, la localizzazione del tumore, l'istotipo secondo i criteri OMS

del 2019, il grading, le caratteristiche istopatologiche secondo i sistemi di classificazione di Lauren e Ming. Sono stati considerati anche l'anno e il centro in cui è stato raccolto il campione. Dai referti patologici sono state raccolte anche informazioni riguardanti lo stadio, la terapia neoadiuvante e lo stato di altri marcatori (PD-L1, stato dMMR/MSI, EBER).

Risultati. È stato possibile valutare lo stato HER2 in 1.189 su1.210 casi. Tra i 1.189 casi valutabili, 710 (59,7%) erano HER2 0, 217 (18,3%) erano HER2 1+, 120 (10,1%) erano HER2 2+ non amplificati, 41 (3,4%) erano HER2 2+ amplificati e 101 (8,5%) erano HER2 3+. La prevalenza di HER2-low è stata complessivamente del 28,3% (95% CI 25,8-31,0%) ed è risultata più elevata nei campioni bioptici (34,9%, 95% CI 31,2-38,8%) rispetto a quelli di resezione chirurgica (21,0%, 95% CI 17,7-24,6%) ($p<0,0001$). Inoltre, la prevalenza di HER2-low variava dal 19,1 al 40,6% tra i centri ($p=0,0005$) e dal 26,6 al 40,6% in base al clone dell'anticorpo utilizzato per la colorazione immunoistochimica ($p=0,01$). È stato inoltre evidenziato che la prevalenza di HER2-low era più bassa negli adenocarcinomi a cellule ad anello con castone puri ($p=0,07$).

Conclusioni. Alla luce della promettente attività di trastuzumab-deruxtecan nei tumori gastroesofagei avanzati a bassa espressione di HER2 e sulla scia delle conoscenze disponibili sui tumori mammari a bassa espressione di HER2, la nuova categoria "HER2-low" nei tumori gastroesofagei può essere il punto di partenza verso una riconsiderazione del mondo dei tumori esprimenti HER2. Tuttavia, questo lavoro mostra come l'espansione dello spettro HER2 possa sollevare problemi di riproducibilità, soprattutto nei campioni bioptici, riducendo la concordanza tra laboratori e tra osservatori. Ciò potrebbe rendere necessaria una migliore definizione della categoria HER2-low.

1. INTRODUCTION

1.1 GENERAL OVERVIEW OF GASTROESOPHAGEAL CANCERS

Gastroesophageal cancers include malignant neoplasms developing from the esophagus and stomach (1). Over 90% of gastric malignancies are adenocarcinomas (2), whereas esophageal cancers are distinguished into two main histological subtypes, adenocarcinoma and squamous cell carcinoma. Although they derive from the same organ, esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC) present several differences with regards to geographical distribution, etiology, genetics and molecular patterns and should be treated as separate entities (1).

Traditionally, adenocarcinomas of the esophagus and stomach have been considered as two separate types of cancers (1). In this context, there has been uncertainty regarding the characterization of adenocarcinomas spanning the area from the lower esophagus to the proximal stomach, comprising the gastroesophageal junction (GEJ). Recent evidences have suggested, however, that the molecular alterations' pattern of EAC in the lower esophagus is very similar to that of gastric adenocarcinoma of the proximal stomach (cardia), particularly a subtype characterized by the presence of chromosomal instability (CIN) (1) (3). From this evidence, a new hypothesis has been proposed, namely that of considering esophageal adenocarcinoma of the lower esophagus and gastric adenocarcinoma of the cardia as a whole pathologic entity, which is named as "gastroesophageal adenocarcinoma". Epidemiological trends in these cancers, which have changed significantly in recent decades, have also highlighted an increase in the incidence of cancers arising at the GEJ, further supporting the hypothesis that esophageal and gastric adenocarcinoma occurring nearby the GEJ have a common pathogenetic origin (4).

Gastroesophageal adenocarcinomas as a whole, comprising both esophageal and gastric adenocarcinoma, represent currently a significant public health concern, mainly because the diagnosis of these tumours arrives often when the diseases is at an advanced unresectable or metastatic stage, resulting in a poor prognosis of patients.

Improved understanding of the molecular mechanisms behind gastroesophageal adenocarcinoma has advanced the landscape of oncologic treatment beyond cytotoxic chemotherapy. Currently, the most important achievement in this context has been the recognition of HER2 receptor as an exploitable molecular biomarker for targeted treatment strategies.

1.2 ESOPHAGEAL CANCER

1.2.1 EPIDEMIOLOGY

Esophageal cancers are less frequent than gastric cancers. According to GLOBOCAN 2020 database, esophageal cancers rank eighth in terms of incidence and sixth in terms of mortality overall, counting 604,000 new cases and almost 544,000 deaths in 2020 (5).

According to data from the report "The Numbers of Cancer in Italy 2020", published by the Italian Association of Cancer Registries (AIRTUM) and the Italian Association of Medical Oncology (AIOM), in Italy in 2020 2,400 new cases of esophageal cancer have been estimated, 1,700 in males and 700 in females. As for mortality, 1,900 deaths have been estimated, again more frequent in males (1,400) than in females (500). On the basis of these data, excluding non-melanoma skin cancers, esophageal cancers represent 0.6% of all malignancies (6).

Esophageal cancer is a pathology of older age, having a pick of incidence between 60 and 70 years (7). With approximately 70% of cases occurring in men, incidence and mortality rates are 2 to 3 times higher among men than among women (5).

The vast majority of esophageal cancers cases are found in Eastern Asia, particularly in China, followed by Southern Africa, Eastern Africa, Northern Europe and South Central Asia (5). We can see the worldwide distribution of esophageal cancer in *Figure 1*. The highest incidence of esophageal cancer is seen in an area that begins from East of Turkey and Northeastern of Iran, and continues to the East Asian countries, including North and Center of China. In this area, which is also called "Asian esophageal cancer belt", the incidence rate is more than 100 cases per 100,000 people annually (7).

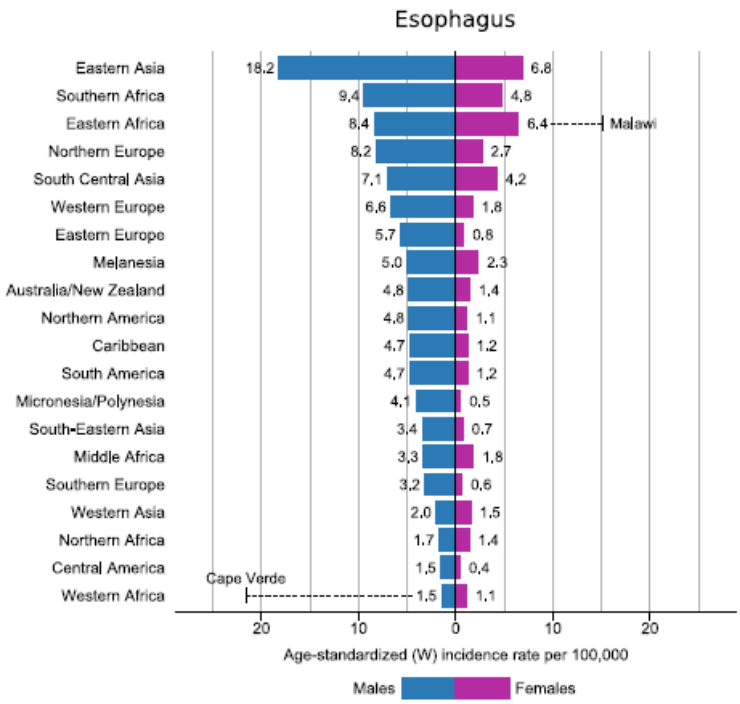

Figure 1. Region-Specific Incidence Rates by Sex for Esophageal Cancer in 2020. Rates are shown in descending order of the world rate among men, and the highest national rates among men and women are superimposed. Source: GLOBOCAN 2020.

The geographic distribution of esophageal cancer's incidence rates differs considering the two most common histological subtypes (squamous cell carcinoma [ESCC] and adenocarcinoma [EAC]) (8).

The results from many populations studies showed that ESCC is the predominant subtype of esophageal malignancy globally, although its proportion, relative to EAC, varies from country to country (8) (9). Since the mid 1970's, the incidence of EAC, which occurs predominantly in the lower tract of esophagus near the gastroesophageal junction (GEJ), has rapidly increased initially in Western countries, especially Northern America, Western Europe and Australia, but then also in some Eastern countries with high-income economy (9). With the increasing of EAC's incidence a parallel decreasing of ESCC has been registered in Western countries. In the US, there has been a complete epidemiologic shift from ESCC, which used to be responsible of more than 90% of all esophageal cancers until 1970, to EAC, which is currently the leading type of esophageal cancer, representing up to 80% of all esophageal cancer cases (7). The main reason behind the increasing trend of EAC is the recent increasing prevalence of obesity and gastroesophageal reflux disease (GERD), which represent significant risk factors

for EAC but not for ESCC and are pathologies that are also increasing in high-income countries (8).

By contrast, ESCC predominates in the upper and mid tracts of esophagus and is associated with different risk factors, such as smoking and alcohol exposure. Although the incidence of ESCC is gradually decreasing worldwide, ESCC remains the most common type of esophageal cancer worldwide and it is the predominant subtype in the "Asian esophageal cancer belt" (9). In lower income countries, including parts of Asia and sub-Saharan Africa, ESCC continues to represent over 90% of all esophageal cancer cases (8).

These trends, with EAC increasing its incidence rate and EAC decreasing, are predicted to continue in the near future, with the prediction that EAC will exceed ESCC in many countries also out of Western world (5).

1.2.2 PATHOGENESIS AND RISK FACTORS

Pathogenesis of esophageal squamous cell carcinoma

Esophageal squamous cell carcinoma (ESCC) arise from the squamous epithelium of the upper and mid tracts of the esophagus through a carcinogenetic process that occur in the presence of risk factors which cause chronic irritation and inflammation. The major risk factors associated with ESCC are smoking and alcohol consumption. The gradual decline of smoking habits, especially in Western countries, is believed to contribute to the decline in incidence of ESCC in this part of the world (7).

Also nutritional factors such as a low consumption of fruits and vegetables which leads to the lack of antioxidant agents and vitamins contribute to the development of ESCC. The habit of using hot beverages is hypothesized to be implicated in the pathogenesis of ESCC in some countries such as Iran (5). The role of human papilloma virus (HPV) is still uncertain, but probably it can contribute to the development of ESCC (5).

The precursor lesion for ESCC is squamous dysplasia, which is a histologic lesion confined to epithelium and characterized by both cytological and architectural abnormalities (10). Squamous dysplasia was traditionally graded as mild (involving up to one-third of the epithelium's thickness), moderate (up to two-thirds) and

severe (involving the entire epithelium's thickness) (11). In 2000, the WHO classification system introduced the term "intraepithelial neoplasia" (IEN) in place of dysplasia (10), and classified IEN in a two-tier system as low-grade or high-grade (11). When less than half of the epithelium's thickness is involved with atypical cells it is graded as low-grade, while when more than half of thickness is involved it is graded as high-grade. In Japan, lesions with full thickness involvement of epithelium (high grade IEN) are also called "squamous cell carcinoma *in situ*" (CIS) or "noninvasive squamous cell carcinoma" (11).

Pathogenesis of esophageal adenocarcinoma

Esophageal adenocarcinoma (EAC) traditionally arises in the context of Barrett's esophagus (BE) which develops in the setting of the gastroesophageal reflux disease (GERD) (10). BE is a metaplastic condition of the lower esophagus characterized by the replacement of the normal esophageal stratified squamous epithelium with a columnar epithelium in response to chronic acid or biliary reflux from the stomach or intestine (12). EAC in the setting of BE develops through a sequential progression which goes from inflammation to metaplasia, dysplasia and ultimately adenocarcinoma (10).

Columnar metaplasia within the esophagus has been traditionally subdivided into three histological subtypes: gastric fundic type, junctional type, and specialized intestinal type metaplasia characterized by the presence of goblet cells (IM) (13). Given this subdivision, the question whether IM must be present for BE's diagnosis has been a controversial point, as some guidelines used to consider IM with goblet cells as a pre-requisite for BE's diagnosis, whereas others considered that also a columnar metaplasia without goblet cells was sufficient to make diagnosis of BE. On the basis of most evidences, IM appears to be the only type of metaplasia that is clearly prone to malignant transformation and so can be considered a pre-neoplastic condition for the development of EAC (12). Currently, The American Gastroenterological Association (AGA), European Society of Gastrointestinal Endoscopy (ESGE) and also Russian Society of Pathologists (RSP) all believe that IM with goblet cells is necessary for the diagnosis of BE. By contrast, British Society of Gastroenterology (BSG), Asia-Pacific Working Group (APWG) and Benign Barrett's

and Cancer Taskforce consensus group (BOB CAT) do not agree and believe that any type of columnar metaplasia in distal esophagus should be considered as BE (so IM is not necessary for the diagnosis of BE) (12). This latter conviction is based on the results of some recent studies that have suggested that also patients with columnar metaplasia without intestinal differentiation and goblet cells can develop EAC (14). Other studies have found that columnar metaplasia without goblet cells contains some molecular abnormalities which appear to be similar to those of columnar metaplasia with goblet cells (15). Therefore, the definition of BE and the precise role of IM is still under debate.

Despite the uncertainties in its definition, BE is considered a premalignant condition for EAC, as the risk of developing EAC is significantly higher in patients with BE compared to the general population. Neoplastic progression in BE goes through the following stages: non-dysplastic BE - low-grade dysplasia (LGD) – high-grade dysplasia (HGD) – EAC. However, only a small minority of patients with BE will develop EAC and the annual risk of developing EAC in patients with BE has reported to be 0.12% (7). The risk of progression, however, increases with male gender, current tobacco smoking, visceral obesity, Caucasian origin and especially with the length of BE. The risk of EAC increases linearly with the length of BE, with a higher risk of developing cancer in long segment BE (> 3 cm) than in short segment BE (7).

1.2.3 SYMPTOMATOLOGY AND DIAGNOSIS

Esophageal cancer is often asymptomatic in the early stages. When it is present, symptomatology includes: dysphagia, as the presence of the mass obstructs the transit of the food bolus; odynophagia and retrosternal chest pain, as the food bolus has to pass by forcing the stenosis, thus causing pain; regurgitation, as the bolus just can't get past the stenosis and comes back; unintentional weight loss, because the patient has actual feeding problems; asthenia and anorexia, because it is a cachetizing neoplastic disease (16).

Diagnosis is made through radiologic and/or endoscopic techniques. The first tests used to initially identify and diagnosis esophageal cancers are upper gastrointestinal tract contrast study (barium X-ray) and upper endoscopy with

biopsy (EGDS) (17). After the histologic cancer diagnosis has been obtained, subsequent studies are performed to determine the stage of the tumour as accurately as possible before any treatment is initiated (17).

The esophageal cancer staging is defined by the eighth edition of the American Joint Committee on Cancer (AJCC) TNM staging system, which includes both esophageal and GEJ cancers (18). This staging system exploits TNM categories in order to obtain information about the depth of invasion of the primary tumor (T), lymph node involvement (N), and extent of metastatic disease (M). AJCC presents three separate classifications: the first is a clinical classification (cTNM) that is based almost exclusively on imaging tests; a second pathological classification (pTNM) is based on the microscopic examination of resection specimens; and a third classification (ypTNM), which represents the novelty of the new edition, is applicable after neo-adjuvant treatment followed by surgical resection.

It is important to note that these 3 classifications are the same for EAC and GEJ adenocarcinomas but not for ESCC, whose cTNM and pTNM have different features. However, for the ypTNM classification, there is no distinction between the two histopathological types of esophageal tumours (18).

1.2.4 TREATMENT AND PROGNOSIS

A multidisciplinary evaluation by surgery and medical oncology is recommended for all patients before any treatment strategy is initiated. Treatment options include local minimally invasive treatments, such as endoscopic mucosal resection (EMR), endoscopic submucosal dissection (ESD) and endoscopic ablation therapies, esophagectomy with lymphadenectomy, chemotherapy associated or not with radiotherapy in both adjuvant and neo-adjuvant settings, and, in particular contexts, molecular targeted therapy and/or immunotherapy. The choice and the order of these treatment options depend on several factors, including the type, stage and grade of cancer, patient's preferences and overall health (16).

For superficial tumors that involve only the mucosa (T1a) local endoscopic treatments should be considered, including EMR or ablation therapies such as cryoablation, radiofrequency ablation, and photodynamic therapy (17). Patients

who have an invasion of submucosa or muscularis mucosae (T1b) are not good candidates for local treatments due to an increased risk of lymph node metastasis and should be subjected to esophagectomy. Otherwise, recent reports have shown that ESD followed by chemoradiotherapy has promising results and might become a new therapeutic approach (19).

The treatment for locally advanced esophageal cancer that does not have distant metastases and is potentially resectable (T3-4aN0, T1-4aN1M0) is highly variable in practice and considers a multimodality therapy with integration of chemotherapy, radiotherapy, and surgical resection (17). Neo-adjuvant chemoradiotherapy followed by surgery currently represents a standard of care for patients with locally advanced potentially resettable esophageal or junctional cancer thanks to the promising results of the CROSS trial (20).

Approximately 50% of patients have evidence of distant metastatic disease at the time of diagnosis. In these cases, a palliative strategy is used and it is based on chemotherapy and/or radiotherapy (17). Although over the past four decades a decreased mortality was registered thanks to significant improvements in cancer treatment, the overall 5-year survival rate of esophageal cancer, of all types, remains poor and approximately lower that 20% (17).

1.2.5 MOLECULAR DISTINCTION OF ESOPHAGEAL CANCERS

In 2017 the Cancer Genome Atlas Research Network performed a comprehensive molecular and genomic profiling study which showed that the two histological subtypes of ESCC and EAC are distinct in their molecular characterization (21).

From these findings ESCC emerges as a disease more similar to other squamous cell carcinomas, such as head and neck squamous carcinomas, while EAC is more similar to gastric adenocarcinomas, rather than to ESCC.

In particular, the amplification of HER2 gene is seen in 32% of EAC compared to 3% of ESCC. On the basis of these results, HER2 status, which is considered a well-established molecular biomarker for gastric cancer, appears to have a potential role as a biomarker also in EAC. At present, humanized HER2-targeting monoclonal antibody Trastuzumab has been approved for the treatment of advanced HER2-positive gastric cancer and cancers of the GEJ. However, given the fact that HER2-

amplification is present in 32% of EAC, HER2-positive EAC can be treated off-label with Trastuzumab (22).

The molecular distinction of the two histotypes of esophageal cancers has important implications for medical treatment. The distinct molecular profiles of EAC compared to ESCC has pointed out that combining adenocarcinoma and squamous subtype patients in the clinical trials, as has happened commonly in the past, can lead to misleading results. Adenocarcinoma and squamous subtype are distinct in their genetic alteration profiles, so they need to be evaluated and treated separately (4).

1.3 GASTRIC CANCER

1.3.1 EPIDEMIOLOGY

According to GLOBOCAN 2020 database gastric cancer represents the fifth most common malignancy and the fourth leading cause of cancer death worldwide, with one million new cases and 769.000 deaths in 2020 around the world (5).

According to data from the report "The Numbers of Cancer in Italy 2020", published by the Italian Association of Cancer Registries (AIRTUM) and the Italian Association of Medical Oncology (AIOM), in Italy in 2020 14.500 new cases of gastric cancer have been estimated, 8.500 in males and 6.000 in females. As for mortality, 8.700 deaths have been estimated, again more frequent in males (5.300) than in females (3.400). On the basis of these data, excluding non-melanoma skin cancers, in Italy gastric cancer accounts for about 4% of all malignancies in both sexes, ranking seventh as incidence in men (4,3% of all cancers in men) and ninth in women (3,9% of all cancers in females). If we consider the age group > 70 years, gastric cancer reaches the fifth place for both men and women in terms of incidence. With about 6% of cancer related deaths, gastric carcinoma occupies the fifth place in both sexes (6).

Gastric cancer has a wide geographical distribution variability around the world. Incidence and mortality rates are higher in Eastern Asia where currently over the 60% of all gastric cancer cases are found (2). Eastern Asia rates are followed by

Central and Eastern Europe, whereas incidence and mortality rates in Northern America and Northern Europe are generally low (*Figure 2*) (5).

In all populations and countries, gastric cancer is rare in persons younger than 50 years (23). On overage, rates for gastric cancer are 2-fold higher in men than in women (5).

The epidemiology of gastric cancer has significantly changed over the past decades. Gastric cancer used to be the most frequent cause of cancer death in the world until the 1980s when it was overtaken by other types of cancers (2). However, according to the Global Cancer Observatory promoted by IARC, still in 1990 gastric cancer was the tumor with the second highest incidence and mortality worldwide (24). The worldwide incidence and mortality of gastric cancer have declined even more rapidly over the recent few years. This decline first took place in countries with low gastric cancer incidence such as the USA and in general the Western countries (where this decline began from the 1930s), while the decline in countries with high incidence like Asian countries was delayed and slower (2). In Italy, this reduction in incidence and mortality rates continues steadily nowadays: between 2008 and 2016, the annual average percentage reduction in both men and women was approximately -1,9%/year and -1,4%/year respectively as a change in incidence and -2,4%/year and -2,7%/year respectively as change in mortality (6).

However, not all types of gastric cancer are declining: cardia and gastroesophageal junction (GEJ) adenocarcinomas are becoming more frequent both in Western countries and Asian countries. Considering the increase in the prevalence of GEJ involvement in recent decades, GEJ adenocarcinoma has become an important public health concern (23).

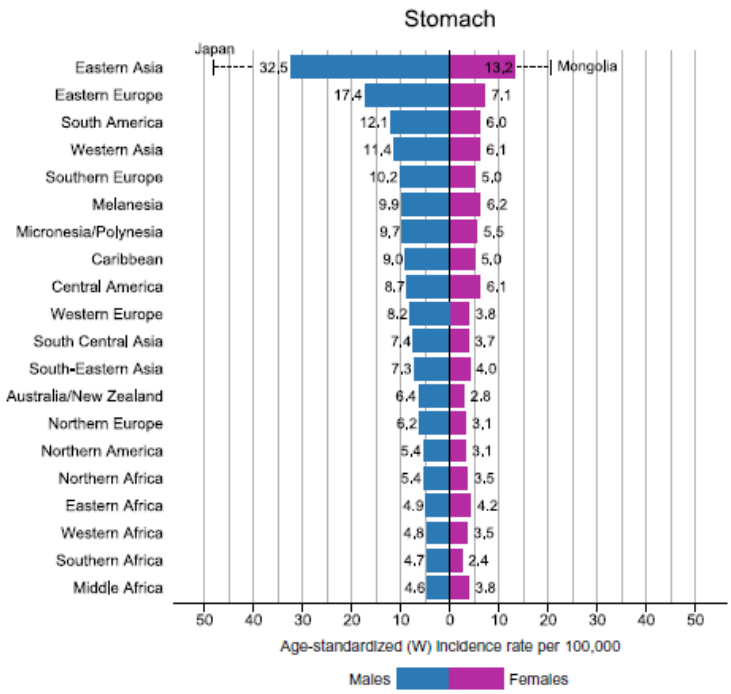

Figure 2. Region-Specific Incidence Rates by Sex for Gastric Cancer in 2020. Rates are shown in descending order of the world rate among men, and the highest national rates among men and women are superimposed. Source: GLOBOCAN 2020.

1.3.2 PATHOGENESIS AND RISK FACTORS

Histologically the vast majority (almost the 90%) of gastric cancers are adenocarcinomas, while other types of tumors (including lymphoma, sarcoma, neuroendocrine tumors) are rare (2). In this study, when we refer to gastric cancer we consider adenocarcinoma.

Gastric adenocarcinoma is traditionally classified according to the anatomic site into two subtypes, cardia and noncardia gastric cancer. Cardia gastric cancer is found in the proximal part of the stomach, near the gastroesophageal junction (GEJ), and it has several features that resemble the esophageal adenocarcinoma (EAC) which develops in the lower tract of the esophagus. Noncardia gastric cancer is found in the mid and distal part of the stomach, including gastric fundus, body and antrum. These pathological entities differ in terms of risk factors, pathogenesis, epidemiologic patterns and geographical distribution (5) (25).

In general terms, the etiology of gastric adenocarcinoma is multifactorial, including both genetic risk factors, among which there are positive family history and inherited predisposition, and environmental and lifestyle-related risk factors. It seems that the variation in gastric cancer's incidence and in the distribution of

cardia/noncardia subtypes worldwide derived from variations in exposure to environmental or lifestyle-related risk factors (25).

Pathogenesis of noncardia gastric cancer

The principal cause of noncardia gastric cancer is represented by chronic *Helicobacter pylori* infection (25). The prevalence of *H. pylori*, a bacterium colonizing in the stomach, varies wildly between and within countries, reaching values from 20% to 50% in developed countries to more than 80% in developing countries (26). Of course, not all patients with *H. pylori* infection will develop gastric cancer: there are several factors that contribute to the process of carcinogenesis, including virulence genetic factors (infection with CagA and VacA viral genotypes confers a higher risk of developing adenocarcinoma), genetic susceptibility of the host, predisposed gastric environment and other environmental factors (26).

Other risk factors beyond *H. pylori* infection for gastric cancer include EBV-infection, alcohol consumption, tobacco smoking, food preserved by salting, high consumption of salt and processed or grilled meat and low intake of fruit and vegetables (25).

From the epidemiologic point of view, noncardia gastric cancer remains the most commonly diagnosed gastric cancer worldwide, however, its proportion, relative to cardia subtype, varies from country to country (*Figure 3*). Incidence and mortality rates of noncardia gastric cancer have been steadily declining over the last half century in most populations, especially in Western high-income countries. These trends are mainly attributed to the effectiveness of eradication strategies for the *H. pylori* infection, but also to improvements in food handling with the introduction of refrigeration, and to the decrease in the use of tobacco and dietary salt (25).

The sequence of changes in the stomach after *H. pylori* infection was formalized by Pelayo Correa in what we call the Correa's cascade (27). Correa's cascade is a model that describes the series of events which lead normal gastric mucosa to turn into gastric adenocarcinoma, specifically of intestinal-type, which is one of the two histologic subtype that have been described by Lauren (28). This cascade

can include initially the *H. pylori* infection, which is the single most common etiologic factor that precipitates the cascade, or other causes of mucosa inflammation. The inflammation can cause a chronic active non-atrophic gastritis, which may persist or evolve into atrophic gastritis. Atrophic gastritis is followed by gastric intestinal metaplasia (IM), which is considered the precancerous lesion for gastric adenocarcinoma of intestinal-type and is defined as the replacement of foveolar and/or glandular gastric epithelium by intestinal epithelium. IM may also progress to dysplasia (also called intra-epithelial neoplasia IEN), distinguished in low-grade dysplasia, which has minimal architectural disarray with mild to moderate cytologic atypia, and high-grade dysplasia, which presents marked cytologic atypia. The progressive acquisition of DNA mutations, molecular alterations and epigenetic dysregulation drives these morphological changes towards IM and dysplasia. The final step of the cascade is represented by invasive adenocarcinoma which develops when neoplastic cells acquire the ability to invade the surrounding stroma (29).

Pathogenesis of cardia gastric cancer

Unlike noncardia subtype, the role of *H. pylori* infection in cardia gastric cancer and adenocarcinoma occurring nearby the GEJ is uncertain. While in Eastern Asia a few studies continue to show a positive association between these types of tumor and *H. pylori* infection (although this association is more modest than that with noncardia gastric cancer), in Europe, USA and Australia most studies of population have reported that this association is null or even inverse, supporting the idea that cardia cancers arise via alternative mechanisms (25).

In fact, in Western countries the risk factors for gastric cancer developing in the cardia closely mirror those for Barrett esophagus and esophageal adenocarcinoma and are represented mainly by obesity and gastroesophageal reflux disease (GERD) (23). As a result of the association with these increasing risk factors, in Western countries the contribution of cardia gastric cancer versus noncardia to the overall burden has been gradually increasing over the past few years (5) (*Figure 3*). These trends, with the incidence of cardia gastric cancer increasing and noncardia decreasing, reflect what is happening in the esophagus, where the

incidence of squamous cell carcinoma in the upper and mid tract of esophagus is decreasing, while the incidence of esophageal adenocarcinoma in the lower tract near GEJ is increasing (8).

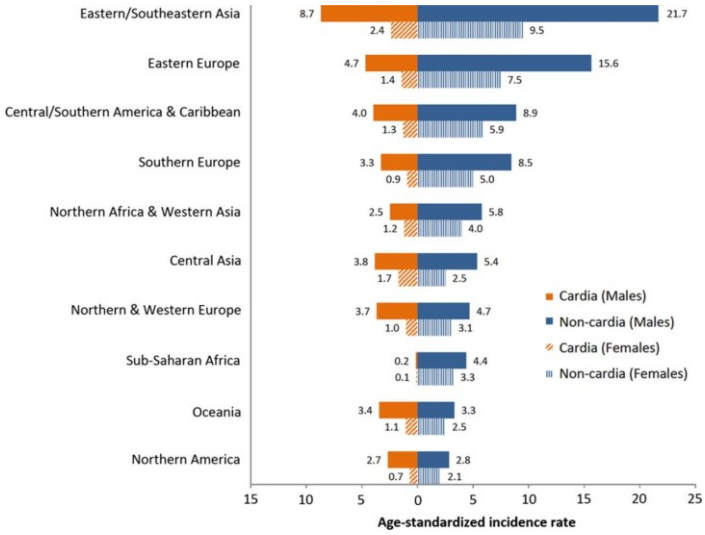

Figure 3. Estimated cardia and non-cardia gastric cancer age-standardized incidence rates (per 100 000) by region and sex, 2012. Colquhoun A, Arnold M, Ferlay J, et al. Gut, 2012

As well as for esophageal adenocarcinoma, also cardia gastric cancer, especially of Lauren's intestinal-type, finds in intestinal metaplasia (IM) its precancerous lesion. Given this evidence, we can see that cardia gastric IM and Barrett esophagus with IM as precancerous lesions are overlapping concepts, with GERD acting as the precursor for both proximal gastric IM and distal esophageal Barrett esophagus (30).

Considering the similarities in the etiology and epidemiological trends, the appropriate demarcation between cardia gastric cancer of intestinal-type and esophageal adenocarcinoma and the correct classification of adenocarcinomas occurring at the GEJ have been topics of debate for a long time (4). The common origin from GERD-related IM, and thus the common pathogenetic process, supports the hypothesis that esophageal adenocarcinoma of the lower esophagus and intestinal-type gastric adenocarcinoma of the cardia region are two forms of the same disease (1). Whether this unique disease might origin from cardia gastric mucosa and spreads to the lower esophagus or origins from esophageal mucosa and spreads to proximal stomach is still under debate. This debate overlaps also with the debate on the origin of Barrett esophagus from esophageal mucosa or

cardia gastric mucosa. What is sure is that there is evidence that these two pathologic entities have a common molecular and genetic basis, as we will see later.

