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TARGETING EPIGENETIC MECHANISMS IN GASTRIC CANCER

Presentata da: Matteo Canale

Coordinatore Dottorato

Manuela Ferracin

Supervisore

Paola Ulivi

Co-supervisore

Pier Luigi Lollini

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Summary

1. Abstract

Gastric cancer (GC) is a hard challenge for medical oncology, with globally over one million of new diagnoses each year and low survival rates. Gastric carcinogenesis is guided by the interaction of several risk factors such as diet, genetics, tobacco smoking and Helicobacter pylori infection, and exerts through sequential histopathologic steps, including chronic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia and cancer. GC is classified on the basis of anatomical, histological or molecular classification, reflecting the wide cancer heterogeneity, also highlighted by the inefficacy of the actual treatment schedules. When feasible, surgery is the recommended treatment option, often accompanied by neo-adjuvant and adjuvant chemotherapy. For advanced disease, several treatment schedules are used, mainly platinum-based or fluoropyrimidines, while trastuzumab is approved for positive Her2 patients.

Epigenetic mechanisms alterations affecting DNA methylation, histone methylation and acetylation, are a recognized hallmark of cancer and stand at the basis of gastric carcinogenesis and tumor development. The pharmacological targeting of these altered mechanisms is an attractive option for new cancer treatments.

Aim of this study was to test the therapeutic potential of the compound CM-272 for GC, a selective and strong dual inhibitor of DNMT1 and EHMT2, which reached important results in pre-clinical models of other gastrointestinal malignancies. Moreover, in a GC patients case series, the expression of the target of the compound was tested, to prove the rationale for inhibition

DNMT1, EHMT2 and their functional adaptor were over-expressed in the majority of GC patients tissues. Through *in-vitro* testing of CM-272 alone and in combination with the most used chemotherapeutic treatments for GC in a panel of GC cell lines, this study demonstrated that the compound has a strong ability in inhibiting GC cells growth, acting at lower doses with respect to chemotherapeutic agents and other epigenetic compounds. It was demonstrated that CM-272 has a synergistic effect when administered together with chemotherapy, while it has an additive effect when administered prior to cytotoxic treatment. Even though not directly inducing apoptosis, CM-272 was able to induce a senescent phenotype in GC cells, and to epigenetically reprogram the transcription of genes involved in phosphorylation cascades and mitochondria metabolism, thus affecting the growth and energetic machinery of cancer cells.In conclusion, the pharmacological targeting of epigenetic mechanisms demonstrated good potential in *in-vitro* pre-clinical models of GC, and further investigations to test *in-vivo* efficacy, and studies focused on the molecular mechanisms compound-induced are needed.

2. Introduction

2.1 Stomach and Gastric cancer

Stomach is a fundamental component of the gastrointestinal system. Topographically, it is divided in 5 regions: - cardia and gastroesophageal junction (GEJ); - fundus; - corpus; - antrum and - pylorus. Fundus and corpus host acid-secreting glands, while the antrum has an alkaline-secreting surface epithelium and endocrine G-cells, which secrete gastrin [1,2]. From the lumen of the organ, the wall of the stomach is divided in four layers: - mucosa; - submucosa; - muscolaris mucosa and - serosa (Figure 1).

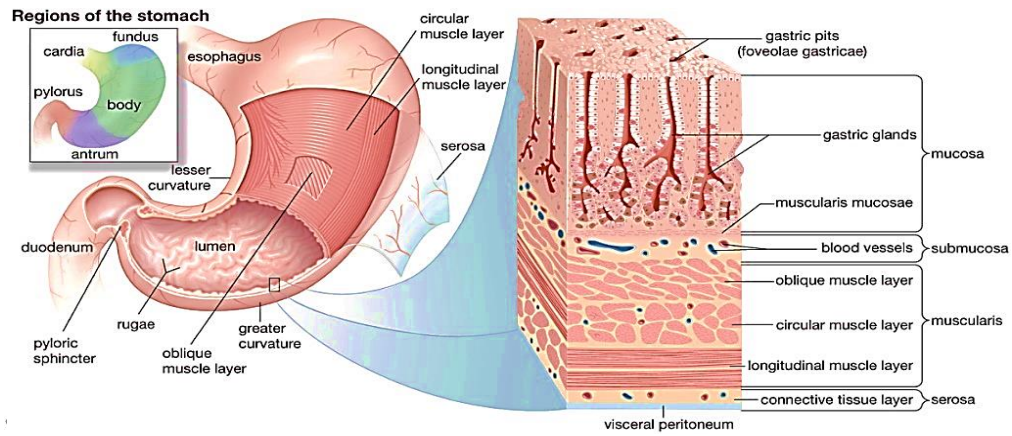

Figure 1. Structure of the stomach and layers of the stomach wall [2].

Gastric cancer (GC) is a hard challenge for medical oncology, with globally over one million of new diagnoses each year and low survival rates. Gastric carcinogenesis is guided by the interaction of several risk factors such as diet, tobacco smoking and Helicobacter pylori infection, and exerts through sequential histopathologic steps, including chronic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia and cancer [3]. GC presents as a wide heterogeneous disease at histopathological, onset location and molecular levels, often diagnosed at late stages, creating a complex scenario for patients clinical management. Treatment algorithm is based on the TNM classification system (table 1), cancer histology and molecular classification and staging of the disease, performed on the basis of the depth of invasion into the wall of the stomach (figure 2).

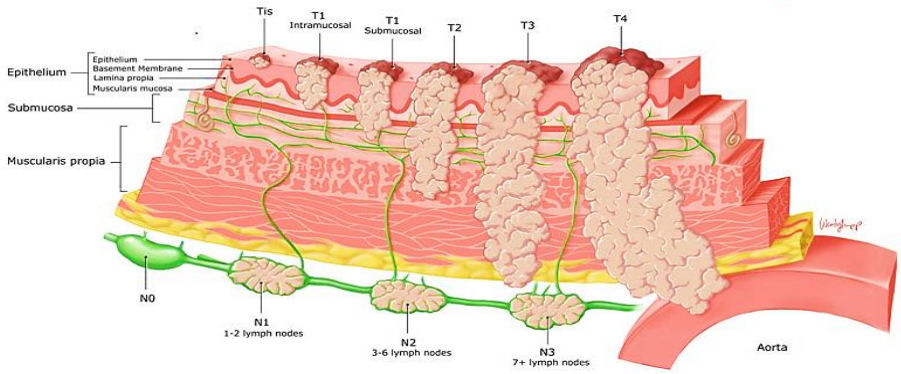

Figure 2. Gastric cancer stadiation.
Rabab Cancer Foundation (2018)

Table 1. The Tumor Node and Metastasis classification system

T (tumor)	describes how deep the cancer has grown through the stomach wall and whether it has grown into nearby tissues
T1	The tumor has grown through some of the inner layers of the wall of the stomach but has not reached the muscle layer of the stomach (muscularis propria).
T2	The tumor has grown into the muscular layer of the stomach (muscularis propria) but has not reached the subserosa
T3	The tumor has grown to reach the subserosa layer.
T4	The tumor has grown into the serosa and may be growing into a nearby organ (spleen, intestines, pancreas, kidney, etc.) or other structures such as major blood vessels. This is further subdivided into:
T4a	The tumor has grown through the stomach wall into the serosa, but the cancer hasn't grown into any of the nearby organs or structures.
T4b	The tumor has grown through the stomach wall and into nearby organs or structures
N (node)	describes whether the cancer has spread to lymph nodes surrounding the stomach.
N0	No spread to nearby lymph nodes.
N1	The cancer has spread to 1 to 2 nearby lymph nodes.
N2	The cancer has spread to 3 to 6 nearby lymph nodes.
N3	The cancer has spread 7 or more nearby lymph nodes. This is further subdivided into:
N3a	The cancer has spread to 7 to 15 nearby lymph nodes.
N3b	The cancer has spread to 16 or more nearby lymph nodes
M (metastasis)	describes whether the cancer has spread to distant sites, such as distant organs or lymph nodes that are not near the stomach (referred to as "metastatic sites")
M0	no signs of cancer spread to distant organs or distant lymph nodes.
M1	The cancer has spread either to lymph nodes far away from the stomach or distant organs/tissues such as the lungs, liver or brain). In addition if peritoneal washings are found to contain cancer cells then this is considered M1 disease even if distant organs or lymph nodes appear to be uninvolved with cancer.

Even though GC often display a wide range of heterogeneity at histopathological levels, and several parameters and classification systems have been adopted to face this heterogeneity (see paragraph 1.4.2), the most representative histology types of GC are the intestinal type and the diffuse type (Figure 3).

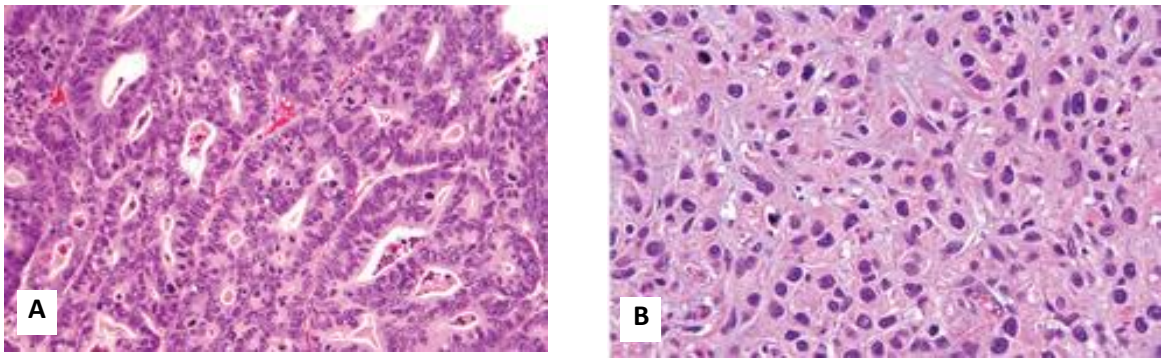

Figure 3. A. Intestinal-type gastric cancer. **B.** Diffuse type gastric cancer

2.2 Epidemiology

GC is the fifth most diagnosed and the fourth most lethal malignancy worldwide, with an estimated more than one million new cases annually, and 769,000 deaths in 2020 [4].

GC is more commonly diagnosed in males than in females (720,000 and 370,000 new cases in 2020, respectively), and higher incidence rates are recorded in Eastern Asia (about 60% of all new diagnoses), Eastern Europe and South America, while in several African countries less than 5 new cases per 100,000 persons/year are reported (Figure 4) [5].

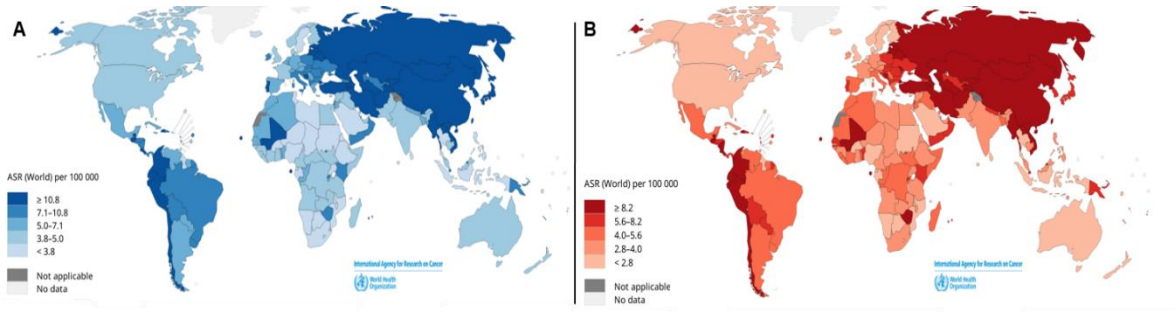

Figure 4. Estimated age-standardized rates (ASR) in 2020 for gastric cancer incidence (A) and mortality (B), both sexes, all ages. *Data source: GLOBOCAN 2020, World Health Organization – International Agency Research on Cancer (http://gco.iarc.fr/today).*

Globally, 8.3% of all cancer deaths are attributable to GC, and the cumulative risk of death from this malignancy, from birth to age 74, is 1.36% for males and 0.57% for females [6]. GC is a highly deadly malignancy, and survival rates largely depend on age and tumour stage at diagnosis, as 5-year survival rate for early stage surgically-treated tumours (stage IA, IB) are 94% and 88%, respectively, while stage IIIC tumours present a 18% rate [7].

GC has been the global most common cause of cancer-related mortality up to the mid-1990s [6].

In the last decades, improved living conditions in developing countries led to an estimated 2% and 0.7% annual decrease in men and women in medium human development index (HDI) countries, while screening programmes in high incidence areas, such as Korea and Japan, led to substantial reductions in gastric cancer-associated mortality [8–10].

2.3 Risk factors

Genetics. The majority of GC are sporadic, but up to 10% of GC show familial aggregation, while 1-3% show genuine hereditary causes. Germline variants in specific genes or set of genes are associated to the hereditary cancer syndrome, defined as a genetic predisposition to malignant conditions. There are three hereditary GC syndromes described: Hereditary Diffuse Gastric Cancer (HDGC), Familial Intestinal Gastric Cancer (FIGC), and Gastric Adenocarcinoma and Proximal Polyposis of the Stomach (GAPPS). Other syndromes include Li Fraumeni, Familial Adenomatous Polyposis and Lynch. Hereditary diffuse gastric cancer (HDGC) is a rare, highly penetrant malignancy characterized by autosomal dominant inheritance of pathological variants of the *CDH1* and genes, which encode the adhesion molecules E-cadherin and α-catenin, respectively (Figure 5) [11,12]. HDGC presents as a poorly differentiated diffuse carcinoma, invading the whole thickness of the gastric wall. *CDH1* mutations include point mutations or small insertions and deletions, with no hotspots highlighted to date; the encoded protein E-cadherin is a type I calcium transmembrane glycoprotein expressed on epithelial tissue and responsible to maintain cell-to-cell adhesion in adherens junctions and cell polarity [13,14].

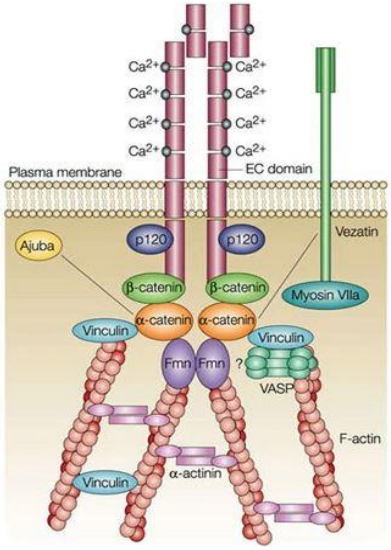

Figure 5. E-cadherin transmembrane structure and intracellular associated components [12].

Helicobater pylori. *Helicobacter pylori* infection is the most well described risk factor for GC. Australian researchers Barry J. Marshall and J. Robin Warren were awarded the Nobel Prize in

Physiology or Medicine in 2005 “for their discovery of the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease" [15]. In 1985, Marshall deliberately infected himself with the bacterium to demonstrate it was the cause of acute gastritis, and to date the 80% of gastric ulcers and 90% of non-cardia GC are recognized to be related to by *H. pylori*, identified as a class I carcinogen by World Health Organization [16–19]. This bacterium produces a variety of virulence factors, of which cytotoxin-associated gene A (CagA) and the vacuolating cytotoxin A (VacA) are the major pathogenic ones. CagA protein is internalized in the target cells via Type IV secretion system (T4SS) and undergoes tyrosine phosphorylation at its glutamate-proline-isoleucine-tyrosine-alanine (EPIYA) motif by Src family kinase. Once activated, it interacts with host proteins activating Ras, mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), nuclear factor kB (NF-kB) and β-catenin pathways, resulting in enhanced proliferation of gastric epithelial cells [20]. CagA also interacts with SH2-containing phosphatase (SHP-2), inducing cytoskeleton and morphological changes via inactivation of focal adhesion kinase (FAK), affecting tissue architecture and cell polarity, thus guiding the so-called “hummingbird phenotype”, involved in gastritis and carcinogenesis processes (Figure 6) [21] . VacA, once internalized by the host cells, is able to create cytosolic vesicles with characteristics of late endosomes/early lysosomes, which can be transferred to mitochondria causing dissipation of membrane potential and activation of pro-apoptotic factor Bcl-2 associated x protein (Bax) [22]. H. pylori provokes inflammatory responses in the gastric mucosa by inducing the expression of pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, IL-8, TNFα, NF-kB [23]. This sustained inflammation within the mucosal microenvironment often becomes chronic, guiding all the steps of gastric carcinogenesis.