Familial gastric cancer

Although the majority of gastric adenocarcinomas are sporadic, a familial aggregation is seen in approximately 10% of cases. Among these familial cases a hereditary cause is determined in only 1-3% cases that are associated with germline mutations in genes involved in molecular pathways of gastric carcinogenesis. The best-known hereditary form of gastric cancer is hereditary diffuse gastric cancer (HDGC) which is an autosomal dominant cancer predisposition syndrome characterized by an increased risk of developing Lauren's diffuse-type gastric cancer, but also lobular subtype breast cancer. It is related to germline heterozygous mutations in the calcium-dependent adhesion (*CDH1*) gene. These mutations lead to an altered or absent expression of E-cadherin protein, which plays an important role in cell polarity and intercellular adhesion. According to the two-hits hypothesis, when the second wild-type allele is inactivated or silenced the process of carcinogenesis can begin. The lifetime risk of developing gastric carcinoma in male carriers is 70% and 56% for female carriers (31).

Patients with familial adenomatous polyposis (*APC* gene), Peutz–Jeghers syndrome (*STK11* gene), Li–Fraumeni syndrome (*TP53* gene) or Lynch syndrome (particularly with *MLH1* or *MSH2* mutations), also have an increased risk of developing gastric cancer (24).

1.3.3 SYMPTOMATOLOGY AND DIAGNOSIS

The symptomatology correlated with gastric adenocarcinoma is very often vague and nonspecific, thus does not prompt patients to pursue further diagnostic investigation. This implies that the disease has time to grow and at diagnosis often already manifests in an advanced stage. When it is present, symptomatology includes mainly epigastric pain, which can occur intermittently and even unrelated to food intake. Usually the non-specificity and mild extent of pain do not make it

attach any particular alarm significance. Other symptoms are: dyspepsia, which refers to a vague digestive disorder, which can be described variously as a feeling of abdominal distension, postprandial fullness, belching, burning, etc.; hypochromic anemia, which is secondary to a chronic blood ooze caused by the presence of a neoplasm that has ulcerated (massive hemorrhage, with hematemesis and melena, is a possible but rare occurrence); unintentional weight loss, which in most cases becomes relevant when the neoplasm is already advanced; dysphagia, which may be present when the neoplasm localizes to the cardia level causing stenosis/sub-stenosis of this tract of the viscera; sense of postprandial stuffiness, belching, nausea and vomiting which may be present when the neoplasm localizes to the antro-pyloric portion; palpable mass which is rarely documented; hepatomegaly and jaundice due to direct metastatic involvement of the liver or involvement of hepatic lymph nodes with obstruction to biliary outflow (obstructive jaundice); ascites, from peritoneal carcinosis (16).
Upper endoscopy (EGDS) is the most common test used to detect stomach cancer. It allows detailed macroscopic typing and, of course, it also allows to obtain adequate biopsy sampling for histological diagnosis. EGDS with biopsy is usually sufficient for diagnosis. The other instrumental investigations are for staging the neoplasm (32). Gastric cancer staging is based on the eighth edition of the American Joint Cancer Committee (AJCC) TNM staging system. As well as for esophageal cancers, also for gastric staging system there are three classifications, clinic (cTNM), pathologic (pTNM) and post-neo-adjuvant therapy (pyTNM).

1.3.4 TREATMENT AND PROGNOSIS

Gastric cancer can be classified, based on staging, into two types: early gastric cancer (EGC) and advanced gastric cancer. EGC is a neoplasm with invasion limited to the mucosa or submucosa regardless of the size of the tumor or the presence/absence of lymph node involvement (33). This category plays an important role in clinical practice. In fact, EGC can be subjected to conservative endoscopic treatment, including two main techniques, endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD). Conservative treatment has numerous advantages for the patient in terms of reduction in

mortality and morbidity of surgery. The five-year survival rate for these cases is 70-90% (33).

For locally advanced gastric cancers that extend beyond the level of gastric submucosa and into the muscle layer resective surgery of the stomach is necessary and consists in total gastrectomy with lymphadenectomy. The five-year survival rate following radical gastrectomy without any further treatment is poor and around 10-30% (30). Several strategies have been developed to improve this survival rate. Recently, the addition of neo-adjuvant chemotherapy to surgery has demonstrated to increase the 5-year survival rate of patients with advanced potentially resectable tumours (30). Adjuvant chemotherapy is recommended in completely-resected gastric adenocarcinoma, particularly in those who did not receive neoadjuvant therapy (33).

Unfortunately, most cases of gastric cancer are diagnosed in an advanced, unresectable or metastatic stage. When the malignancy has exceeded the limits of curability or is metastatic, systemic chemotherapy remains the standard of care for most patients, except for those with tumors harboring specific molecular alterations. Molecular targeted therapies and/or immunotherapy based on the new information available on the molecular characterization of the tumor have significantly changed the landscape of metastatic and unresectable gastric cancers (32).

1.3.5 HISTOPATHOLOGICAL CLASSIFICATION

Gastric adenocarcinoma represents a highly heterogeneous disease from several points of view, first of all from the morphological standpoint. The numerous histopathological classifications that have been proposed during time reflect such a high heterogeneity. Moreover, the coexistence of different morphological components within the same tumour, is frequent, adding complexity to histological classifications. Some morphological classification schemes that have been proposed are those of Lauren (1965), Ming (1977) and the World Health Organization (2019).

LAUREN'S CLASSIFICATION

The most commonly used classification system was proposed by Lauren in 1965, in which gastric cancer is divided in two main types, the intestinal-type (53%) and the diffuse-type (33%), on the basis of the histological characteristics which were found in the samples. The remaining 17% of gastric adenocarcinomas are classified as mixed or indeterminate type (28). Intestinal and diffuse-type gastric cancer present different characteristics concerning not only histological features, but also epidemiologic profiles, clinical aspects, genetic and molecular features and prognostic significance.

Intestinal type gastric cancer

Histologically, the intestinal-type is characterized by the presence of neoplastic cells with a high cohesive capacity which form well differentiated glandular structures and occasionally papillary or solid components. This behavior on the part of the cells also accounts for the macroscopic appearance of the lesions that are usually exophytic and often ulcerating. Depending on glandular architecture, cellular pleomorphism and nuclear morphology, pathologists can define three degrees of differentiation: well differentiated, moderate differentiated and poorly differentiated/undifferentiated (34).

There is evidence that intestinal-type gastric cancer is associated with intestinal metaplasia (IM), which is, as we have seen before, the precancerous lesion for both cardia and noncardia gastric cancer, deriving from a multi-step process that starts with chronic inflammation of the gastric mucosa caused by GERD or *H. pylori* infection, respectively. IM may progress to low and high grade dysplasia and then to invasive adenocarcinoma, as part of gastric multi-step carcinogenesis as explained by Correa (34) (*Figure 4*).

Intestinal-type gastric cancer is the most common histologic variant found in the proximal stomach, including the gastric cardia and GEJ. It is more common in male and in older patients and has usually a better prognosis compared to diffuse-type gastric cancer (34).

Diffuse-type gastric cancer

In diffuse-type gastric cancer neoplastic cells have a little tendency for cohesion, having lost cell-to-cell interactions: these cells infiltrate the gastric wall as single cells or small cellular nests and typically do not form glandular structures. By definition, diffuse type adenocarcinoma is poorly differentiated/undifferentiated (34). Because of this behavior, diffuse-type gastric cancer does not form exophytic lesions, but typically evokes a response in the surrounding extracellular matrix microenvironment which is clinically termed the "desmoplastic response". This response leads to a stiffening of the gastric wall. When large areas of infiltration are present, the gastric wall becomes rigid and thickened: this pattern is called "Linitis plastica" and represents a well-established negative prognostic factor (35). Diffuse-type gastric cancer is more prone to disseminate into the peritoneum, when compared to intestinal-type which tends to metastasize haematogenously.

Unlike the intestinal-type which is highly correlated with inflammation cascade towards IM, the diffuse-type appears to be less associated with inflammation and environmental factors, with, by contrast, a stronger relevance of genetic factors (36). Diffuse-type gastric cancer seems to arise from normal gastric mucosa and the carcinogenetic process does not involve a specific carcinogenic sequence (*Figure 4*).

The existence of different carcinogenic pathways distinguishing intestinal and diffuse-type is further confirmed by several publications which have described important molecular differences among them. The 50% of diffused-type gastric cancer are characterized by mutations in CDH1 gene which lead to an abnormal expression of E-cadherin (37).

Diffuse type is more frequent in younger patients and it has a poorly prognosis than intestinal type. With regard to gender differences, some studies reported that diffuse-type is more frequent in female, some others suggested that its proportion is similar between men and women (34).

Although the Lauren classification system dates back to 1965, it is still widely accepted and used by pathologists and represents a simple but robust classification approach.

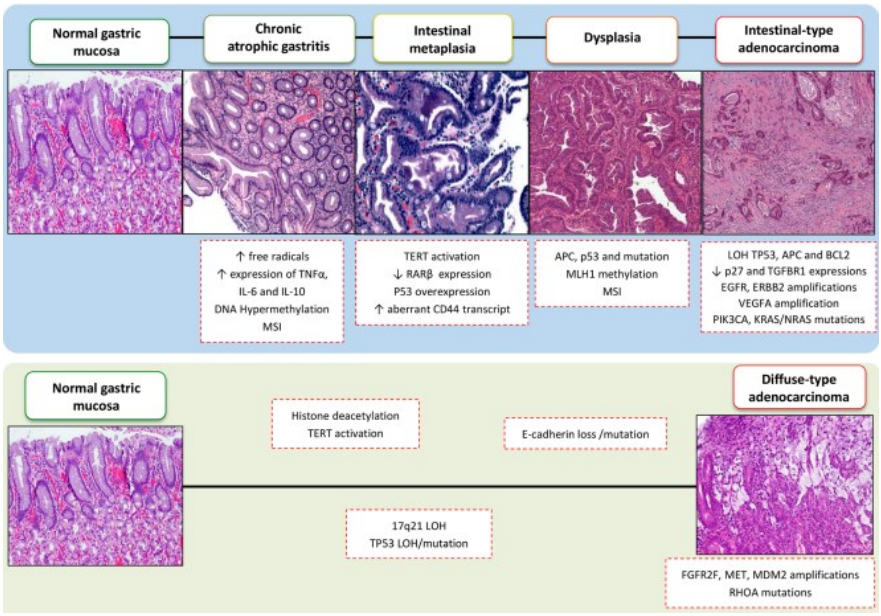

Figure 4. Sequential morphologic, genetic and epigenetic alterations in both intestinal-type and diffuse-type gastric cancer. This figure summarizes the sequence of molecular events that have been characterized for intestinal-type and diffuse-type GC according to the Correa cascade model. MSI: microsatellite instability; GS: genomically stable; EBV: Epstein-Barr virus; CIN: chromosomally unstable; LOH: loss of heterozygosity. Source: Riquelmen I., Oncotarget, Vol.6, 2015

MING'S CLASSIFICATION

According to Ming's classification, which was presented in 1977, gastric cancer can be divided into two types: expanding (67%) and infiltrative (33%) gastric cancer. These two subtypes differ from each other on the basis of the observed growth pattern: expanding carcinomas grow by expansion resulting in the formation of masses or tumor nodules, which consist of glandular structures more or less differentiated, whereas in infiltrative carcinomas tumor cells invade gastric wall individually or in form of small cellular nests without forming glandular structures. According to Ming, Lauren classification system presents a basic inconsistency, because the terms "intestinal" and "diffuse" refer to two different aspects of the tumor: the term "intestinal" describes the morphological structure of the tumor, while the term "diffuse" indicates the distribution of the tumor. With his classification Ming placed the focus not so much on architectural and histological aspects but rather on the biological behavior of the two subtype of tumors, overcoming in this way what he called "the pitfall" of Lauren classification.

However, the tumor types in Lauren's and Ming's classifications correspond to each other closely. In fact, generally, the vast majority of expanding tumors have

features of intestinal-type cancers, while infiltrative tumors have features of diffuse type cancers (38).

WHO 2019 CLASSIFICATION

The fifth edition of the WHO classification of digestive tumors, which was edited in 2019, divides gastric cancer into five histologic subtypes: tubular, papillary, mucinous, poorly cohesive (including signet ring cell phenotype) and mixed carcinomas, plus uncommon histologic variants. The first three subtypes are generally considered to be sub-variants of intestinal-type gastric cancer according to Lauren's classification system, whereas poorly cohesive subtype has features resembling diffuse-type. This classification is based on the predominant histologic pattern of the carcinoma, although there are some tumors which present different histologic components creating the mixed phenotype (11).

Tubular adenocarcinoma

Tubular adenocarcinoma is the most frequent histologic type of gastric carcinoma, with a relative frequency ranging from 45% to 64%. It is characterized, macroscopically, by the formation of polypoid or fungating masses that protrude into gastric lumen, whereas, histologically, it presents irregularly distended, fused or branching tubular glands of various sizes. For tubular, papillary and mucinous gastric adenocarcinomas, which resemble Lauren's intestinal type, a grading system is also provided. Traditionally, three grades are recognized, well, moderately and poorly differentiated, based on tubular glands formation, but, according to the 2019 edition of the WHO classification, grading is preferably performed using a two-tiered system: low-grade (formerly well or moderate differentiated) versus high grade (formerly poorly differentiated tumors) (11).

Papillary adenocarcinoma

Papillary adenocarcinoma is another common histologic variant of gastric cancer, accounting for 2,7-9,9% of all gastric cancers (11). It is characterized by the presence of epithelial finger-like projections lined by columnar or cuboid neoplastic cells surrounding a fibro-vascular central core. It is more frequent in older patients and occurs usually in the proximal stomach. In some papillary

tumors there are micropapillary or tubular components, creating a mixing phenotype (39).

Reporting of this phenotype is clinically important because it is associated with a high frequency of liver metastasis and lymph node involvement, so it has an important prognostic role. Some studies have reported that patients with papillary carcinomas have a worse prognosis than those with tubular ones (40).

Mucinous adenocarcinoma

Mucinous adenocarcinoma has been reported to account for 2,1-8,1% of gastric cancers (11). The WHO classification has defined mucinous adenocarcinoma as a type of gastric cancer where more than the 50% of total tumor volume is represented by extracellular mucin. Neoplastic cells can form glandular structures surrounded by interstitial mucin or irregular cell clusters or nests dispersed in mucinous pools (39).

Mucinous tumors usually have a larger size than other phenotypes and present at a more advanced stage. They also have more frequently lymphatic invasion and seem to have a poorer prognosis than non-mucinous tumors. However, a mucinous histology is not considered as an independent prognostic factor, which means that the poorer prognosis of this type of tumor is not related to the histology but, rather, to the advanced stage at which tumors are found (41).

There is also evidence that HER2 protein expression and HER2 amplification gene is lower than that observed in other histologic phenotypes (41).

Mixed adenocarcinoma

Mixed adenocarcinoma has a relative reported frequency of 6-22% (11). This subtype displays two or more distinct histological components, glandular (which can be both tubular and/or papillary) and poorly-cohesive and/or signet ring cells. Available data suggest that patients with mixed adenocarcinomas have poorer prognosis than those with only one component (11).

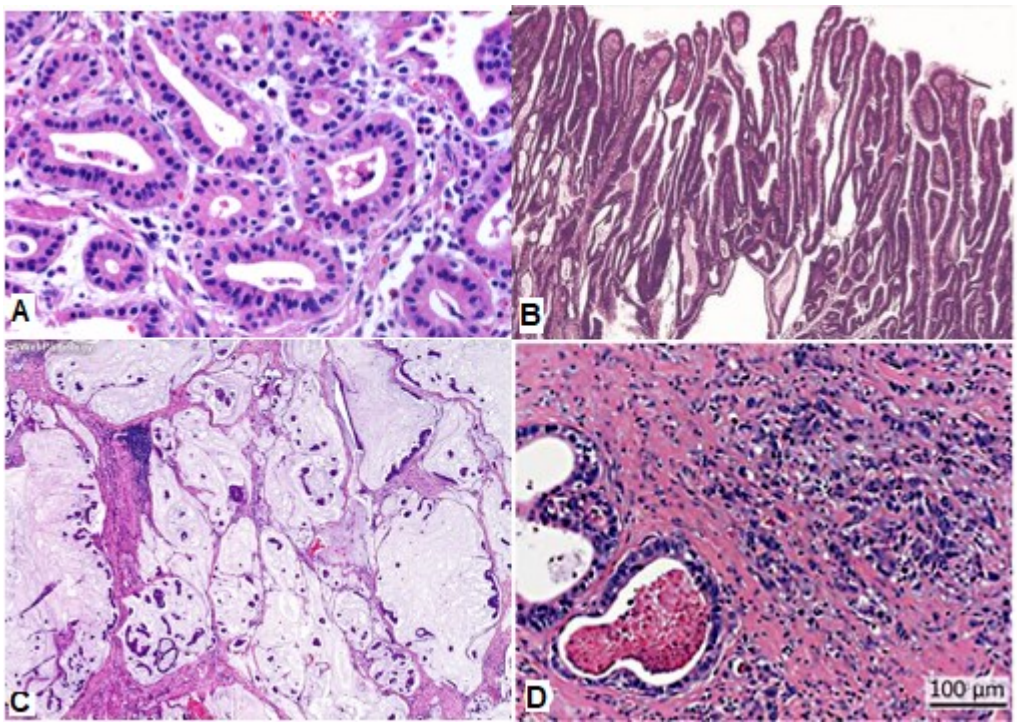

Figure 5. ***A****. Tubular adenocarcinoma. The tumor is composed of dilated tubular glands. Source: WebPathology* ***B****. Papillary adenocarcinoma. The tumor consists of finger-like processes with fibrovascular connective core, lined by columnar or cuboid cells. Source: 2019 WHO classification of tumours, 5th edition.* ***C****. Mucinous adenocarcinoma. The tumor consists of malignant glands or isolated cellular nests flooting in extracellular mucin pools. Source: WebPathology.* ***D****. Mixed adenocarcinoma. The tumour displays one glandular/intestinal component (left side) and one poorly cohesive/diffuse component (right side). Source: Gullo I. et al, Pathobiology, 2018*

Poorly cohesive gastric cancer and signet ring cells phenotype: a challenge for classification

Poorly cohesive cells gastric cancer (PCC) accounts for 20-54% of all gastric cancers. PCC gastric cancer is defined by the WHO classification as a phenotype composed of neoplastic cells that lack cellular cohesion and are isolated from each other or arranged in small aggregates (11).

In the context of PCC gastric cancer two different patterns can be distinguished: signet ring cells (SRC) type and poorly cohesive non-signet ring cells (PCC-NOS) type. SRC type is characterized by the presence of neoplastic cells which contain a large amount of mucin that displace the nucleus to the cell periphery: the central pool of mucin, which appears optically clear in hematoxylin and eosin stained specimens, mimics the appearance of a finger hole whereas the nucleus mimics the appearance of the bezel ring in profile. In PCC-NOS type non-signet ring neoplastic cells have the morphological aspect of histiocytes, lymphocytes or plasma cells (42).

Due to the cells' lack of cohesion and tendency to invade gastric wall, PCC gastric cancers, including SRC phenotype, fall into the diffuse-type according to Lauren's classification system (39). This correspondence is also supported by the fact that PCC and SRC gastric seem to share no risk factors with conventional intestinal-type gastric cancers such as *H.pylori* infection. E-cadherin deficiency due to *MLH1* gene mutations seems to be involved in the carcinogenesis of a significant proportion of SRC cases. These mutations can be both genetic-determined in the contest of HDGC and sporadic. Although the loss of cell-to-cell adhesion molecules is thought to be involved in earlier tumour initiation, the mechanisms and pathways underlying mucin accumulation in cells are not well recognized (43).

Considering the epidemiology of SRC, it is more frequent in women than non-SRC, occurring among younger patients of age ranging from 55 to 61 years, 7 years before the occurrence of non-SRC (43). The interest of pathologists towards these type of tumour is due to recent epidemiological data which reported that in the last few years there is an increasing incidence of PCC and SRC cancers diagnosis (44).

In the past few years, there was a lack of a standardization in the definition and terminology for these types of tumor, as the terms "diffuse type", "poorly cohesive" and "signet ring cell" gastric cancer were often used indiscriminately (42) (44). The definition of signet ring cells (SRC) gastric cancer has evolved in the different published editions of the WHO classification.

In 2017, the European Chapter of International Gastric Cancer Association (IGCA) has promoted a workshop in Verona, in which a multidisciplinary expert team tried to reach a consensus on the classification of PCC and SRC carcinomas. Verona consensus proposed to classify as SRC gastric cancer only those cancers with more than 90% of cells presenting a signet ring morphology. All other types that do not meet this definition are subdivided into poorly cohesive carcinoma non otherwise specifies with signet ring component (PCC-NOS/SRC), when the percentage of signet ring cells is < 90% but > 10%, and poorly cohesive non otherwise specified carcinoma (PCC-NOS), when the percentage of signet ring cells is <10% (45).

This new classification system is important because it contributes to achieving a standardization in the interpretation of studies' results and to facilitating comparison between studies.

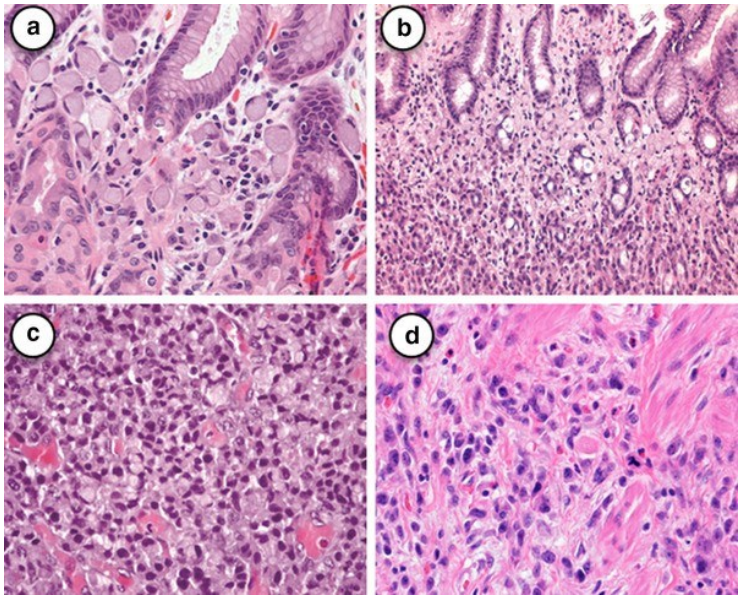

Figure 6. Poorly cohesive gastric carcinoma, examples of morphology. ***a*** *Signet ring cell carcinoma (SRCC) (> 90% of signet ring cells): classical signet ring cells are seen at the superficial layer of gastric mucosa;* ***b*** *combined PCC-NOS and SRCC (PCC-NOS/ SRC) (< 90% but > 10% of signet ring cells): this case has two components, the superficial part is composed of classical signet ring cells and the deeper part is composed by poorly cohesive, non-signet ring cells;* ***c*** *combined PCC-NOS and SRCC (PCC-NOS/ SRC) (< 90% but > 10% of signet ring cells): in this case, the two cell types (signet ring and poorly cohesive cells) are intermingled;* ***d*** *poorly cohesive carcinoma NOS (PCC-NOS) (< 10% of signet ring cells): the poorly cohesive, non-signet ring cells, are invading the muscle layer (H&E, original magnifications ×200–400). Source: Mariette C. et al, Gastric cancer, 2019*

A recent study has evaluated the prognostic impact of Verona consensus classification of SRC gastric cancer. The results of this study have shown that the long-term survival was significantly higher in SRC-type (> 90% SRC) compared with PCC-NOS/SRC (< 90% but > 10% of SRC) and PCC-NOS (< 10% of SRC) tumors. Particularly, the percentage of SRC was found to be inversely related to tumor aggressiveness, with lower depth of invasion, pT-stage at diagnosis and number of positive nodes. By contrast, PCC-NOS cancers seem to be associated with unfavorable clinical factors, a greater depth of invasion, a more advanced stage at diagnosis and more frequent lymph node metastasis (46). In reality, a meta-analysis published by *Zhao et al.* in 2021 revealed that at early stage SRC exhibited better prognosis than non-SRC, while at advanced-stage SRC exhibited poorer prognosis than non-SRC. According to this meta-analysis the better prognosis of SRC cancer may be due to the tendency of this tumour to be diagnosed at an early

stage and at younger age, and not to an intrinsic better biologic behavior. The typical intracytoplasmic accumulation of mucin, which compresses nuclei in the corner, may explain the tendency of these tumours to be larger and expand superficially to mucosal and submucosal layers, thus leading to the early diagnosis of SRC (43). A stage-dependent prognostic role of SRC was also confirmed by studies on Western population (45). The question whether SRC morphology could be considered as an independent prognostic factor is still debated.

Other and rare histological subtypes

Gastric adenocarcinoma with lymphoid stroma: this subtype has been reported to account for 1-7% of all gastric adenocarcinomas. It is also known as lymphoepithelioma-like carcinoma and medullary carcinoma. The main feature of this subtype is its prominent lymphocytic infiltrate with intra-epithelial lymphocytes. It is more frequently located in the proximal stomach and is more common in males. It has been reported that a variable percentage ranging from 22,5% to 100% of cases is correlated to EBV-infection detected by performing EBER-in situ hybridization.

Hepatoid adenocarcinoma: this subtype is predominantly composed of large polygonal eosinophilic hepatocyte-like neoplastic cells and it represents 0,3-2% of all gastric cancers.

Micropapillary adenocarcinoma: this subtype is characterized by the presence of small clusters of tumour cells without fibrovascular cores protruding into clear spaces. This subtype usually accompanies tubular or papillary adenocarcinomas, although a pure micropapillary carcinoma has been described in the GEJ. The presence of a micropapillary component represents a negative prongnostic-factor, as it is associated with frequent lymph-node metastasis.

Other rare variants: encompass gastric adenocarcinoma of fundica-gland subtype, mucoepidermoid carcinoma, Paneth cell carcinoma, parietal cell carcinoma, adneosuqmous carcinoma, adenocarcinoma with enteroblastic differentiation (11).

1.3.6 MOLECULAR CLASSIFICATION

THE CANCER GENOME ATLAS CLASSIFICATION

Understanding the molecular characteristics of gastric cancers is critical to develop new treatment strategies. In 2014, The Cancer Genome Atlas (TCGA) project realized a comprehensive molecular characterization of gastric adenocarcinoma and proposed a new molecular classification system for this type of tumour which comprises four subtypes: tumours positive for Epstein-Barr virus (EBV), microsatellite instability tumours (MSI), genomically stable tumours (GS) and tumours with chromosomal instability (CIN) (3). This classification system highlighted the main molecular alterations specific to each subtype of tumor.

It is important to note that TCGA classification overlaps substantially with histopathological classifications made by Lauren and Ming, suggesting that the genetic and molecular substrate may condition the tumor morphology, thus explaining the great morphological heterogeneity observed in these tumours (47). Although the original TCGA study did not investigate the relationship between each subtype and clinical outcomes, some subsequent studies evaluated this relationship. The clinical implications of TCGA classification, however, depend on the possibility of exploiting molecular biomarkers in order to create a personalized treatment for each type of tumour. Here, we will describe the genetic and clinic-pathological features of each subtype of tumour according to the TCGA classification, focusing on the main biomarkers that are currently associated, or supposed to be associated, with efficacy of certain therapies in clinical practice.

Epstein Barr Virus (EBV)-positive subtype

EBV-positive tumors represent 8,8% of gastric cancer's cases among those which were analyzed by TCGA project (3).

EBV is a transforming pathogen which acts in the process of oncogenesis through the interaction between the expression of its own genes and host cells' genome.

EBV enters gastric epithelial cells mainly via cell-to-cell contact with EBV-infected B lymphocytes. The EBV genome does not integrate into the host genome and is maintained as plasmids called episomes which, in the absence of an autonomous transcription system, are transcribed together with the host genome.

When EBV genome is transcribed, it expresses a series of latency genes, which are different among different EBV-associated malignancies. Considering specifically gastric oncogenesis, EBV genome's transcription produces a series non-coding RNA, including EBV-encoded small RNA 1/2 (EBER1/2). EBER 1/2 can be exploited as molecular biomarkers and detected by in situ-hybridization technique (48).

The TCGA study has reported that EBV-positive gastric cancers are characterized by a high level of CpG island methylator phenotype (CIMP). The hypermethylation of DNA is more extreme in EBV-associated gastric cancer than in any other type of cancer (48). DNA methylation in a promoter region is an important epigenetic mechanism for the downregulation (silencing) of gene expression (47). Infection with EBV induces the hypermethylation of both host and viral genomes, but the precise mechanism which leads EBV infection to hypermethylation remains unclear (48).

All EBV-positive tumours which have been assayed in the TCGA cohort displayed *CDKN2A* promoter hypermethylation (3). The cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene on chromosome 9p21 is a tumor suppressor gene. It is responsible for inhibiting various cyclin-dependent kinases and plays an important role in cell cycle regulation by decelerating cell cycle progression at the G1/S phase (49).

Approximately 80% of EBV-positive tumors exhibit mutations in the *PIK3CA* gene, which encodes the PI3Kα protein. This protein can be targeted by PI3K inhibitors. (3). These cancers show also inactiving mutations in AT-rich interactive domain-containing protein 1A (*ARID1A*) (55%) and B-cell lymphoma 6 Corepressor (*BCOR*) (23%, also muted in leukemia and medulloblastoma) (3).

The 15% of EBV-positive subgroup present a recurrent amplification at 9p24.1, a chromosomal region that contains *JAK2* gene which can be a potential therapeutic target for JAK2 inhibitors. Two other genes located in this locus are *CD274* and *PDCD1LG2* that encodes programmed death-ligand 1 and 2 (PD-L1, PDL-2) proteins which are overexpressed. The high level of PDL1 and PDL2 protein expression makes this type of tumor targetable with immune checkpoint inhibitors (3).

Most EBV-associated gastric cancers have been found in the gastric fundus or body and in male patients (47,48). Regarding EBV prognostic role, a cohort study data from the TCGA found that the prognosis of EBV-positive gastric cancers was the best in terms of overall survival compared with MSI, GS, and CIN subtype (47). This result is confirmed by a meta-analysis conducted in 2014 in which EBV-associated tumours were found to be associated with lower tumour–node–metastasis (TNM) stage and a lower mortality rate (50).

Microsatellite instability (MSI) subtype

The MSI subtype represents 21,7% of all gastric cancers in the TCGA report (3).

Also MSI tumors are characterized by a high level of DNA hypermethylation, but this methylator phenotype, unlike EBV-associated cancers, regards typically the MutL homolog 1 (*MHL1*) promoter (3). *MLH1* is one of the genes involved in DNA mismatch repair (MMR) system. Hypermethylation of the *MLH1* gene promoter is responsible for the loss of MLH1 expression and for the deficiency in MMR functioning (3).

Mismatch repair (MMR) system is a complex of proteins that preserves DNA homeostasis and as such is an evolutionary guarantee of genomic stability. The main actions of the DNA MMR system are to recognize and repair erroneous single base mismatches and short insertions-deletions that can arise during DNA replication and recombination (51,52) . The most important proteins which are included in the MMR system are: MLH1 (mutL homologue 1), MSH2 (mutS homologue 2), MSH6 (mutS homologue 6) and PMS2 (postmeiotic segregation increased 2) (53). These four proteins form two heterodimers complexes, namely MLH1-PMS2 and MSH2-MSH6, where MLH1 and MSH2 are obligatory partners of these heterodimers. In fact, PMS2 and MSH6 can only form a heterodimer with MLH1 and MSH2, respectively. On the other hand, MLH1 and MHS2 can form heterodimers with other MMR proteins, namely MSH3, MLH3 and PMS1 (52).

The biallelic inactivation of one or more of these genes leads to the inefficacy of this system which is a guarantor of DNA stability (i.e. defective MMR, dMMR) and consequently to the accumulation of mismatching inaccuracies and frame-shift mutations (either through insertions or deletions) with an increased mutational burden of the tumour (54). This biallelic inactivation can result from mutations

(either germline or somatic) or from epigenetic silencing (55). Epigenetic silencing includes also promoter hypermetilation.

Microsatellites are repetitive DNA sequences (1–6 nucleotides) that are distributed along the genome of both coding and noncoding regions and are particularly sensitive to DNA mismatching errors (53). When MMR system is deficient (dMMR), there is an accumulation of alterations in microsatellites typically consisting of repeat length alterations: this situation is called microsatellite instability (MSI), which represents, therefore, an indirect evidence of a dMMR (54).

Deficiency MMR and MSI are genetic features best-known for their association with Lynch syndrome, but Lynch syndrome associated with gastric and esophageal adenocarcinoma is rare. In fact, the frequency of gastric cancer in Lynch syndrome patients is estimated to be 1,6%, while esophageal cancers do not develop in the contest of this syndrome (55). The vast majority of MSI esophageal and gastric cancers are sporadic forms. The epigenetic silencing of MLH1 by promoter hypermethylation represents the leading cause of MMR deficiency in both sporadic and familial MSI tumours, while mutations of MLH1 and MSH2 are relatively rare (56).

Generally speaking, MSI phenotype with dMMR can be seen in up to 5% of esophageal adenocarcinoma (55) and in 11-13% of gastric adenocarcinoma (54). Regarding the association between MSI status and Lauren's classification, there is a higher prevalence of this molecular subtype for intestinal-type gastric cancer (47). In the TCGA cohort and also in other subsequent studies, MSI subtype is more frequent in patients with advanced age and female gender and it usually occurs in the antral region of the stomach (54,55).

It is notable that the MSI/dMMR status in gastric cancer is a positive prognostic factor, as it is correlated with a better survival compared with microsatellite stability (MSS) tumours, with less frequent lymph node involvement and lower stage at diagnosis (56). Additionally, the TCGA study, as well as other subsequent studies conducted on MSI tumours, revealed that this subtype is commonly associated with alterations in major histocompatibility complex class I genes, including B2M and HLA-B (47), a dense intra- and peri-neoplastic lymphocyte

infiltration (54) and a widespread expression of immune-checkpoint proteins, such as PD-L1 (3).

Due to their intrinsic mutational burden and expression of immune checkpoint proteins, such as PD-L1, MSI/dMMR status are considered as important molecular hallmarks which are predictive for the efficacy of immunotherapy (47) . Other mutations regard *TP53*, *KRAS*, *ALK* and genes involved in PI3K/PTEN/mTOR signaling (3).

Genomically stable (GS) subtype

The GS subtype has been found in 19,7% of gastric cancer cases in TCGA cohort (3). GS tumors have a lower mutational burden compared with other gastric cancer subtypes and are characterized by diffuse-type histology (47). The main molecular features of the GS subgroup are represented by somatic mutations in *CDH1* gene (37% of cases) that regulates the expression of E-cadherin, and in Ras homolog family member A (RHOA) gene (15%) (3). *CDH1* germline mutations are associated with hereditary diffuse gastric cancer syndrome. *RHOA* gene encodes a member of the Rho family of small GTPases, which cycle between inactive GDP-bound and active GTP-bound states acts as a molecular switch in signal transduction cascades (3).

Another molecular alteration which can be found in GS tumors is an inter-chromosomal translocation leading to the fusion between *CLDN18* gene, which encodes Claudine-18 protein, a component of tight junction adhesion structures, and *ARHGAP26* or *ARHGAP6*, which encodes a GTP-ase activating protein (GAP 6 OR 26) that facilitates conversion of Rho GTPases to the inactive GDP state. The resulting chimeric protein affects ARHGAP's regulation of Rho protein and cell motility. Furthermore, this fusion may also interfere with Claudine-18's function and affect cellular adhesion. Interestingly, CLDN18-ARHGAP26 fusion is mutually exclusive with RHOA mutations (3).

All these alterations affecting genes which are involved in cellular adhesion and motility may contribute to determine the diffuse growth pattern and lack of cell cohesion that are hallmarks of diffuse-type gastric cancer according to Lauren's classification system. In fact, over the 70% of GS gastric cancer have the characteristics of diffuse-type tumors (47).

Chromosomal instability (CIN) subtype

The most frequent molecular subtype in the TCGA cohort is represented by chromosomal instability (CIN) subtype, which accounts for 49,8% of all cancer cases analyzed in this study. This subtype has the special feature of being located more frequently in the proximal stomach, gastroesophageal junction and cardia region, and of having intestinal histology according to Lauren's classification (3,47).

Chromosomal instability (CIN) refers to the property of displaying and acquiring several types of chromosomal changes, including segmental or whole-chromosome aneuploidies and architectural chromosomal aberrations. This property derives from abnormalities due to various cellular defects occurring in chromosome segregation process during cell division. The high rate of chromosome segregation errors, characteristic of this type of cancer, ultimately leads to aneuploidy in the resulting progeny of cancer cells. Besides such numerical changes, also structural chromosomal aberrations like translocations, deletions, segmental duplications, and gene amplifications are also part of CIN subtype (57). Particularly, CIN tumors displayed frequent amplifications of genes encoding receptor tyrosine kinases (*RTKs*) or other genes involved in RAS pathways, such as *ERBB2/HER2* (24%), *ERBB1/EGFR* (10%), *ERBB3* (8%), *FGFR2* (8%), *MET* (8%) (3). Amplifications of *ERBB* genes, including *ERBB2/HER2* and *ERBB1/EGFR*, can be targetable by molecular target antibodies, such as HER2-targeting monoclonal antibody Trastuzumab for HER2 amplification, or Cetuximab or Panitumumab for EGFR amplification, or small molecules tyrosine kinase inhibitors, such as Laptinib, also for HER2 amplification (47).

Recurrent amplifications of the gene encoding ligand VEGFA is also notable, leading to the possibility of using VEGFR2-targeting monoclonal antibody Ramucirumab or VEGF-targeting monoclonal antibody Bevacizumab (47).

Additionally, frequent amplifications of cell cycle mediators' genes (*CCNE1, CCND1* and *CDK6*) suggest the potential for therapeutic inhibition of cyclin-dependent kinases (47).

Another molecular hallmark for CIN gastric tumors is represented by the high frequency of *TP53* mutations (73%). Phosphorylation of EGFR (pY1068) is also

significantly elevated in the CIN subtype, consistent with the detection of *EGFR* amplification in this subtype (3).

Given the possibility of using different types of targeted therapies, CIN subtype has the greatest survival benefit with adjuvant chemotherapy (47).

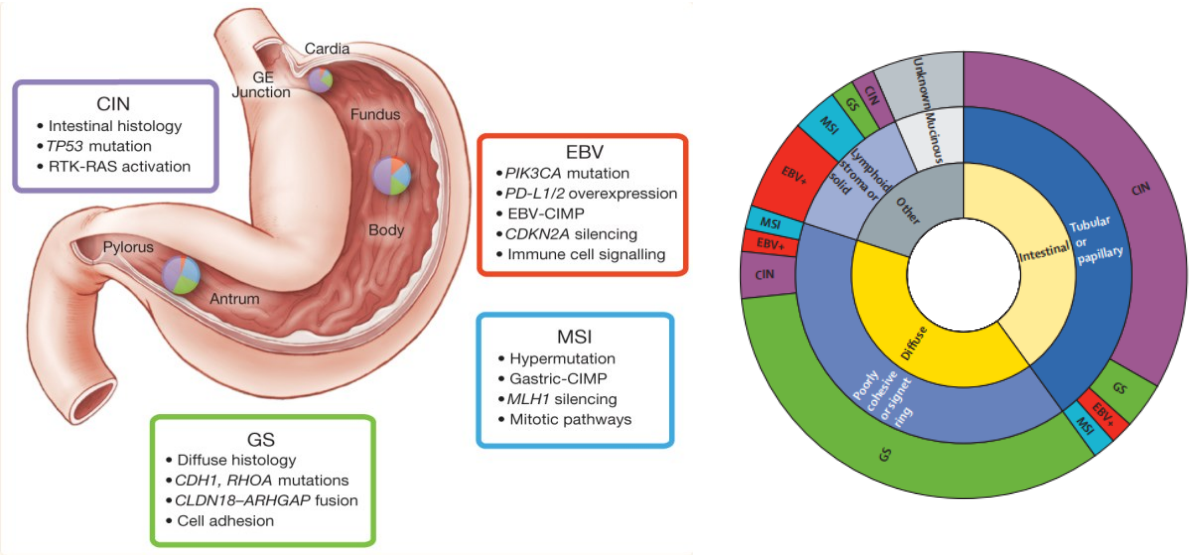

Figure 7. On the left, the figure shows some of the main molecular alterations associated with each of the four molecular subtypes of GC according to TCGA classification. Distribution of molecular subtypes in tumours obtained from distinct regions of the stomach is represented by inset charts. Source: Wang Q., Gastroenterology Research, 2019. On the right, graphical depiction of overlapping classifications. Smyth E. et al., The Lancet, 2020

THE ASIAN CANCER RESEARCH GROUP CLASSIFICATION

Beyond the TCGA molecular classification, the Asian Cancer Research Group (ACRG) has also categorized gastric cancers by studying a set of Korean cases, and proposed four subtypes of gastric adenocarcinoma with a certain level of overlap with TCGA subtypes: MSI (microsatellite instability subtype), MSS/EMT (microsatellite stability with epithelial-to-mesenchimal-transition), MSS/TP53-deficient subtype, and MSS/TP53-active subtype (*Table 1*). The MSS/TP53-deficient subtype resembles the TCGA CIN subtype due to substantial aneuploidy and focal amplification of HER2/ERBB2, EGFR/ERBB1, CCNE1 and CCND, and is frequently observed in proximal stomach, nearby the GEJ (58).

It is interesting to note that, compared with the TCGA set of tumours coming mainly from the US and Western Europe, a much larger proportion of the ACRG set from Korea were diffuse-type tumours and many fewer were proximal or junctional tumours (59).

	GC					OAC	
Lauren	Diffuse type	Intestinal type				Intestinal type in most cases (~95%)	
TCGA	GS	EBV	MSI	CIN	Others*	Non-MSI	MSI
	• *CDH1* and *RHOA* mutations • *CLDN18–ARHGAP26* fusion • Cell adhesion pathways • Younger patients	• DNA hypermethylation • *PIK3CA* mutation • *PDL1* and *PDL2* overexpression • Recurrent *JAK2* and *ERBB2* amplification • *CDKN2A* silencing • Immune cell signalling • Common in the corpus • Frequent *ARID1A* and *BCOR* mutation • Rare *TP53* mutation	• Hypermutation • *MLH1* silencing • KRAS or NRAS activation • *RASA1* and *PTEN* inactivation • Mitotic pathways • Older patients • Less A->C transversion	• RTK–RAS activation (*ERBB2*, *EGFR*, *MET*, *VEGFA* and *KRAS* or *NRAS*) • *TP53* mutation • Amplifications of cell cycle mediators (*CCNE1*, *CCND1* and *CDK6*), *GATA4* and *GATA6* • Common in GOJ and cardia cancer		• *TP53* mutation from early BO lesion • Progressive loss of tumour suppressor genes (*TP53*, *CDKN2A*, *SMAD4* and *ARID1A*) • RTK–RAS activation (*EGFR*, *ERBB2*, *VEGFA* and *KRAS*) • Amplifications of cell cycle mediators (*CCND1*, *CCNE1* and *CDK6*), *MYC*, *GATA4* and *GATA6*	• ~7% of OACs • *MSH3* or *MSH6* mutations
ACRG	MSS/EMT	MSS/*TP53*⁺	MSI	MSS/*TP53*⁻			
	• *CDH1* silencing • Younger patients • Worst prognosis	• Intact *TP53* • *MDM2* amplification • EBV infection • Enrichment with	• Common in the antrum • Best prognosis • Hypermutation • *MLH1* silencing	• *TP53* mutation • Genomic instability • Recurrent amplification (*ERBB2*, *EGFR*, *GATA6*			

Table I. Molecular classification systems of Gastric cancer according to The Cancer Genome Atlas (TCGA) classification and the Asian Cancer Research Group (ACRG) classification. Each subtype carries specific genomic alterations. Most oesophageal adenocarcinomas (OACs) are MSS, whereas OACs with MSI are less common. Gastric cardia cancer and OAC share CIN signatures with a high frequency of TP53 mutation, activation of receptor tyrosine kinase (RTK)– RAS pathways, and frequent amplifications in genes encoding cell cycle mediators. Source: Hayakawa Y. et al., Nature Reviews Cancer, 2016

1.4 GASTROESOPHAGEAL ADENOCARCINOMA AS A WHOLE ENTITY

1.4.1 GEJ CANCER: IS IT GASTRIC OR ESOPHAGEAL?

The definition of the adenocarcinoma occurring at the gastroesophageal junction (GEJ) has been an area of controversy and disagreement for many years mainly because it was not clear whether this tumor was of gastric or esophageal origin. Esophageal adenocarcinomas (EAC) located just above the GEJ have been traditionally considered separate from gastric adenocarcinoma occurring in the proximal part of the stomach (cardia gastric) (4). This view follows the model according to which EAC originates from Barrett's Esophagus (BE) that is, as explained before, a metaplastic condition characterized by the replacement of the normal esophageal squamous epithelium with a columnar epithelium with an intestinal differentiation (IM) (12). In the past years, one of the most important theories that attributes the origin of EAC from BE is based on the assumption that the metaplastic epithelium arises from a direct conversion of squamous cells to columnar cells, due to a process called trans-differentiation. Trans-differentiation represents an irreversible metaplastic conversion from one fully differentiated state into another (60). This assumption leads to the concept that BE and consequently EAC develop directly from squamous epithelium, and so EAC has been characterized as a distinct entity from cardia gastric adenocarcinoma, which develops from columnar epithelium of cardia gastric mucosa. According to this

view, EAC and cardia gastric cancer were seen as two distinct pathologic entities with different origins, although they have lots of similarities (4).

However, in 2011 an interesting study conducted on BE mouse models supported the hypothesis that BE might originate from embryonic remnant cell populations located at the GEJ and at the basement membrane of the proximal stomach. Wang et al., in fact, observed that a small population of residual embryonic columnar cells are maintained at the squamo-columnar junction in adult mice; these cells can migrate proximally in order to replace the squamous epithelium which has been eroded by GERD and so they might give rise to BE (61).

In 2012, another study on BE mouse models suggested that BE might arise from a progenitor cell lineage located in cardia gastric mucosa. This progenitor cell lineage can be activated by chronic acid-dependent inflammation, and, through clonal expansion, migrate to the distal esophagus where it gives rise to the columnar metaplasia of BE (62). It is important to note that also Norman Barrett himself, who firstly described that morphological change we call BE in 1950, initially assumed that BE resulted from a proximal migration of stomach epithelium just below the GEJ (63).

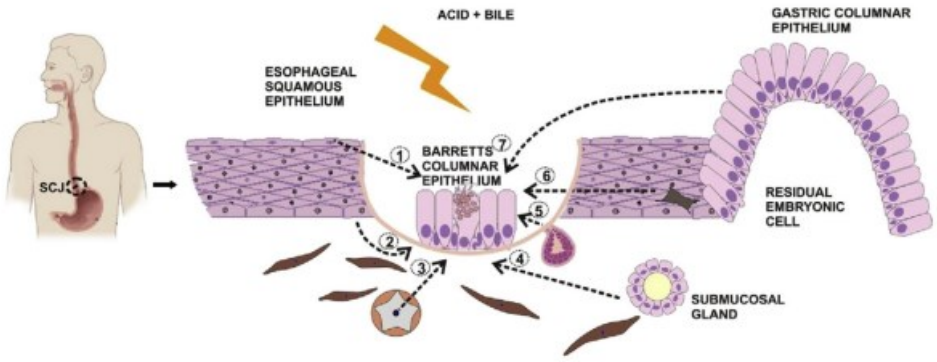

Figure 8. Different theories proposed to explain cell of origin of BE: (1) transdifferentiation of native squamous cells; (2) transcommitment of resident squamous stem cells; (3) colonization by circulating bone marrow-derived stem cells; (4) reparative emergence of submucosal glandular stem cells; (5) luminal unfolding of esophageal retention cysts; (6) residual embryonic cells at the transitional zone; (7) migration of cells from gastric epithelium. BE, Barrett's esophagus; SCJ, squamocolumnar junction.
Source: Harit Kapoor et al., The journal of laboratory and clinical medicine, 2015

These alternative explanations of the origin of BE lead also to a change in the interpretation of EAC and of its distinction with cardia gastric cancer. In fact, if BE does not actually originate from squamous epithelium through a process of trans-differentiation, but from undifferentiated stem or progenitor cells located at the GEJ, the hypothesis of a different origin for esophageal and gastric

adenocarcinoma, which by the way also have other similarities, would be disproved.

1.4.1 SIEWERT CLASSIFICATION: A PRACTICAL POINT OF VIEW

The most commonly used classification system for cancers of the GEJ in Western countries is the Siewert classification which was introduced in Germany by *Siewert et al.* in 1987 and was published in 1998. Siewert classification system is applied to adenocarcinomas in which the tumor center locates within 5 cm proximally and distally the GEJ and distinguishes junctional tumours into three categories. This classification was proposed in order to evaluate the exact location and extent of the cancer so that the best surgical approach can be chosen. Siewert type I adenocarcinoma represents a tumour in which the tumor center is located within 1-5 cm from the proximal side of the GEJ line; this type of tumor is considered as an "adenocarcinoma of the lower esophagus" and invades GEJ from above. Siewert type III adenocarcinoma is a gastric side adenocarcinoma in which the tumor center is located within 2-5 cm from the distal side of the GEJ line; this type of tumor is called "sub-cardiac adenocarcinoma" and invades GEJ from below. Siewert type II adenocarcinoma is called also "true adenocarcinoma of the GEJ" or "true adenocarcinoma of the cardia" as the tumor center is located in the region between types I and III (within an area from 1 cm proximal to 2 cm distal of the GEJ) (64) (*Figure 9*).

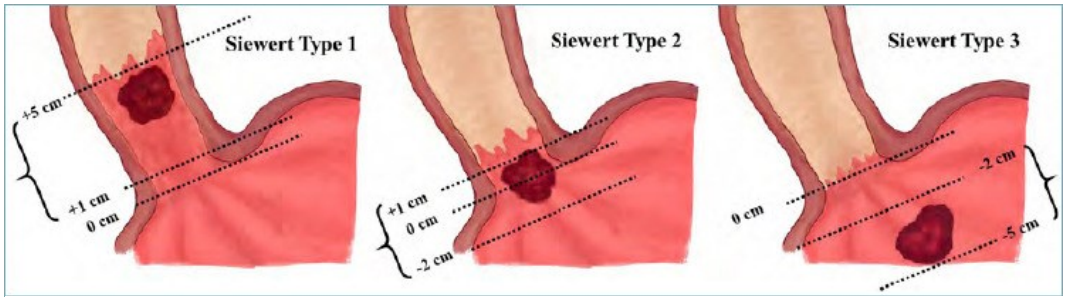

Figure 9. Schematic representation of the Siewert macroscopic classification of gastro-oesophageal junction tumours. Source: Grillo F. et al, Pathologica, 2020

The eighth edition of the AJCC TNM staging manual has re-defined GEJ tumours precisely by using the Siewert classification: cancers with GEJ invasion that have their epicenter within the proximal 2 cm of the GEJ (Siewert type I/II) should be staged using the TNM for the esophagus/GEJ. Cancers whose epicenter is more

than 2 cm distal from the GEJ (Siewert type III), even if the GEJ is involved, should be staged with gastric cancers (65).

From a therapeutic point of view, the most appropriate surgical approach is different for the three types of tumors, because Siewert type I cancers are treated as esophageal cancers, so with transthoracic esophagectomy, whereas Siewert type III adenocarcinomas are treated as gastric cancers, so with gastrectomy with transhiatal extension. The optimal surgical approach for type II cancers, in which a gastrectomy as well as an esophagectomy is technically possible in many cases, still remains under discussion (66).

1.4.2 THE TCGA STUDY'S BREAKTHROUGH

In 2017 The Cancer Genome Atlas Research Network performed a comprehensive molecular and genomic profiling on a series of esophageal (both squamous [ESCC] and adenocarcinoma [EAC]) and gastric (both cardia and noncardia subtypes) cancers including also some GEJ adenocarcinomas of indeterminate origin (21). This study, in addition to evaluate the molecular differences and similarities between EAC and ESCC, as explained before in the paragraph "Molecular distinction of esophageal cancers", also proposed to assess whether it was possible to establish an appropriate demarcation between EAC and cardia gastric adenocarcinomas on the molecular standpoint.

Evaluating EAC jointly with gastric cancers, the study showed that almost all (71 of 72) the tumours categorized as EAC could be classified as chromosomal instability tumours (CIN). In other words, in the cohort of cancers considered by TCGA, EAC could not be distinguished from the CIN class of gastric cancers from the molecular point of view. The notable molecular similarity between EAC and CIN gastric cancer supports the hypothesis of a common origin of these tumours and indicates that gastric cancers and EAC tumors should be considered as a singular entity, named as a whole "gastroesophageal adenocarcinoma", analogously to colorectal adenocarcinoma. Particularly, considering esophageal and gastric adenocarcinoma as a whole, the TCGA study showed that the proportion of CIN tumors has a gradual increasing gradient which goes from the lowest percentage in gastric tumours that develop in the distal part of the stomach to a higher

percentage in the proximal gastric cardia, to the point of representing almost 100% in esophageal adenocarcinomas. By contrast, GS, MSI, and EBV^{+}-subtype are more common in the distal part of the stomach and are practically absent in esophageal adenocarcinomas. This anatomic gradient of molecular subtypes, which challenges the firmer separation of cancers in the anatomic esophagus or stomach, is represented in the *Figure 10* (21).

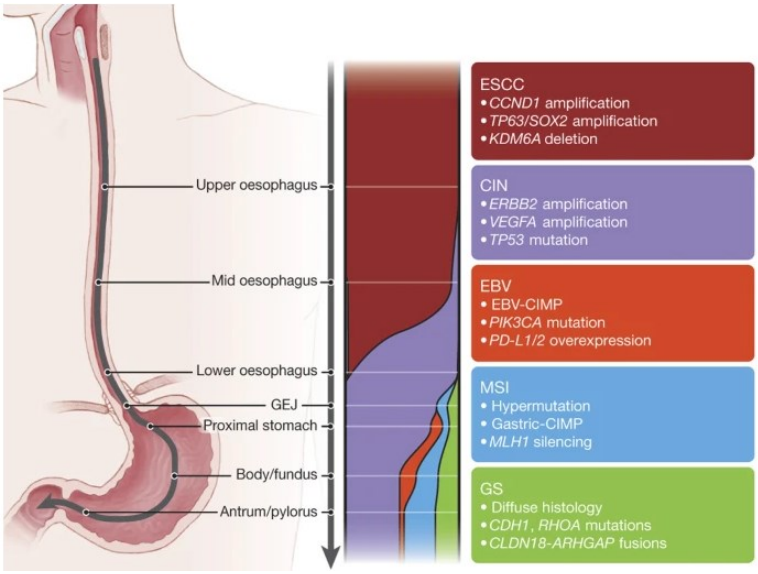

Figure 10. Anatomic gradient of molecular subtypes of gastroesophageal adenocarcinomas. Source: The Cancer Genome Atlas Network, Integrated genomic characterization of oesophageal carcinoma, Nature, 2017

The findings of the TCGA study have led to a fundamental change in the way gastric and esophageal cancers have always been considered, suggesting that whether the tumor originates in the esophagus or stomach is less relevant than the molecular characteristics of the individual tumors. Furthermore, pathologists seem to have overcome the dichotomous view that considers EAC of the lower tract of esophagus and cardia gastric cancer of CIN subtype as distinct tumour types, calling into question also the traditional demarcation of lower esophagus and upper stomach. Future clinical trials conducted to evaluate the effectiveness of targeted therapy on gastric cancers should absorb the idea of esophageal and gastric adenocarcinomas as a common entity (4).

1.5 BIOMARKER-TARGETED THERAPIES: TRANSLATION INTO PRECISION ONCOLOGY TREATMENT

Most of gastroesophageal cancers patients are diagnosed when the tumor is at an advanced stage, defined as unresectable or metastatic disease. For these patients the treatment is mainly palliative and based on systemic chemotherapy: although a large number of combined chemotherapy regimens have been tested in randomized studies, survival rates of patients with advanced cancer treated with these therapies remain low, with a 5-year survival not exceeding 20% (67).

The better understanding of molecular mechanisms of gastroesophageal carcinogenesis and the definition of a molecular classification of cancers have made possible the recognition of new molecular biomarkers and the development of rationally designed molecular targeted therapies (68).

Molecular biomarkers are useful not only for categorizing patients or for doing a prognostic evaluation of each type of tumor. Some of these biomarkers can also be predictive of response to therapy as they may be exploited as molecular targets to novel targeted treatments. HER2 has been the first routinely targeted biomarker used in the context of gastroesophageal cancers in the wake of the successes achieved in the better-known breast cancer (68). In this context, one of the most important molecular innovation achieved in recent years comes from a randomized phase III trial, also known as trastuzumab for gastric cancer (ToGA trial). In this study, HER2-positive advanced gastric and GEJ cancers have demonstrated to respond positively to HER2-targeting monoclonal antibody trastuzumab when this antibody is added to first-line chemotherapy treatment (69). Trastuzumab and chemotherapy have since become the new standard of treatment for patients with HER2 positive advanced gastric and GEJ cancers. The ToGA trial paved the way for other important studies in the direction of providing patients with personalized and precision oncology treatment which is essential for a modern approach to oncological patients.

Recently, also microsatellite instability and mismatch repair system deficiency (MSI/dMMR) status, programmed death ligand 1 (PD-L1) expression and EBV-positivity have been considered as useful molecular biomarkers as they are found to be predictive of response for immunotherapy, particularly for immune

checkpoint inhibitors, such as pembrolizumab. HER2 positivity, MSI/dMMR status and PD-L1 expression, along with EBV-positivity (EBER-ISH positivity), are currently the most important biomarkers associated with the efficacy of some targeted therapies in patients with gastroesophageal advanced cancers (68).

1.6 HER2: THE FIRST BIOMARKER USED IN CLINCAL PRACTICE

1.6.1 GENERAL INFORMATION

Human epidermal growth factor receptor 2 (HER2) is a 185 kDa transmembrane tyrosine kinase (TK) receptor and a member of the epidermal growth factor receptors (EGFRs) family. This family is composed of four members: HER1 (also known as the EGFR), HER2 (also known as neu/ErbB-2), HER3 (also termed ErbB-3), and HER4 (also termed ErbB-4). All these receptors share the same molecular structure which consists of an extracellular ligand-binding domain, a short transmembrane domain, and an intracellular domain with tyrosine-kinase activity (excepting the HER3). The binding of ligands to the extracellular domain triggers a signal transduction cascade that leads to the activation of various intracellular pathways. The consequences of these pathways' activation are increased cell proliferation, longer cell survival through apoptosis evasion, loss of cell cycle control, greater dedifferentiation and increased cell migration. Ligand binding induces HER proteins homodimerization or heterodimerization with other types of HER proteins: dimerization leads to HER activation which occurs by a process of trans-autophosphorylation in which a phosphorylated tyrosine localized in the tyrosine kinase domain of one of the partner mediates the tyrosine phosphorylation of the other partner and vice versa. HER2 is particular because it does not bind to any known ligand, but it is the preferred heterodimerization partner for other members of the HER family (70).

HER2 protein is encoded by the proto-oncogene *ERBB2* which is located on chromosome 17q21. In gastroesophageal cancers, *ERBB2* acts as an oncogene, mainly through a mechanism of gene amplification: the amplification of the gene induces the correspondent protein overexpression on the cellular membrane's surface. The overexpression of HER2 molecules facilitates the spontaneous formation of dimers on the surface of the tumor cell, leading to the acquisition of

advantageous properties by cells with alterations in the regulation of differentiation and proliferation processes (67).

Amplification of the HER2 gene and overexpression of its product were first discovered in breast cancer and the clinical interest in HER2 targeting potentialities remained focused on breast cancer for many years (71). In the recent years, many studies have demonstrated that HER2 overexpression is also present in several other malignancies, including colorectal, ovarian, endometrial, uterine cervix, lung, bladder and, particularly, gastric and gastroesophageal cancer (70).

1.6.2 HER2 OVEREXPRESSION INCIDENCE

In gastroesophageal adenocarcinomas, the frequency of HER2 overexpression varies wildly in literature ranging from 4,4% to 53,4 %, with a mean of 17,9% (72). One of the main reasons that may explain such a high variability in HER2 positivity rates is that the early studies on this subject were conducted using the same HER2 testing and scoring systems which are used for breast cancers. There are several evidences which show that HER2 overexpression and gene amplification are much more heterogeneous in gastric cancer compared to breast cancer. Therefore, specific testing and scoring methods for gastric cancer are required (72).