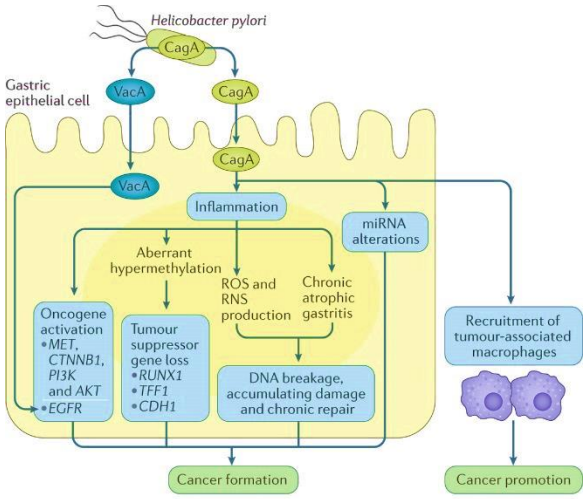

Figure 6. *Helicobacter pylori* internalization and effects in a stomach cell [19].

H. pylori infection is highly prevalent, with over half of the world’s population actually infected, and genomic analyses highlighted a long history of co-evolution with the human host, suggesting the interaction of the bacterium with host genetics and diet in enhancing gastric ulcers and cancer risk [6,24,25]. In fact, among infected individuals, approximately 10% develops peptic ulcer disease, 1–3% progresses to gastric cancer (GC), and 0.1% develops gastric mucosa-associated lymphoid tissue

(MALT) lymphoma [20]. Moreover, a large study demonstrated that GC risk was almost twice than expected in patients with gastric ulcers, while patients with duodenal ulcers had a 40% decreased risk for GC [26]. Of note, *H. pylori* infection increases the risk of non-cardia GC by promoting gastritis, but it is known to reduce acid secretion in the cardia of the stomach, thus preventing gastritis in the oesophagus and cardia. This results in the fact that this bacterium protects against cardia cancer [16].

Epstein-Barr virus (EBV). EBV infections are associated to 5-10% of GC, with higher incidence when co-infection with H- pylori is present; the role of EBV in the gastric carcinogenesis has still to be completely clarified [27,28].

Gastroesophageal reflux disease (GERD). GERD reported significant association with severe gastric atrophy and GC; moreover, incidence rates in reflux-related cardia cancer and oesophageal cancers are very similar, suggesting that these two malignancies show similar aetiology and pathophysiological processes [29,30].

Diet. the effects of intake of salt- and saturated fats-rich aliments, and known gastric carcinogens such as N-methyl-N-nitro-N-nitrosoguanidine (MNNG), are able to erode the mucosal barrier, contributing to inflammation processes [31]. On the contrary, carotenoids, folate, phytochemicals and vitamin C contained in fruits and vegetables are able to modulate xenobiotic metabolism, supplying antioxidants to prevent metabolic damage; a case control study highlighted that high intake of fruit and vegetables reduce of 37% the risk of GC [31].

Tobacco, alcohol consumption. Life habits of tobacco and alcohol consumption are proved to increase the risk of GC development; in particular, 11% of all GC are attributable to smoking, while heavy alcohol intake leads to mucosal erosion and gastritis, and has a positive correlation with GC incidence [6,32].

2.4 Gastric cancer classification

GC is a heterogeneous disease with different histological characteristics (phenotypes) and genotypes, often associated to tumor location, and this heterogeneity results in several classification systems proposed.

2.4.1 Anatomical classification

GC can be classified basing on tumor location. This classification system substantially identifies cardia (gastroesophageal junction) and non-cardia (stomach) tumors. These differs in terms of incidence, regional distribution and prognosis [33]. This classification follows the Siewert-Stein parameters, identifying cardia GC as type II or type III, depending on the epicentre of the tumor and the level of involvement of gastroesophageal junction, while type I malignancies extend in the oesophagus (Figure 7) [19,34]. More recently, in the TNM staging system introduced further parameters to classify cardia cancers, that consider both the epicentre of the tumor and where the tumor extends [35].

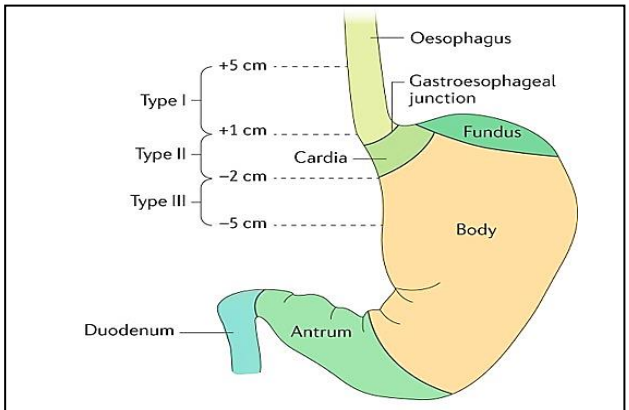

Figure 7. Gastric cancer classification by Siewert-Stein [34].

2.4.2 Histological classification

The first classification system based on histological characteristics was by Lauren. Later, two similar classification systems have been proposed by the Japanese Association for Gastric Cancer (JGCA) and the World Health Organization (WHO) [36,37]. The Lauren classification distinguishes intestinal subtype, diffuse type, and indeterminate or unclassifiable subtype; this classification is mainly based on the tissue architecture and glandular patterns [38]. While intestinal type tumors are moderate to differentiate tumors, with a glandular structure, not strictly related to a specific risk factor, diffuse type tumors are non-cohesive and poorly differentiated cancers, with no gland formation.

The WHO classification identifies five subtype, mainly depending on the histological patterns of the tumor, that is, tubular, papillary, mucinous, and poorly cohesive subtypes and rare variants. Tubular carcinomas are poorly differentiated tumors with low- to high-grade nuclear atypia, the papillary subtype presents finger-like processes of cuboidal or cylindrical cells; mucinous carcinomas are characterized for a 50% extracellular mucin, while poorly cohesive tumors present small aggregates or

alone cancer cells (*e.g.* signet-ring cancer cells) [36]. The two classification systems overlaps (i.e. tubular and papillary subtypes correspond to intestinal subtype, and poorly cohesive is associable to diffuse-type, and the WHO classification has been sometimes criticised for its complexity and the large number of subtypes identified, some of which are very rare [39], Table 2.

Table 2. Gastric cancer classification systems basing on histological characteristics (SRC: signet-ring cell carcinoma, NOS: not otherwise specified)

Lauren classification (1965)	**JGCA (2017)**	**WHO (2018)**
Intestinal type	Papillary adenocarcinoma Tubular, well differentiated Tubular, moderately differentiated	Papillary adenocarcinoma Tubular, well differentiated Tubular, moderately differentiated
Indeterminate type	Poorly differentiated (solid)	Tubular, poorly differentiated (solid)
Diffuse type	Signet ring cell carcinoma Poorly cohesive (non-solid)	Poorly cohesive, SRC Poorly cohesive, NOS
Intestinal/diffuse/indeterminate	Mucinous carcinoma	Mucinous carcinoma
Mixed type	Components description	Mixed carcinoma
Not defined	*Special types:* Adeno-squamous carcinoma Squamous cell carcinoma Hepatoid adenocarcinoma Carcinoma with lymphoid stroma Undifferentiated carcinoma Adenocarcinoma with enteroblastic differentiation Adenocarcinoma of fundic gland	*Histological variants:* Adeno-squamous carcinoma Squamous cell carcinoma Hepatoid adenocarcinoma Carcinoma with lymphoid stroma Undifferentiated carcinoma Adenocarcinoma with enteroblastic differentiation Adenocarcinoma of fundic gland Micropapillary adenocarcinoma

2.4.3 Molecular classification

In the last years, molecular approaches for gastric cancer classification have been attempted, especially relating cancer molecular features to histological phenotypes [40,41]. The most comprehensive genome-wide analysis, including DNA copy number alterations, mutations, mRNA, miRNA and protein patterns and alterations, was performed by The Cancer Genome Atlas (TCGA) research network. The results classified GC in four molecular distinct subtypes: 9% of EBV-positive (EBV+), 21% of microsatellite instable (MSI), 20% of genomically stable (GS) and 50% of chromosomal unstable (CIN) tumors [42]. Interestingly, these subtypes are often associated to tumor location: EBV+ tumors are mostly located in the fundus or body of the stomach, with 81% prevalence in men; CIN are

more frequently diagnosed in the gastroesophageal junction , while GS tumors are more often associated to diffuse type histology (Figure 8A). Another molecular classification by the Asian Cancer Research Group (ACRG) was performed basing on transcriptome molecular signature and clinically relevant features of 300 GCs. This classification identified MSI hypermutated tumors and microsatellite stable (MSS) tumors, with the latter divided into mesenchymal and epithelial subtype, which were further divided into MSS/TP53+ and MSS/TP53- (Figure 8B) [43]. MSI were often characterized by intestinal subtype, major location in the antrum, best prognosis and lower rates or recurrence. Among MSS subtypes, mesenchymal tumors displayed worse prognosis, epithelial TP53- showed intermediate and TP53+ showed worse patients' prognosis.

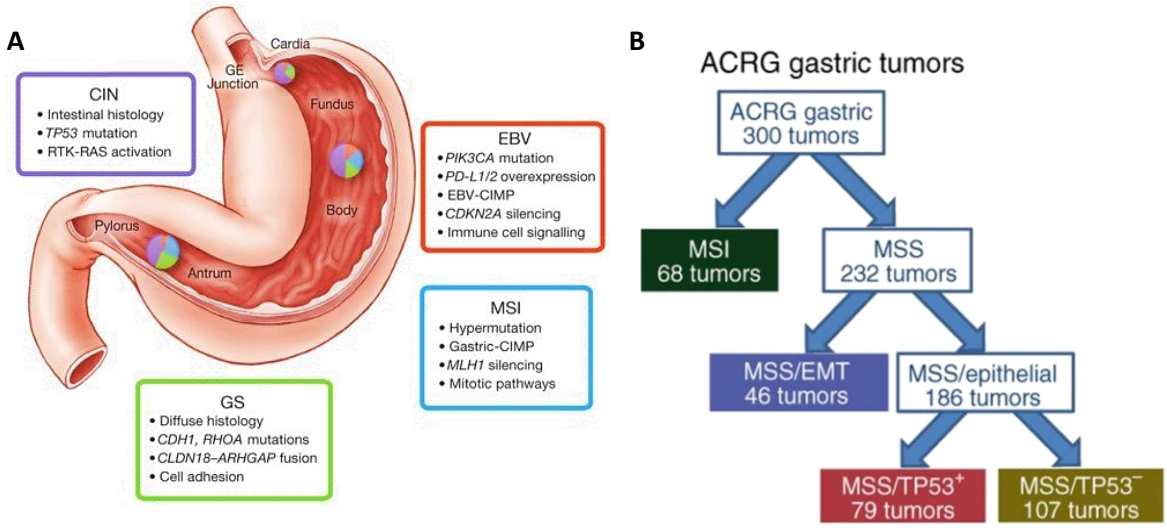

Figure 8. A. Tumor location basing on The Cancer Genome Atlas classification. **B.** Asian Cancer Research Group classification based on transcriptome profiling [42, 43].

Even though the different classification systems highlighted different molecular features of analysed tumors, the subgroups identified often overlap each other, and molecular characteristics are identified in the different histological subtypes previously identified (Figure 9B).

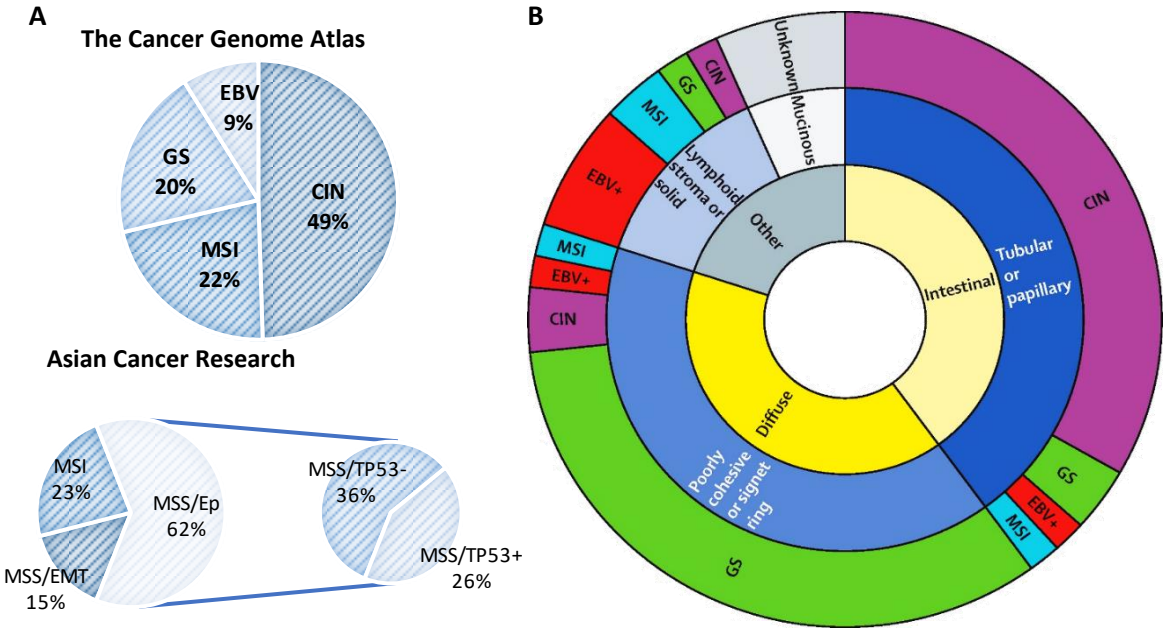

Figure 9. A. The Cancer Genome Atlas and Asian Cancer Research Group classification subgroups with incidence rates; **B.** Overlapping of histological and molecular subgroups [39].

2.5 Gastric cancer clinical management

Gastric cancer is a hard challenge for medical oncology, with more than one million new diagnoses and almost 800,000 deaths in 2020 [44]. Tumor heterogeneity remains a substantial obstacle for systemic therapies, and targeted therapies are available only for a few patients and current therapeutic treatments cannot face the pathological heterogeneity of the disease. In fact, the only approved targeted therapy as a first line treatment to date is trastuzumab for HER2+ patients. Treatment decision making is based on a multidisciplinary team including surgeons, medical and radiation oncologists, gastroenterologists, radiologists, dieticians and nurse specialists, and depends on staging and molecular features of the disease.

2.5.1 Clinical management of localised gastric cancer

When feasible, surgery is always recommended. Very early tumors (stage IA) could be removed with endoscopic or surgical intervention. For more advanced stages, as stages IB-III, radical gastrectomy is indicated, with >3 cm of proximal margin for tumors with an expansive growth patterns (as intestinal hystotypes) and ≥5 cm for tumors with infiltrative growth patterns (as poorly cohesive/diffuse phenotypes) [45,46]. The burden of nodal dissection is still a debated issue. D1 resection implies the

removal of peri-gastric lymph nodes and those along the left gastric artery; additional lymph nodes along the proper or common hepatic artery, splenic artery or coeliac axis are involved in D1+ and D2 lymphadenectomy. A D2 resection is recommended, but in Western countries it is suggested in specialised high-volumes centres [47]. Laparoscopic surgery has the potential benefit or reduced post-operative morbidity and recovery time, with lymph nodes resection comparable to open surgery. Moreover, robot-assisted laparotomy is an acceptable approach for gastrectomy, suggesting that gastric surgery will become minimally invasive, using rapidly developing robotic technologies [39].

In a German phase II-III trial, perioperative 5' fluorouracil (FU)-leucovorin-oxaliplatin-docetaxel (FLOT) regimen demonstrated benefit in overall survival (OS) in resected patients, and it is now recognised as the standard of therapy for patients fitting for a triple chemotherapy regimen [48]. Adjuvant chemotherapy has been larger accepted in Asia than in Europe, where the use of peri-operative chemotherapy is routinely used. Even though a large meta-analysis demonstrated a 6% absolute benefit in 5-years OS for 5-FU-based chemotherapy compared to surgery alone ($p<0.001$) [44], peri-operative approach is often preferred because it is better tolerated than adjuvant therapy, and it reaches tumor down-sizing, allowing for more curative resections [44]. Adjuvant chemo-radiotherapy seems not to add any benefit in PFS nor in OS, according to phase III CRITICS trial [49]. This result was also confirmed by ARTIST and ARTIST II trials, which enrolled patients undergoing gastrectomy with D2 lymphadenectomy [50,51]. Thus, adjuvant chemo-radiotherapy could be considered only in patients who did not receive a pre-operative cytotoxic agent or have not undergone an appropriate D2 lymphadenectomy [52].

Microsatellite instability (MSI) is the only recognized biomarker to be considered for localised GC, as it has prognostic implications. Patients undergone radical resection with MSI-high (MSI-H) tumors usually show a better prognosis with respect to non-MSI-H patients, with apparent no benefit reaching by chemotherapy addiction, even though patients receiving FLOT peri-operative regimen showed a better clinical response than those undergone platinum-5-FU in a small case series [53], suggesting that if tumor down-staging is necessary, FLOT is the regimen of choice. Society of Medical Oncology (ESMO) clinical practice guidelines for treatment of localised GC is shown in Figure 10.

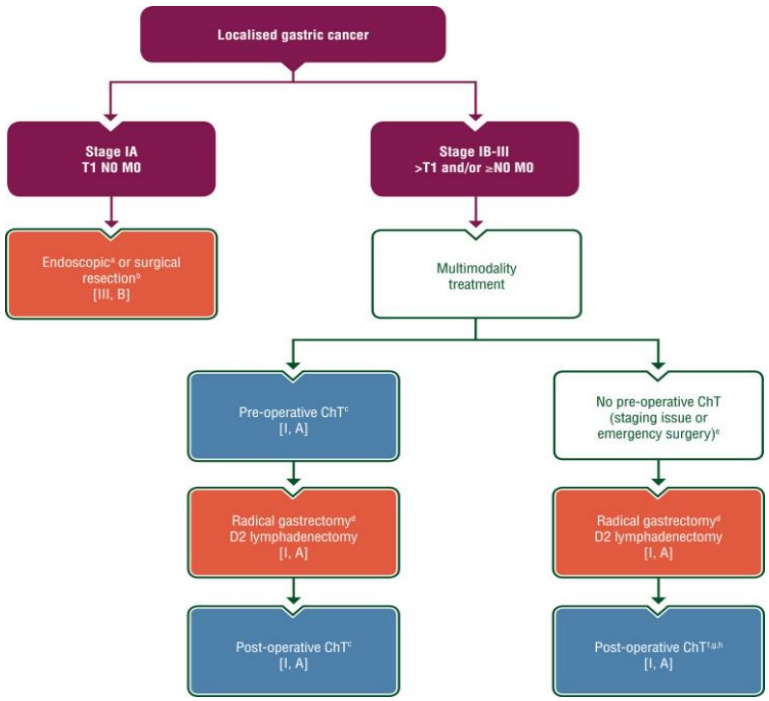

Figure 10. European Society of Medical Oncology (ESMO) Clinical Practice Guidelines for treatment of localised gastric cancer [44].

2.5.2 Clinical management of advanced and metastatic gastric cancer

Advanced disease has a very poor prognosis, usually <1 year in non-Asian countries [39]; on the other hand, recent improvements have reached a slight prolongation in patients' survival, especially in the field of immunotherapy and targeted therapy.

ESMO proposed algorithm for first-line therapy is shown in Figure 11A. Standard chemotherapy is a platinum-fluoropyrimidine doublet. In older patients, oxaliplatin has a better safety profile than cisplatin and should be preferred [54]. Fluoropyrimidines could be used as an infusion (5-FU) or as an oral solution (tegafur-gimeracil-oteracil (S-1), often used in Asia) [55]. The addiction of a taxane to a platinum doublet demonstrated better radiological responses rates but with increased toxicity [56]. A Japanese phase III trial compared cisplatin-S-1 to cisplatin-S-1-docetaxel, observing no differences in radiological response rate, PFS or OS, and an increased toxicity in the taxane-treated group, confirming that a triplet approach is not recommended as the standard [57]. Irinotecan-5FU could be used instead of cisplatin-5-FU for patients who do not tolerate platinum, as demonstrated prolonged time to treatment failure with respect to epirubicin-cisplatin-capecitabine (ECX) [58]. The ToGA trial demonstrated higher response rates and prolonged OS (HR 074; 95% CI 0.60-0.91, p<0.01) for

patients with Her-2 positive tumors (immunohistochemistry (IHC) 3+ or IHC 2+/FISH positive) treated with the addition of trastuzumab to standard chemotherapy [59]. Immunotherapy-based treatment is a feasible option as a first-line therapy. The phase trial III CheckMate 649 demonstrated that the addition of nivolumab to chemotherapy improved OS (HR 0.71; 98.4% CI 0.59-0.86; p<0.001) and PFS (HR 0.68; 98% CI 0.56-0.81; p<0.001) versus chemotherapy alone in patients with a programmed death-ligand 1 (PD-L1) combined positive score (CPS) ≥5 [60]. The phase III trial Keynote-062, pembrolizumab monotherapy demonstrated non-inferiority in OS compared to chemotherapy in patients with CPS≥1, even though associated with inferior PFS [61]; pembrolizumab is approved for patients with CPS score ≥10, basing on the results of the phase III Keynote-590 [62]. Anti-Programmed death-1 (PD-1) monotherapy treatment increases response rates and long-term survival in patients with MSI-H [63]. The phase III REGATTA trial demonstrated that gastrectomy added to chemotherapy without resection of metastases for patients with oligometastatic disease did not improve survival with respect to chemotherapy alone [64], while the phase II AIO-FLOT3 demonstrated favourable outcomes for oligometastatic disease patients undergoing FLOT induction followed by gastrectomy with resection of metastases [65]. New landscapes are represented by the addition of hyperthermic intraperitoneal chemotherapy (HIPEC) to cytoreduction in patients with limited peritoneal carcinomatosis, which improved clinical outcomes, and by pressurised intraperitoneal aerosol chemotherapy (PIPAC), often performed during laparoscopic intervention, that could represent a strategy for patients with unresectable peritoneal disease [66,67].

In second-line and later-line treatments (ESMO proposed algorithm is shown in Figure 11B), the standard chemotherapy options are paclitaxel, docetaxel and irinotecan, which present similar efficacies but different toxicity profiles [68].

The anti-vascular endothelial growth factor receptor 2 (VEGFR2) antibody, ramucirumab, improves ORR, PFS and OS when added to chemotherapy, as demonstrated by RAINBOW trial [69], reaching clinical benefit only in survival as a monotherapy in the phase III REGARD trial [70]. In the phase III Keynote-061 trial, pembrolizumab monotherapy demonstrated improvement in survival only for subgroup of patients with MSI-H [71], while in the phase II Keynote-158 trial pembrolizumab monotherapy demonstrated a 45.8% ORR and a mPFS of 11 months in pre-treated MSI-H patients, with a mOS still not reached [72], therefore this treatment is the standard of care for this subgroup of patients. Even though the addition of lapatinib and trastuzumab emtansine had negative results in patients progressed to trastuzumab, the HER-2 antibody conjugate, trastuzumab deruxtecan, demonstrated a survival benefit and better response rates in the same patients setting compared to chemotherapy alone [39,73]. In the third-line setting, the phase III TAGS trial established the trifluridine-

tipiracil as the standard of care, with the possibility to administrate a taxa ne or irinotecan to chemo-refractory patients [68,74].

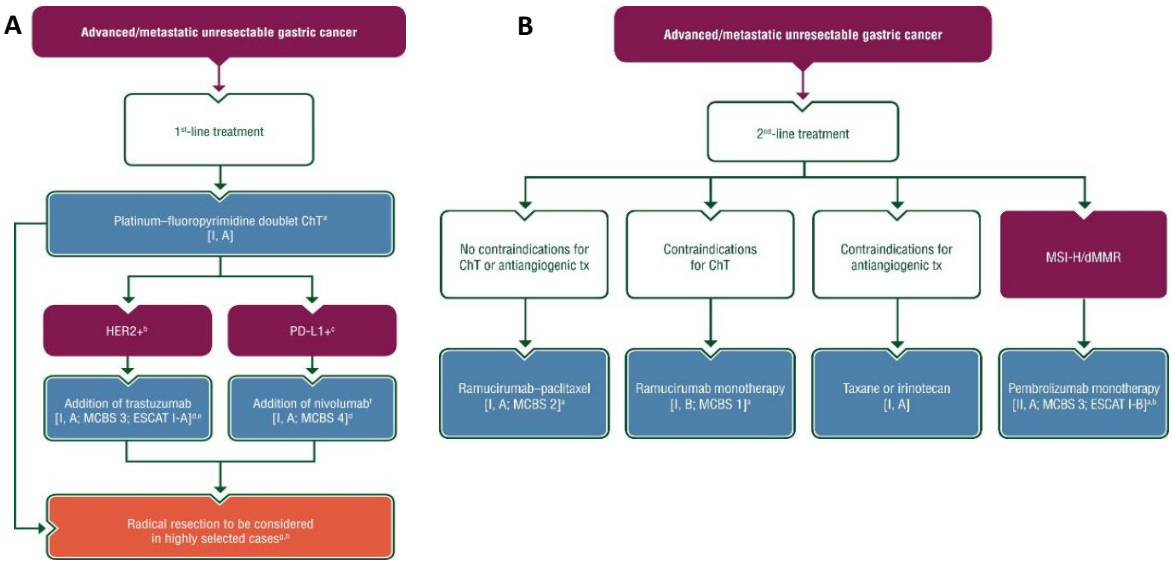

Figure 11. European Society of Medical Oncology (ESMO) Clinical Practice Guidelines of first-line **(A)** and second-line **(B)** for treatment of advanced gastric cancer [44].

3. Epigenetic mechanisms

The term "epigenetic" was historically referred to as events that could not be explained by genetic principles. The term and its first definition was coined by Conrad Waddington in 1942, who defined epigenetics as "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being" [75]. Epigenetics could be intended as a bridge between genotype and phenotype, influencing the final outcome of a genic locus without changing its DNA sequence. Epigenetic mechanisms are at the basis of many cellular processes, such as cellular differentiation and disease, and consist in a plethora of covalent and non-covalent modifications to DNA, histone proteins and mRNA [75]. All epigenetic modifications resume the "epigenome", literally what stands above the genome. Epigenomic marks are recognized to be responsive to environment, and there is a lot of interest in their potential role as non-genetic risk factors for disease, and targets for drug development, such as histone deacetylase and DNA methyltransferase inhibitors [76].

Among the different epigenetic changes there are: chromatin remodeling, which ensures genome packaging and unpackaging, thus controlling accessibility to DNA regulatory elements and regulating gene transcription, DNA replication, repair and recombination. Four families of chromatin remodeling

complexes were identified, including switching defective/sucrose non-fermenting SWI/SNF) family, imitation switch (ISWI) family, chromodomain, helicase, DNA binding (CHD) family, and Inositol requiring 80 (INO80) family.

(Non-coding RNAs, representing over about 98% of genetic transcripts, are small and long non-coding RNAs operate post-transcriptional modifications to messenger RNA, altering its translation thus playing an important role in regulating gene expression [77].

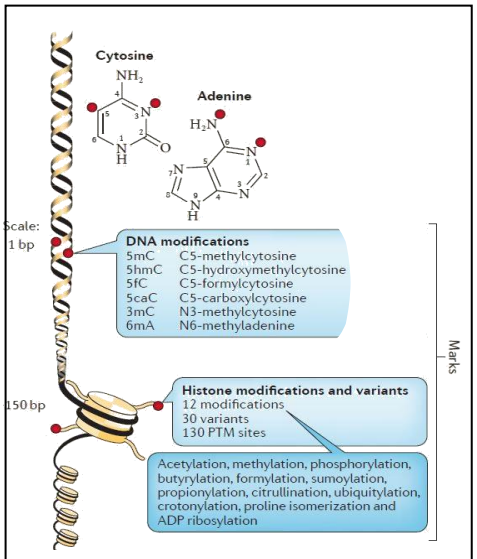

Figure 12. Main epigenetic DNA and histone modifications [76].

DNA methylation, which occur predominantly in specific gene regions including promoters, intra and intergenic enhancers, exons and introns, in particular in the so called CpG islands, that are sequences that contain a high frequency of CG dinucleotide repeats [78,79]. DNA methylation is performed by DNA methyltransferases (DNMTs) enzymes, which are able to de novo methylate or maintain DNA in methylated form. DNA methylations is mainly reversed by ten-eleven translocation (TET) enzymes, leading to cytosine stepwise modifications, *i.e.* hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5- carboxylcytosine (5caC) [79]. DNA methylation can affect also repetitive genomic regions (*e.g.* Alu sequences, LINE, satellite DNA, centromeric and epicentromeric tandem repeats). The hypomethylation of such regions can activate the aberrant expression of some proto-oncogenes and the reactivation of transposable elements and retroviruses, inducing chromosome instability and gene activation. Hypermethylation of normally unmethylated promoter CpG islands or the global hypomethylation of DNA may be considered a hallmark of cancer.

Histones are evolutionary highly conserved basic proteins, organized in octamers to wrap DNA into nucleosomes. Covalent modifications at the tails of the four core histones (H2A, H2B, H3 and H4) are able to change the interactions of these alkaline proteins with DNA, influencing chromatin architecture, thus affecting various pathophysiological cellular functions such as carcinogenesis, inflammation and epithelial-mesenchymal transition. Histones are subjects to methylation, acetylation, phosphorylation, ribosylation, ubiquitination and sumoylation, for a 130 post-transcriptional modifications (PTM) [76] (Figure 12). Histone acetylation results from a dynamic balance between the activities histone acetyltransferases (HATs) and histone deacetylases (HDACs). In general, histone acetylation is associated with gene activation. HATs and HDACs not only target histone proteins, but they can also interact with non-histone proteins, including transcription factors and proteins involved

in DNA repair, cellular signalling, metabolism, cytoskeletal dynamics, apoptosis, nuclear import, and protein folding [80]. HDACs are implicated in various functions other than gene expression control, including protein stability, protein translocation, enzymatic activity, protein-protein interaction, and DNA binding affinity via acetylation of non-histone proteins. They can also regulate gene transcription by deacetylating other epigenetic proteins such as DNMTs and HATs [81]. Histone methylation is regulated by writers (Histone Methyl Transferases: HMTs), readers (Histone Methylation Recognizing Proteins), and erasers (Histone Demethylases: HDMs). It can occur at arginine (R) and lysine (K) residues and it is catalyzed by HMTs (KMTs or RMTs). K residues can be mono-, di- or tri-methylated, while R residues can only be mono- or di-methylated [81]. Histone methylation can promote or inhibit gene expression. For instance, methylation of histone H3K4, H3K36, and H3K79 is associated with transcriptional activation, while di- and tri-methylation of H3K9 and H3K27 are associated with transcriptional repression [82]. Histone demethylation is catalyzed by two HDMs; amine oxidase type lysine-specific demethylases (LSDs or KDM) that remove methyl group only from mono- and di-methylated lysines, and JumonjiC (JMJC)-domain- containing histone demethylases that demethylate the three-methyl states in lysine and arginine residues [82].