In the ToGA trial, the rate of HER2 overxpression was 22,1% (69).

1.6.3 CORRELATION WITH HISTOPATHOLOGICAL VARIABLES

HER2 status varies depending on histologic characterization and anatomic location of the tumor. Regarding the first feature, HER2 overexpression and gene amplification are observed predominantly in Lauren's intestinal-type gastric cancer rather than in diffuse-type (73)(71). In the ToGA trial's cohort of cancers HER2 positivity rates were 31,8% for intestinal-type tumours, and 6,1% for diffuse-type cancers (69). Moreover, mixed-type HER2-positive cases display HER2 IHC positivity in the intestinal component, supporting the strong correlation between HER2 expression and intestinal histological type. It is interesting to note that also in breast cancer HER2 gene amplification is a common feature of ductal invasive carcinomas and uncommon in lobular invasive carcinomas (70). In this context, a

study published in 1998 suggested that there is an inverse association between HER2 gene amplification and E-cadherin mutations, which are typical of diffuse-type gastric adenocarcinoma and lobular invasive breast carcinoma, while are rare in intestinal-type gastric adenocarcinoma and ductal invasive breast carcinomas (74).

Regarding the association between HER2 expression and anatomic location of tumours, HER2 overexpression and gene amplification rates are higher in those tumours which are located in the proximal part of the stomach (cardia gastric) in close proximity to gastroesophageal junction (GEJ). In this region the percentage of HER2-positive adenocarcinomas is up to 30% (68); particularly, in the cohort of gastric and GEJ tumours considered in the ToGA trial this percentage was 32,2% (69) . By contrast, this percentage decreases proceeding to the distal part of the stomach, representing the 21,4% of all samples considered in the ToGa trial's cohort (69). This association between HER2 expression and the tumour's site is consistent with the association between HER2 expression and tumour's histology, because, as we have seen before, GEJ cancers are generally of intestinal-type. Unlike the histological characterization, differences in incidence according to the anatomic location of the tumor are generally absent in breast cancers (73).

The increasing gradient of HER2-positivity incidence proceeding from the distal stomach to the GEJ reminds us the similar gradient of the proportion of CIN molecular subtype among adenocarcinomas occurring in the gastroesophageal tract. As we explained in the paragraph "The TCGA study breakthrough" regarding the results of 2017 TCGA study, the proportion of CIN tumours is low in the distal part of the stomach and grows in the proximal part up to represent practically the 100% of all adenocarcinomas occurring near to the GEJ (21). It is interesting to note that HER2-positivity is associated more frequently with CIN gastroesophageal tumours rather that with other molecular subtypes of gastroesophageal cancers (73).

1.6.4 HER2 IN GASTROESOPHAGEAL CARCINOGENESIS

As we have seen before, esophageal adenocarcinoma developed on BE and intestinal-type gastric adenocarcinoma of the cardia region have a common

molecular characterization (CIN subtype) and a probable common pathogenetic origin. Both esophageal adenocarcinoma and cardia gastric adenocarcinoma of intestinal-type develop through a multistep process in which a major role is played by chronic inflammation due to acid-reflux from the stomach (GERD). In both types of cancer, intestinal metaplasia (IM) represents the “carcinogenic field” in which intra-epithelial neoplasia (IEN, thus dysplasia is currently named, and proceeds from low-grade to high-grade IEN) can develop and even possibly progressing to adenocarcinoma (71).

Some studies have investigated the occurrence of HER2 amplification and overexpression in esophageal and gastric precancerous lesions, thus IM and IEN. In one of these studies (75), a consecutive series of 275 samples of stomach and esophagus tissues was studied. HER2-status was assessed in the whole spectrum of phenotypic changes involved in the carcinogenetic cascade leading to both intestinal-type gastric adenocarcinoma and esophageal adenocarcinoma. HER2 status was assessed by immunohistochemistry (ICH) and silver in situ hybridization (SISH). The results showed that in both esophageal and gastric samples, the rate of HER2 overexpression rose significantly from low-grade to high-grade IEN to adenocarcinoma. Neither native nor metaplastic mucosa samples (obtained from either stomach or esophagus) showed *HER2* amplification. This study demonstrates that HER2 dysregulation is early involved in the neoplastic transformation of both gastric and esophageal mucosa.

Recent studies on the role of microRNAs (miRNAs) further reinforce this hypothesis. Particularly, in a study conducted in 2013 (76), pathologists studied the association of the expression of HER2 protein with that of two miRNAs, miR-125a-5p and miR125b in a series of biopsy samples which represented the whole spectrum of lesions in the carcinogenic cascade leading to both intestinal-type gastric adenocarcinoma and esophageal adenocarcinoma. MicroRNAs (miRNAs) are a class of short, noncoding RNAs that bind to target messenger RNAs, blocking their translation in the correspondent protein. HER2 status was assessed by ICH and chromogenic in situ hybridization (CISH), whereas miR-125a-5p/125b expression was assessed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). miRNAs qRT-PCR-levels were tested also in a series of gastric

and esophageal adenocarcinoma, which included HER2-negative and HER2-positive cases. Comparing HER2 and miRNAs expression levels, a significant mutual exclusion of miR-125/HER2 expression has been shown in the journey from IM to low-grade – high-grade IEN neoplasia to invasive adenocarcinoma. Both in gastric and esophageal tissue samples, miR-125a-5p and miR-125b levels show a progressive decrease throughout the spectrum of lesions that go from normal mucosa, to IM, low-high-grade IEN and lastly invasive adenocarcinoma. In both gastric and esophageal mucosa, miR-125a-5p and miR-125b levels were significantly lower in HER2-positive than in HER2-negative carcinomas. By contrast, HER2 expression and amplification significantly increased from IM, low-high grade IEN to cancer. These results showed that the down-regulation of miR-125a-5p and/or miR-125b might be (at least in part) responsible for HER2 protein overexpression in gastroesophageal tumors. Therefore, although the main mechanism leading to HER2 overexpression seems to be the amplification of the correspondent gene, other molecular processes might lead to the same result.

Moreover, miR125a-5p/125b might be considered as possible molecular biomarker for therapeutic target in HER2-positive tumors. Studies in this direction are already ongoing in the context of breast cancer. Scott and colleagues demonstrated that infecting HER2-positive breast cancer cell lines with retroviral constructs expressing miR-125a-5p and/or miR-125b results in HER2 transcript suppression (77). This suppression through retroviral constructs might be exploited in order to obtain a therapeutic advantage. Breast cancer continues to be the furrow on which pathologist proceed in acquiring new information on the role of HER2 in gastroesophageal cancer pathogenesis.

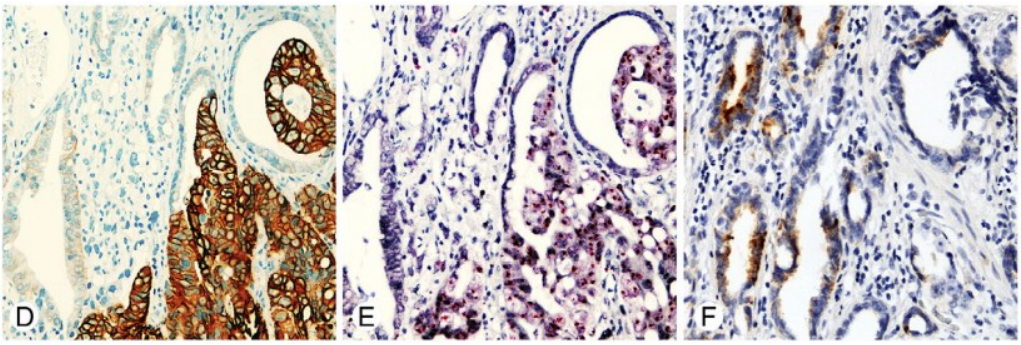

Figure 11. In HER2-positive cases, miR-125a5p expression (D) and HER2 up-regulation (at both gene [E] and protein [F] level) are mutually exclusive; original magnifications ×20 and ×40. Source: Fassan M, et al., Human Pathology, 2013

1.6.5 HER2 EVALUATION IN PRACTICE

In order to correctly select patients who may be eligible and may benefit from Trastuzumab treatment, it is imperative to accurately determine HER2 status. The experience gained in breast cancer has highlighted the importance of defining precise HER2 testing and scoring systems which results have to be correctly interpreted.

In 2016, the College of American Pathologists (CAP), American Society for Clinical Pathology (ASCP) and the American Society of Clinical Oncology (ASCO) organized an international expert panel in order to develop an evidence-based guideline to establish recommendations for HER2 testing in gastric and gastroesophageal adenocarcinomas. The Panel proposed 11 recommendations for both clinicians and pathologists. The first recommendation given by ASCO/ASCP/CAP guidelines is that all patients who has been diagnosed with advanced gastroesophageal adenocarcinoma and who may be good candidates for HER2-targeting therapies should have their tumor tested for HER2 overexpression/amplification (78).

HER2 assessment methods

HER2 status can be assessed by immunohistochemistry (IHC) and in situ hybridization assays (ISH) testing either formalin-fixed and paraffin-embedded (FFPE) biopsy or surgical resection specimens (79). IHC evaluates membranous HER2 protein expression on cancer cells, whereas ISH, which encompasses fluorescence in situ hybridization (FISH), identifies the presence or absence of HER2 gene amplification. Together, IHC and FISH are the most commonly used methods of determining HER2 status in routine diagnostic settings. FISH is an accurate assessment method, however, due to its high costs and time consumption, it is generally used as a confirmation test for HER2 IHC equivocal cases. The high concordance between FISH and IHC that is reported in literature supports the use of ICH as the first screening method for HER2 evaluation, with ISH used as a confirmation test, as it is recommended by the ASCO/ASCP/CAP guidelines (78).

Pre-analytical issues for HER2-testing

HER2 testing can be conducted both on endoscopic biopsy or surgical resection samples. Tumor samples can be obtained from both primary and metastatic site. Most patients with gastroesophageal cancer present with advanced disease and are not surgical candidates (at least in Western countries, in Asian countries, such as Japan, surgical specimens are more common (79)); for this reason, endoscopic biopsy sampling is usually the only diagnostic material available (72)(71). Important pre-analytical considerations are necessary to be done in order to gain a HER2-assessment which would be adequate and representative of all the tumour. First of all, endoscopic biopsy sampling should be conducted with an adequate number of tumour fragments in order to take into account the problem of intra-tumour heterogeneity of HER2-expression. The definition of the optimal minimum set of biopsies which should be submitted to evaluation has not been given yet. According to the ASCO/ASCP/CAP guidelines, for biopsy specimens, a minimum of 5 biopsy fragments, optimally 6 to 8, is needed (78), whereas the National Comprehensive Cancer Network guidelines recommend more than 6 samples to be taken (80). Also for surgical resection specimens, a multi-block analysis is recommended, as it has shown to increase HER2-testing accuracy than one-block analysis (71). In order to obtain representative blocks on surgical resection tissue, it is important for pathologists to select or include at least one gland-forming, well-differentiated area, with intestinal-type histology, as HER2-positivity is usually associated with gland-forming, intestinal-type adenocarcinomas (79).

Furthermore, pathologists should ensure that biopsy and resection specimens for HER2-testing are rapidly placed in fixative. In fact, tumours specimens need to prompt fixation for ideal histology, IHC and ISH testing. Delay in formalin fixation after specimen collection and/or prolonged fixation times may affect HER2-testing results. Delay to formalin fixation (DFF), also known as cold ischemia, is defined as the length of time between removal of the specimen from the patient and the time the pecimen is stabilized in formalin (ie, the biological activity in the tissue is stopped by fixing). The term “cold ischemia” actually refers to a room temperature environment, in contrast to “warm ischemia” following devascularization of the

tissue while still at body temperature (81). The ASCO/ASCP/CAP guidelines recommend that tissue specimens should be fixed in formalin within 1 hour and maintained in formalin for a total fixation time of no less of 8 hours and no more than 48 hours (78). In particular, endoscopic biopsy samples, which are more sensible to the effect of cold ischemia and tend to dry very quickly, should be immediately placed into formalin already in the endoscopy suite, or, at least, within 20 minutes (72). Both prolonged DFF/cold ischemia time and fixation time beyond 10 days promote the decrease of HER2 staining intensity and HER2-positive cells (72) (78). Also the type of fixative which is used could affect negatively the results of ICH and ISH testing; in order to avoid problems of poor fixation or hyperfixation, the preferred fixative is 10% neutral-buffered formalin (72). Always according to the ASCO/ASCO/CAP guidelines, testing on fine needle aspiration (FNA) specimens is considered as an acceptable alternative for those patients with advanced and metastatic cancer who are not candidates for surgical resection and are too ill for undergoing upper GI endoscopy (78).

Differences between HER2 expression in breast and gastric cancer

The determination of HER2 status in gastric cancer was initially based on the experience gained with HER2 testing in breast cancer. HER2 testing protocol in breast cancer includes IHC testing as the primary method of choice to determine HER2 status. HER2 IHC scoring system in breast cancer is based on intensity of reactivity (distinguished in three categories, i.e., faint, moderate and intense), completeness or incompleteness of membranous ICH staining, and percentage of reactive cells. HER2 expression patterns are scored as IHC 0 (negative), IHC 1+ (negative), IHC 2+ (equivocal) or IHC 3+ (positive) for HER2 overexpression. Samples scored as IHC 2+ are retested with FISH or other ISH methods, according to the testing algorithm (82).

This IHC scoring system is validated for breast cancer only and does not take account of histological differences between gastric and breast tissues.

Thus, gastric cancer presents unique characteristics regarding HER2 expression compared with breast cancers. The main difference is the pattern of expression of HER2 protein on the membrane of neoplastic cells: this pattern is predominantly

circumferential in breast cancer, creating a “chickenwire” staining pattern, whereas in gastric cancer it is incomplete and predominantly basolateral (creating an “U shaped” staining pattern) or lateral (lateral membranous staining with linear staining at contact sites between 2 cells creates a “parallel lines” pattern) (72). This is due to the fact that, unlike breast carcinomas, gastric carcinomas are gland-forming, mucin-producing carcinomas and mucin granules accumulate at the luminal pole of the cells where there are no HER2 receptors expressed. This demonstrates incomplete, basolateral or lateral staining patterns. For this reason, the completeness and circularity of membrane IHC staining, which are required for considering breast cancers as HER2 positive IHC 3+ (82), are not a criterion for HER2 IHC scoring in gastric cancer (72).

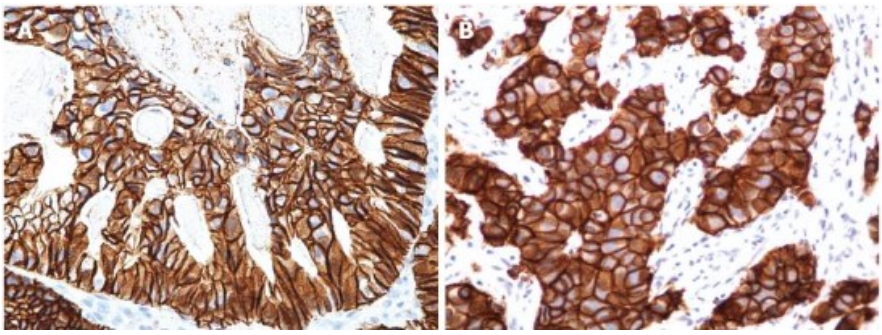

Figure 12. A: HER2-positive (3+) case of gastric adenocarcinoma; the cytoplasmic membranous immunostaining is incomplete and predominantly basolateral (× 400); B: HER2-positive (3+) case of invasive ductal carcinoma of the breast; the cytoplasmic membranous staining is fully circumferential (× 400).
Abrahao-M. et al. World Journal of Gastroenterology, 2016

The second difference regards intra-tumour heterogeneity, defined as the presence of areas with different HER2 ICH scores in the same tumor, which is frequent in gastric cancer, but rarely encountered among breast cancers. This latter characteristic not only made it necessary to create a HER2 IHC scoring system unique for gastric cancer, but may cause sampling errors when randomly samples biopsies are examined (72).

Finally, another difference between IHC staining in breast cancers and gastric cancers is the variation of incidence of HER2 expression with the anatomic location of the primary tumor, as HER2 overexpression rate is found to be higher in GEJ adenocarcinomas rather than in tumors located in more distal part of the stomach. By contrast, in breast cancer there is no difference in HER2 pattern of expression depending on the anatomical location of the tumor (73).

Given these differences, it was not possible just transferring breast cancer HER2 IHC scoring system to gastric and gastroesophageal cancers, therefore an appropriate and standardized diagnostic system needed to be performed.

Immunohistochemistry scoring criteria

The method of HER2 status evaluation which was used in the ToGA trial was essentially based on a separate validation study (the so-called pre-ToGA) which was conducted in 2007 (83). In this validation study, a panel of experts, among them also Manfred Hofmann, met in order to establish a HER2 assessment system specific for gastric cancer to identify suitable patients for enrolment in the ToGA trial. The panel concurred that HER2 testing protocol currently used in breast cancer with IHC as the first screening method for HER2 evaluation and ISH as a method to confirm HER2 positivity in equivocal cases (2+) could be applied also to gastric cancer. However, regarding the possibility of using breast cancer's HER2 IHC scoring system also for gastric cancer, some modifications were necessary to be done in order to take account of the incomplete reactivity of cell membranes and the intra-tumour heterogeneity observed in gastric cancer samples (79), (83). The modified ICH scoring system defined by Hofmann et al. in 2007, on which the ToGA trial was based, is still being used today and these gastric specific ICH scoring criteria are also known as the "Hofmann criteria". The 2016 ASCO/ASCP/CAP guidelines recommend the use of these criteria for the interpretation of HER2 IHC staining assessment (78).

The Hofmann criteria, besides being specific for gastric tumors, also distinguishes biopsies from surgical specimens. One of the most important modifications introduced by Hofmann is the abolition of the cut-off of at least 10% IHC stained tumor cells, as originally proposed for HER2 scoring in breast cancer, in gastric cancer biopsies. Thus, gastric cancer biopsies can be accepted as HER2 positive if they contain at least one cluster of ≥ 5 tumor cell with positive membrane reactivity. In resection specimens, the 10% cut-off continues to be used (83). Consequently, IHC scoring procedure is different for biopsies and resection specimen in gastric cancer.

Another difference is that, unlike for breast cancer, circularity of IHC staining is no longer a criterion for HER2 IHC scoring in gastric cancer: a strong membranous reactivity, although basolateral or lateral, is still considered positive (3+) if such reactivity is assessed in at least one cluster of ≥5 tumour cells for biopsies and in ≥10% of tumour cells for surgical specimens (83). By contrast, in breast cancer circularity and completeness of staining is a must for the tumor to be considered positive with an IHC score of 3+ (84).

In conclusion, the Hoffmann HER2 ICH scoring criteria define what we can see in *Figure 13*. In surgical specimens, a score of 0 is determined if there was no reactivity or membranous reactivity in <10% of tumor cells. A score of 1+ is determined if faint or barely perceptible membranous reactivity occurs in ≥10% of tumor cells, and the cells are reactive only in part of the membrane. A score of 2+ is determined if weak to moderate complete or basolateral membranous reactivity occurs in ≥10% of tumor cells. A score of 3+ is determined if moderate to strong, complete or basolateral membranous reactivity occurs in ≥ 10% of tumor cells. In biopsy specimens, when membrane reactivity is observed in at least one cancer cell cluster (≥5 cells), scores of 1+, 2+, and 3+ are determined according to the intensity of membrane reactivity (faint, moderate or intense), regardless of the percentage of immunoreactive area and the completeness of reactivity (78).

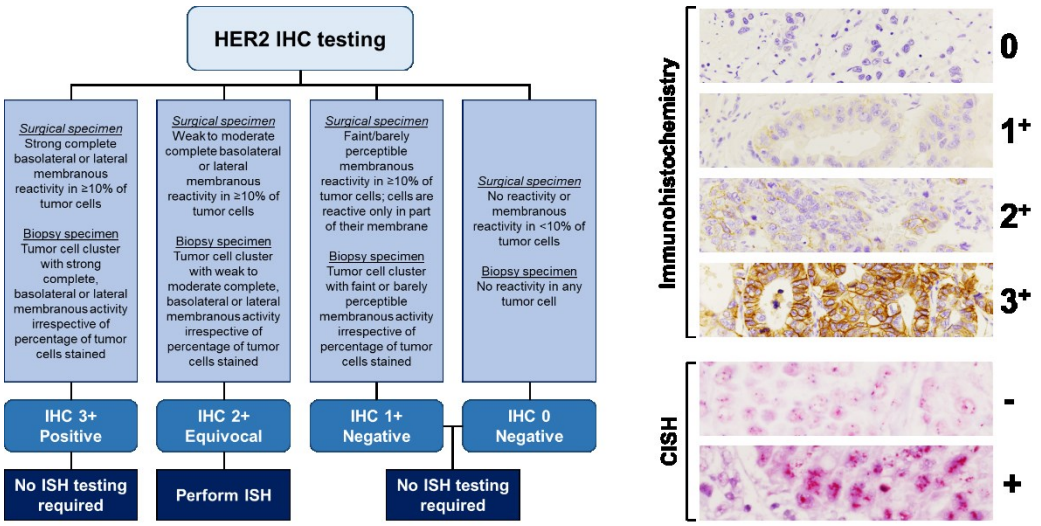

Figure 13. HER2 testing in gastroesophageal adenocarcinomas. (A) Diagnostic algorithm proposed by ASCO/ASCP/CAP guidelines: the ASCO/ASCP/CAP guidelines recommend the use of the modified Hofmann criteria in the evaluation of HER2 IHC staining. (B) Representative immunohistochemical examples of a negative (0) case showing no reactivity in any of the tumor cells, a negative (1+) case with faint/barely perceptible membranous staining, an equivocal 2+ immunoreaction and a strongly and diffuse 3+ positive case. CISH examples of a HER2 non-amplified and an amplified case are also shown.
Source: Fassan M, et al. – Pathologica 2020

In situ hybridization

Following the HER2 testing algorithm proposed by the ASCO/ASCP/CAP guidelines (78), those samples classified as IHC 2+ should be retested by in ISH methods in order to assess HER2 gene amplification. Positive (3+) or negative (0 or 1+) IHC results do not require further ISH testing (84).

ISH assays can use a single-probe method in order to determine the absolute HER2 gene copy number present in the sample. However, most assays use a dual-probe method. In the dual-probe method the first probe targets the HER2 locus in order to identify the number of gene copies present, whereas the second probe acts as a control probe which targets the centrometric portion of chromosome 17 (CEP17). ISH results are expressed as the ratio between the number of copies of HER2 gene and the number of copies of chromosome 17 within the nucleus counted in at least 20 cancer cells. According to the ASCO/ASCP/CAP guidelines the definition of ISH positivity in gastric cancers is a HER2:CEP17 ratio of ≥ 2 (78) . Applying Hofmann criteria for HER2 IHC scoring an excellent correlation between HER2 overexpression assessed by IHC and HER2 amplification assessed by ISH methods has been observed (79).

In addition to FISH, also other techniques can be used, such as chromogenic in situ hybridization (CISH), silver-enhanced in situ hybridization (SISH). Unlike FISH technique which requires the use of a fluorescent microscope, these methods allow to use a conventional bright field ordinary microscope and have shown excellent correlation with results obtained by FISH (79).

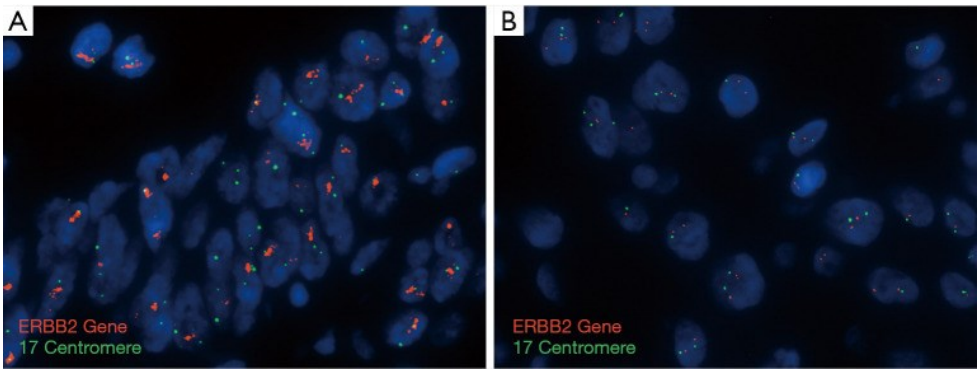

Figure 14. Human epidermal growth factor receptor 2 (HER2) expression by fluorescence in situ hybridization (FISH) in gastroesophageal junctional adenocarcinoma. The HER2 (ERBB2) probe is shown in red, while the chromosome 17 enumeration probe (CEP17) is noted in green. (A) A tumor with HER2 amplification as demonstrated by a HER2(ERBB2): CEP17 ratio of ≥2. (B) HER2 non-amplified tumor exhibiting a HER2(ERBB2): CEP17 ratio of ≤2. Images are acquired at 400× magnification. Source: Dhakras P. et al., Translational Gastroenterlogy and Hepatology, 2020.

1.6.6 HER2 AS A THERAPEUTIC TARGET – THE ToGA TRIAL

HER2 overexpression represents currently the most useful therapeutic biomarker for patients with gastroesophageal cancers. The recognition of this important biomarker has enabled the introduction in clinical practice of Trastuzumab, a fully humanized monoclonal antibody which targets specifically the extracellular domain of HER2 protein. Trastuzumab inhibits HER2-mediated signaling through several antitumor mechanisms: prevention of heterodimerization of HER2 receptor on cancer cells' surface, blockade of downstream signals depending on PI3KCA and AKT kinases, internalization and ubiquitin-dependent degradation of HER2 protein, down-modulation of HER2 expression, activation of apoptotic signals of tumor cells, activation of antibody-dependent cell-mediated cytotoxicity (67) (70).

Trastuzumab was approved as far back as 1998 for the treatment of HER2-positive metastatic breast cancer. Trastuzumab in breast cancers is currently used also in neoadjuvant and adjuvant settings, expanding the possibility of exploiting all the potentialities of this drug.

The clinical benefit of Trastuzumab in advanced gastroesophageal cancers was first demonstrated in the ToGA (Trastuzumab for Gastric Cancer) trial (69). The ToGA trial was an open-label, international, phase 3, randomized controlled trial undertaken in 2010 in 122 centers of 24 countries in Asia, Central and South America and Europe, testing 594 patients. In this trial patients with HER2-positive unresectable locally advanced, recurrent or metastatic gastric and GEJ adenocarcinoma who have not received other prior therapies were randomly assigned in a 1:1 ratio to receive chemotherapy in combination with Trastuzumab or chemotherapy alone (which regimen consists of capaecitabine plus cisplatin or fluorouracil plus cisplatin). Tumours were tested for HER2 status with immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH). HER2 positivity required to assess the eligibility of patients in the study was identified as IHC staining 3+ (defined as strong complete or basolateral membrane reactivity) and/or FISH positive (defined as HER2/centromeric probe for chromosome 17 ratio ≥ 2).

The primary endpoint of the study was the overall survival in all randomized patients, while other secondary endpoints were progression-free survival, time to progression, overall tumor response rate, duration of response and safety.

The results of this trial have completely changed the therapeutic approach to these tumors, as it showed clearly that the addition of trastuzumab to chemotherapy can significantly improve the overall survival of patients compared to chemotherapy alone. The subgroup of patients who were assigned to chemotherapy plus trastuzumab had a median overall survival of 13,8 months compared with the median overall survival of 11,1 months of those assigned to chemotherapy alone (*Table 2*).

On the basis of these findings, in January 2010 the European Medicine Agency (EMA) approved trastuzumab in combination with cisplatin plus capaecitabine or cisplatin plus fluorouracil for the first-line treatment of patients with HER2-positive advanced gastric and GEJ adenocarcinoma. The EMA limited the approval to patients whose tumours have HER2 overexpression as defined by IHC score 2+, confirmed by a positive FISH, or by IHC score 3+ (73). Approvals are later granted also in the United States (October 2010) and Japan (2011).

Currently there is no evidence to support trastuzumab beyond progression after first-line chemotherapy or before surgery in patients with resectable gastric cancer (32).

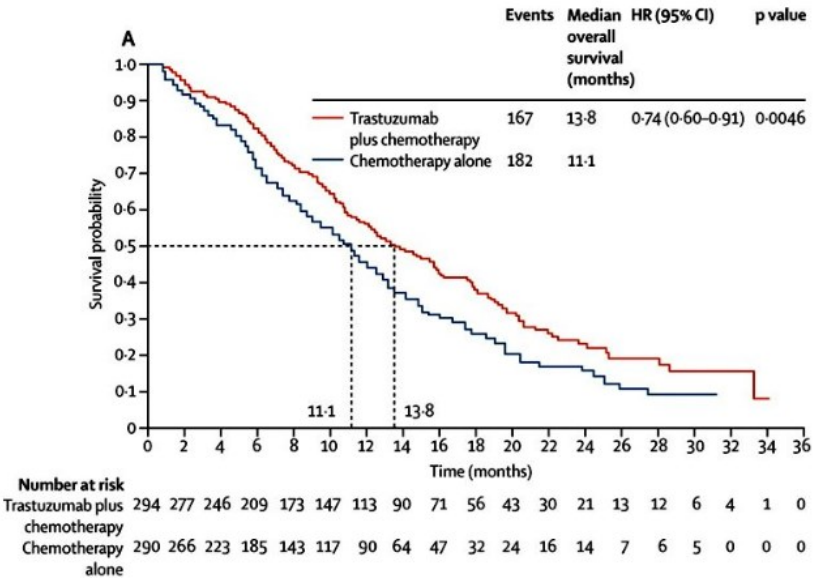

Table II. Overall Survival of patients enrolled in the ToGA trial and defined as HER2-posotive (FISH+ or IHC3+). Source: Bang, Y. Lancet, 2010 ToGA

Interestingly, a pre-planned analysis according to HER2 expression level showed that patients with high HER2 expression (defined as IHC 3+ and FISH+) gained the greatest benefit from Trastuzumab plus chemotherapy with a median overall survival of 17,9 months (versus 12,3 months of patients with the same expression level but assigned to chemotherapy alone). By contrast, patients with low HER2 expression (defined IHC 2+ with FISH +) showed a median overall survival of 12,3 months (versus 10,8 months of patients with the same expression level but assigned to chemotherapy alone). Patients with lower HER2 expression (IHC 1+ and FISH +) had an even lower benefit from trastuzumab, with a median overall survival of 8,7 months.