3.1 Epigenetics of Gastric Cancer

Alterations in epigenetic mechanisms are recognized to be both as early- and advanced stage events in GC, and epigenetic perturbations seem to precede genetic instability during the tumor transformation [83,84]. As epigenetic mechanisms are influenced by environmental factors, agents like diet, age, smoking habits, chronic inflammation consequent to *H. pylori* and EBV infections, they are crucial in remodelling gastric epigenetic machinery, paving the way for gastritis and ulcer development until metaplasia, dysplasia, and tumor development [83]. In particular, pathogens invade host cells and cause epigenetic changes, such as DNA methylation, making it a safer environment for themselves. This allows the infection to persist and promotes the development of GC [85].

Although eliminating HP significantly decreases methylation of tumor suppressor genes, DNA methylation does not return to the same level as that in individuals who are never infected so that individuals with ongoing presence of aberrant DNA methylation would face a higher risk of GC even after the eradication of HP [86]. EBV infection directly induces hypermethylation in both the viral genome and the host genome and give rise to poorly differentiated cancer tissue due to loss of function in critical tumor suppressor genes and cell cycle regulation genes in differentiation [82].

Recently, epigenetic mechanisms have been proposed as markers for new GC classifications, identifying profiles to predict patients prognosis and risk of metastatic behaviour of GC [87–89]. These investigations reflect the need to resolve cancer heterogeneity, especially considering that current

treatments for GC are suboptimal and are not able to face tumor heterogeneity, creating the opportunity to design new treatment strategies. Principal epigenetic marks found in GC are resumed in Figure 13.

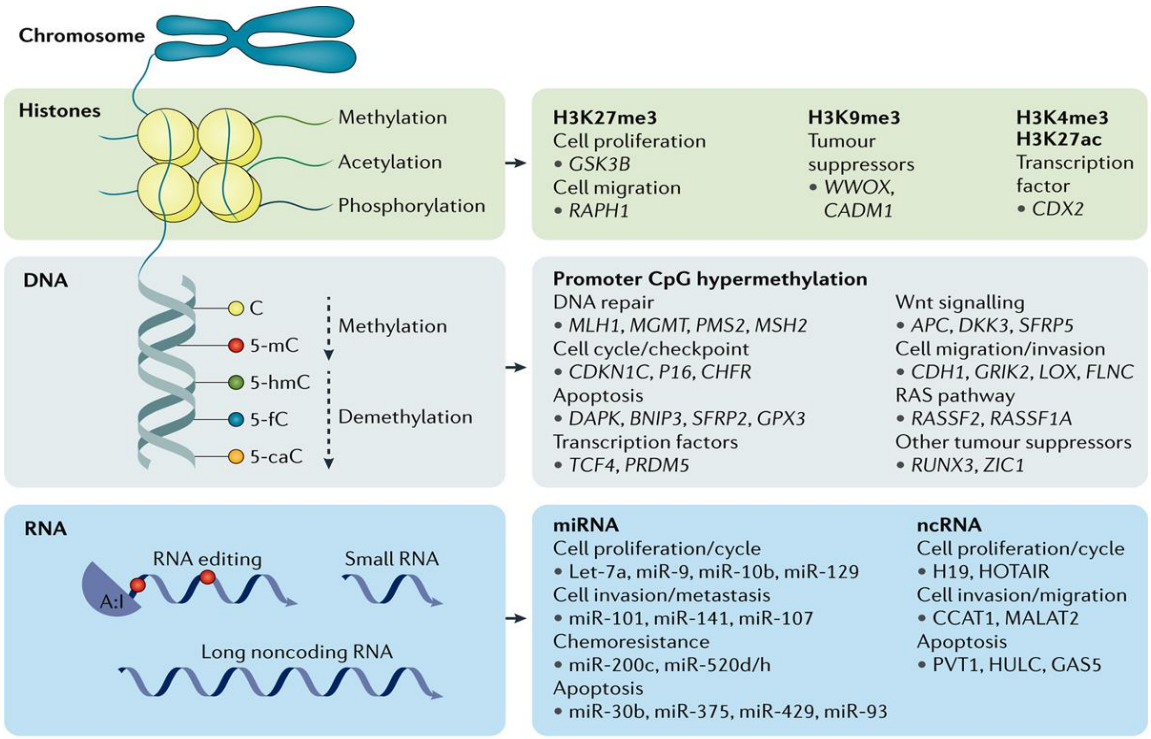

Figure 13. Main epigenetic marks highlighted in gastric cancer [89].

3.1.1 DNA methylation

As other malignancies, GCs are characterized by a global DNA hypomethylation, associated to global transcription and proto-oncogene activation, and a focal hypermethylation, responsible for turning off tumor suppressor genes. DNA methylation is an active process highlighted in GC tumorigenesis, and different DNA methylation patterns have been highlighted in the four TCGA subgroups, identified as CpG island methylator phenotype (CIMP) subgroups, *i.e.* EBV positive and MSI subtypes (Figure 14). Accordingly to Matsusaka' classification GC can be divided into extremely high-methylated epigenome type which overlaps with the EBV+ class of TCGA classification; high-methylated epigenome type, which overlaps with the microsatellite instable (MSI) class and low-methylated epigenome type, corresponding to GS and CIN tumors [90]. Interestingly, it has been recently found a new unique subtype with extremely low methylation characterised by poor prognosis that is not associated with inflammation-associated elevation of DNA methylation levels [91].

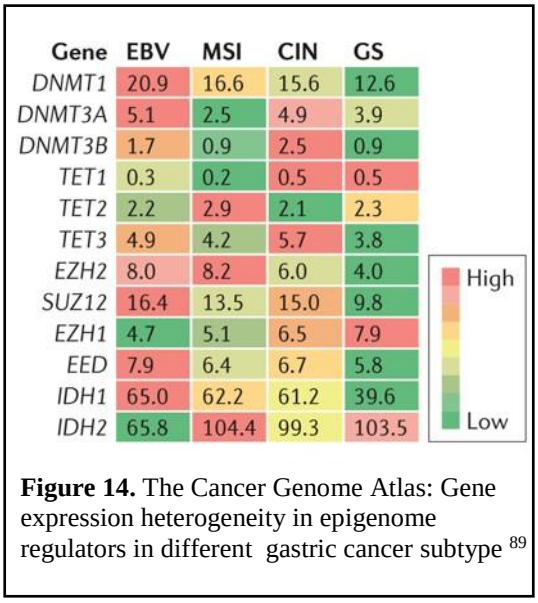

Figure 14. The Cancer Genome Atlas: Gene expression heterogeneity in epigenome regulators in different gastric cancer subtype [89]

Aberrant DNA hypermethylation usually happens in the promoter of tumor suppressor genes in GC like p16, RASSF1A and hMLH1, resulting in gene silencing. In addition, hypomethylation at gene body regions impact on cancer progression and are associated with shortened survival time in patients with advanced gastric cancer [92,93]. Promoter hypermethylation, loss of heterozygosity (LOH) and somatic alterations are responsible for loss of oncosuppressor CDH1, the encoding gene for E-cadherin, triggering tumorigenic pathways such as β-catenin and Wnt-, EGFR-, and mTOR signalling [94,95]. Loss of *CDH1* is common to both intestinal and diffuse GC, and *CDH1* germline mutations are recognized to be the genetic cause of hereditary diffuse GC, as they are present in the 50% of cases, and usually act as a second hit after genomic alterations to definitively silence the gene [96]. Loss of CDH1 gene also has clinical implications, as it is strictly related to H. pylori infection, and predicts worse OS and Disease-Free Survival (DFS) of patients [95,97]. Gastric tumor suppressor frequently silenced through promoter methylation in GC are RUNX3 and RASSF1A [98,99], such as p16, a tumor suppressor involved in cell cycle regulation, for which methylation of its encoding gene CDKN2A is associated with *H. pylori*, EBV infections, and pre-cancerous lesions [100].

On the other hand, several proto-oncogenes are epigenetically activated by promoters hypomethylation, such as the component of RAS pathway HRAS, and c-MYC [101].

3.1.2 Histone modifications

A plethora of covalent modifications to core histones tails influence gene expression by changing chromatin accessibility to RNA polymerase II and transcription factors [102].

Histone methylation, mainly occurring by mono- (me1), di- (me2) and tri-methylation (me3) to lysine residues, is a reversible epigenetic mechanism promoted by histone methyltransferase (HMTs) and reverted by histone demethylases (HDMs). Histone methylation could favour or repress gene expression, depending on the specific amino acid residue and the number of methyl groups bound [82]. Deregulation in histone modification has been linked to gastric carcinogenesis and tumor progression, *e.g.* H3K9me3 expression has been associated to T stage and gastric cancer recurrence and worse prognosis [103]. Histone methylation alterations are associated to tumor growth and metastasis, and

worse prognosis in GC patients [104,105]. EHMT2 (G9a) is able to promote methylation at H3K9 and H3K27, and has been associated to carcinogenesis in several malignancies, including GC. It is associated to TNM staging, lymphatic invasion and prognosis of GC patients [82]. G9a is able to interact or be recruited to form transcriptional regulatory complexes with DNA methylatransferases and histone deacetylases (HDACs) [106].

Histone acetyltransferases (HATs) and HDACs are responsible for reversible and balanced acetylation/deacetylation of histone tails, mainly occurring at lysine residues; acetylation favours the euchromatin conformation and gene expression activation; this epigenetic mechanism has been highlighted in several cancer histologies, including GC [107]. HDACs are a family of four classes of proteins (I-IV) depending of preferred cellular localization [108]. In GC, global hypoacetylation has been associated to HDAC class I, which also correlate with TNM stage and lower OS [82].

4. Clinical and translational significance

In the last years, epigenetic mechanisms have been investigated as therapeutic targets in several malignancies, also in GC. Many epigenetic drugs are nowadays under development to reprogram the epigenome of GC, in order to ensure the control of tumor growth, block invasion, and metastasis, and treat tumor resistance [109,110]. The clinical benefit of epigenetic drugs in treating solid tumours is still emerging, but their use at low dosage for epigenetic priming in combination with other therapies seems to be the best promising approach [109,111]. In fact, epi-drugs have been demonstrated to enhance the activity of anticancer therapies such as radiotherapy, cytotoxic chemotherapy, DNA damage repair-targeting therapies, hormonotherapy, targeted therapies and immunotherapies leading to sensitization to treatment and/or reversal of resistance [112]. DNMT inhibitors (DNMTi) and HDAC inhibitors (HDACi), synergise with genotoxic and/or cytotoxic therapies by increasing DNA damage and disruption of the DNA damage response (DDR) pathway. Treatment of cancer cells with enhancer of zeste homologue 2 inhibitors (EZH2i), lysine-specific histone demethylase 1A inhibitors (LSD1i), or DNMTi favours chemokine-dependent T cell attraction and stimulate type I interferon response. Epi-drugs can also modulate immune cell transcriptional programmes, elicit functional alterations in regulatory T cells, enhance the cytotoxicity of effector T cells, decrease myeloid-derived suppressor cell activity and favour a M1-like macrophage-mediated antitumour immune response [112]. Despite numerous preclinical studies, these therapies have not reached clinical practice in solid malignancies yet, except for some few epigenetic drugs that are approved by Food and Drug Administration (FDA) [111]. The use of epigenetic agents on gastric cancer has been deeply studied in preclinical models and their effect on the reduction of cell growth and invasiveness on CIMP cell lines has been demonstrated

[113,114]. On the other hand, still issues are present about which drug concentration and which treatment schedule could be the most effective for a right translation into humans. In fact, only a single phase I study has been completed on the use of demethylating agents for epigenetic priming before the standard neoadjuvant chemotherapy in resectable gastric cancers [115]. Other epigenetic inhibitors, particularly HDACi and HMTi, have been or are being tested in preclinical and phase I settings in gastric cancer. Used as a single agent or in combination with other therapies, either in in vitro and in vivo models, they have been shown to decrease cancer cell proliferation and migration, trigger apoptosis, re-establish tumor suppressor gene expression and act synergically with chemotherapy in cytotoxic response [116–118].

In a phase II clinical trial combining the same epigenetic drug with capecitabine–cisplatin in advanced GC patients an objective response rate of 42% was reached, not appearing to improve the clinical outcome of patients, and with a considerable rate of grade 3–4 adverse events [119].

Finally, the combination of immune checkpoint inhibitors (ICIs) with epigenetic agents are starting to show considerable synergy in *in vivo* models of cancer [120]. Moreover, clinicals trial to test a combination regimen of ICIs along with epigenetic agents will be of particular interest in the future, in particular in PDL1-negative patients, and there even combining epi-drugs could be a walkable option in the future (Figure 15).

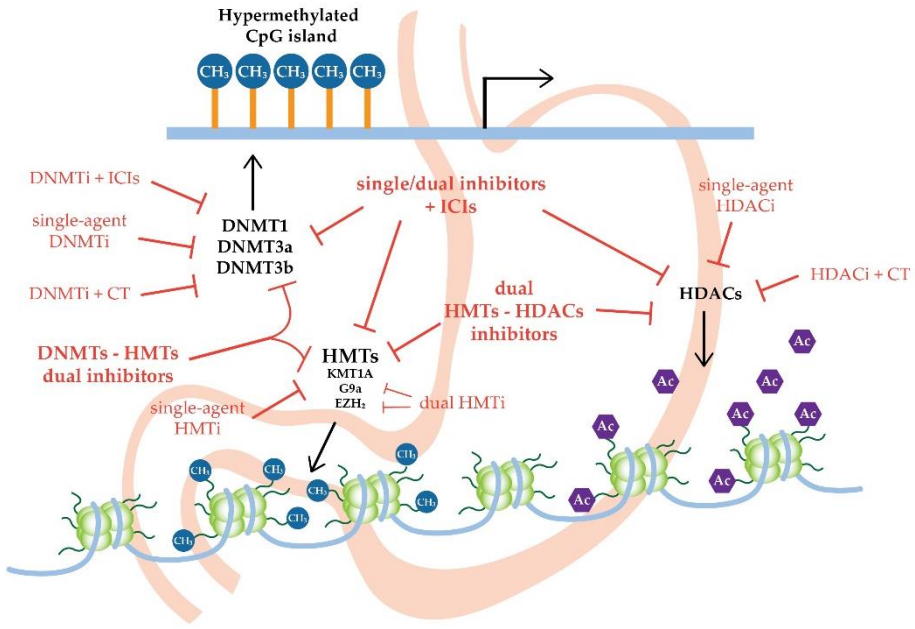

Figure 15. Promising new epigenetic strategies for gastric cancer treatment. CT: chemotherapy; DNMTs: DNA methyltransferases; DNMTi: DNA methyltransferase inhibitor; HDACs: histone deacetylases; HDACi: histone deacetylase inhibitor; HMTs: histone methyltransferases; HMTi: histone methyltransferase inhibitor; ICIs: immune-checkpoint inhibitors [82].

5. Study aims

Gastric carcinoma is characterized by a wide heterogeneity at the histopathological, onset location and molecular levels, resulting in a complex scenario for patients' clinical management and prognosis. Current treatment algorithms are not able to face this medical challenge, creating the need for new therapeutic approaches. In fact, precision medicine is a passable option only for a subgroup of selected patients, and immunotherapy, even though brought a revolution in patients' clinical management, lacks of predictive biomarkers. GC is defined by remarkable epigenetic profiles, which play active roles in carcinogenesis until advanced disease. Targeting epigenetic mechanisms could be therefore a new approach for GC treatment, and several studies are focusing on new epigenetic therapies for gastrointestinal diseases [110]. Despite this, to date no epigenetic therapies are available for GC clinical management.

Recently, at the Centro de Investigación Médica Aplicada (CIMA) in Pamplona, Spain, an active compound with epigenetic activity has been synthetized, namely CM-272, which is a potent and dual reversible inhibitor of G9a and DNMT1, and of their adaptor UHRF1 (Figure 16) [121]. This molecule already reached important results in haematological malignancies and gastrointestinal disease, such as hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) pre-clinical models [121–123]. In fact, it has been demonstrated that G9a, DNMT1 and UHRF1 were upregulated in HCC and CCA tissues with respect to non-tumoral tissues, and that pharmacological targeting with CM-272 had strong anti-tumoral effect both *in-vitro* and *in-vivo* [122].

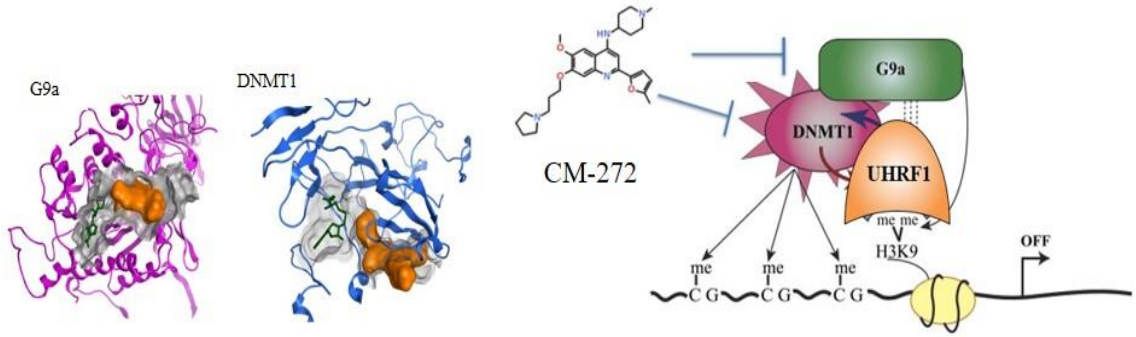

Figure 16. A Plausible binding models proposed by docking studies using known crystal structures (3RJW.pdb for G9a and 4DA4.pdb for DNMT1) for CM-272. B. Dual targeting of G9a and DNMT1 by CM-272 compound [118].

The aim of this project is to evaluate the potential of CM-272 in the treatment of pre-clinical models (in-vivo and in-vitro) of GC. Moreover, a case series of GC patients has been analysed, to assess the expression of the targets of this compound in GC tissue, with the aim to confirm the rationale for a

pharmacologically inhibition of these specific epigenetic molecules. The expression of G9a, DNMT1 and UHRF1 has been assessed also in relation to clinical outcome of patients and to cancer histological type and features, to assess whether specific epigenetic patterns are more present in specific histological subtypes of GC.

For *in-vitro* evaluation of the therapeutic potential of CM-272, a panel of GC cell lines has been considered. In particular, cell lines with a known molecular profile, and representative for the different cancer histologies, have been selected. CM-272 molecule has been tested alone and in combination with standard cytotoxic agents used in the clinical practice for GC management, comparing its efficacy with respect to other epigenetic compounds (*i.e.* azacitidine as anti-DNMT1 and BIX-01294 as anti-G9a). Several doses and treatment schedules of these drugs have been tested, to establish which dose of single agent/combination of agents is the most effective.

The mechanism of action and the pharmacological profile of CM-272 has been investigated at cellular and molecular levels, to better elucidate which epigenetic patterns stand at the basis of GC cells, and how this compound is able to reprogram and remodel cellular epigenetic machinery and gene expression.

Aim of the project was also to verify the CM-272 activity using *in-vivo* xenograft murine models of GC, alone or in combination with cytotoxic agents, with the best treatment schedule highlighted *in-vitro*.

This project has been conducted through an active research network involving several research Institutes, such as the Istituto Romagnolo per lo Studio dei Tumori (IRST) “Dino Amadori”, the University of Bologna and the San Raffaele Institute in Italy and the Centro de Investigación Médica Aplicada (CIMA) in Spain

The expected results of this study were to provide the preclinical rationale to test epigenetic drugs and inhibitors in GC patients, and to better understand the biology of the epigenetic pathways related to carcinogenesis and cancer development.

6. Materials and methods

6.1 Patients case series

A total of 67 patients with a diagnosis of GC were enrolled at the Medical Oncology Units of the Romagna catchment area (Area Vasta Romagna, AVR). Different stages of disease and subtypes were considered, and all patients underwent surgical intervention for GC. Patients characteristics were retrieved using medical and radiographic records including age at diagnosis, gender, histology, and clinical follow-up information.

Patients tissue samples were retrieved by the Pathology Units of AVR. For each patient, 5 unstained formalin-fixed paraffin embedded (FFPE) sections of 4μM were retrieved for both cancerous and matched non-cancerous tissues, together with a hematoxylin eosin slide for tumor selection by an expert pathologist. The study was approved by Ethic Committee of Romagna (CEROM), study code IRST-B121.

6.2 Immunostaining

G9a, DNMT1 and UHRF1 expression on cancer tissues were assessed by immunohistochemistry (IHC) on FFPE slides. All analyses were performed on a Ventana BenchMark Ultra platform using Optiview DAB detection kit (Ventana Medical Systems Inc, Tucson, AZ, USA). Sections were stained with pre-diluted antibodies from Abcam (Cambridge, UK): G9a (EPR18894, dilution 1:200), DNMT1 (EPR18453, dilution 1:100) and UHRF1 (EPR18803, dilution 1:100) and counterstained with Hemoatoxylin II (Ventana Medical Systems). Immunohistochemical scores were evaluated in blind by an expert pathologist, considering the following expression cut-off: ≤10%: negative, >10%: positive. Stained slides were scanned by Aperio CS2 platform and images analysed with Aperio Imagescope software (Leica, Wetzlar, DE).

For assessment of senescence status of GC cell lines, the senescence β-galactosidase staining kit (Cell Signaling Technology, Danvers, MA, USA) was used, according to the manufacturer's instructions. Briefly, GC cells at a concentration $5x10^3$/1ml/well in a 24 well plate were seeded and treated or not for 72 hours with relative IC_{50} dose of CM-272 (see below). Cells were then fixed in 1X fixative solution for 15 minutes at room temperature and stained overnight at 37°C in absence of CO_2 with β-galactosidase solution at pH 6.0. Fixed cells were washed with PBS 1X and images were acquired using an inverted Olympus IX51 microscope (Olympus Corporation, Tokyo, Japan) at a 20X magnification, equipped with a Nikon Digital Sight DS-Vi1 camera (CCD vision sensor, square pixels of 4.4 μM side length, 1600 × 1200-pixel resolution, 8-bit grey level) (Nikon Instruments, Tokyo, Japan). To assess the senescence status, for each experimental condition, 4 fields containing at least 100 cells were taken into consideration. Stained senescent cells were counted for each field, and

percentage was annoted. The average value of the four fields was established to assess the senescent in every experimental condition, and Student's t-test has been performed to assess statistical significance among experimental conditions. The experiment has been repeated three times, and each experimental condition has been tested in triplicate.

6.3 Gastric cancer cell lines

Several GC commercially available cell lines were used. In particular, cell lines from GC (AGS, YCC-1, YCC-2, SNU-1, SNU-5, KATOIII) and distal oesophageal carcinoma (KYAE-1, FLO-1) were used. Cells were maintained in F12-K with 10% foetal bovine serum (FBS) (AGS cell line), RPMI 1640 with 10% FBS (SNU-1, KYAE-1, YCC-1, YCC-2 cell lines), IMDM with 20% FBS (KATO III, SNU-5 cell lines), DMEM with 10% FBS (FLO-1 cell line), all supplemented with 1% penicillin-streptomycin, and cultured at 37°C in a 5% CO_2 atmosphere.

6.3.1 Tumor cells growth assay

Tumor cells growth was assessed by MTT assay. Briefly, 3000 cells/100 µL/well were seeded in a 96-well plate. Cells were treated with CM-272, cisplatin, azacitidine, BIX-01294 or 5-fluorouracil the day after seeding. All compounds were dissolved in the growth medium of each cell lines, and aliquots for treatment were prepared the same day of treatment. After 72 hours of exposures to compounds, cells were incubated with 0.5 mg/ml of MTT solution (Sigma Aldrich, Darmstadt, Germany) dissolved in phosphate buffered saline (PBS) 1X, pH 7.4, for 2 hours at 37°C. Absorbance was determined at 550 nm using a Synergy H1 plate reader (Biotek, Agilent, Santa Clara, CA, USA).

For each cell line, half maximal inhibitory concentration (IC_{50}) was calculated through serial dilutions of drugs, *i.e.* CM-272 (from 1 nM to 10 µM), azacitidine (from 10 nM to 100 µM), BIX-0194 (from 1 nM to 50 µM), cisplatin (from 3.32 mM to 332 nM) and 5-fluorouracil (from 10 nM to 100 µM). All doses for drug administrations, including drug combinations, were arbitrary selected basing on IC_{50} doses, considered as reference.

For drug combination assays, cells were seeded in a 96-well plate and treated for 72h with contemporary administration of two or more drugs, at different concentrations. For epi-sensibilization assays, cells were seeded and pre-treated for 24 or 48 hours with the epigenetic compound, and then treated with only cytotoxic compound for 72 hours.

All experiments were conducted in triplicate, and all analyses and graphics were performed using GraphPad Software v.8.4.3 (GraphPad Software Inc., La Jolla, CA, USA). For drugs synergism assessment, CompuSyn software v. 1.0 (ComboSyn INC, Paramus, NJ, USA) was used.

6.4 Apoptosis and cell cycle assays

Induction of apoptosis was assessed by AttuneNext cytometer (ThermoFisher, Waltham, MA, USA) with the Annexin V Apoptosis Detection kit (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. GC cell lines were seeded $3x10^5$ cells/well/2.5ml in a 6-well plate and treated with CM-272 for 72 hours at relative IC_{50} concentrations. The experiments were performed in triplicate, in at least three independent experiments. For caspase 3 and 7 activation assessment, the Caspase-Glo 3/7 assay system (Promega Corporation, Madison, WI, USA) was used, according to manufacturer instruction. Briefly, cells were seeded in at a concentration of $3x10^3$ cells/well/100µl a 96-wells plate compatible with luminometer and treated with the compounds. The Caspase-Glo 3/7 substrate was added to its specific buffer and then spotted to the wells. After mixing and incubating for 30 minutes, luminescence was assessed at 700nm by a Synergy H1 plate reader (Biotek, Agilent, Santa Clara, CA, USA).

For cell cycle analysis, the same cell lines were seeded and treated at the same drug concentrations. The effect of CM-272 on cell cycle was evaluated using propidium iodide staining mix (Sigma-Aldrich, St.Louis, MO, USA). Quantitative analyses were performed using FlowJo software (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

6.5 RNA-Sequencing

6.5.1 RNA extraction

For RNA extraction, cells were seeded $1x10^6$ in 10 ml in a Petri dish. The day after cells were treated with three different doses of CM-272 (around IC_{50}) for 72 hours. After treatment, cells were counted, and RNA was extracted using RNeasy mini kit (Qiagen, Hilden, Germany), using manufacturer's instructions. RNA yields were assessed with Qubit 3 instrument using RNA High Sensitivity kit (Waltham, MA, USA). All experimental conditions were performed in triplicate.

6.5.2 RNA library preparation

RNA libraries were performed starting from 900 ng of purified total RNA, using the Illumina Stranded Total RNA Prep with Ribo-Zero Plus (Illumina, San Diego, CA, USA) on an automatic Hamilton STARlet platform (Reno, NV, USA). Briefly, total RNA was depleted, and total RNA was fragmented and denatured. The cDNA two strands were synthetized in two separated steps, followed by adenylation at 3' ends and anchors ligation. Generated fragments were cleaned up and libraries were generated by amplification. After a clean up step, libraries quantity and quality were assessed on Qubit

instrument and BioAnalyzer 2100 (Santa Clara, CA, USA). All libraries were sequenced on a single run on a NovaSeq 6000 platform (Illumina) with a NovaSeq 6000 S2 Reagent Kit v1.5, and two steps of 101 cycles of paired-end sequencing were performed.

6.5.3 Reads alignment and bioinformatics

Transcript-level read count was performed with kallisto v0.46.2, then raw counts were collapsed to gene-level with tximport v1.12.1, then Differential Expression Analysis (DEA) was performed with DESeq v1.22.1 [124–126]. The same software was used to produce PCA plots, while for heatmaps and hierarchical clustering seaborn v0.12.1 was used. Gene Set Enrichment Analysis (GSEA) was performed with the package GSEApy v0.9.16 [127–129]. For DEA and GSEA analyses, the counts for the three different CM-272 doses were compared among the three technical replicates for each experimental condition. For QC analyses, DESeq2's Variance Stabilizing Transformation (VST) was applied to raw read counts, and the 500 genes with highest variance across the dataset were selected to perform PCA and hierarchical clustering; VST-transformed counts were also used as input for GSEA, after removing genes < 1 across all samples. GSEA were analysed using three different genomic repositories, *i.e.* Kyoto Encyclopedia of Genes and Genomes (KEGG) Human Pathway Database v. 2019, Reactome v. 2016 and Gene Ontology (GO) Biological Process, v. 2018.

Full workflow for RNA Sequencing is shown in Figure 17.

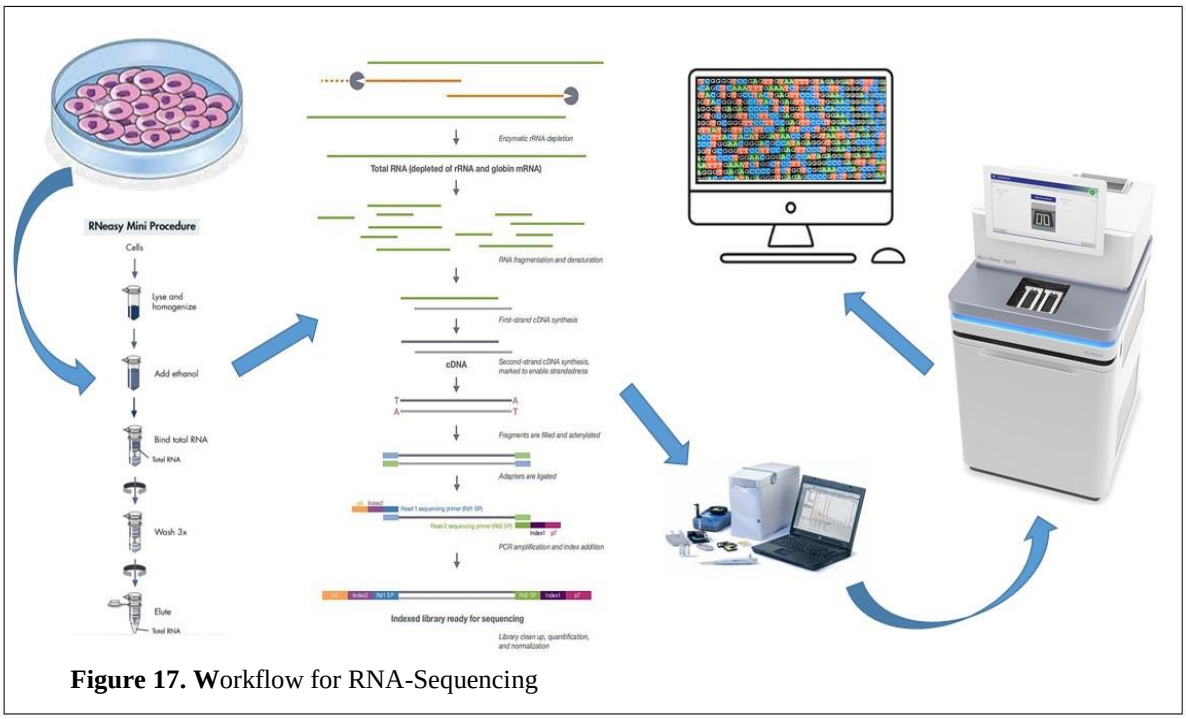

Figure 17. Workflow for RNA-Sequencing

6.6 Statistical analyses

For in-vitro-experiments each experiment was repeated at least three times, with three technical replicates for each condition. GraphPad Software v.8.4.3 was used to perform all analyses.