To further explore this finding, a post-hoc exploratory subgroup analysis was conducted. In this context all patients were divided into two large groups, one with high HER2 expression (defined as IHC 3+ or IHC 2+ and FISH+) and one another with low HER2 expression (IHC 0 and FISH+ or IHC 1+ and FISH +). The post-hoc analysis confirmed the previous results, as patients with high HER2 expression and treated with Trastuzumb plus chemotherapy had an overall survival of 16 months (versus 11,8 months of patients with the same expression level but assigned to chemotherapy alone), while patients with low HER2 expression and treated with Trastuzumab plus chemotherapy had an overall survival of 10 months (versus 8,7 of patients with the same expression level but assigned to chemotherapy alone). These results highlighted that there is a correlation between HER2 expression levels and the response to Trastuzumab therapy (69) (73). (*Table 3*)

At present it is recommended that HER2 status should be evaluated in all gastric and gastroesophageal junction adenocarcinomas at time of diagnosis, in order to select the patients that might benefit from treatment with trastuzumab.

Although no esophageal adenocarcinoma has been evaluated in the ToGA trial, these results can be applied also to advanced HER2-positive EAC due to the fact that, as demonstrated by the already oft-mentioned 2017 TCGA study (21), there is no difference between esophageal and gastric adenocarcinoma on the molecular standpoint.

		n	Median OS		HR	95% CI
			Trastuzumab + chemotherapy	Chemotherapy only		
Overall population						
FISH+ or IHC 3+		584	13.8	11.1	0.74	0.6–0.91
Pre-planned exploratory analysis						
FISH+	IHC 0	61	10.6	7.2	0.92	0.48–1.76
	IHC 1+	70	8.7	10.2	1.24	0.7–2.2
	IHC 2+	159	12.3	10.8	0.75	0.51–1.11
	IHC 3+	256	17.9	12.3	0.58	0.41–0.81
FISH–	IHC 3+	15	17.5	17.7	0.83	0.2–3.38
Post-hoc exploratory analysis						
FISH+	IHC 0, 1+	131	10.0	8.7	1.07	0.7–1.62
FISH+ or IHC 3+	IHC 2+	446	16.0	11.8	0.65	0.51–0.83

Abbreviations: OS, overall survival; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; HR, hazard ratio; CI, confidence intervals.

Table III. Results from pre-planned and post-hoc exploratory analysis. Source: Gastrointestinal Cancer Targets and Therapy · July 2011

1.6.7 HER2 AS A PROGNOSTIC FACTOR

With the publication of the ToGA trial's results, the predictive role of HER2 in gastric and gastroesophageal junction cancers has been positively proven. Regarding the prognostic value of this biomarker in gastric cancer the issue is still controversial, as many studies have suggested that HER2-positivity is associated with poor outcomes, more aggressive disease and higher frequencies of recurrence, whereas others do not lead to the same conclusion (73). This inconsistency is not present in breast cancer, where HER2 overexpression is recognized to be a marker of poor prognosis with HER2-positive cancers having a more aggressive behavior compared to HER2-negative breast cancers (85).

1.6.8 HER2 TARGETING BEYOND THE ToGA TRIAL

After the initial success seen with ToGA trial, other HER2-targeted therapeutic strategies have been tested, such as the dual inhibition with trastuzumab and pertuzumab, the antibody-drug conjugate (ADC) trastuzumab emtansine (T-DM1) and the tyrosine-kinase inhibitor lapatinib.

Pertuzumab is a humanized monoclonal antibody that binds to the dimerization domain of HER2 and inhibits HER2 heterodimerization with other receptors of the same family, switching off the downstream signaling cascade. Pertuzumab was approved in HER2-positive metastatic breast cancers for the first-line combination therapy with trastuzumab and docetaxel thanks to the phase III CLEOPATRA study

(86). The phase III JACOB trial evaluated the effect of adding pertuzumab to trastuzumab plus chemotherapy in first-line setting for HER2-positive advanced gastric and GEJ cancer patients. The addition of pertuzumab failed to achieve the primary endpoint of overall survival as no statistically significant difference was found in the overall survival between the two groups of patients (87).

Trastuzumab emtansine (T-DM1) is an antibody-drug conjugate composed of trastuzumab and the cytotoxic agent DM1, which is a tubulin polymerization inhibitor. T-DM1 has been approved in second-line setting for patients with previously treated HER2-positive advanced or metastatic breast cancers thanks to the results of the phase III EMILIA and TH3RESA trials (88) (89). On the basis of what has been seen in breast cancer, the GATSBY trial compared the effect of T-DM1 to that of taxane chemotherapy in second-line setting for HER2-positive advanced gastric and GEJ cancers progressed during or after first-line trastuzumab-containing therapy. Also this study did not reach the primary endpoint of overall survival (90). In this study, however, there was a basic problem, because it did not include a re-evaluation of HER2 status prior to trial entry in order to assess whether HER2 overexpression was still present. One of the main problems of HER2-targeting is the temporal heterogeneity in HER2 expression which can lead to the loss of HER2 expression. This loss of expression may represent one of the reasons of the disease's progression after first-line therapy with trastuzumab, but it can also lead to the lack of response to second-line therapy with T-DM1, interfering with the study's results.

Lapatinib is a dual small molecule tyrosine kinase inhibitor targeting both HER2 and EGFR receptors. It has been approved for use in combination with capecitabine in second-line setting for HER2-positive advanced breast cancer after progression on prior trastuzumab-containing therapy (91). Given the effect seen in breast cancers, lapatinib has been investigated in first (phase III TRIO-013/LOGiC trial (92)) and second-line (phase III TyTAN trial (93)) settings in advanced gastric gastroesophageal cancer patients with *HER2* amplification by FISH. In both studies, the primary endpoint, which was the overall survival, was not statistically improved with the addition of lapatinib to chemotherapy.

In conclusion, after the first ToGA trial's promising success, the following studies on HER2-targeting in gastroesophageal adenocarcinomas, which were conducted in the same vein of those for breast cancer, did not lead to results that pathologists had expected. Why these HER2-targeted agents, which have been demonstrated to be efficacy on HER2-positive breast cancer, did not provide the same level of benefit for patients with gastroesophageal adenocarcinoma, is not entirely understood. Although much has yet to be studied, intra-tumour heterogeneity of HER2 expression seems to be one of the most important contributing factors.

1.6.9 INTRA-TUMOR HETEROGENEITY: THE ACHILLES'S HEEL OF HER2-TARGETING

HER2 inter- and intra-tumor heterogeneity

Gastric and gastroesophageal cancers are notoriously heterogeneous diseases. The morphological and molecular heterogeneity of gastric cancers encompasses not only inter-tumor heterogeneity, but also intra-tumor heterogeneity. While inter-tumor heterogeneity is found among patients with different histotypes or molecular subtypes of the same tumor, intra-tumor heterogeneity is found within a single tumor (94).

The problem of tumor heterogeneity affects also HER2 expression. We have seen that HER2 status varies depending on tumor locations (proximal gastric cancer and GEJ adenocarcinoma are more frequently positive), histological subtypes (the HER2-positive rate is higher in intestinal compared to diffuse gastric cancer), and molecular classification (CIN tumors are characterized by the highest incidence in HER2 amplification and expression). All these associations are examples of inter-tumor heterogeneity applied on HER2-expression (94).

Intra-tumor heterogeneity of HER2 expression is even a much important issue. The concept of intra-tumor heterogeneity comprises both spatial and temporal heterogeneity. Spatial heterogeneity refers to the heterogeneity occurring among different geographical regions of the same tumor. This type of heterogeneity is due to the presence of different subpopulations of neoplastic cells inside the same tumor, resulting in the presence of a combination of HER2-positive and HER2-negative regions or regions with different IHC staining scores (94). Temporal

heterogeneity refers to the variations affecting HER2 status and occurring over time during tumor progression (71). Temporal heterogeneity results in different levels of expression between primary tumor and metastasis and/or recurrent disease or among different metastatic lesions (intra-metastatic heterogeneity) (71) (*Figure 15*). The tumor is a dynamic entity that can change during progression. The tumor evolution is a result of additional genetic alterations acquired during cancer progression but also as a consequence of treatments which can select resistant clones resulting in temporal intra-tumor or intra-metastases heterogeneity (95).

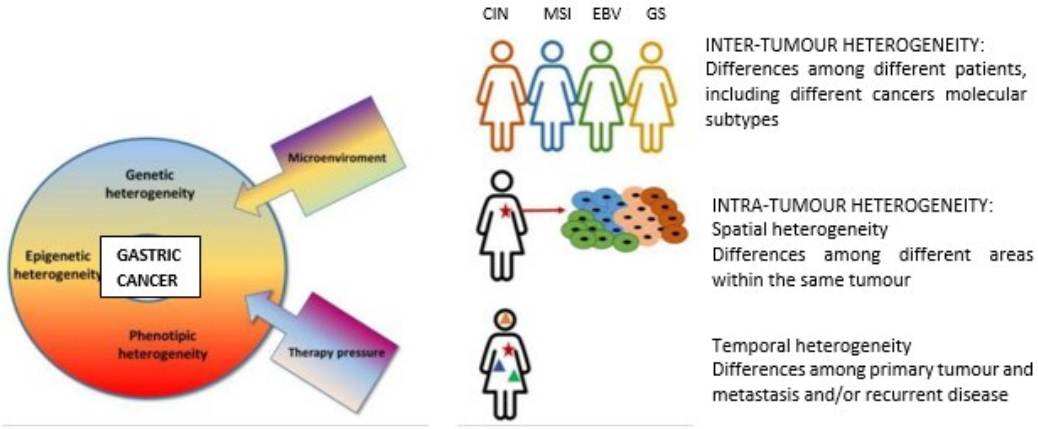

*Figure 15. Gastric cancer heterogeneity. (**A**) Factors that contribute to gastric tumor heterogeneity; (**B**) Type of gastric cancer heterogeneity: Inter-tumor and intra-tumor heterogeneity. Star: primary tumor, Triangles: relapses. Source: Fumagalli C. et al., Diagnostics, 2021*

The problem of HER2 expression intra-tumour heterogeneity is complex because the frequency of spatial heterogeneity of HER2 expression assessed by IHC within primary tumors varies widely across studies, ranging from 5 to 79% of HER2-positive gastric and gastroesophageal junction cancers (94) (68) (96) (71). These discrepancies can be explained by the lack of a universally accepted definition of heterogeneity. For example, *Van Cutsem et al.* identified the cut-off value of 30% between HER2 homogeneity and heterogeneity: those samples with ≤30 % of stained tumor cells were considered heterogeneous for HER2-expression, whereas those samples with >30 % of tumor cells stained were considered homogenous (97). In the study performed by *Motoshima et al.* HER2 heterogeneity was defined as the presence of ≥ 10% but ≤ 90% of tumor cells showing HER2 overexpression in samples with an IHC score of 3+ and or an IHC

score of 2+ with ISH positive status. HER2 homogeneity was defined as >90% of tumor cells showing HER2 overexpression in samples with an IHC score of 3+ (98).

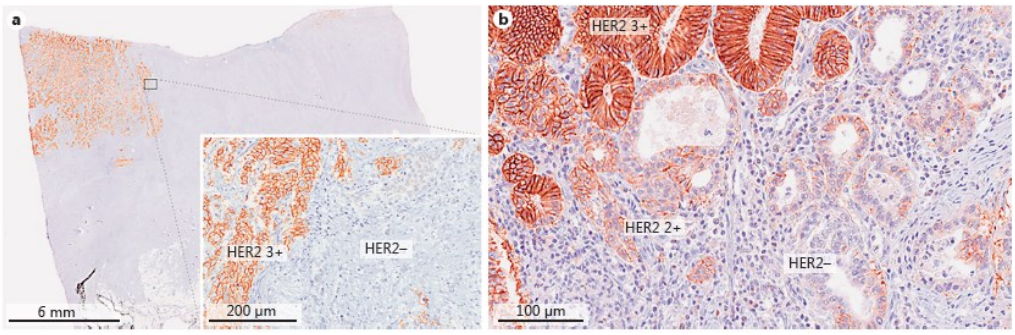

Figure 16. Spatial intra-tumor heterogeneity of HER2 expression in gastric cancer. Source: Gullo I. et al., Pathobiology, 2018

Consequences of HER2 intra-tumor heterogeneity: diagnosis

Intra-tumor heterogeneity of HER2-protein expression has important implications from two main points of view, diagnosis and therapy.

From a diagnostic point of view, because HER2 intra-tumor heterogeneity along with incomplete membrane staining are much more frequent, as explained before, in gastric cancer in comparison to breast cancer, breast cancer's HER2 testing and scoring systems cannot be applied for gastric cancer and new scoring systems specific for gastric cancer needed to be defined (71).

Furthermore, another interesting diagnostic problem is that of sampling errors, as HER2 expression evaluated in a portion of the tumor might not be representative of the HER2 status in the whole tumor (94). This problem becomes more relevant when HER2 status assessment is performed on endoscopic biopsy specimens (71). The HER2 status in advanced gastric carcinoma is usually assessed on the biopsy samples from the primary tumor because patients with metastatic disease rarely undergo surgery or biopsy from the metastatic disease. There, an important question is whether endoscopic biopsy samples are sufficient to obtain a HER2 assessment which could be representative of the entire tumour (71). This question can be answered by considering two issues: the concordance of HER2 status between biopsy and surgical samples and the concordance between primary tumor and metastatic lesions. These issues can potentially give rise to discordant results between different samples obtained from the same patient, leading to false-negative interpretation and potential undertreatment.

Intra-tumour heterogeneity of HER2 expression in gastric cancer represents the major explanation for the discordance between the results of HER2 assessment in biopsy and surgical resection specimens. Most of studies conducted on matched biopsy and surgical resection specimens have shown a variable concordance in HER2 status between the biopsy specimen and the resection specimens, ranging from 45,5 to 94% (99). Some of these studies showed that discordant cases were more frequently HER2 negative at biopsy but HER2 positive on the correspondent surgical specimen, whereas others studies showed an opposite tendency. The most probable explanation for false negative HER2 status on biopsy is intra-tumour heterogeneity, whereas HER2 positivity on biopsy and not on surgical resections may be due to prolonged cold ischemia and/or over or under-fixation in larger specimens (71). The HER2-scoring system specific for gastric and gastroesophageal cancer, in part, takes into account this issue when considers HER2 evaluation in biopsy specimens, thus considering a cluster of at least 5 neoplastic stained cells to define the biopsy as positive (71). However, the possibility of finding this discordance highlights the necessity of obtaining multiple biopsy specimens. Although defining the optimal number of biopsy specimens which endoscopist must submit for evaluation is fundamental in order to predict HER2 status in gastric cancer, conflicting reports have suggested a different number of tissue fragments for adequate assessment in biopsies. The ASCO/ASCP/CAP guidelines recommend a minimum of 5 biopsy specimens, optimally 6 to 8 (78), whereas National Comprehensive Cancer Network guidelines recommend more than 6 samples to be taken (80).

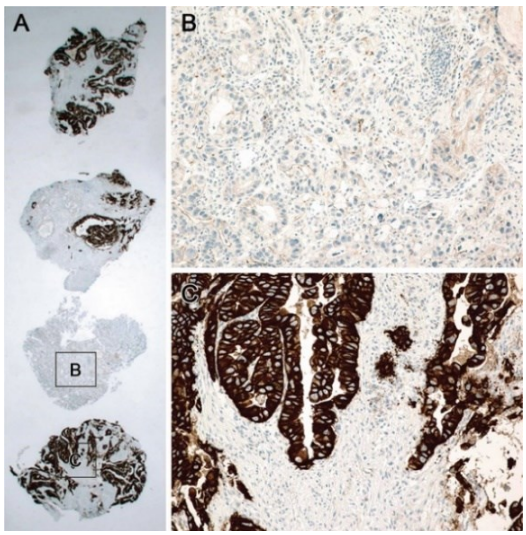

Figure 17. Heterogeneity of HER2 IHC staining in a biopsy specimen. ***A.*** *Four endoscopic biopsy fragments with tumor cells showing heterogeneous expression. One fragment (star) shows no staining in tumor cells* ***B.*** *while other three fragments stained strongly* ***C.*** *Source: Ahn S. et al., Onocotarget, 2015*

Another diagnostic issue regarding HER2 intra-tumour heterogeneity is represented by the discordance of HER2 positivity between primary and metastatic lesions. This discordance has been observed in 1-14% of gastric and GEJ cancers (68,71). Both positive (negative in primary tumour and positive in metastasis) and negative (positive in primary tumour and negative in metastasis) conversion are possible and are likely associated with intra-tumour heterogeneity (71).

HER2 status is more frequently assessed in primary gastric tumors and that is the result used to guide therapy for recurrent or metastatic disease; however, discordance in HER2 positivity between primary and metastatic tumors developing either synchronously or metachronously could be misleading. In fact, positive conversion of HER2 expression may lead to the exclusion from the targeted treatment of a percentage of patients with a HER2-negative primary tumour but developing HER2-positive metastatic disease. Regarding this conversion, data from the GASTHER1 study have demonstrated that patients with HER2 positivity assessed in recurrent and/or metastatic sites of an initially HER2-negative primary tumour gain a similar benefit from trastuzumab-containing first line therapy than those patients with HER2-positive disease on initial assessment (100). The main implication of this study is that the repetition of HER2 assessment

in recurrent and/or metastatic sites is recommended in those patients with advanced gastric and GEJ cancer whose initial evaluation was HER2 negative (100).

Consequences of HER2 intra-tumor heterogeneity: therapy

From a therapeutic perspective, the heterogeneous HER2 expression patterns have implications on HER2 targeted treatments' efficacy, representing one of the major mechanisms that can attenuate the response to anti-HER2 treatment and also one of the major obstacles that may have impeded the development of further HER2-targeted therapies as well as for breast cancers (68).

HER2-targeted therapies might eradicate HER2-expressing neoplastic cells, but are not effective on HER2-negative clones, which can emerge and drive tumor progression resulting on one hand to the inefficacy of these therapies and on the other hand to the recurrence of the tumor. It was reported by several studies that the heterogeneous expression of HER2 within the primary tumor is associated with lower survival benefits on first-line trastuzumab-containing regimens compared to patients with homogenous expression (68) (101) (102). These studies showed that HER2 heterogeneity is a negative predictor for HER2-targeted therapies efficacy. However, beyond intra-tumour heterogeneity, other mechanisms of resistance can potentially reduce the inhibitory effect of trastuzumab-containing first line treatment, such as such as phosphatase and tensin homolog (PTEN) deficiency, PI3K mutations, hyperactivation of the hepatocyte growth factor (HGF)/mesenchymal epithelial transition factor (MET) pathway, co-existing EGFR overexpression, and MET/KRAS amplifications (103).

The selective eradication of HER2-overexpressing neoplastic cells clones by trastuzumab-based first line therapy might also lead to the loss of HER2 positivity as only HER2-negative cellular clones remain in the tumour. Several studies have reported that the loss of HER2 positivity occurs in 24–35% of patients with HER2-positive advanced or metastatic gastric cancer, after trastuzumab-based first line therapy (104). The precise biological change that underlies the loss of HER2 positivity after trastuzumab-based chemotherapy remains unclear, but intra-tumour heterogeneity and treatment-induced clonal selection are likely the two main mechanism implicated in this phenomenon. Whatever its cause, the loss of HER2 positivity might be a mechanism promoting resistance to second line HER2-

targeted therapy. The GASTHER3 study tried to assess the relationship between post-progression HER2-positivity loss and response to second-line HER2-targeted therapy based on T-DM1. In reality, this study found only a modest difference in the overall survival and progression free survival between patients who have conserved HER2 positivity and patients who have lost HER2 positivity (104). Another study that had the same aim did not observe a difference in relapse-free survival between the maintained HER2 expression group and the loss of HER2 expression group (105). These results contrasted with those of the studies conducted on breast cancers, in which patients showing loss of HER2 expression after trastuzumab-based chemotherapy have a lower relapse-free survival rate than those who maintained HER2 expression. Additional research is needed to clarify the biological mechanism and clinical significance of HER2 loss in patients with advanced HER2-positive gastric cancer. The only conclusion we have reached at the moment is that the re-examination of HER2 status before initiating second-line anti-HER2 treatment may be reasonable (104).

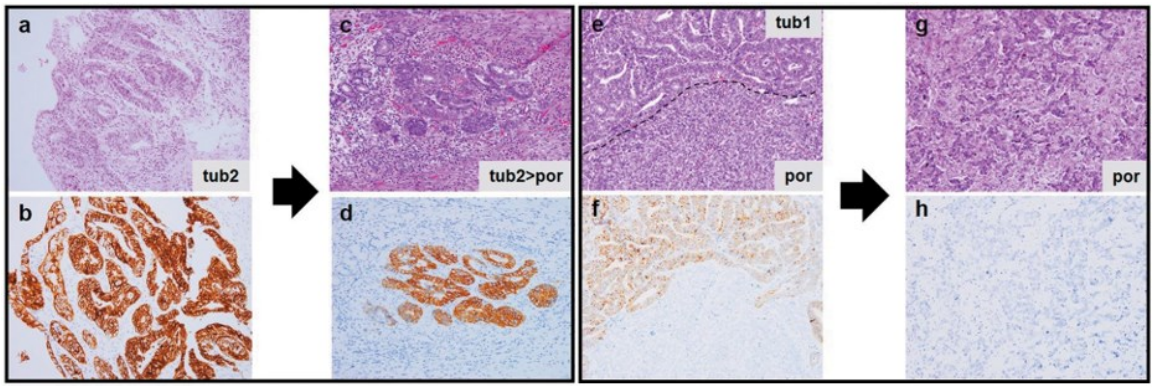

Figure 18. Representative staining for HER2 status. (a, b) Biopsied specimens before trastzumab-based therapy and (c, d) resected specimens after trastuzumab-based chemotherapy in the maintained HER2 expression group (Case 1). (e, f) Resected gastric specimen before trastuzumab-based chemotherapy and (g, h) resected liver metastatic specimens after chemotherapy in the loss of HER2 expression group (Case 2). It is interesting to note that in the maintained HER2 expression group there is no change in the predominant histological subtype, as the tumor which was predominantly of well-differentiated tubular histology in the pre-treatment specimen (a) conserved its predominant histology in the post-treatment specimen with the difference of having a poorly differentiated component (c). Comparing the EE stained specimen (a, c) with immunochemistry (b, d), we note that those cells which maintained HER2-overexpression maintained also the organization in tubular structures, while the other cells lost tubular organization. In the loss of HER2 expression group, the pre-treatment specimen stained with EE (e) shows a mixed phenotype, whit a tubular component visible upon the dotted line, and a poorly cohesive component visible below the dotted line; whereas the post-treatment specimen (g) showed a unique poorly differentiated phenotype. We note that in the pre-treatment specimen the well differentiated tubular component corresponds to those cells which are positive for HER2 overexpression while the poorly differentiated component corresponds to those cells which are negative for HER2 overexpression (f). In the post-treatment specimen, HER2 overexpressing cells lose both HER2 positivity and tubular histology (h). This is a visible example of the association between histology/morphology and genetics/molecular pattern with HER2 expression being more frequent in Lauren's intestinal-type cancers, which corresponds to tubular or papillary histotype according to the WHO classification, than in diffuse type, which are poorly differentiated. Source: Kijima et al., Anticancer Research, 2020

1.6.10 HER2-TARGETING AND IMMUNOTHERAPY

PD-L1, MMR/MSI and EBV as predictive biomarkers for immunotherapy

The immune system can selectively identify and kill pathogens and tumour cells by coordinating responses by its innate and adaptive components. T-lymphocytes are the main actors of this anti-tumour action. In this complex system, there are numerous checkpoints that control immune response so that this response does not mistakenly destroy healthy cells together with neoplastic cells. Cancer cells frequently develop an immune evasion system by upregulating these immune checkpoint proteins (55). Among these checkpoints, there is programmed death ligand 1 (PD-L1) which is one of the ligands of the programmed cell death 1 receptor (PD1). The interaction between PD-L1 on cancer cells and PD-1 receptor on immune cells can acts by blocking immune response activities such as T cell activation and T cell proliferation, thus attenuating the host immune response to tumor cells. The blockade of this combination can awaken the immune system and, through the induction of T-cell proliferation and cytotoxic response, leads to an objective tumour response (55). PD-L1 is overexpressed in various subtypes of tumour, including about 40% of gastroesophageal cancers (55), manly those tumours associated with EBV infection and dMMR/MSI tumours (54). These findings have provided a rationale for immunotherapy with anti-PD-1/PD-L1 drugs in many recent clinical trials in advanced gastroesophageal cancers.

Immunohistochemistry (ICH) represents the gold standard for PD-L1 expression evaluation. In gastroesophageal settings, PD-L1 staining regards not only tumor cells, but also immune cells within the stroma of the tumour, specifically lymphocytes and macrophages. As such, in gastroesophageal cancers, results of IHC PD-L1 testing are reported through a combined positive score (CPS) in order to take into account all the cell types that are stained when the sample is place in contact with detecting antibodies. CPS is obtained from the number of PD-L1 stained cells, including tumor cells, lymphocytes and macrophages, dived by the total number of viable tumor cells, multiplied by 100. CPS is reported as a single number with a maximum score of 100. CPS is considered positive when the final score is ≥ 1, while CPS is considered negative when the score is ≤ 1. CPS should be

evaluated in a tissue sample with at least 100 viable tumour cells, in order to obtain a representative result (55).

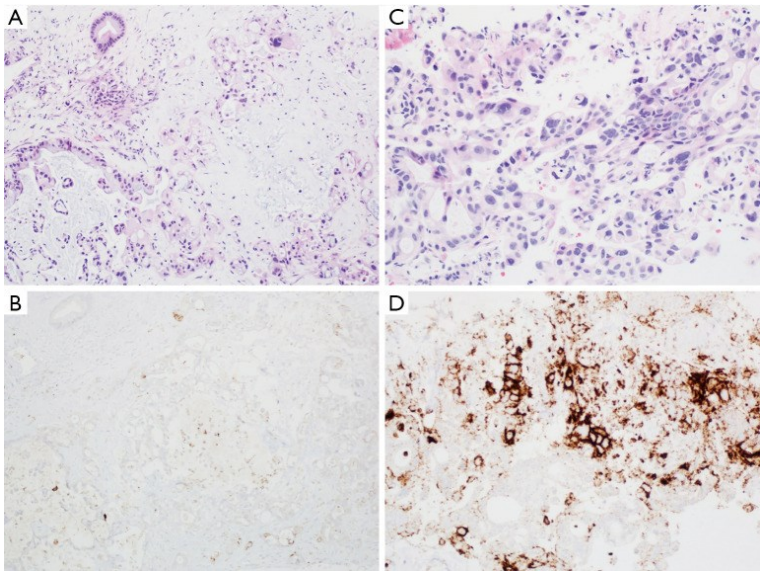

Figure 19. PD-L1 (clone 22C3) expression in esophageal adenocarcinoma by immunohistochemistry. (A) Hematoxylin and eosin stained tumor section confirming presence of at least 100 tumor nuclei. (B) Rare, PD-L1 positive, membranous expression is noted and thus has a CPS ≤1 (negative) (C) Hematoxylin and eosin stained section from a different tumor, again confirming at least 100 tumor nuclei. (D) Strong PD-L1 positive, membranous expression is noted within the tumor cells and in lymphocytes and thus has a CPS of 20–25 (positive). Source: Dakhras P. et al., Translational gastroenterology and hepatology, 2020

In the phase II trial KEYNOTE-059 patients with PD-L1 positive (defined as a tumour with CPS ≥ 1 using the Dako 22C3 pharmDx assay) gastric and gastroesophageal cancers were enrolled and treated with monotherapy pembrolizumab as third or later line of therapy. These patients were found to have an advantage in terms of overall response rate and overall survival rate compared to those who had PD-L1 negative cancers (106). On the basis of these results, this antibody has been approved by FDA for patients with advanced esophageal, gastric and gastroesophageal junction PD-L1 positive (defined as well as in the study as CPS ≥1) adenocarcinoma who have previously received two prior lines of therapy (55,68). In the Asian phase III trial (ATTRACTION-2), another anti-PD1 monoclonal antibody, Nivolumab, significantly increased the overall survival compared to placebo in third line or later treatment of patients with advanced gastroesophageal cancers, leading to approval of the use of nivolumab in Japan, South Korea, and Taiwan (107).

Pembrolizumab in monotherapy or in combination with chemotherapy was tested also for second (KEYNOTE-061) and first line (KEYNOTE-062) of therapy and

compared to chemotherapy or chemotherapy alone, but these studies failed to meet their endpoints, thus limiting the approval to third or later line of therapy.

Despite these failures, post-hoc analysis of both studies revealed that the treatment effect was greater in patients with a PD-L1 CPS ≥10 than CPS ≥1. The cutoff value for our decision is of great importance to clinical practice, but the question remains unanswered (108).

Along with PD-L1 expression, other biomarkers, including deficiency mismatch repair system (dMMR)/microsatellite instability (MSI) and Epstein–Barr virus (EBV) infection status, have been proposed to identify susceptibility to anti-PD-L1/PD1 immune checkpoint inhibitors (109). Both dMMR/MSI and EBV-associated gastroesophageal cancers represent one of the four molecular subtypes of tumours according to the TCGA classification, and are characterized by a prominent immune infiltrate, a high tumour mutational burden and a widespread overexpression of PD-L1 (3). In particular, PD-L1 expression was observed in approximatively 50% and 94% of tumor cells and immune cells in the EBV subtype and in approximatively 33% and 45% of tumor cells and immune cells in MSI-H tumors (110).

dMMR/MSI has been well described in several types of human cancers, most frequently in colorectal (17%), endometrial (20%), and gastroesophageal (11-13%) adenocarcinomas (54). The evaluation of dMMR/MSI status can be performed with two different methods. The first one is MSI testing by polymerase chain reaction (PCR) which is used to detect instability in microsatellite repeats; the second one is IHC which is used to detect the presence or absence of nuclear expression of one or more of the MMR proteins (55). Due to the high concordance rate among IHC and PCR (55), IHC analysis is usually preferred over microsatellite instability testing, as it is a lower time-consuming method and allows to obtain a direct and rapid response (52). In ICH assay, MMR protein expression is interpreted as retaines, when a moderate to strong expression (similar to that observed in the stromal cells as internal control) is present in ≥ 10% tumour cells; loss, in case of complete loss of nuclear expression in cancer cells; indeterminate, when ICH staining intensity in tumour cells is lower than the internal control or

the tumour is positive in < 10%. Indeterminate ICH results should be subjected to MSI testing (54).

Depending on the level of microsatellite instability determined by PCR, MSI tumours can be categorized as high or low (MSI-H and MSI-L, respectively). Conversely, tumors without instability at any microsatellite loci are categorized as microsaellite stable tumors (MSS) (56). Retrospective analyses and large clinical trials suggest that dMMR or MSI gastric cancers have a favourable prognosis compared with mismatch repair proficient (pMMR) or MSS gastric cancer (32).