For patients case series analyses, data were summarized using median and first (IQ) and third (IIIQ) quartile and minimum and maximum values, for continuous variables. Categorical variables were reported as natural frequency and percentage. Correlation among variables was measured through the Spearman correlation coefficient. The association between continuous and categorical variables was assessed using the Wilcoxon-Mann Whitney test or Kruskal Wallis test, as apropriate, whereas that between categorical variables using the Pearson Chi-square test or the Fisher Exact test, as appropriate.

The association between the biomarkers and the time-to-event endpoints was investigated by means of the Kaplan-Meier estimator as well as the Cox proportional hazards model. The endpoint considered were the DFS, defined as the time since surgery until disease progression or death from any cause, whichever occurred first (alive patients withour recurrence were censored at the time of last contact); the OS defined as the time since surgery until death from any cause (alive patients were censored at the time of last contact); time-to-recurrence (TTR) defined as the time since surgery until disease progression (patients not experiencing recurrence were censored at the time of death, if any, or at the last contact).

7. Results

7.1 G9a, DNMT1 and UHRF1 expression in patients case series

A case series of 67 GC patients has been enrolled (Table3). Of all patients, 42 were males and 25 were females, with a median age at diagnosis of 71 years (range 42-89.5 years). Stage III (58.2%) and intestinal type according to Lauren (68.6%) were the most common diagnosed cancer characteristics in the case series.

	n	%
Gender		
M	42	62.69
F	25	37.31
Age at diagnosis, y		
Median [IQ - IIIQ]	71 [60 - 76]	
Min-max	42 – 89.5	
Stage		
IB	1	1.49
IIA	6	8.96
IIB	21	31.34
IIIA	11	16.42
IIIB	16	23.88
IIIC	12	17.91
WHO		
Poorly differentiated	44	65.67
Signet-ring	15	22.39
Papillary	3	4.48
Mucinous	2	2.99
Tubular	3	4.48
Lauren		
Intestinal type	46	68.66
Diffuse type	16	23.88
Mixed type	5	7.46
Tumor localization		
Antrum	35	53.03
Fundus	6	9.09
Cardias	4	6.06
Angolus	3	4.55
Body	12	18.18
pylorus	5	7.58
missing	1	
Adjuvant therapy		
No	7	10.45
Yes	60	89.55

Given the retrospective case series, no patient underwent neo-adjuvant treatment, while almost all patients (60/67, 89.5%) were treated with an adjuvant therapy; this information is critical for the study aims, as the possibility to investigate surgical specimens without damage of neo-adjuvant chemotherapy, empower the biomarkers expression data significance. All clinical data were retrieved from patients medical records.

Table 3. Patients characteristics.
WHO: World Health Organization

7.1.1 Expression of G9a, DNMT1 and UHRF1 in cancerous and non-cancerous tissues

Expression of G9a, DNMT1 and UHRF1 was assessed by IHC. Expression of G9a (>10% of positive cells in the sample) was present in 40.3% (27/67), DNMT1 was present in 17.9% (12/67) and UHRF1 was expressed in 87.8% (58/66) of patients. Six patients had contemporary co-expression of the three markers, while only 5 patients (7.4%) had a negative tumor for all the three markers (Figure 18). Moreover, considering the positivity of markers as a continuous variable, a significant positive correlation existed between the expression of G9a and UHRF1 (Spearman correlation 0.445), with was not observed for the other markers. G9a, DNMT1 and UHRF expression in cancer and non-cancer tissues is shown in figure 18.

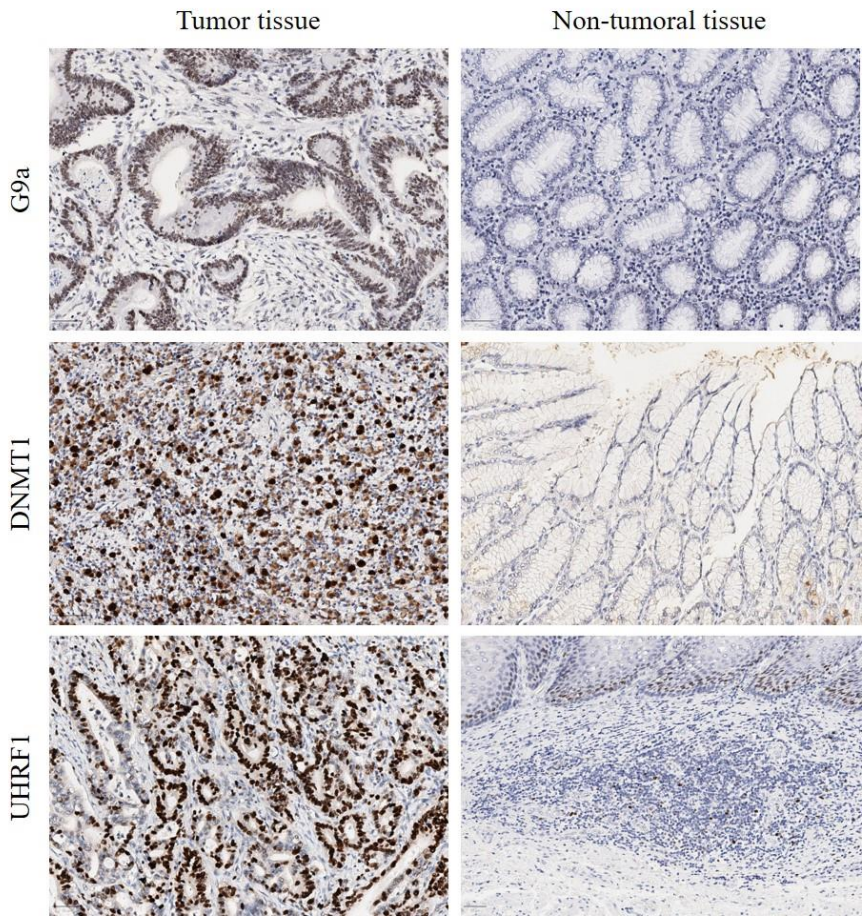

Figure 18. G9a, DNMT1 and UHRF1 immuno-histochemical expression in tumoral and matched non-tumoral tissues of gastric cancer patients (20x magnification).

7.2 Expression of UHRF1

Considering the whole case series, UHRF1 was the most expressed biomarker among the patients. When expressed, it had a strong IHC staining in almost totality of the cancerous cells in analyzed samples. Intriguingly, UHRF1 was also expressed in non-cancerous tissues of patients, especially in follicular lymphoid tissues, intestinal metaplasia and high-grade dysplasia (figure 19), suggesting it is involved in the malignant transformation of gastric mucosa.

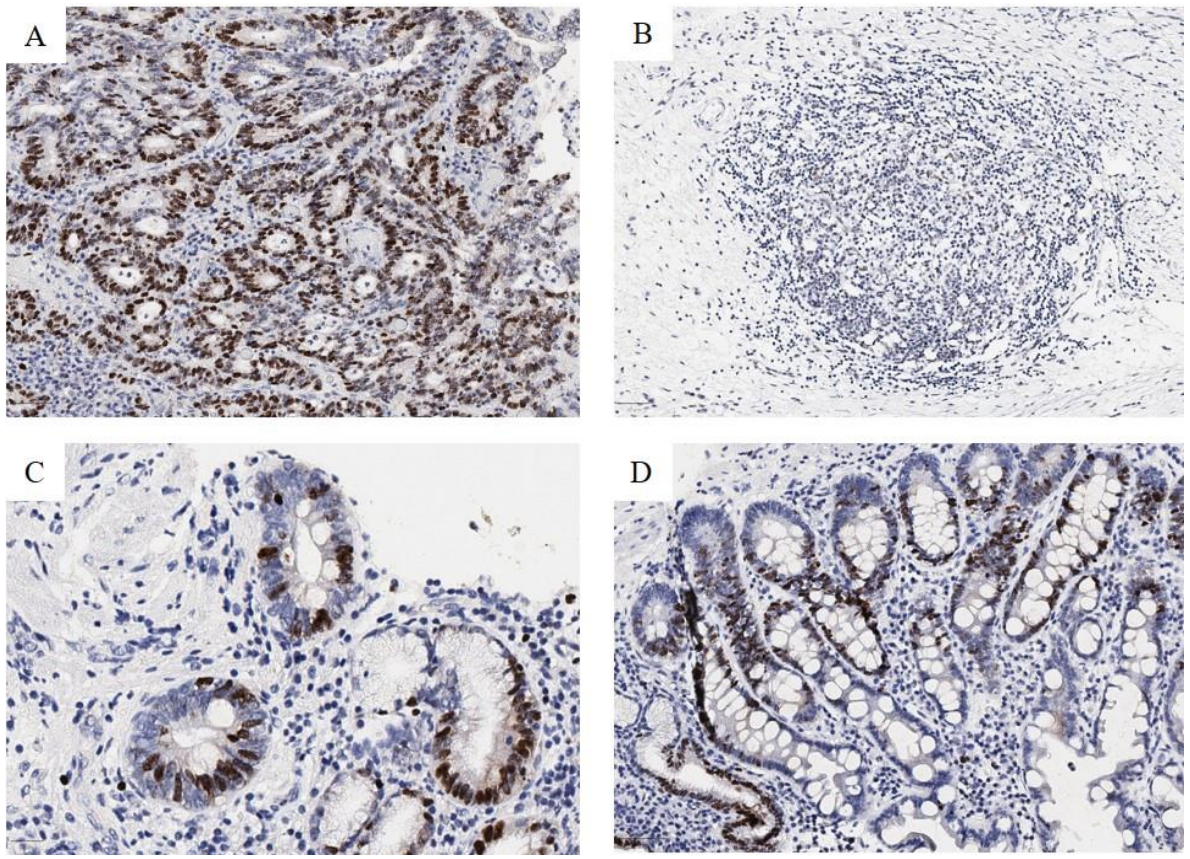

Figure 19. UHRF1 immuno-histochemical expression in pre-cancerous lesions in gastric cancer patients. **A:** high grade dysplasia (20X magnification); **B:** follicular lymphoid tissue (20X magnification); **C:** metaplasia with regeneration aspects (200X magnification); **D:** intestinal metaplasia (200X magnification).

7.3 Association of G9a, DNMT1 and UHRF1 with clinical characteristics of patients

Of the 65 patients with clinical information about tumor relapse after surgical treatment, 14 (21.5%) relapsed, while 51 (78.5%) did not. No associations were observed between the expression of the three biomarkers and the tumor location. Considering Lauren classification, G9a was found significantly less expressed in the diffuse type cancer patients (p=0.02), (Figure 20). On the other hand, no statistically significant associations were found between the three biomarkers and the classification of GC according to WHO (Figure 21). No statistically significant associations have been found between biomarkers expression and time-to-relapse (TTR) after surgical treatment.

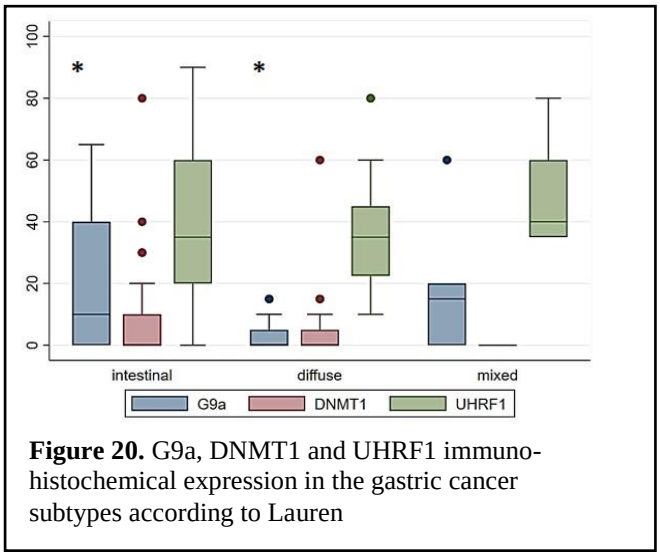

Figure 20. G9a, DNMT1 and UHRF1 immuno-histochemical expression in the gastric cancer subtypes according to Lauren

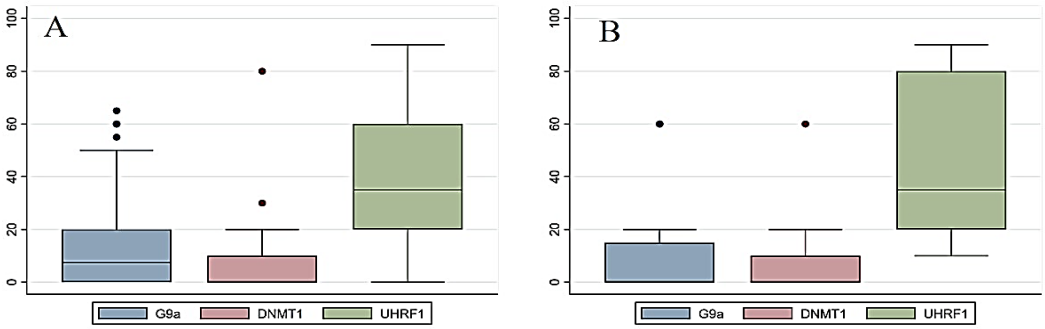

Figure 21. G9a, DNMT1 and UHRF1 immuno-histochemical expression in the gastric cancer subtypes according to World Health Organization. **A:** Poorly differentiated; **B:** Signet-ring cell carcinoma

7.4 Gastric cancer cells sensitivity to CM-272, azacitidine, BIX-01294, cisplatin and 5-fluorouracil

IC_{50} for different compounds was established in a panel of gastric and distal oesophageal cancers. In particular, the compound CM-272, the DNMT1 inhibitor azacitidine, the G9a inhibitor BIX-01294, and two cytotoxic compounds largely used in the clinical practice for GC, *i.e.* cisplatin and 5-fluorouracil. Each cell line was seeded at a concentration of 3000 cells per well in 100 µl, and after an overnight time to permit cells attachment, single compounds were administrated for 72 hours. As shown in figure 22, CM-272 demonstrated to have a strong cancer cells inhibition in all tested cell lines, with IC_{50} concentrations at nM order. Interestingly, it demonstrated a potent activity especially in FLO-1 and KYAE-1 cell lines, isolated from distal oesophageal carcinoma, with IC_{50} of 102nM and 11nM, respectively.