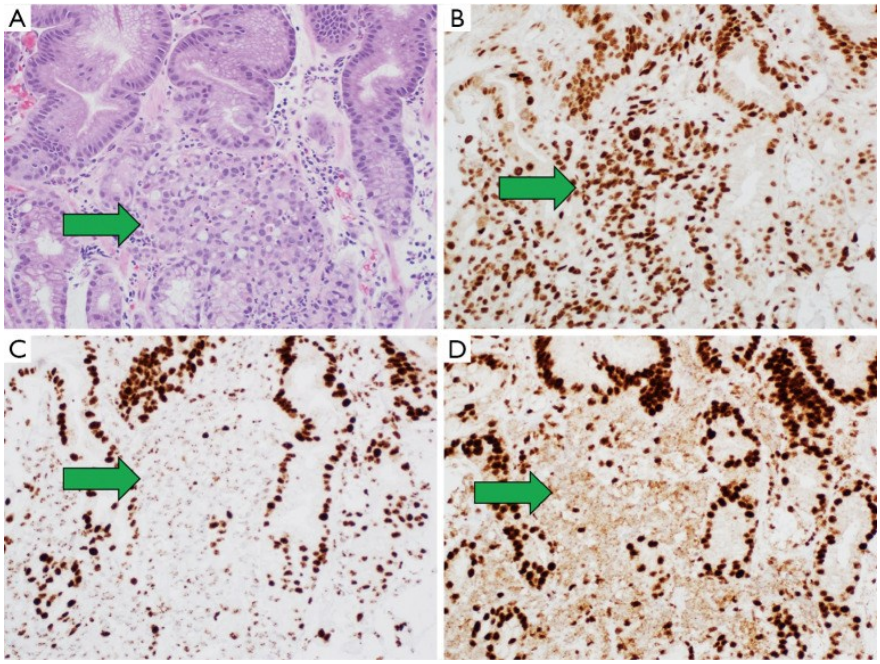

Figure 20. Mismatch repair (MMR) by immunohistochemistry in gastric adenocarcinoma. (A) Hematoxylin & eosin (H&E) stained slide exhibiting normal gastric foveolar glands and in the center of the image (green arrow) is a focus of adenocarcinoma with loss of gland formation. (B) Immunohistochemistry for MSH6 shows intact nuclear expression in normal and tumor nuclei. (C) MLH1 immunoexpression shows a faint, dot-like peri-Gogli staining pattern that is interpreted as loss of nuclear expression in the tumor cells. (D) PMS2 nuclear staining is also lost in the tumor cells. Images acquired at 200× magnification. Source: Dakhras P. et al., Translational gastroenterology and hepatology, 2020

A series of disease-specific multicenter clinical trials (KEYNOTE-016, KEYNOTE-164, KEYNOTE-012, KEYNOTE-028, and KEYNOTE-158) reported the potential efficacy of pembrolizumab in treating tumors with MSI-H/dMMR, confirming that dMMR/MSI-H status is a useful predictive biomarker for immunotherapy in various solid tumours. On the basis of these findings, in 2017 the FDA granted the approval for pembrolizumab in patients with unresectable or metastatic solid tumors with positive MSI-H or dMMR biomarkers, irrespectively of the tumour type and site (111).

Finally, regarding EBV-positivity, EBV-associated gastric carcinoma comprises about 9% of gastric cancers. The gold standard method for EBV identification is the detection of EBV-encoded small RNAs (EBER) by in situ hybridization (ISH) in paraffin-embadded samples. This method localizes the viral infection to the malignant cells with a moderate to strong staining (54). Although the exact mechanisms by which EBV infection might influence the immune system and so might impact on immune checkpoint blockade remain to be clarified, there has been a growing interest in EBV as an emerging biomarker for the prediction of immunotherapy efficacy in gastroesophageal cancers (112).

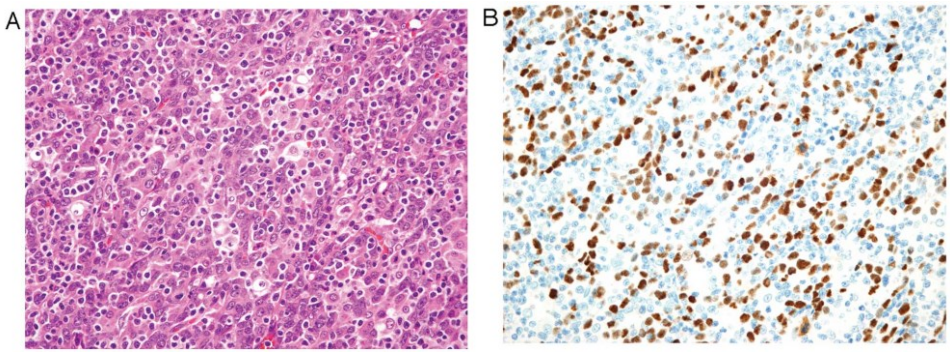

Figure 21. EBV-associated gastric carcinoma exhibiting a typical lymphoepithelioma-like carcinoma morphology. (A) H&E staining. Poorly differentiated carcinoma with prominent lymphocytic infiltration. (B) EBER-ISH highlights carcinoma cells (stained brown). Note the infiltrating lymphocytes are EBER-negative. Source: Shinozaki-Ushiku et al., International Journal of Oncology, 2015

HER2-directed immunotherapy

Several studies have demonstrated that there is a synergistic antitumour activity between anti-HER2 targeting agents (trastuzumab) and anti-PDL1/PD1 immune checkpoint inhibitors (pembrolizumab), as they have shown to act together by enhancing each other's anti-tumor effect. First of all, HER2-positive tumors seem to have a particular tumor microenvironment, with higher tumor-infiltrating lymphocytes (TILs) and PD-L1 expression levels compared with patients with normal HER2 status (113). Second, on one hand, trastuzumab and other anti-HER2 targeting agents have been shown to upregulate expression of PD-1 and PD-L1, induce expression of TILs, and modulate expression of major histocompatibility complex class II (114); all factors that are beneficial to enhance the efficacy of immunotherapy; on the other hand, a study conducted on HER2-positive mouse models has shown that anti-PD-1 antibody could significantly improve antitumor

activity of trastuzumab with the implementation of antibody-dependent cellular cytotoxicity (ADCC) (114). Third, among the mechanisms which are supposed to contribute to trastuzumab resistance there is also the up-regulation of PD-L1, which can be eliminated by immune checkpoint inhibitors (113). Given these theoretical basis, some studies proved that the combination of immunotherapy and HER2-targeted therapy with or without chemotherapy may bring some extra survival benefit for HER2-positive tumors. The phase III KEYNOTE-811 trial evaluated the efficacy of pembrolizumab or placebo in combination with trastuzumab and chemotherapy as first-line treatment for patients with advanced unresectable or metastatic HER2-positive gastric or GEJ adenocarcinoma. In the first interim results, the addition of pembrolizumab showed a statistically significant improvement in objective response rate compared with trastuzumab and chemotherapy alone, with a total effective rate of 74.4% in the pembrolizumab group and 51.9% in the placebo group (trastuzumab and chemotherapy) (114). KEYNOTE-811 trial is still ongoing to evaluate overall survival and progression free survival, however, the promising activity data of the combination of pembrolizumab, trastuzumab, and CT presented in this work have already enabled FDA accelerated approval of pembrolizumab in this setting (May 2021) (115). Although in KEYNOTE-811 patients were recruited irrespective of PD-L1 status, the objective response rate of patients with PD-L1 CPS ≥ 1 was significantly higher than that of patients who were PD-L1 negative. However, it would be interesting to see the effect of PD-L1 expression levels and also HER2 expression levels in relation to the efficacy of this combination (113), (68). Although significant challenges remain and other immunotherapy-based approaches are still being studied, the integration of immunotherapy combined with HER2 targeted therapy into the treatment of gastroesophageal cancers, based on current and emerging evidence, is hoped to improve outcomes for patients in this setting.

1.7 HER2-LOW: MIGHT IT HAVE A PREDICTIVE ROLE IN GASTROESOPHAGEAL CANCERS?

1.7.1 A LOOK AT HER2-LOW IN BREAST CANCERS

Although HER2-positive gastroesophageal cancers are quite different from breast cancers, all the information generated from breast cancer research can provide meaningful information relevant to gastroesophageal tumors. Due to the numerous studies conducted on the role of HER2 for breast carcinomas, for which HER2 represents a well-established prognostic factor in addition of being predictive of HER2-targeting therapies' efficacy, breast cancer is far better-known than gastroesophageal ones. Since the concept of HER2-low has yet to be defined for gastroesophageal cancers, it is worth considering what is known for breast cancers. Breast cancer is traditionally classified as HER2-positive when HER2 expression is scored as 3+ assessed by IHC or 2+ by ICH with gene amplification by ISH. For these tumors there is a strong recommendation for anti-HER2 targeted agents, as they have shown to own a great benefit from these agents. By contrast, tumors with ICH scores 0 and 1+, or 2+ with negative ISH, are considered HER2-negative and no HER2-targeted therapy is recommended (84).

Recently, a potential new nomenclature has been proposed for the cases with IHC 1+ or 2+ with negative ISH: these tumors with low levels of HER2 expression are named HER2-low breast cancers (116). The need to introduce this new definition is due to the fact that several studies have shown that, with a percentage of HER2-positive cancers of 15-20%, a great proportion of patients (up to the 60%) traditionally considered as HER2-negative shows in reality a low expression of HER2 and, by virtue of this low expression, they need to be classified differently, mainly because this low expression might still be targetable (116) (117).

Although from a theoretical point of view it makes sense to try to target HER2 even in HER2-low tumors, in the past clinical trials, trastuzumab (118) and other HER2-targeting therapies (pertuzumab (119), trastuzumab-emtansine T-DM1 (120) and anti-HER2 vaccine nelipepimut-S (121)), have failed to improve the outcomes of patients with HER2-low breast cancer. On the basis of these previous results, HER2-low breast cancers are currently considered altogether with those with 0+

at IHC as HER2-negative for the purpose of current treatment decisions (i.e., non-eligible for anti-HER2 therapies) (116). In other words, although among HER2-negative cancers there is in reality a wide spectrum of HER2-expression levels, in common clinical practice the treatment decision process in terms of access to anti-HER2 targeted agents is still driven by the dichotomization in HER2-positive vs negative tumours.

Recently, this landscape has been challenged in the light of the promising results seen with novel anti-HER2 antibody-drug conjugate (ADCs) with a quite different mechanism of action from that of the traditional HER2-targeting agents. Particularly, one of these novel anti-HER2 agents that have changed the landscape of HER2-low breast cancers is Trastuzumab-deruxtecan (T-DXd or DS-8201a) which belongs to the category of ADCs (122). The phase 3 DESTINY-Breast04 trial (123) included 557 patients with HER2-low (as defined as IHC scored 1+ 2+ with ISH negative) metastatic breast cancer, previously treated with one to two prior lines of chemotherapy. Participants were randomly assigned, on a 2:1 basis, either to treatment with T-DXd or the physician's choice of several standard chemotherapy drugs. The results of this study showed that patients with HER2-low cancer who received T-DXd versus standard chemotherapy, had a significantly improved overall survival (23,9 months versus 17,5 months) and a progression-free survival nearly doubled (10,1 months versus 5,4 months). On the basis of this trial, on August 2022, the FDA definitely approved T-Dxd as the first targeted therapy for patients with HER2-low metastatic or unresectable breast cancer who have received a prior chemotherapy (124). Other clinical studies on HER2-low breast cancer patients are currently ongoing with other ADCs, such as trastuzumab-duocarmazine, or bispecif antibodies (125). The development of these new anti-HER2 agents for HER2-low breast cancer has the potential to improve the treatment armamentarium for this subgroup of patients traditionally considered not good candidates for HER2-targeted therapy.

Pathologists and oncologists are currently trying to better define HER2-low breast cancer category in order to obtain the most accurate stratification of patients.

1.7.2 T-DXd: A POSSIBILITY FOR HER2-LOW TARGETING

Trastuzumab-deruxtecan (T-DXd or DS-8201a) is an ADC which consists of a humanized monoclonal anti-HER2 antibody bound through an enzymatically cleavable linker to the topoisomerase I inhibitor deruxtecan (a derivative of exatecan) which has cytotoxic action. The cleavable peptide linker used to bind the antibody and the cytotoxic agent deruxtecan distinguishes T-Dxd from other members of its class, such as T-DM1, which has a non-cleavable linker attaching trastuzumab to the cytotoxic maytansine derivative. Furthermore, in addition of being cleavable, this linker allows the conjugation of seven to eight molecules of the topoisomerase I inhibitor per molecule of the anti-HER2 monoclonal antibody. Considering the mechanism of action, trastuzumab binds to the extracellular domain of HER2 receptors on the surface of neoplastic cells overexpressing HER2. Once bound to HER2 receptors, the antibody is internalized by the cell, carrying the bound deruxtecan along with it. Inside the cell, linker cleavage occurs through the actions of lysosomal enzymes and, once released, deruxtecan can enter the nucleus and interfere with the action of topoisomerase, leading to DNA damage when the cell attempts to replicate itself, thus causing apoptotic cell death. The higher antibody-drug ratio (7-8:1) allows that a greater amount of deruxtecan molecules reaches the targeted cells, resulting in a more potent cytotoxic effect. Then, thanks to the cleavage of the peptide linker and to its high membrane permeability, deruxtecan can freely diffuse through the cell's membrane layer and exert its cytotoxic effect on tumor cells in close proximity to targeted cells, regardless of their HER2 expression levels (116). This antitumor effect occurring not only on HER2-overexpressing cells but also on neighboring HER2-negative or HER2-low expressing cells has been called "bystander killing effect" (*Figure 22*).

In the study performed by *Takegawa et al.* in Japan in 2019, this bystander killing effect has been confirmed both in in vitro and in vivo colorectal cancers HER2-negative cells. These cells essentially negative for HER2 expression are killed in the presence of HER2-expressing cells (126). Ultimately, the bystander killing effect

explains the success of T-Dxd in targeting HER2-low tumors, despite their lower degree of HER2 expression (116).

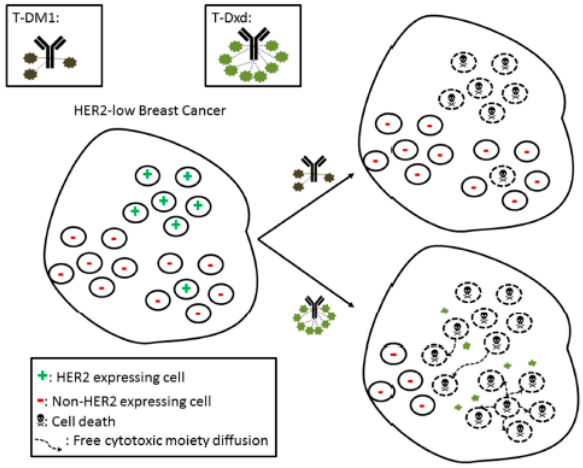

Figure 22. Schematic representation of HER1-low breast cancer being exposed to T-DM1 (with non cleavable linker) and T-Dxd (with cleavable linker and diffusible cytotoxic agent). While DM1 is trapped inside the trastuzumab-targeted cells, Dxd is freely diffusible and able to kill also non-expressing HER2 cells. The same mechanism of action is seen also in gastric and gastroesophageal cancers. Source: Eiger. D., et al., Cancers, 2021

1.7.3 HER2-LOW IN GASTROESOPHAGEAL CANCERS

In the wake of the results obtained for breast cancers, T-Dxd's efficacy in advanced gastroesophageal cancers has been evaluated in an open-label, three cohort, multicenter, randomized, phase II trial (DESTINY-Gastric01) (127). In this trial, T-Dxd was compared to the physician's choice chemotherapy (irinotecan or paclitaxel) in patients with HER2-positive advanced or metastatic gastric or GEJ cancer. The primary cohort consisted of a total of 188 patients from Japan and Korea with HER2-positive disease defined, according to the current definition of HER2-positivity in gastroesophageal cancer, as ICH 3+ or IHC 2+ with ISH positive, who progressed after two or more previous therapies including trastuzumab. Additionally, this study also contained two exploratory cohorts comprising tumours treated with at least two prior regimens, but anti-HER2 therapy naïve, which resulted as HER2-negative according to the current guidelines and thus should not be considered eligible for trastuzumab treatment. The first cohort included tumours classified as IHC 2+ with ISH negative, while the second cohort IHC 1+.

The primary endpoint was the objective response rate, while among secondary endpoint there were overall survival, progression free survival and safety. The results of the study showed that in the primary cohort objective response, overall survival and progression free survival were significantly higher in the group treated with T-Dxd than in the chemotherapy group (51% versus 14% of objective response rate, 12,5 months versus 8,4 months of median overall survival and 5,6 months versus 3,5 months of median progression free survival) (*Table IV*). Regarding safety, the most frequent adverse events of T-DXd were neutropenia (51,2%), anemia (37,6%) thrombocytopenia (11,2%) and interstitial lung disease (ILD)/pneumonitis (9,6% of patients). Most ILD were low grade (127).

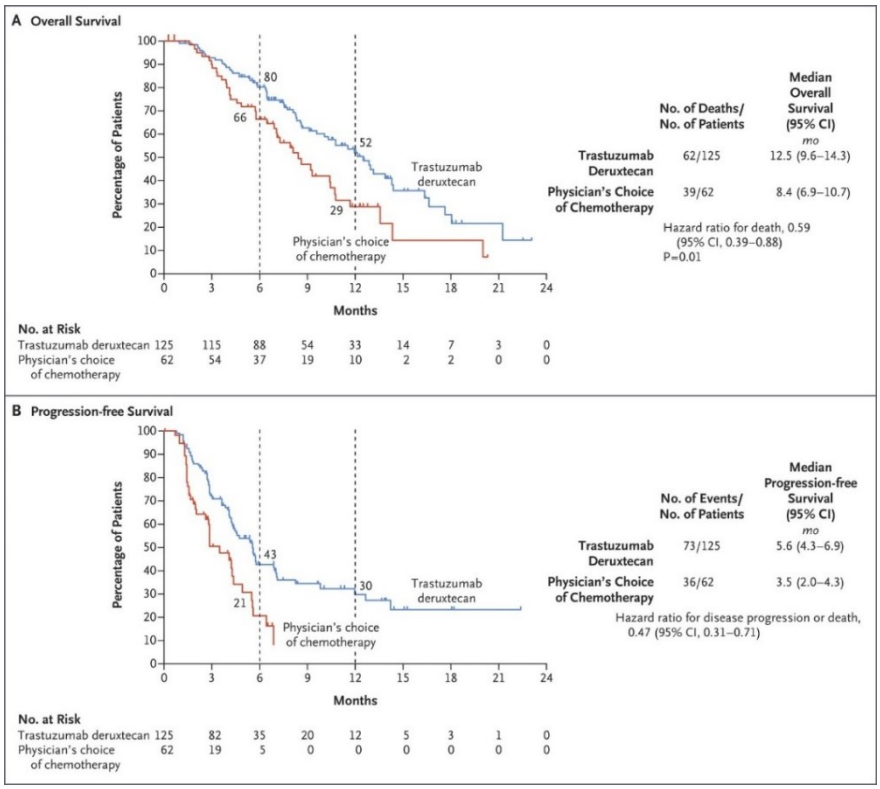

Table IV Overall Survival and Progression-free Survival, two secondary endpoints, in the primary cohort of patients of Destiny01gastric trial. Panel A. Overall survival was significantly longer in the trastuzumab deruxtecan group than in the physician's choice group (median, 12.5 months vs. 8.4 months; Panel B. The median progression-free survival was 5.6 months in the trastuzumab deruxtecan group and 3.5 months in the physician's choice group. Source: Kohei Shitara, M.D. et al. N Engl J Med 2020

Given this promising results, on January 2021, the FDA approved T-DXd for patients with locally advanced or metastatic HER2-positive gastric or gastroesophageal (GEJ) adenocarcinoma who have received a prior trastuzumab-based regimen (128).

On November 2022, also the European Medicines Agency's Committee for Medicinal Products for Human Use (CHMP) has recommended the approval of trastuzumab deruxtecan as monotherapy for the treatment of patients with advanced HER2-positive gastric or GEJ adenocarcinoma who have received a prior trastuzumab-based regimen (129). The CHMP based its favorable opinion on the updated results of the DESTINYGastric02 trial which were presented at ESMO (European Society of Medical Oncology) congress in July 2022. This latter study is a phase II single-arm trial conducted on Western patients (European and Northern American) with HER2-positive unresectable or metastatic gastric or GEJ cancer who progressed after trastuzumab-containing regimen. Updated results from this latter study confirmed the substantial clinical benefit and of T-DXd on Western population, with a confirmed objective response rate of 41.8%, a median overall survival of 12,1 months and a progression free survival of 5,6 months. Unlike DESTINYGastric01, DESTINYGastric02 does not have further exploratory cohorts with low levels of HER2 expression (130).

Returning to DESTINY-Gastric01 trial, this study included two exploratory cohorts of patients with advanced or metastatic gastric or GEJ cancers and a low expression of HER2 protein (exploratory cohort 1, IHC 2+ and ISH–; exploratory cohort 2, IHC 1+). These patients have been previously treated with at least two prior regimens, but were anti-HER2 treatment naive. Results from these exploratory cohorts were reported by *Yamaguchi et al*. in an article published on November 2022. In this article the authors reported that, even though the effect was weaker than in high HER2-strongly positive patients, T-DXd had substantial activity even in HER2-low gastric or GEJ cancer. Cohort 1 had a confirmed objective response rate of 26,3%, and a median progression free-survival and overall survival of 4,4 and 7,8 months, respectively; cohort 2 had a confirmed objective response rate of 9,5%, and a median progression free-survival and overall survival of 2,8 and 8,5 months, respectively (*Table V*) (131).

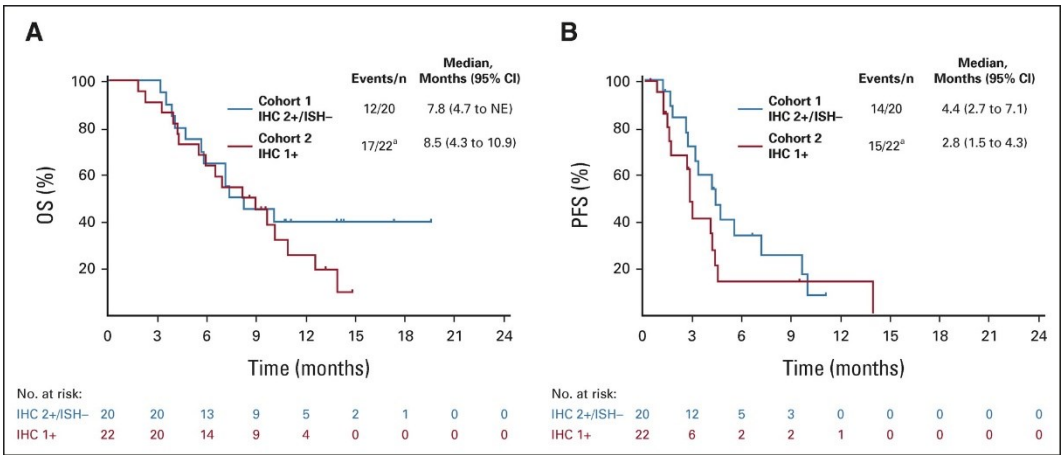

Table V. (A) OS and (B) PFS on the basis of ICR (full analysis set). Vertical lines show censored data: (A) eight patients (40%) in cohort 1 and five patients (23%) in cohort 2 had their data censored; (B) six patients (30%) in cohort 1 and seven patients (32%) in cohort 2 had their data censored. [a]Two patients were excluded from the analysis because of a missing HER2 status by central laboratory assessment. Source: Kohei Shitara, M.D. et al. N Engl J Med 2020

T-DXd proved to be effective, even if to a lower degree, in patients with HER2-low disease, with a safety profile similar to that in the HER2+ primary cohort. Activity in HER2-low tumors might be attributed to the high membrane permeability of T-DXd, which enables it to permeate neighboring cells that do not express HER2 or express it at low levels. The authors also wrote in the background that the proportion of patients with HER2-low gastric cancer defined as IHC 2+/ISH– or IHC 1+ is not well documented but estimated at 5.4% or 18.6%, respectively. This study had great limitations, due to the small patient numbers, the lack of comparator in the exploratory cohorts and the origin of patients only from Japan and Korea. Another limitation was represented by the low concordance rate (of 56.1%) between local and central scoring (131).

2. AIM OF THE STUDY

The new antibody-drug conjugated Trastuzumab-Deruxtecan, in addition of being valuable in overcoming the problem of intra-tumour heterogeneity of HER2 expression, has proved to be effective also in HER2-low diseases, defined as those cancers that are scored as 1+ at immunohistochemistry or 2+ with negative in situ hybridization assessment.

While HER2 overexpression has been largely investigated, few studies have provided data on the prevalence of HER2-low cancers. The purpose of this study is to evaluate the prevalence of HER2-low expression in a large real-world and multi-institutional series of cases of gastric and gastroesophageal cancers. In addition to the prevalence analysis, the study also aims to evaluate the correlation between this low expression rate with several clinical and histopathological features, including other biomarkers' status such as MMR/MSI status, EBER and PD-L1 expression levels. The definition of HER2-low category, with its characteristics, might pave the way toward a new way of considering the concepts of positivity and negativity of HER2 expression.

3. MATERIALS AND METHODS

3.1 STUDY DESIGN

In this study we retrospectively evaluated a total of 1.210 formalin-fixed paraffin-embedded (FFPE) samples of gastroesophageal adenocarcinoma which were analyzed by IHC for HER2 protein expression from January 2018 to June 2022. The participating centers were 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), Pisa University Hospital (Pisa, Italy), Santa Chiara Hospital (Trento, Italy), Fondazione IRCCS Policlinico San Matteo (Pavia, Italy), and Santa Maria della Misericordia University Hospital (Udine, Itay).

Our series included 627 (52,7%) biopsy specimens and 562 (47,3%) surgical resection specimens. Both in surgical and biopsy specimens HER2 protein expression was assessed by immunohistochemistry (ICH), and for those samples for which ICH analysis resulted to be equivocal (2+) a further evaluation of HER2 gene amplification was performed by in situ hybridization (ISH) technique.

Original slides were also re-evaluated focusing on cell morphology and architecture, in order to determine the histologic variant histotype and grading according to WHO 2019 criteria and the morphological characterization according to the historical Lauren and Ming classification systems. A special consideration was used for those tumours with poorly cohesive histological phenotype which were sub-classified according to the criteria formulated in the Verona consensus by the European Chapter of International Gastric Cancer Association (IGCA).

Information regarding the age and gender of patients, whether the samples have been collected from surgical or biopsy specimens, and the number of biopsy fragments which were available was collected from the pathology reports.

Further information includes the location of the tumour, the stage of the disease at diagnosis according the UICC/AJCC TNM 2016 staging system, whether neoadjuvant therapy has been performed and other biomarkers' status (PD-L1, MMR/MSI status, EBER). Regarding the assessment of these biomarkers, PD-L1

and deficient mismatch repair (MMRd) status were evaluated by ICH, EBER status was assessed by ISH, and microsatellite instability status (MSI) was assessed by multiplex amplification with fluorescent primers and subsequent DNA length fragment analysis on an automated sequence.

3.2 BIOMARKERS' ASSESSMENT BY IMMUNOHISTOCHEMISTRY

IHC staining was performed using 4 µm thick FFPE sections which were incubated with the primary antibodies for HER2 (4B5, Ventana; CB11, ThermoFisher; A0485, Dako), MLH1 (ES05, Dako; M1, Ventana), MSH2 (FE11, Dako; G219-1129, Ventana), MSH6 (EP49, Dako; SP93, Ventana), PSM2 (EP51, Dako; A16-4, Ventana), PD-L1 (22C3; Dako and SP263; Ventana), EBV7LMP (CS.1-4, Dako). For each type of antibody, we define the clone and the source.

IHC for HER2 protein expression

Immunoreactivity for HER2 was studied using three different IHC staining systems which exploit different types and clones of primary antibody in order to detect HER2-expression. Specifically, two monoclonal antibodies, 4B5, Ventana, and CB11, ThermoFisher, and one polyclonal antibody, A0485, Dako, were used.

PATHWAY anti-HER-2/neu (clone 45B) is a rabbit monoclonal antibody directed against the internal domain of the HER2 protein. This antibody is included in the UltraView Universal DAB Detection Kit, an indirect, biotin-free system which detects specific mouse and rabbit primary antibodies bound to an antigen in paraffin-embedded tissue sections stained on the Ventana BenchMark ULTRA automated IHC staining system. The specific antibody is located by a cocktail of secondary antibodies labeled with the horseradish peroxidase (HRP) enzyme (HRP Multimer) that bind to the primary antibody. The complex is then visualized adding *3-3'-diaminobenzidine (DAB)*, a derivate of benzene, which is the substrate for HRP enzyme. HRP catalyzes reaction between the substrate and H2O2, leading to the production of an intense, alcohol-resistant, brown stain that is readily observed by light microscopy (*Figure 23*).

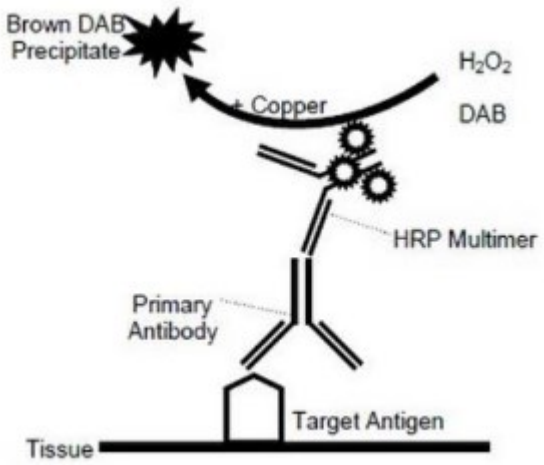

Figure 23. UltraView Universal DAB Detection Kit Reaction

Thermo Fisher Scientific's anti-HER2 monoclonal antibody (clone CB11) is a mouse monoclonal antibody which targets the internal domain of the HER2 oncoprotein and is located by a goat anti-mouse superclonal secondary antibody, Alexa Fluor® 488 conjugate which produces fluorescence outputs.

A0435, Dako is a rabbit polyclonal antibody. Unlike monoclonal antibodies, polyclonal antibodies have affinity for different parts (epitopes) of an antigen and they consist of a mix of different antibodies molecules.

For the evaluation of HER2 IHC stained tissue, the traditional four-tier score (0, 1+, 2+, 3+) was adopted; according to the available guidelines, the IHC scoring criteria formulated by Hofmann were used (83). For those tumours with the HER2 IHC score of 2+ (equivocal) further analysis with fluorescent in situ hybridization (FISH) technique was performed in order to test for HER2 gene amplification. Tumorus scored as HER2 IHC 1+ and IHC 2+ with FISH negative, which are traditionally considered as HER2-negative, were reclassified as HER2-low, while HER2 IHC 2+ with FISH positive and HER2 IHC 3+ cases were classified as HER2-high.

IHC for MMR STATUS

Deficient mismatch repair (MMRd) status was assessed by testing MSH2, MSH6, MLH1 and PSM2 expression through the use of primary antibodies MLH1 (ES05, Dako; M1, Ventana), MSH2 (FE11, Dako; G219-1129, Ventana), MSH6 (EP49, Dako; SP93, Ventana), PSM2 (EP51, Dako; A16-4, Ventana). Samples were tested with two antibodies each time in order to evaluate the maintenance or loss of function

of each eterodimer. Samples were defined as deficient mismatch repair (MMRd) when one or both proteins from a functional couple resulted negative, according to national and international guidelines.

IHC for PD-L1 expression

PD-L1 expression was assessed through the use of anti-PD-L1 primary antibodies on two different PD-L1 ICH assays, VENTANA SP263 and Dako 22C3 IHC assays. Results were expressed by using the Combined Positive Score (CPS). Thresholds of CPS 1 and 10 were used for the analysis.

3.3 BIOMARKERS' ASSESSMENT BY ISH

HER2 FISH

In this study, the evaluation of HER2 gene amplification was made by using fluorescent in situ hybridization (FISH) assay, which is a procedure that detects specific DNA sequences location in chromosomes in metaphase o interphase cells, using fluorescent probes. According to the available guidelines, FISH was performed only for equivocal cases (78), thus those tumours which were scored as HER2 ICH 2+. HER2 ICH 2+ with FISH positive were considered HER2-high, whereas ICH 2+ with FISH negative were considered HER2-low.

EBER-ISH

Epstein Barr Virus encoded RNA (EBER) is abundantly expressed in latent EBV infection. EBER transcripts are non-polyadenlyated and remain untranslated (non-coding RNA); EBER detection by ISH is considered a sensitive method for the detection of latent EBV infection. Three probes were used to detect EBV infection, the BOND Ready-to-Use ISH EBER Probe (Leyca Biosystems), the Fluorescein-Conjugated EBV PNA Probe (Dako) and the ZytoFast EBV Probe (PF29) (ZytoVision).

3.4 MSI ANALYSIS

In 25 cases MSI analyses was performed by using the Titano MSI test (Diatech Pharmacogenetics). The Titano MSI kit allows the analysis of DNA extracted from

fresh, frozen or PFFE and from peripheral blood leukocytes. In this study, Titano MSI test was performed on DNA derived from tumour and corresponding normal mucosa or blood leukocytes. The procedures of MSI status evaluation consists in a multiplex PCR amplification with fluorescent primers and subsequent DNA length fragment analysis on an automated sequencer. Starting from 20 ng of extracted DNA, this tool is able to detect variation in the number of repetitive sequences for 10 different microsatellite loci (*BAT25*, *BAT26*, *D2S123*, *D17S250*, *D5S346*, *BAT40*, *D18S58*, *NR21*, *NR24* and *TGFβRII*) by comparing peak profiles generated from the capillary electrophoresis run of the tumor and the corresponding normal tissue samples for each patient.

3.5 STATISTICAL ANALYSIS

Categorical data were summarized as number and percentage, and continuous data as median and interquartile range (IQR). HER2-low prevalence was compared in the strata using Chi Square test and Fisher's exact test. The estimated prevalence of HER2-low was reported with the 95% confidence interval (CI). All tests were 2-sided and a p-value < 0,05 was considered statistically significant. Statistical analysis was performed using R 4.1 (R Foundation for Statistical Computing, Vienna, Austria) (132)

4. RESULTS

Clinical and histopathological features in the overall study cohort

21 out of 1.210 samples were excluded because they were HER2 2+ with unavailable information on gene amplification.

The analysis included 1.189 cases of gastroesophageal adenocarcinoma, 800 males and 389 females. Overall, the median age of patients was 71 years (IQR 61-78). The male-to-female ratio was 2.06.

Out of 1.189 samples analyzed, 627 (52,7%) were biopsy specimens, whereas 562 (47,3%) were surgical resection specimens; among biopsy specimens, for 440 (71,8%) there were <6 biopsy fragments, whereas for 173 (28,2%) there where ≥ 6 fragments; for 14 biopsies data regarding the number of biopsy fragments were not available. Overall, in our case series 1.151 (96,8%) were primary tumors, and 38 (3,2%) were metastasis; 781 (67,0%) were gastric adenocarcinomas and 384 (33,0%) were GEJ adenocarcinomas. In 24 samples, whether the primary tumor was gastric or gastroesophageal was unknown.

Among gastric adenocarcinomas, 249 (31,9%) were localized in the corpus/fundus, 492 (63%) in the antrum/angulus and 40 (5,1%) cases in the antrum/corpus. For 87/384 GEJ adenocarcinoma information regarding the tumour location was available: 22 were Siewert type I tumours (adenocarcinoma of the lower esophagus), 52 were Siewert type II tumours (real adenocarcinoma of the GEJ) and 13 were Siewert type III tumours (adenocarcinoma of the sub-cardiac).

With regard to the histotype according to WHO 2019 classification, 553 (47,7%) of cases were tubular, 39 (3,4%) were papillary or tubular-papillary, 34 (2,9%) were mucinous, 269 (23,2%) were poorly cohesive, 236 (20,4%) were of mixed histotype, 15 (1,3%) were carcinomas with lymphoid stroma, 13 (1,1%) cases were of other histotype (including 6 adenosquamous carcinomas, 4 undifferentiated histotype, 2 mixed neuroendocrine-non-neuroendocrine neoplasm (MiNeN) and 1 mucoepidermoid). For 30 cases data regarding hystotipe were not available.

269 cases of poorly cohesive phenotype were classified according to the ICGA classification of poorly cohesive gastric carcinomas: 80 (32,1%) were classified as PCC-NOS (with < 10% of neoplastic cells with signet ring phenotype), 124 (49,8%) as PCC-NOS/SRC (>10% but <90%), and 45 (18,1%) as pure SRC (>90% of neoplastic

cells with signet ring phenotype). ICGA classification of poorly cohesive gastric carcinomas was not applicable in 20 poorly cohesive cases.

When investigating the distribution across Lauren's classes, 610 (52,7%) of cases were of intestinal-type, 279 (24,1%) were diffuse-type, 239 (20,7%) were mixed, 29 (2,5%) were indeterminate and in 32 cases the histotype according to Lauren's was not assessable. With regard to Ming classification, 192 (24,8%) of cases were expansive, 582 (75,2%) were infiltrative, and 415 were not assessable.

As to the grading, only tubular, papillary and mixed (tubular component) cases were graded. Out of 828 cases, 435 (61,3%) were high-grade, 274 (38,7%) were low-grade and 119 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: 21 (3,7%) were pTX, 55 (9,8%) were pT1, 60 (10,7%) were pT2, 266 (47,3%) were pT3, 160 (28,5%) were pT4.

When investigating lymph node involvement in surgical specimens, 32 (5,7%) were pNx, 149 (26,5%) were pN0, 96 (17,1%) were pN1, 109 (19,4%) were pN2, 176 (31,3%) were pN3.

188 patients (15,8%) were subjected to neoadjuvant chemotherapy; for 39 (3,3%) weather patients were subjected to neoadjuvant chemotherapy was not possible to establish.

All these clinical and histopathological feature in the study cohort were presented in the first column *Table VI*.

Strata	**Total (n = 1.189)**	**HER2 0 (n = 710)**	**HER2-low (n = 337)**	**HER2-high (n = 142)**	**Comparison of HER2-low prevalence in the strata (p-value)**
Type of Specimen:					**<0,0001**
Surgical Resection	562 (47,3%)	389 (69,2%)	118 (21,0%)	55 (9,8%)	
Biopsy	627 (52,7%)	321 (51,2%)	219 (34,9%)	87 (13,9%)	
Number of biopsy fragment: [a]					0,09
<6	440 (71,8%)	226 (51,4%)	144 (32,7%)	70 (15,9%)	
≥6	173 (28,2%)	89 (51,4%)	70 (40,5%)	14 (8,1%)	
Type of sample:					0,99
Primary tumor	1151 (96,8%)	689 (59,9%)	326 (28,3%)	136 (11,8%)	
Metastasis	38 (3,2%)	21 (55,3%)	11 (28,9%)	6 (15,8%)	

Site of primary tumor: [b]					0,21
Gastroesophageal junction	384 (33,0%)	231 (60,2%)	99 (25,8%)	54 (14,0%)	
Stomach	781 (67,0%)	467 (59,5%)	230 (29,6%)	88 (10,9%)	
Stomach site:					0,04
Corpus/fundus	249 (31,9%)	145 (58,2%)	78 (31,4%)	26 (10,4%)	
Antrum/angulus	492 (63,0%)	290 (58,9%)	147 (29,9%)	55 (11,2%)	
Antrum/corpus	40 (5,1%)	32 (80,0%)	5 (12,5%)	3 (7,5%)	
Lauren Classification: [d]					0,28
Intestinal	610 (52,7%)	346 (56,7%)	180 (29,5%)	84 (13,8%)	
Diffuse	279 (24,1%)	185 (66,3%)	69 (24,7%)	25 (9,0%)	
Mixed	239 (20,7%)	152 (63,6%)	64 (26,8%)	23 (9,6%)	
Indeterminate	29 (2,5%)	14 (48,3%)	11 (37,9%)	4 (13,8%)	
Ming Classification: [e]					0,91
Expansive	192 (24,8%)	120 (62,5%)	51 (26,6%)	21 (10,9%)	
Infiltrative	582 (75,2%)	366 (63,0%)	150 (25,8%)	65 (11,2%)	
2019 WHO classification					0,36
Tubular	553 (47,7%)	312 (56,4%)	165 (29,8%)	76 (13,8%)	
Papillary	39 (3,4%)	21 (53,8%)	11 (28,2%)	7 (18,0%)	
Poorly cohesive	269 (23,2%)	179 (66,5%)	66 (24,5%)	24 (9,0%)	
Mixed	236 (20,4%)	150 (63,6%)	63 (26,7%)	23 (9,7%)	
Mucinous	34 (2,9%)	24 (70,6%)	8 (23,5%)	2 (5,9%)	
C. with lymphoid stroma	15 (1,3%)	7 (46,7%)	6 (40,0%)	2 (13,3%)	
Others	13 (1,1%)	6 (46,2%)	5 (38,4%)	2 (15,4%)	
ICGA classification of PCC					0,07
PCC-NOS	80 (32,1%)	46 (57,5%)	22 (27,5%)	12 (15,0%)	
PCC-NOS/SRC	124 (49,8%)	82 (66,1%)	32 (25,8%)	10 (8,1%)	
SRC	45 (18,1%)	39 (86.7%)	5 (11,1%)	1 (2,2%)	
Grading (in tubular, papillary and mixed histotypes): [f]					0,79
High-grade	435 (61,3%)	262 (60,2%)	125 (28,7%)	48 (11,1%)	
Low-grade	274 (38,7%)	155 (56,6%)	82 (29,9%)	37 (13,5%)	
pT stage in surgical specimens:					0,17
pTX	21 (3,7%)	16 (76,2%)	3 (14,3%)	2 (9,5%)	
pT0	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
pT1	55 (9,8%)	45 (81,8%)	5 (9,1%)	5 (9,1%)	
pT2	60 (10,7%)	40 (66,7%)	12 (20,0%)	8 (13,3%)	
pT3	266 (47,3%)	175 (65,8%)	62 (23,3%)	29 (10,9%)	
pT4	160 (28,5%)	113 (70,6%)	36 (22,5%)	11 (6,9%)	
pN stage in surgical specimens:					0,63
pNX	32 (5,7%)	23 (71,9%)	5 (15,6%)	4 (12,5%)	
pN0	149 (26,5%)	112 (75,2%)	26 (17,4%)	11 (7,4%)	
pN1	96 (17,1%)	65 (67,7%)	21 (21,9%)	10 (10,4%)	
pN2	109 (19,4%)	69 (63,3%)	25 (22,9%)	15 (13,8%)	
pN3	176 (31,3%)	120 (68,2%)	41 (23,3%)	15 (8,5%)	
Neoadjuvant therapy	188 (15,8%)	122 (64,9%)	49 (26,1%)	17 (9,0%)	0,50

Table VI.Clinical and histopathological features in the overall study cohort and according to HER2-status. Data summarized as n (%) or median (IQR). Percentages are calculated by column for the whole series and by row for the HER2 groups. Other histotypes included adenosquamous (n=6), undifferentiated histotype (n=4), mixed neuroendocrine-non-neuroendocrine neoplasm (MiNeN, n=2) and mucoepidermoid (n=1). ICGA classification of poorly cohesive gastric carcinomas was not applicable in 20 poorly cohesive cases. Data not available in [a]14, [b]24, [c]30, [d]32, [e]416, [f]119 cases.

HER2-positivity and HER2-low prevalence in overall cohort of samples and in biopsy versus surgical resection specimens

Among 1189 assessable cases, HER2 IHC expression was scored as follows: 710 (59,7%) cases were HER2 0, 217 (18,3%) cases were HER2 1+, 120 (10,1%) were not amplified HER2 2+, 41 (3,4%) were amplified HER2 2+, and 101 (8,5%) were HER2 3+. According to the available guidelines which consider as HER2-positive those tumours with HER2 ICH scores of 3+ or 2+ with amplification certified by FISH positivity, HER2-positivity prevalence among the 1.189 assessable cases was 11,9%. By introducing HER2-low category and subdividing cancers in three groups as HER2 0, low and high, HER2 status was classified as follows: 710 (59,7%) were HER2 0, 337 (28,3%) were HER2-low and 142 (11,9%) were HER2-high. The overall prevalence of HER2-low was 28,3% (*Table VII*). The prevalence of HER2-positivity and negativity and of HER2-category (0, low, high) in the overall cohort of assessable samples is showed in *Figure 24*.

<table>
<tr><th colspan="7">Overall cohort of samples (n=1.189)</th></tr>
<tr><td rowspan="3">Negative</td><td>0</td><td>710 (59,7%)</td><td rowspan="3">1047 (88,1%)</td><td>HER2-0</td><td>710 (59,7%)</td><td>710 (59,7%)</td></tr>
<tr><td>1+</td><td>217 (18,3%)</td><td>HER2-low</td><td>217 (18,3)</td><td rowspan="2">337 (28,3%)</td></tr>
<tr><td>2+/ISH-</td><td>120 (10,1%)</td><td></td><td>120 (10,1%)</td></tr>
<tr><td rowspan="2">Positive</td><td>2+/ISH+</td><td>41 (3,4%)</td><td rowspan="2">142 (11,9%)</td><td rowspan="2">HER2-high</td><td>41 (3,4%)</td><td rowspan="2">142 (11,9%)</td></tr>
<tr><td>3+</td><td>101 (8,5%)</td><td>101 (8,5%)</td></tr>
</table>

Table VII. Distribution of HER2-IHC scoring in the overall cohort of assessable samples. On the left the table shows the subdivision according to the current dichotomic distinctin of HER2-positive and negative cases. On the right the table shows the subdivision according to the three-category classification of HER2 0/low/high cases.

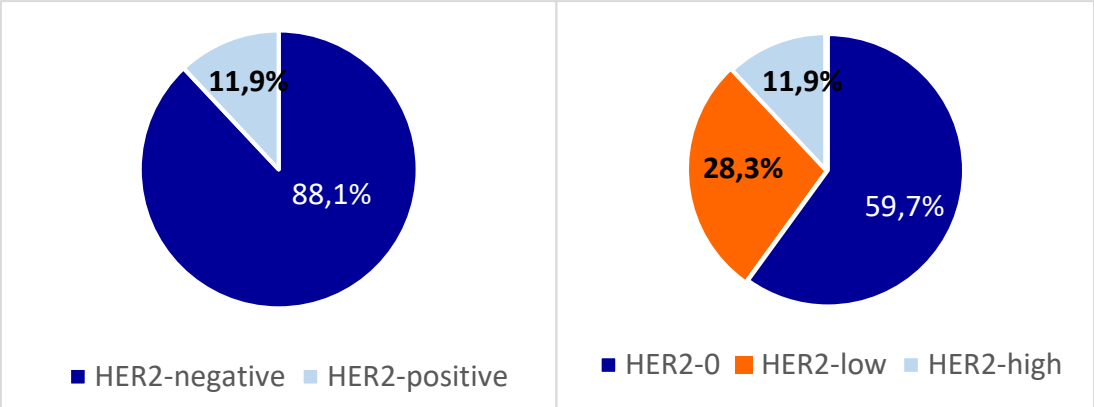

Figure 24. On the left, prevalence of HER2-positivty and negativity in the overall population of assessable samples. On the right, prevalence of HER2-status according to the three-category classification system (0, low, high) in the overall population of assessable samples. Reporting a HER2-low prevalence of 28.3% may be important in clinical practice in the light of the promising results of DESTINYGastric01 trial, which may extend the possibility of access to T-DXd targeted treatment also to this subset of patients.

Out of the 627 biopsies, HER2-status was detected as follows: 321 (51,2%) cases were HER2 0, 141 (22,5%) were HER2 1+, 78 (12,4%) were non amplified HER2 2+, 23 (3,7%) were amplified HER2 2+, and 64 (10,2%) were HER2 3+. By introducing HER2-low category, 321 (51,2%) were HER2 0, 219 (34,9%) were HER2-low, 87 (13,9%) were HER2-high. The prevalence of HER2-low in biopsies was 34,9%.

Out of the 562 surgical resection specimens HER2-status was detected as follows: 389 (69,2%) were HER2 0, 76 (13,5%) were HER2 1+, 42 (7,5%) were non amplified HER2 2+, 18 (3,2%) were amplified HER2 2+, 37 (6,6%) were HER2 3+. By introducing HER2-low category, 389 (69,2%) cases were HER2 0, 118 (21,0%) were HER2-low, and 55 (9,8%) were HER2-high. The prevalence of HER2-low in surgical resection specimens was 21,0%. All these data are shown in *Tables VIII and IX*.

Overall, HER2-low prevalence was higher in biopsy specimens (34.9%, 95% CI 31.2 to 38.8%) compared to surgical resection specimens (21.0%, 95% CI 17.7 to 24.6%) ($p<0.0001$). The difference in the prevalence of HER2-low category in biopsy and surgical resection specimens is shown in *Figure 25*.

		Surgical resection specimens (562)		Biopsy specimens (627)		Total (1189)	
Negative	0	389 (69,2%)	507 (90.2%) :	321 (51,2%)	540 (86.1%)	710 (59,7%)	1047 (88,1%)
	1+	76 (13,5%)		141 (22,5%)		217 (18,6%)	
	2+/ISH-	42 (7,5%)		78 (12,4%)		120 (10,1%)	
Positive	2+/ISH+	18 (3,2%)	55 (9.8%)	23 (3,7%)	87 (13.9%)	41 (3,4%)	142 (11.9%)
	3+	37 (6,6%)		64 10,2%)		101 (8,5%)	

Table VIII. Stratification of HER2 IHC scores according to the type of specimen, with grouping of HER2 status according to currently binary system HER2 positivity vs negativity

		Surgical resection specimens (562)		Biopsy specimens (627)		Total (1189)		P-value for HER2-low distribution
HER2 0	0	389 (69,2%)	389 (69,2%)	321 (51,2%)	321 (51,2%)	710 (59,7%)	710 (59,7%)	
HER2-LOW	1+	76 (13,5%)	118 (21.0%)	141 (22,5%)	219 (34.9%)	217 (18,6%)	337 (28,3%)	P<0,0001
	2+/ISH-	42 (7,5%)		78 (12,4%)		120 (10,1%)		
HER2-HIGH	2+/ISH+	18 (3,2%)	55 (9,8%)	23 (3,7%)	87 (13,9%)	41 (3,4%)	142 (11,9%)	
	3+	37 (6,6,%)		64 (10,2%)		101 (8,5%)		

Table IX. Stratification of HER2 IHC scores according to the type of specimen, with grouping of HER2 status according to three-category system HER2 0/low/high

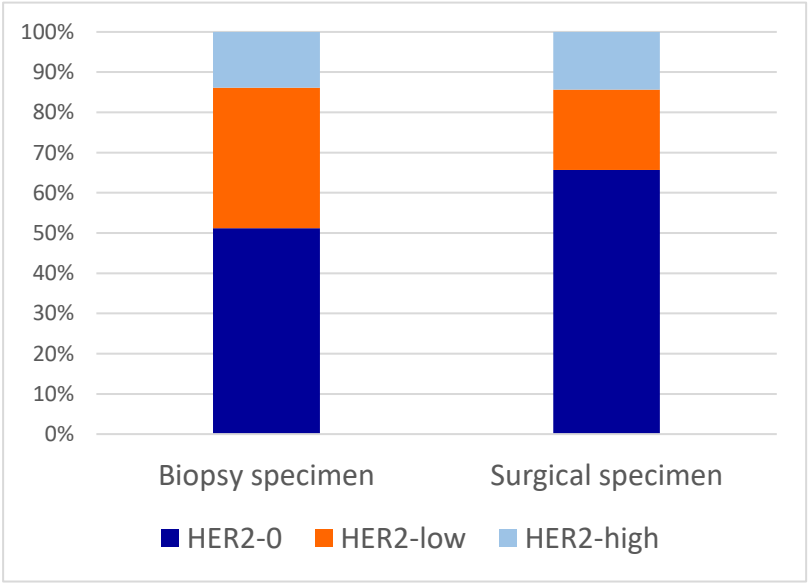

Figure 25. Distribution of HER2-0, HER2-low and HER2-high according to the type of specimens

When using 6 biopsies as a cut-off value, a significant difference in the distribution of HER2 0, low and high was found between cases with <6 biopsy fragments and cases with ≥6 biopsy fragments. HER2-low prevalence was 40.5% (95% CI 33.1 to 49.1%) in cases with ≥6 fragments and 32.7% (95% CI 28.4 to 37.3%) in cases with <6 fragments (p=0.09) (*Table X* and *Figure 26*).

		<6 biopsy fragments (440)		**≥6 biopsy fragments (173)**		**P-value for HER2-low distribution**
HER2 0	0	226 (51,4%)	226 (51,4%)	89 (51,4%)	89 (51,4%)	p=0,09
HER2-LOW	1+	93 (21,1)	144 (32,7%)	47 (27,2%)	70 (40,5%)	
	2+/ISH-	51 (11,6%)		23 (13,3%)		
HER2-HIGH	2+/ISH+	19 (4,3%)	70 (15,9%)	4 (2,3%)	14 (8,1)	
	3+	51 (11,6%)		10 (5,8%)		

Table X. Stratification of HER2 IHC scores according to the number of biopsy fragments, with grouping of HER2 status according to three-category system HER2 0/low/high.

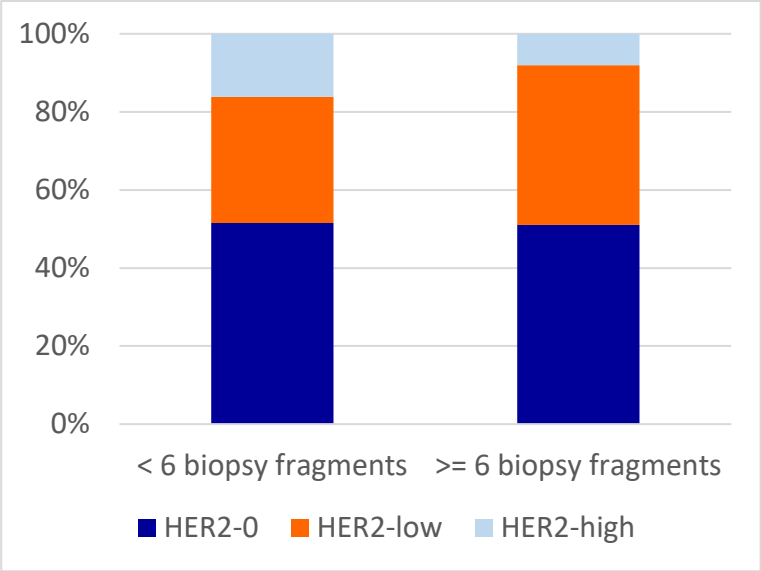

Figure 26. Distribution of HER2-0, HER2-low and HER2-high according to the number of biopsy fragments

HER2-low association with clinical and histopathological features

Out of 1.151 primary tumor samples, 689 (59,9%) were HER2 0, 326 (28,3%) were HER2-low and 136 (11,8%) were HER2-high. Out of 38 metastatic samples, 21 (55,3%) were HER2 0, 11 (28,9%) were HER2-low, and 6 (15,8%) were HER2-high.

Out of 384 GEJ adenocarcinomas, 231 (60,2%) were HER2 0, 99 (25,8%) were HER2-low and 54 (14%) were HER2-high.

Out of 781 gastric adenocarcinomas, 467 (59,5%) were HER2 0, 230 (29,6%) were HER2-low and 88 (10,9%) were HER2-high. Out of 249 gastric cases from the corpus/fundus, 145 (58,2%) were HER2 0, 78 (31,4%) were HER2-low and 26 (10,4%) were HER2-high. Out of 492 gastric cases from the antrum/angulus, 290 (58,9%) were HER2 0, 147 (29.9%) were HER2-low and 55 (11,2%) were HER2-high. Out of 40 gastric cases from the antrum/corpus, 32 (80,0%) were HER2 0, 5 (12,5%) were HER2-low and 3 (7,5%) were HER2-high.

Regarding the histotype according to the WHO 2019 classification, among the 553 tubular adenocarcinomas, 312 (56,4%) were HER2 0, 165 (29,8%) were HER2-low and 76 (13,8%) were HER2-high. Out of the 39 papillary adenocarcinomas, 21 (53,8%) were HER2 0, 11 (28,2%) were HER2-low and 7 (18%) were HER2-high. Out of the 269 poorly cohesive carcinomas, 179 (66,5%) were HER2 0, 66 (24,5%) were HER2-low and 24 (9%) were HER2-high. Out of the 236 mixed adenocarcinomas, 150 (63,6%) were HER2 0, 63 (26,7%) were HER2-low and 23 (9,7%) were HER2-high. Out of the 34 mucinous adenocarcinomas, 24 (70,6%) were HER2 0, 8 (23,5%) were HER2-low and 2 (5,9%) 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, among 80 PCC-NOS cases (<10% of cells with signet ring morphology), 46 (57,5%) were HER2 0, 22 (27,5%) were HER2-low and 12 (15%) were HER2-high. Out of the 124 PCC-NOS/SRC (>10 but < 90% of cells with signet ring phenotype), 82 (66,1%) were HER2 0, 32 (25,8%) were HER2-low, and 10 (8,1%) were HER2-high. Out of 45 SRC (> 90% of cells with signet ring morphology), 39 (86,7%) were HER2 0, 5 (11,1%) were HER2-low, and 1 (2,2%) was HER2-high. The lower prevalence of HER2-high cases in PC-NOS appeared to be statistically significant (p=0,07). The difference of HER2-low prevalence in PCC-NOS, PCC-NOS/SRC and SRC is shown in *Figure 27*.

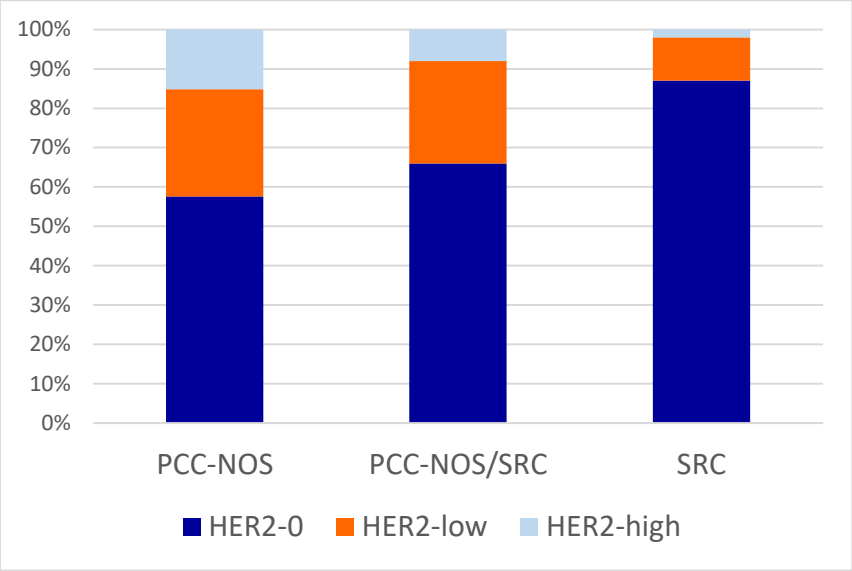

Figure 27. Distribution of HER2-0, HER2-low and HER2-high according to the category of poorly cohesive gastric cancers defined by the European Chapter of International Gastric Cancer Association (IGCA.

Regarding Lauren classification, among 610 intestinal cases, 346 (56,7%) were HER2 0, 180 (29,5%) were HER2-low and 84 (13,8%) were HER2-high. Out of the 279 diffuse cases, 185 (66,3%) were HER2 0, 69 (24,7%) were HER2-low and 25 (9%) were HER2-high. Out of the 239 mixed cases, 152 (63,6%) were HER2 0, 64 (26,8%) were HER2-low and 23 (19,6%) were HER2-high. Out of the 29 indeterminate cases, 14 (48,3%) were HER2 0, 11 (37,9%) were HER2-low and 4 (13,8%) were HER2-high.

Regarding Ming classification, among 192 expansive cases, 120 (62,5%) were HER2 0, 51 (26,6%) were HER2-low and 21 (10,9%) were HER2-high. Out of 582 infiltrative cases, 366 (63%) were HER2 0, 150 (25,8%) were HER2-low and 65 (11,2%) were HER2-high.

Out of the 435 high-grade cases, 262 (60.2%) were HER2 0, 125 (28,7%) were HER2-low and 48 (11,1%) were HER2-high. Out of the 274 low-grade cases, 155 (56,6%) were HER2 0, 82 (29,9%) were HER2-low and 37 (13,5%) were HER2-high.