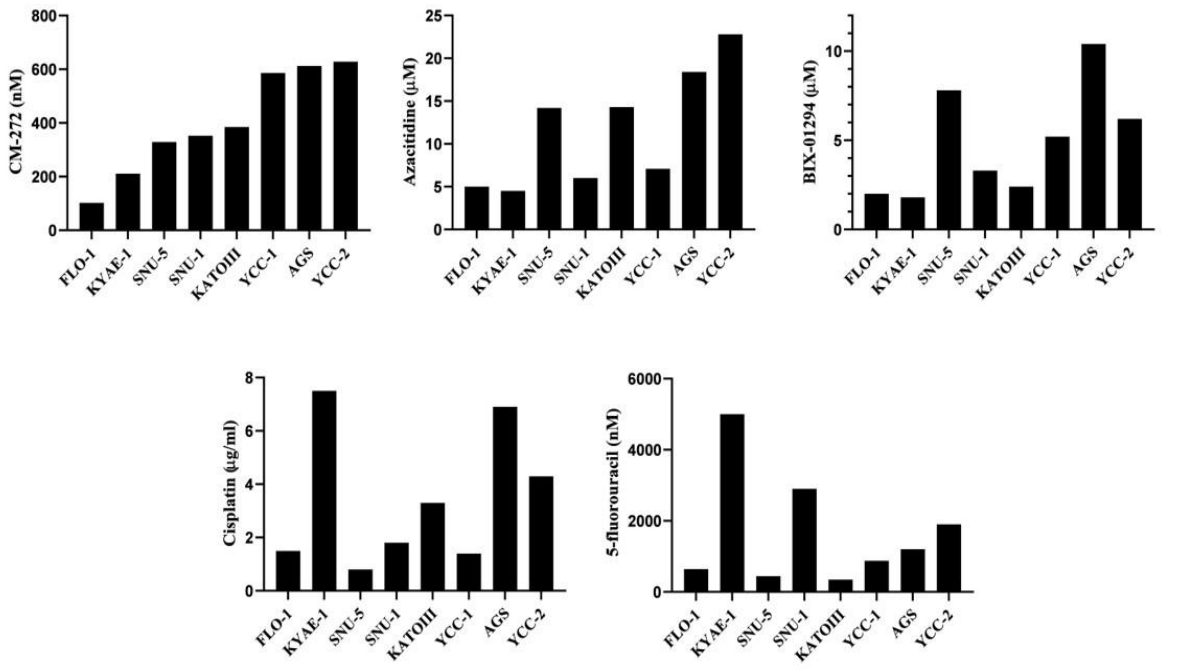

Figure 22. IC_{50} of a panel of gastric and distal oesophageal cancers for epigenetic compounds (CM-272, azacitidine and BIX-01294) and cytotoxic agents (cisplatin and 5-fluorouracil).

This efficacy emerges when comparing CM-272 effects with respect to other single-target epigenetic compounds, *i.e.* azacitidine and BIX-01294, which display an IC_{50} at µM order. For cytotoxic compounds, IC_{50} was established for each cell line in order to have experimental specific assessments to consider as a reference for combination treatments with CM-272 and other epigenetic compounds.

Basing on these concentrations, the focus of the following experiments was mainly addressed on SNU1, KATOIII and AGS cell lines, which are GC cell lines representative of the different disease subtypes, and with a medium/high resistance to CM-272.

7.5 Drug synergistic effect and epi-sensitization of CM-272 and chemotherapy

Previous works highlighted that CM-272 had a chemo-sensitizing effect in CCA cells, also demonstrating a synergic activity when the two drugs were administered together [123]. In the present study, several combinations at different doses of CM-272 with cytotoxic agents used in the clinical practice for GC patients management have been tested in GC cell lines. Treatment schedules were administered in GC cell lines seeded as for IC_{50}, and analyses were carried out after 72 hours exposures to compounds. Compounds concentrations were mainly lower than single IC_{50} for each cell line, to observe synergic effect. As shown in figure 23A, CM-272 has a strong synergic effect with cisplatin, especially when it was administered at low doses. On YCC-1 cell lines, the reverse effect was then observed, as a synergistic effect was observed, but only at higher doses of CM-272 and cisplatin simultaneous administration.

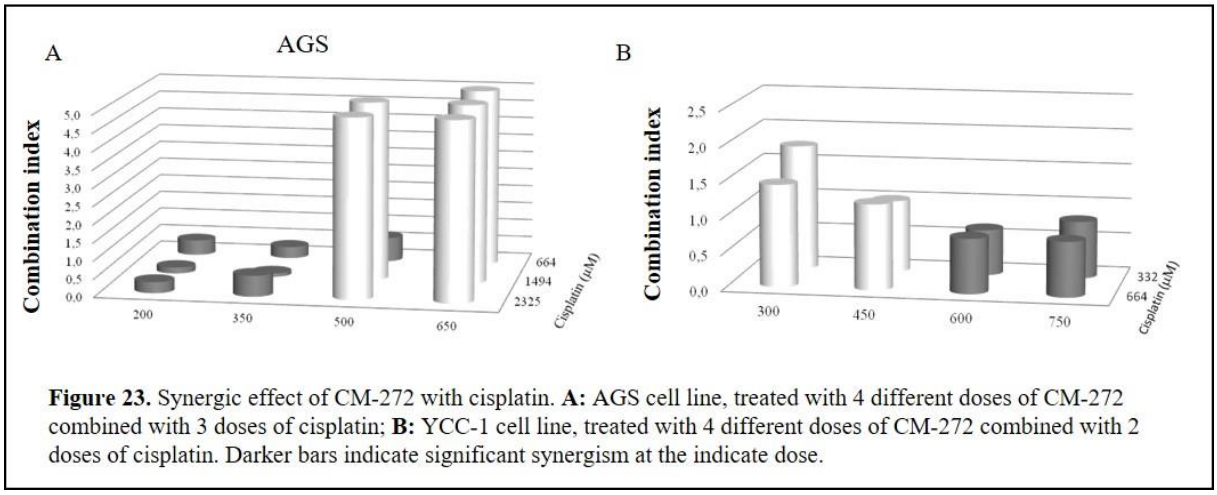

Figure 23. Synergic effect of CM-272 with cisplatin. **A:** AGS cell line, treated with 4 different doses of CM-272 combined with 3 doses of cisplatin; **B:** YCC-1 cell line, treated with 4 different doses of CM-272 combined with 2 doses of cisplatin. Darker bars indicate significant synergism at the indicate dose.

Moreover, a possible effect of epi-sensitization to chemotherapeutic agents has been tested. The rationale was possibly to re-establish the expression of genes possibly involved in chemotherapy efficacy prior to cytotoxic effect. GC cell lines were seeded in 96-wells plates as described above, and pre-treated with 24 or 48 hours with CM-272, or kept with normal medium; then fresh medium with serial dilutions of cytotoxic compound were added to cells for further 24 hours, and cells viability was compared between pre-treated cells and no pre-treated cells (Figure 24). Even though not statistically significant, the synergic effect of CM-272 and cisplatin emerged, becoming to be evident at low doses of cisplatin. Conversely, CM-272 was not able to chemo-sensitize cancer cells to 5-fluorouracil.

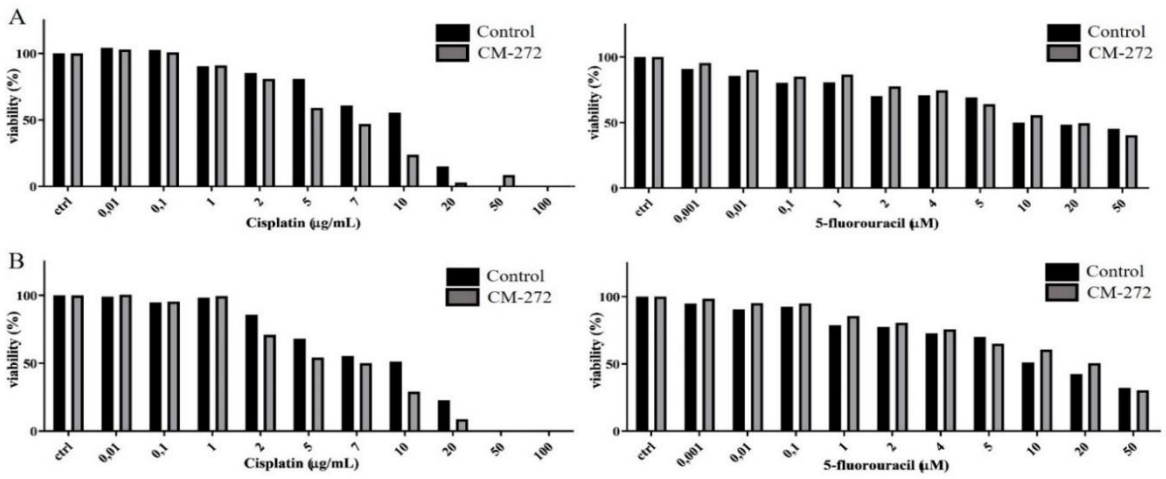

Figure 24. Epi-sensitization assessment in AGS cell line with CM-272 and chemotherapy. The cells were pre-treated with CM-272 at 400nM concentration (two-third of IC_{50}) for 24 hours (**A)** or 48 hours (**B**).

7.6 Growth inhibition induced by CM-272 and pro-apoptotic pathway activation

As shown, CM-272 alone has a potent inhibitory effect on GC cells growth, and the possible mechanism of action of the compound was investigated. Cells were seeded in a 6-wells plate and treated (or not) with two-third of IC_{50} dose of CM-272, and cell growth inhibition was visually assessed after 72 hours of treatment. As shown in Figure 25, CM-272 alone was a potent inhibitor of cancer cells growth.

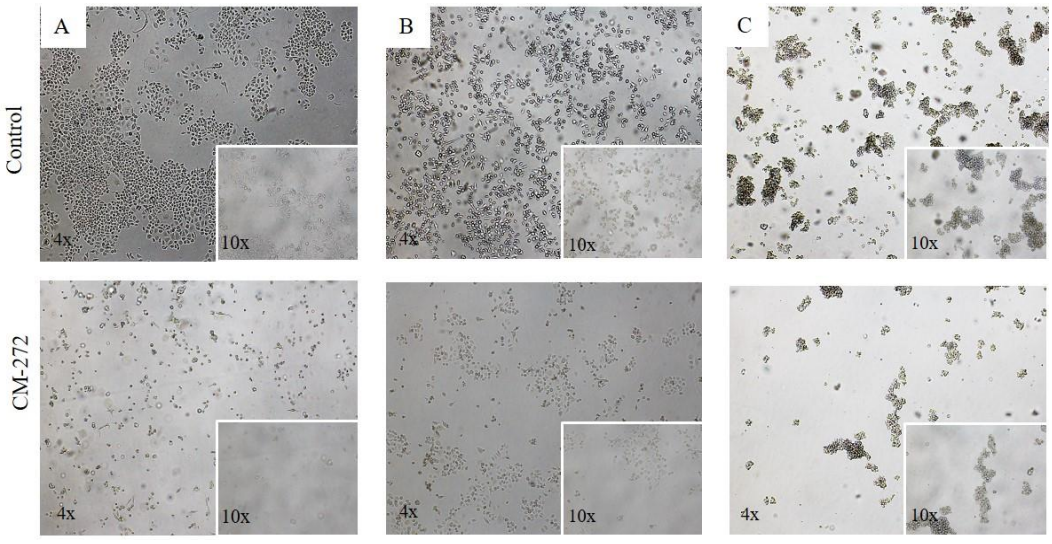

Figure 25. Visual assessment of cell growth inhibition. **A:** AGS cell line control and CM-272 at 400nM for 72 hours (4x and 10x magnification); **B:** KATOIII cell line line control and CM-272 at 300nM for 72 hours (4x and 10x magnification); **C:** SNU-1 cell line control and CM-272 at 300nM for 72 hours (4x and 10x magnification).

Considering this potent and evident effect, evaluation of induction of apoptotic process of the compound alone was tested. AGS, KATOIII and SNU-1 were seeded in a 6-wells plate and treated with CM-272 at different doses, for 72 hours. Cells were then counted and stained for annexin-V expression for cytofluorimetric analyses. As shown in Figure 26, the compound alone was slightly able to induce pro-apoptotic cascades in the analyzed cells, as only slight differences were observed in cellular subpopulations percentages.

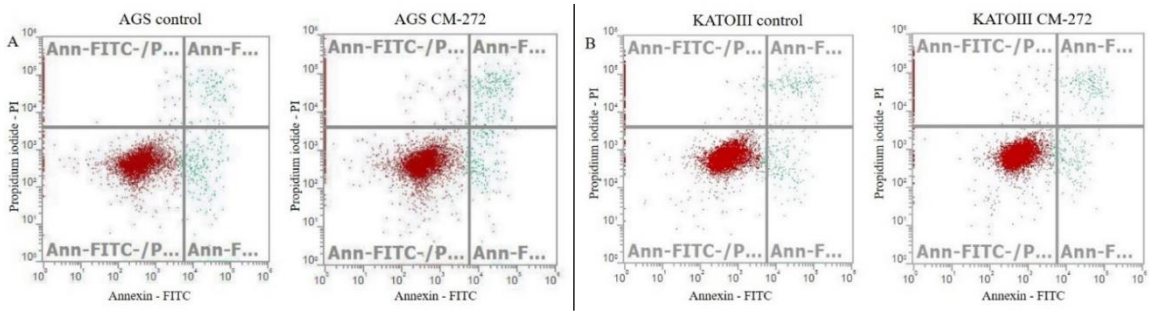

Figure 26. Apoptosis assay by cytometer evaluation of annexin expression. **A:** AGS cell line control and CM-272 at 400nM for 72 hours. **B:** KATOIII cell line control and CM-272 at 300nM for 72 hours.

Nonetheless, an evaluation of early activation of pro-apoptotic cascade has been tested. In particular, a luminescent assay for caspase 3 and 7 activation was performed. AGS cells were seeded in a 96-wells plate at a concentration of 3000 cells/well/100µL and treated or not with CM-272 alone. At 8, 16 and 24 hours, each sample triplicate was evaluated for caspase 3 and 7 activation. Contemporary, another sample triplicate was used to assess cell viability by MTT assay. As shown in Figure 27, CM-272 was a potent activator of caspase 3 and 7, in a time-dependent manner. At the same time, cells viability decreased accordingly to caspase activation. This result and the evidence from annexin assay suggest that the compound could act as an activator of early apoptotic processes, but not of late apoptosis.

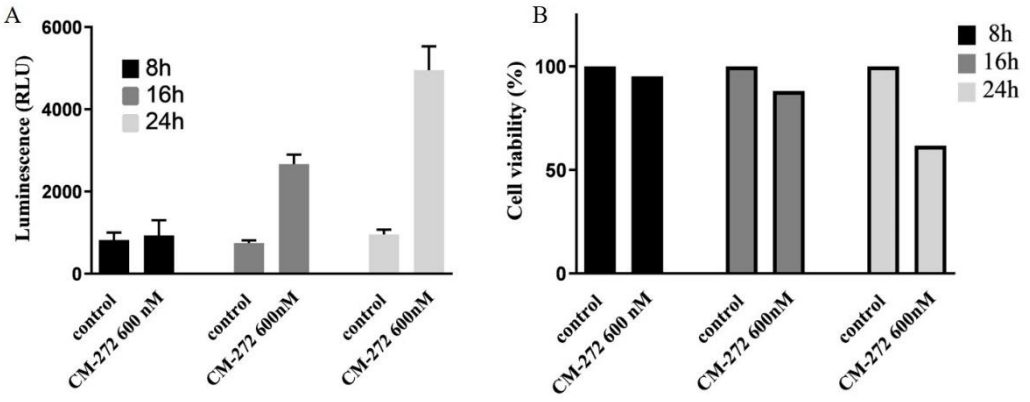

Figure 27. Induction of caspase 3 and 7 by CM-272 in AGS cell line. **A:** Chemo-luminescence at different time-points denoting a time-dependent activation of caspases. **B:** Contemporary assessment of cell viability by MTT assay at the same time-points.

7.7 Senescence induction

The induction of cell cycle arrest was then investigated in gastric cancer cell lines, highlighting that the compound was not able to induce cell cycle arrest (data not shown).

AGS and KATOIII cell lines were then cultured and treated with relative IC_{50} of CM-272 to investigate the potential of epigenetic induction of cellular senescence. After being fixed, cells were fixed, and stained to assess β-galactosidase expression, indicator of cellular senescence. Cellular senescence is a state of non-proliferative and metabolic inactivation, leading to a quiescent phenotype. The induction of senescence by CM-272 was then investigated in GC cell lines at various exposure times. Interestingly, it was demonstrated that CM-272 is a potent inductor of cellular senescence in particular in AGS cell line ($p<0.05$), while it was not statistically significant in KATOIII. Nonetheless, in KATOIII cell line, typical cellular senescence aspects was observed in several cells, consisting in increased cell size and flat cells with huge cytoplasm (appearance of a fried egg), and increased cytoplasmic granularity (Figure 28).