Regarding tumor extent of the surgical specimens, among the 55 surgical specimens classified as pT1, 45 (81,8%) were HER2 0, 5 (9,1%) were HER2-low, and 5 (9,1%) were HER2-high. Out of the 60 surgical specimens classified as pT2, 40 (66,7%) were HER2 0, 12 (20%) were HER2-low, and 8 (13,3%) were HER2-high. Out of the 266 surgical specimens classified as pT3, 175 (65,8%) were HER2 0, 62 (23,3%) were HER2-low, and 29 (10,9 %) were HER2-high. Out of the 160 surgical

specimens classified as pT4, 113 (70,6%) were HER2 0, 36 (22,5%) were HER2-low, and 11 (6,9%) 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 149 surgical specimens classified as pN0, 112 (75.2%) were HER2 0, 26 (17,4%) were HER2-low, and 11 (7,4%) were HER2-high. Out of the 96 surgical specimens classified as pN1, 65 (67,7%) were HER2 0, 21 (21,9%) were HER2-low, and 10 (10,4%) were HER2-high. Out of the 109 surgical specimens classified as pN2, 69 (63,3%) were HER2 0, 25 (22,9%) were HER2-low, and 15 (13,8%) were HER2-high. Out of the 176 surgical specimens classified as pN3, 120 (68,2%) were HER2 0, 41 (23,3%) were HER2-low, and 5 (8,5%) were HER2-high.

Out of 188 pre-treated samples, 122 (64,9 %) were HER2 0, 49 (26,1%) were HER2-low and 17 (9%) were HER2-high.

All these clinical and histopathological associations are represented in the last three columns of *Table VI*.

HER2-low association with other biomarkers' status

In subsets of cases, further information was available about PD-L1 expression (n=250), EBER expression (n=229) and MMR/MSI status (n=612).

Among 250 cases investigated for PD-L1 expression, 47 (18,8%) cases were CPS<1, 77 (30,8%) were 1≤CPS<10, 126 (50,4%) were CPS≥10. Out of the 47 cases with CPS<1, 33 (70,2%) were HER2 0, 10 (21,3%) were HER2-low, and 4 (8,5%) 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.

EBER expression was investigated in 229 of 1189 assessable cases; 219 (95,6%) were EBER negative and 10 (4,4%) were EBER positive. Out of the 219 EBER negative cases, 128 (58,4%) were HER2 0, 65 (29,7%) were HER2-low, and 26 (11,9%) were HER2-high. Out of 10 EBER positive 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 612 of 1.189 assessable cases; among them 540 (88,2%) were MMRp/MSS and 72 (11,8%) were MMRd/MSI. Among the 72

MMRd/MSI cases, 43 (59,7%) were HER2 0, 24 (33,3%) were HER2-low, and 5 (7,0%) were HER2-high. Among the 540 MMRp/MSS, 306 (56,7%) were HER2 0, 166 (30,7%) were HER2-low, and 68 (12,6%) were HER2-high.

No statistically significant associations were found between HER2-low and PD-L1 expression (p=0,62), EBER expression (p=0,99) or MMR/MSI status (p=0,75).

All these associations between HER2-status and other biomarkers' status are shown in *Table XI*.

Strata	Total (n=1,189)	HER2 0 (n=710)	HER2-low (n=337)	HER2-high (n=142)	Comparison of HER2-low prevalence in the strata (p-value)
PD-L1 (CPS): [a]					0,62
CPS<1	47 (18,8%)	33 (70,2%)	10 (21,3%)	4 (8,5%)	
1≤CPS<10	77 (30,8%)	54 (70,2%)	17 (22,1%)	6 (7,8%)	
CPS≥10	126 (50,4%)	73 (57,9%)	34 (27,0%)	19 (15.1%)	
EBER: [b]					0,99
Negative	213 (100%)	128 (58,4%)	65 (29,7%)	26 (11,9%)	
Positive	10 (100%)	5 (5,0%)	3 (30,0%)	2 (20,0%)	
MMR/MSI status: [c]					0,75
MMRp/MSS	540 (100%)	306 (56,7%)	166 (30,7%)	68 (12,6%)	
MMRd/MSI	72 (100%)	43 (59,7%)	24 (33,3%)	5 (7,0%)	

Table XI. . Association between HER2-status and the other tested biomarkers. Data summarized as n (%). Percentages are calculated by column for the whole series and by row for the HER2 groups. Data not available in a 939, b 960 and c 577 cases. CPS: combined positive score; MMRp: mismatch repair proficient; MMRd: mismatch repair deficient; MSI: microsatellite instability; MSS microsatellite stable. MMRd, MSI, MMRd/MSI included MLH1/PMS2 loss (n=62), MSH2/MSH6 loss (n=3), MLH1/MSH6 loss (n=1), PMS2 loss (n=1) and MSI (n=5).

HER2 low prevalence according to year of testing, center of evaluation, and the antibody clone used in IHC analysis

The 1.189 samples included in the studies were selected from the surgical pathology units of eight centers dating back to 2018.

The prevalence of HER-low cases among years was: 59/206 (28,6%) in 2018, 79/301 (25,4%) in 2019, 67/225 (29,8%) in 2020, 103/340 (30,3%) in 2021, 29/117 (24,7%) in 2022. In conclusion, the prevalence of HER2-low ranged from 24,7% to 30,3% in the period 2018-2022, with a difference in the distribution of HER2-low cases over the years defined by p=0,68, not statistically significant.

The prevalence of HER2-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, 39/100 (39,0%) in Center 5, 28/69 (40,6%) in Center 6, 7/35

(20,0%) in Center 7, 8/24 (33,3%) in Center 8. In conclusion, the prevalence of HER2-low ranged from 19,1% to 40,6% among centers, with a statistically significant difference in the distribution of HER2-low cases among various centers (p=0,0005). This difference was also found in the subgroups of biopsy specimens (HER2-low prevalence ranged from 14,3 to 45,7%, p=0,01) and surgical resection specimens (HER2-low prevalence ranged from 0.0 to 37,7%, p<0,0001).

The prevalence of HER2-low was highest when using CB11 (Leica) (40,6% vs 33,0% with A0485 (Dako) and 26,6% with 45B (Ventana), p=0,01).

All these associations between HER2-status and year, center and antibody used are shown in *Table XII*.

	Total (n=1.189)	**HER2 0 (n=710)**	**HER2-low (n=337)**	**HER2-high (n=142)**	**Comparison of HER2-low prevalence in the strata (p-value)**
Year:					0,68
2018	206 (17,3%)	121 (58,8%)	59 (28,6%)	26 (12,6%)	
2019	301 (25,4%)	183 (60,8%)	79 (26,2%)	39 (13,0%)	
2020	225 (18,9%)	128 (56,9%)	67 (29,8%)	30 (13,3%)	
2021	340 (28,6%)	202 (59,4%)	103 (30,3%)	35 (10,3%)	
2022	117 (9,8%)	76 (65,0%)	29 (24,7%)	12 (10,3%)	
Center:					0,0005
Center #1	402 (33,8%)	263 (65,4%)	94 (23,4%)	45 (11,2%)	
Center #2	302 (25,4%)	180 (59,6%)	87 (28,8%)	35 (11,6%)	
Center #3	147 (12,4%)	74 (50,3%)	53 (36,1%)	20 (13,6%)	
Center #4	110 (9,3%)	75 (68,2%)	21 (19,0%)	14 (12,7%)	
Center #5	100 (8,4%)	48 (48,0%)	39 (39,0%)	13 (13,0%)	
Center #6	69 (5,8%)	37 (53,6%)	28 (40,6%)	4 (5,8%)	
Center #7	35 (2,9%)	20 (57,1%)	7 (20,0%)	8 (22,9%)	
Center #8	24 (2,0%)	13 (54,2%)	8 (33,3%)	3 (12,5%)	
HER2 antibody clone:	69 (5,8%)	37 (53,6%)	28 (40,6%)	4 (5,8%)	0,01
CB11 (Leica)	182 (15,3%)	94 (51,6%)	60 (33,0%)	28 15,4%)	
A0485 (Dako)	938 (78,9%)	579 (61,7%)	249 (26,6%)	110 (11,7%)	
4B5 (Ventana)					

Table XII. Distribution of her 2 0, HER2-low and HER2-high according to year of evaluation and center where the evaluation was performed. Data summarized as n (%). Percentages are calculated by column for the whole series and by row for the HER2 groups.

HER2 evaluation in matched samples

For 32 patients, both the biopsy specimen and the surgical resection specimen were available. The IHC and FISH results of these 32 paired biopsy and resection specimens were analyzed.

In biopsy specimens, 15 cases were ICH HER2 0, 13 were HER2 1+, 2 were non amplified HER2 2+, 2 were amplified HER2 2+, 0 were HER2 3+. According to the available guidelines on HER2-positivity definition, among biopsy specimens, 30 cases were considered as HER2-negative (ICH 0, 1+, non amplified 2+), and 2 were positive (ICH 3+ or 2+ with FISH positive); HER2-positivity prevalence was 6.3%. By subdividing matched cases in three categories (0, low, high), in biopsy specimens, 15 cases were HER2 0, 15 cases were HER2-low, 2 cases were HER2-high.

In surgical specimens, 28 cases were HER2 0, 0 were HER2 1+, 1 was non amplified HER2 2+, 0 were amplified HER2 2+, 3 were HER2 3+. Among surgical specimens, 29 cases were considered as HER2-negative, and 3 were considered as HER2-positive; HER2 positivity prevalence was 9,4%. by subdividing samples in three categories, in surgical specimens 28 cases were HER2 0, 1 was HER2-low and 3 were HER2-high. Also in matched samples, the prevalence of HER2-low categories is higher in biopsy specimens (46,9%) than in surgical resection specimens (3,1%).

Out of all the 32 couples of specimens, 15 had a concordant ICH HER2 score (46,9%), and all of these concordant cases were HER2 0; the remaining 17 (53,1%) cases were discordant and were labelled 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. In 13 of 17 discordant cases (76,5%) the assessment in the biopsy specimen overestimated the assessment in the surgical resection specimen.

The concordance rate of HER2 ICH score between biopsy specimens and surgical specimens was 46,9%. However, if we group all the cases as HER2-psotivie or negative according to the available guidelines, we find that the concordance rate of HER2-status between biopsy specimens and surgical specimens is 90.6%.

By using the three-category classification of HER2 expression (0, low, high), among biopsy specimens, 15 cases were HER2-0, 15 were considered as HER2-low, and 2 were considered as HER2-high. Among surgical specimens, 28 cases were HER2 0, 1 was considered as HER2-low, and 3 were considered as HER2-high. If we group all the cases as HER2-0/low/high, we find that the concordance rate of HER2-status between biopsy and surgical specimens is 56,3% and the discordance rate is 43,8%. All these results were shown in *Tables XIII* and *XIV*. Concordance and discordance rates between biopsy specimens and surgical resection specimens are summarized in Sankey diagrams in *Figure 28*.

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.

Biopsy specimens (32)		**Surgical resection specimens (32)**					**Prevalence of HER2-positivity and negativity among matched biopsy specimens**
		Negative			Positive		
		0	1+	2+/ISH -	2+/ISH +	3+	
Negative	0	15	0	0	0	0	93,75%
	1+	11	0	1	0	1	
	2+/ISH-	2	0	0	0	0	
Positive	2+/ISH+	0	0	0	0	2	6,3%
	3+	0	0	0	0	0	
Prevalence of HER2-positivity and negativity among surgical specimens		90,6%			9,4%		
Concordance rate of HER2-status (positive/negative): 96.9% Discordance rate of her2-status (positive/negative): 3.1%							

Table XIII.. Concordance and discordance rate of matched biopsy and surgical resection specimens according to the HER2 positive vs negative classification system

		Surgical resection specimens (32)					Prevalence of HER2-category among biopsy specimens
Biopsy specimens (32)		HER2-0	HER2-LOW		HER2-HIGH		
		0	1+	2+/ISH -	2+/ISH +	3+	
HER2-0	0	15	0	0	0	0	46.9%
HER2-LOW	1+	11	0	1	0	1	46.9%
	2+/ISH-	2	0	0	0	0	
HER2-HIGH	2+/ISH+	0	0	0	0	2	6.3%
	3+	0	0	0	0	0	
Prevalence of HER2-category among surgical specimens		87.5%	3.1%		9.4%		
Concordance rate of HER2-status (0/low/high): 56.3% Discordance rate of HER2-status (0/low/high): 43.8%							

Table XIV. Concordance and discordance rate of matched biopsy and surgical resection specimens according to the three-category (0/low/high) classification system

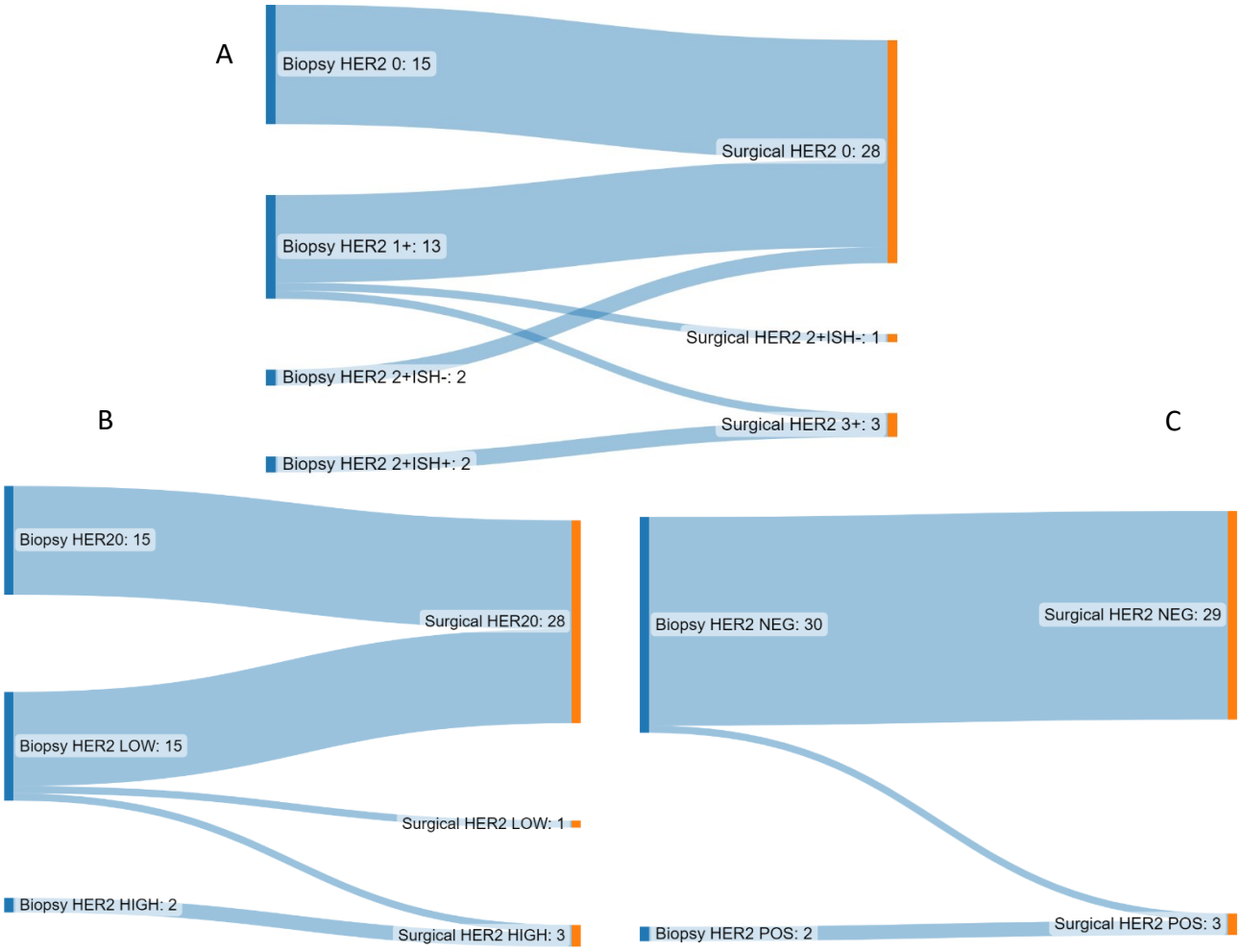

Figure 28. These are Sankey diagrams showing A. the concordance of HER2-category (HER2 0, 1+, 2+ ISH -, 2+ ISH +, 3+), B the concordance of HER2-status, by using the current dichotomous division between HER2- positive and negative, C. the concordance of HER2-status, by using a three-categories classification system (HER2-0, low, high) between biopsy and surgical resection specimens.

5. DISCUSSION

Trastuzumab deruxtecan (T-DXd) is a novel HER2-targeted antibody-drug conjugate containing an anti-HER2 antibody and a cytotoxic topoisomerase I inhibitor (116). Traditionally, according to the available ASCO/ASCP/CAP 2016 guidelines and based on the four-category ICH scoring system, HER2 status can be defined as positive (HER2 3+ or HER2 IHC 2+ with ISH positive) or negative (HER2 IHC 0, 1+ and 2+ with ISH negative) in order to identify those patients who might benefit from Trastuzumab (79). Results from patients in exploratory cohorts in the DESTINY-Gastric01 trial who were confirmed to have HER2-low tumors (IHC 2 + /ISH- [cohort 1] or IHC 1 + [cohort 2]) demonstrated that T-DXd had some anti-tumor activity also in these tumours. Cohort 1 had a confirmed objective response rate of 26,3%, and a median progression free-survival and overall survival of 4,4 and 7,8 months, respectively; cohort 2 had a confirmed objective response rate of 9,5%, and a median progression free-survival and overall survival of 2,8 and 8,5 months, respectively. T-DXd proved to be effective, even if to a lower degree, in patients with HER2-low disease (133). The important results of the DESTINY-Gastric01 trial have pointed out that a subset of patients with low levels of HER2 expression and no detectable HER2 gene amplification, traditionally considered as HER2-negative and so not good candidates for HER2-targeted agents, derive some benefit from the alternative pharmacological mechanism of the antibody drug-conjugate T-DXd. HER2-low may be identified as a new molecular subgroup of HER2-expressing gastric and gastroesophageal adenocarcinomas, thus questioning the current dichotomic system that consider tumours as positive or negative, without intermediate levels of expression.

While HER2 overexpression has been largely investigated, few studies have provided data on the prevalence of HER2 1+ and not amplified HER2 2+ cases.

Considering the HER2 screening data from the ToGA trial, the prevalence of HER2-low cases (defined as HER2 IHC 1+ or HER2 IHC 2+ with ISH negative) among the total of 3280 patients which have been tested was 23,9% (97). Cappellesso and colleagues investigated HER2 status in 1040 gastric and gastroesophageal junction adenocarcinomas using tissue microarrays (TMAs) and two different IHC assay protocols, PATHWAY HER2/neu (clone 4B5) and Oracle HER2 Bond IHC system

(clone CB11). The prevalence of HER2-low cases was quite different for the two protocols, as it is 19,9% using CB11 protocol and 17,5% using 4B5 protocol (134). In two smaller cohorts the prevalence was 18,5% (83) and 12,9% (135). In a study published in November 2022, Yang T. et al. analyzed 157 patients with early-stage gastric cancer and found a prevalence of HER2-low tumours of 31,8% (136). The variability of HER2-low prevalence among the different studies can be explained by several factors, such as 1) the enrichment in either biopsy samples or surgical specimens, 2) the enrichment of only gastric tumours, or both gastric and GEJ tumours, 3) the size of the study population, 4) the enrichment of tumours at different stages, primary tumours and/or metastasis, 5) the use of TMAs *versus* whole slides sections, 6) the use of different IHC assay method and primary antibody clones and 7) inter-observer variability. In the current study, the prevalence of HER2-low expression in the overall cohort of assessable cases was 28,3% (95% CI 25,8 to 31,0%). If we consider that the prevalence of HER2-positivity (defined by current guidelines as HER2 ICH 3+ or IHC 2+/FISH positive) among the overall cases which were analyzed was 11,9%, it is clear that extending the possibility of treating with T-DXd also those cancers classified as HER2-low would mean to treat much more patients than that with the current dichotomic system HER2-positivity versus HER2-negativity.

In this study, beyond the analysis of the overall prevalence of HER2-low cancers, three main statistically significant results were reached. The first regards the discrepancies of HER2-low prevalence between biopsies and surgical resection ($p<0,0001$); the second regards the inconsistency of concordance and discordance rate between matched biopsy and surgical resection specimens when evaluating HER2 status according to a binary (positive vs negative) or a three-category (0/low/high) classification system; the third regards the issue of inter-observer and inter-laboratories agreement variability in the assessment of HER2-status ($p=0,0005$), with discrepancies in HER2 ICH classification by using different monoclonal antibodies ($p=0,01$). Although they were not strictly statistically significant (statistical significance is defined as a p value <0,05), we have also found discrepancies of HER2-low prevalence between biopsy samples with <6 or ≥6

biopsy fragments (p=0,09) and a lower HER2-low and HER2-high prevalence among pure signet ring cell adenocarcinomas (p=0,07).

In locally advanced unresectable or metastatic gastric and GEJ cancer patients, the evaluation of HER2 status is usually based on endoscopic biopsy specimens. Due to the high levels of HER2 expression heterogeneity, the NCCN guidelines recommend that for the correct assessment for HER2 status more than 6 biopsy fragments should be taken and analyzed (80). According to our results, when compared to the surgical specimens the biopsy samples were enriched in HER2-low cases, (34,9% *versus* 21,0%; p<0,0001). The prevalence of HER2-low was also higher between cases with ≥ 6 biopsies (40,5%) than cases with < 6 biopsy fragments (32,7%). This difference might be attributed to the fact that, according to the available guidelines on HER2-testing in gastroesophageal setting, in biopsy samples membranous staining is evaluated in a minimum of 5 cohesive cells while in surgical specimens it is evaluated in ≥10% of the neoplastic cells. This means that only 5 cells with a faint or barely perceptible (HER2 IHC 1+) or weak to moderate (HER2 IHC 2+), although incomplete, membrane staining, are sufficient for considering the biopsy sample as HER2-low expressing.

Furthermore, while previous works evaluated the concordance of HER2 status between biopsy and surgical specimens using a positive/negative classification system, the introduction of a three-category classification 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 previous studies, concordance rates of HER2 status between paired biopsy and surgical resections have been found ranging from 45,5% to 94% (99). In our study, we considered 32 couples of matched biopsy and surgical resection: among them, 15 couples of samples (46,9%) had a concordant HER2 ICH score between biopsy and surgical specimen, while the other 17 couples of samples (53,1%) had discordant IHC scores. If we apply the dichotomous division between HER2-positivity and negativity the concordance rate of HER2-status between biopsy specimens and surgical specimens was 96,9%, with a discordance rate of 3,1%, weather if we apply a three-categories subdivision between HER2 0, HER2-low and HER2-high, the concordance rate of the interpretation of HER2-status diminished

to 56,3% and the discordance rate rises to 43,8%. This means that, despite the fact that the absolute concordance rate of HER2 IHC scoring between biopsy and surgical specimens (46,9%) was partially in line with that of previous studies, if we group cases no longer through a positive-negative dichotomic system, but with a three-categories system based on three levels of HER2-expression, we obtain a percentage of discordance between biopsy and surgical specimens much more high. In conclusion, we see that the impact of the type of specimen on HER2-evaluation is higher when we consider HER2-low category. This discordance may be also attributed to the different cut-off (5 cluster cells in biopsies and 10% in surgical resection specimens) used in the two types of specimen. Regard this issue, it is necessary also to consider that pre-analytical issues such as hyperfixation and cold ischemia are more common in biopsies and may lead to unreliable HER2 evaluation. This warrants some caution in relying on HER2 IHC/ISH of endoscopic biopsy specimens alone to identify HER2-low patients who may benefit from targeted treatment regimens. It would be interesting to further explore this result on a larger cohort of matched samples and to consider also clinical data of response to trastuzumab-deruxtecan therapy, stratifying patients based on the concordance/discordance of HER2-status interpretation on biopsied and surgical samples.

Lastly, the use of neoadjuvant treatment could impact on HER2-concordance between biopsy and matched surgical samples. The concordance of HER2-status (positive or negative) between biopsy and surgical specimens was 38% in pre-treated sample and 63% in treatment naïve patients. This concordance is likely to be amplified by the transition to a three-tiered scoring system.

Another challenge for HER2-low identification is represented by inter-observer and inter-laboratories agreement variability when evaluating HER2-expression at IHC. IHC is a semi-quantitative assay and may be influenced by inter-observer variability and other pre-analytical and analytical factors. The effect of inter-observer and inter-laboratories agreement variability in the assessment of HER2-low expression has been investigated in invasive breast cancer. One study evaluating the discrepancies in local and centralized assessment of HER2 reported that up to 85% of the patients with tumours originally scored as IHC 0 actually

were 1+ or 2+, suggesting that an intrinsic difficult to distinguish these categories (137). Other studies have reported that the lowest agreement rate when different pathologists evaluate IHC-stained samples is between HER2 0 *versus* HER 1+ cases, while HER2 0 versus HER2 3+ cases were found to have the highest agreement (138). In our study, we found a statistically significant distribution of HER2 categories and HER2-low prevalence (p=0,0005) among the eighth centers where the evaluation was performed. HER2-low prevalence ranged from 19,1 to 40,6% among centers (p=0,0005). The difference remained significant even when considering biopsy or surgically specimens alone. In particular, among biopsies HER2-low prevalence ranged from 14,3% to 45,8%, while among surgical resection specimens HER2-low prevalence ranged from 0% to 37,7%.

However, when applying a positive/negative scoring system to the same cohort, the difference in the distribution of HER2-positive and HER2-negative cases among centers was significantly lower. In particular, HER2-positivity ranged from 77,1% to 94,2%, while HER2-negativity ranged from 5,8% to 22,9%. These data suggest that a three-tiered scoring system might result in lower inter-laboratory agreement and lower reproducibility of HER2 IHC assay.

HER2-low prevalence also from 26,6 to 40,6% according to the clone of the antibody used for the immunohistochemical staining (p=0,01).

No significant differences in HER2-low prevalence emerged concerning other clinic-pathologic features such as patients' age, sex, primary tumour versus metastasis, gastric *versus* gastroesophageal localization, Siewert class, corpus/fundus versus antrum/angulus. Furthermore, no statistically significant association between HER2-status and WHO 2019 histotype, Lauren classification, Ming classification, stage and grading was found. However, the fraction of HER2-positive (HER2-high) cases was higher in tubular and papillary adenocarcinomas and in Lauren intestinal adenocarcinomas. The lowest proportion of HER2-positivity was found in mucinous adenocarcinoma (23,5%), as well as reported also by previous studies. We also demonstrated that HER2-low status can be found in rare histotypes, such as carcinoma with lymphoid stroma.

When applying the IGCA classification to poorly cohesive carcinomas, we found a significantly lower prevalence of HER2-low (11,1%) as well as HER2-high (2,2%)

among “pure” signet ring cell carcinomas (SRC, defined as a tumour of poorly cohesive histotype with > 90% of cancer cells with signet ring morphology) than poorly cohesive cancers (PCC-NOS and PCC-NOS/SRC). 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, a consistent proportion of PCC and SRC adenocarcinomas, as well as in general diffuse-type gastric cancers, are characterized by the deficiency of E-cadherin membrane expression (43). E-cadherin is essential for cell-to-cell contacts and is crucial for the assembly of tight junctions, which not only act as selective permeability barriers but also form a fence that physically separates the apical membrane domain from the basolateral domain in epithelial cells (apicobasal membrane polarity). Loss of tight junctions can result in the disruption of this apicobasal membrane polarity and lead to a change in antigen expression by the cell membrane, thus changing also IHC staining patterns. In gastroesophageal cancers HER2-expression is predominantly basolateral or lateral; the loss of E-cadherin expression and cell polarity in diffuse type gastric cancers including SRC subtype leads to the migration of this antigen along the cell membranes, with a more homogenous IHC membranous pattern of HER2 expression. Signet ring cells have a center cytoplasmic vacuole which pushes the cytoplasm more toward the membranes of the cell. As such, cytoplasm and membranes are close together, thus complicating visual differentiation between cytoplasmic staining and homogenous membranous staining in IHC scoring (139). In HER2 evaluation of gastric cancers, membranous staining is considered positive whereas cytoplasmic staining with/without nuclear staining is considered non-specific (140). Furthermore, since a minimum of 5 clustered stained tumor cells is necessary in biopsy samples in order to obtain an adequate IHC scoring, these stained clusters are harder to identify in poorly cohesive and signet ring tumours, because of the loss of cell-to-cell adhesion (139). All these factors might undermine HER2 evaluation, not identifying or over/underestimating HER2 IHC results, as well as increasing inter-observer agreement variability and also discordance between ICH results obtained with different antibodies (140).

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 (27%) and HER2-high (15,1%). KEYNOTE-811 phase III clinical trial investigated whether the addition of pembrolizumab to chemotherapy and trastuzumab in HER2-overexpressing cases might give a clinical benefit for patients with these cancers. The results of this landmark study demonstrated improved efficacy of the triple therapy compared with chemotherapy and trastuzumab double therapy. Although in KEYNOTE-811 patients were recruited irrespective of PD-L1 status, the objective response rate of patients with PD-L1 CPS ≥ 1 was significantly higher than that of patients who were PD-L1 negative. The biomarkers assessment of this study found that PD-L1 expression and also MSI status could be the potential biomarkers for immune checkpoint inhibitors combined with HER2 targeted therapies in HER2-positive gastroesophageal cancers (114). However, it would be interesting to see the effect of PD-L1 expression levels and also HER2 expression levels in relation to the efficacy of this combination.

6. CONCLUSION

In the light of the significant clinical benefits of trastuzumab-deruxtecan (T-Dxd) in advanced HER2-low expressing gastric and gastroesophageal junction cancers and in the wake of the knowledge available on HER2-low breast cancers, the new "HER2-low" category is emerging as a novel distinct entity, and this could be the starting point toward a reconsideration of the world of HER2 expressing cancers. First of all, the introduction of HER2-low category would call into question one of the fundamental pillars on which the routine use of HER2 as a molecular biomarker is based, that is the dichotomous division of HER2-positive and HER2-negative cancers. This shift from a binary (positive versus negative) to a three-tiered scoring system (0, low, high) has clear implications on clinical practice. In fact, if a patient moves from the category of HER2-negative to that of HER2-low, therapeutic approach changes radically and treatment options inevitably expand. 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 re-define the assessment of HER2-low gastroesphageal cancers exist. Future perspectives include: 1) a modification of existing HER2 assays to increase reproducibility, 2) the delivery of specific training for gastrointestinal pathologists, 3) the incorporation of quantitative analysis, such as HER2 RNA levels tested by qRT-PCR, in the workflow and 4) the introduction of complementary biomarkers.

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