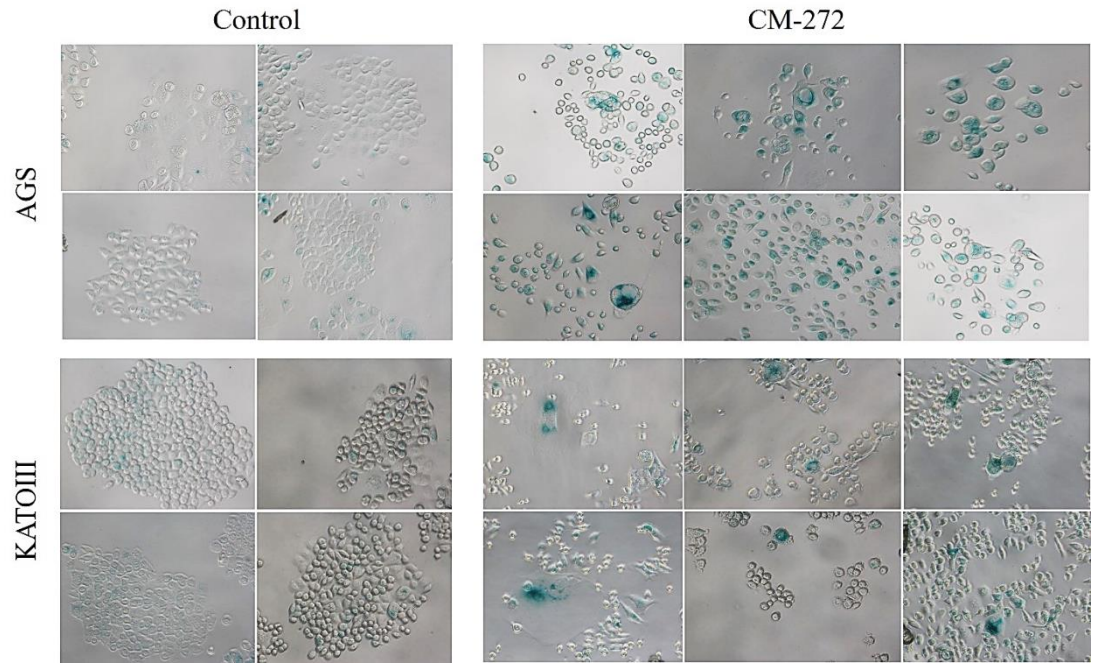

Figure 28. β-galactosidase assay to assess cancer cells senescence. **A:** AGS cell line control and treatment with CM-272 400nM for 72 hours; **B:** KATOIII cell line control and treatment with CM-272 400nM for 72 hours.
Green stained cells indicate β-galactosidase. All images are acquired at 20x magnification, in brightfield.

7.8 CM-272 affects cancer cells proliferation through inhibition of multiple pathways

AGS, KATOIII and SNU-1 cell lines analyses were treated with three different doses (50%, 100% and 200% of IC_{50}) and gene expression using total RNA was performed. After DEA analyses, the KEGG, the GO and the Reactome genome repositories were used to analyse GSEA. As shown in Figure 29, the compound CM-272 was able to reprogram the transcriptomic profile in a cell line-specific fashion.

In particular, for AGS cell line, an inhibition of pathways related to intra-cellular phosphorylation cascades has been observed. Specifically, CM-272 at 400nM for 72 hours was able significantly to reduce expression of genes of RAS, ErbB, PI3-Akt, and MAPK signalling pathways, p values <0.001 for all pathways. The same pathways resulted downregulated using CM-272 at 600nM for 72 hours (all p values <0.001), while at the higher dose of CM-272, the RAS and ErbB signalling pathways remained strongly significant, p=0.02 and p<0.001, respectively. All together, these altered pathways represent the main phosphorylation cascade mechanisms through which cancer cells are able to display several cancer hallmarks, *i.e.* evade growth suppressors, activate invasion and metastasis, resisting cell death and sustain proliferative signalling.

In the KATOIII cell lines, KEGG database highlighted a significant modulation of cellular metabolism, mainly linked to ATP production. In particular, at 400nM concentration for 72 hours, CM-272 was able to significantly down-regulate the valine, leucine and isoleucine degradation, the pyruvate metabolism, the oxidative phosphorylation, and the pentose phosphate pathway, p<001, p=0.007, p<0.001, and p<0.001, respectively. At compound higher dose, the former two pathways resulted significantly down-regulated (p<0.001, and p=0.005).

In the SNU1 cell line, similar results have been demonstrated. In particular, at low dose of CM-272 (200nM) for 72h, the mithocondrial tricarboxylic acid cycle (TCA) pathway and the oxidative phosphorylation pathways were significantly up-regulated (both p<0.001), suggesting a strong addiction of the cell line to mithocondrial respiration and the inefficacy of the compound at this dose. Surprisingly, at 400 nM dose (IC_{50}), both this pathways were strongly down-regulated (both p<0.001), highlighted a deep epigenetic reprograming of all intermediates. Taken together, all these pathway collaborate to carbon metabolism, in maintaining the redox potential within the cell and in producing energy within the cell.

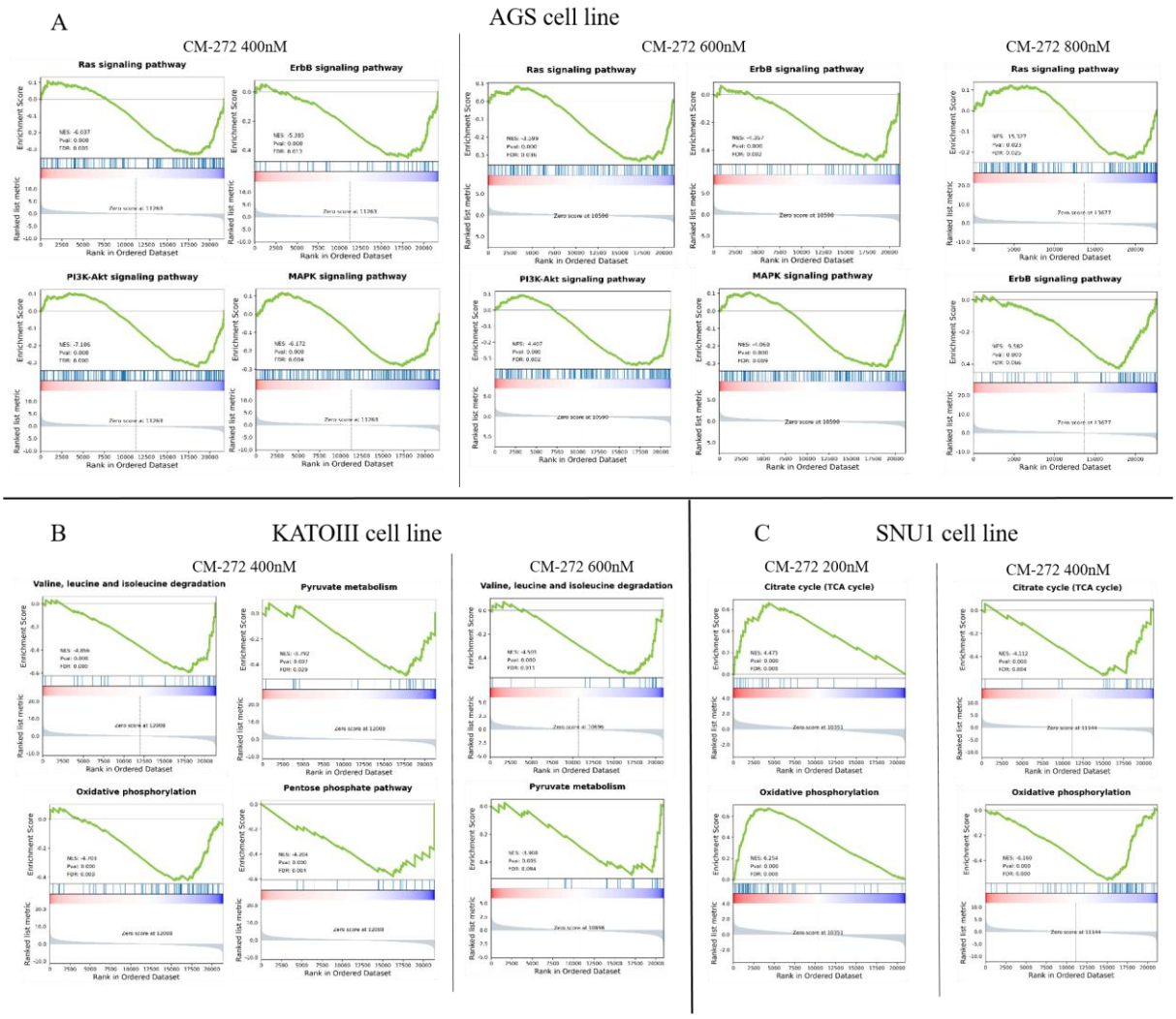

Figure 29. Gene set enrichment analysis (GSEA) using Kyoto Encyclopedia of Genes and Genome (KEGG) in AGS (**A**), KATOIII **(B)** and SNU1 **(C)** cell lines treated with IC_{50}, 50% and 200% IC_{50} of CM-272, for 72 hours.

8. Discussion

Targeting epigenetic mechanisms is an attractive option for the treatment of several malignancies, including gastric cancer. The complex scenario at aetiological and histopathological level of this disease is reflected in the fact that clinical current treatment algorithms are often ineffective. Several studies investigated molecular feature of gastric cancer, identifying epigenetic patterns altered in the different subtypes of disease, also proposing new possible classification system based on epigenetic machinery alterations [42]. Thus, the aim of this study was to target simultaneously two different epigenetic modifiers, namely G9a and DNMT1, known to participate in an intense crosstalk activated in carcinogenesis [130]. CM-272, a small molecule inhibiting G9a and DNMT1 in a reversible manner, showed remarkable results in haematological malignancies, and demonstrated therapeutics improvement in pre-clinical models of hepatocellular carcinoma and cholangiocarcinoma [121]. The main aim of this study was then to test CM-272 efficacy in pre-clinical models of gastric cancer, investigating the potentiality of targeting epigenetic mechanisms in this malignancy.

Firstly, we enrolled a retrospective case series of surgically resected gastric cancer patients affected by different disease histology, to assess the expression of G9a, DNMT1 and their functional adaptor UHRF1 in cancer tissues, to demonstrate the rationale of targeting these biomarkers [131]. As expected, all the three biomarkers were expressed in cancerous tissue of patients with respect to adjacent disease-free tissues. This was consistent with previous results that identified that G9a, DNMT1 and UHRF1 are overexpressed in gastric cancer patients tissue, and are associated with carcinogenesis, triggering of the metastatic process and predictors of poor prognosis [132–135]. In this study, possibly dependent on the clinical characteristics of the enrolled case series (*i.e.* non-metastatic patients), no association among biomarkers expression and clinical outcome was observed. Interestingly, UHRF1 was expressed in almost all case series, and it was also expressed in pre-cancerous lesions such as high grade dysplasia and intestinal metaplasia. This evidence was not observed for the G9a and DNMT1, suggesting that UHRF1 expression could be associated to early carcinogenesis and gastric transformation, as another study found a modest expression of UHRF1 in patients with gastritis [136].

The sensitivity of a panel of gastric cancer cell lines to CM-272 treatment was then assessed, demonstrating that the compound was a potent inhibitor of cancer cell growth, especially with respect to single-target other epigenetic therapies, confirming the efficacy in upper gastrointestinal cancer cell lines highlighted in previous works [122,123]. Interestingly, a strong sensitivity to the compound was demonstrated by two cell lines of distal oesophageal carcinoma; even though it was not the aim of the present study, this evidence merits a deeper testing of the efficacy of such compound also in gastro-oesophageal junction and oesophageal tumours.

Pharmacological epigenetic priming efficacy has been demonstrated to improve chemotherapy efficacy in gastric cancer cell lines, and brought first interesting results also in gastric cancer patients, being tested as a strategy in clinical trials [137,138]. In this study, the potential of CM-272 in improving cytotoxic treatment was evaluated both with concomitant and previous administration of the compound with respect to chemotherapy. Even though it was not observed in all the tested cell lines, with contemporary administration, CM-272 was able to synergize with chemotherapy treatment, especially cisplatin; interestingly, it improved chemotherapy treatment when administered at low or high doses in different cell lines, suggesting that it is able to induce an epigenetic reprogramming and restoring of cellular pathways involved in response to cytotoxic treatment. This synergic effect was consistent with previous results highlighted in CCA cell lines [123]. On the other hand, CM-272 epigenetic priming did not show significant improvements in cytotoxic efficacy, suggesting its effect was additive rather than synergic.

Consistent with previous results, inhibition of cancer cells growth was not accompanied by the induction of apoptosis, or cell cycle arrest [122]. On the other hand, it was demonstrated that CM-272 was a potent inductor of cellular senescence. Cellular senescence is a stable and terminal state of cell, that is associated with a pro-inflammatory and hypersecrectory phenotype and in cell cycle arrest [139,140]. It is often induced by several therapeutic agents that exerts proper mechanism of action by causing DNA damage, such as radiation and cytotoxic compounds [139]. In this study, we found that induction of senescence could be epigenetically regulated. Recently, it has been highlighted that cellular senescence is a dynamic and reversible process, and that epigenetic mechanisms are involved as key regulators in inducing non-DNA damage dependent senescent phenotypes[141,142].
Moreover, it has been demonstrated that CM-272 is a modulator in epigenetically-induced phenotypes, as it was able to reactivate genes related to a more adult and differentiated phenotype in HCC cell lines [122].

Epigenetic reprograming on GC cancer cells has been investigated in this study through a wide transcriptome sequencing and genome database analyses, to highlight which pathways were altered by the epigenetic pharmacological treatment. Interestingly, it has been highlighted that the inhibition of cancer cells growth in-vitro is exerted affecting multiple pathways altered in cancer, such as phosphorylation patterns and metabolic dysregulation, in a cell line-specific manner.

In AGS cell line, a significant down-regulation of RAS, ErbB, MAPK and PI3K-Akt pathways has been highlighted. All these pathways are well recognized to be up-regulated or affected by activating mutations in several solid tumors, especially in the EGFR-HER2/RAS/RAF/MEK/ERK pathway [143]. They are strongly inter-connected, and by activation of ErbB trans-membrane receptors mainly by

growth factors (GFs), the activation of a huge number of kinases promote phosphorylation cascades that promote cell survival, uncontrolled growth, resistance to therapy and metastasis. Downstream activation of RAS, RAF and MEK in that order converge in the activation of the ERK1/2 transcription factor activator. The PI3K/Akt/mTOR cascade can also be activated via ErbB receptors and RAS, and its main implications are related to metabolic signalling and protein synthesis that sustain cell growth [144]. Several agents inhibiting one or more of these kinases are under investigation or already in clinical practice in many solid malignancies, resulting in the fact that these pathways represent the most cancer-addiction altered mechanisms [144]. Even though GC is not a commonly defined oncogene-addicted cancer, as the only targeted therapy approved to date is trastuzumab plus chemotherapy in ErbB2 amplified advanced stage tumors (less than 10% of all advenced tumors in frequency), epigenetic silencing of these pathways could be a fascinating strategy to stop cancer growth [145]. Moreover, the CM-272 compound demonstrated to simultaneously down-regulate the expression of several genes involved in these pathways, even at low dose, and to induce a concomitant multiple inhibition of the principal phosphorylation pathways.

In this study, a metabolic reprograming has been highlighted in two cell lines. In particular, in KATOIII and in SNU1 cell lines, the compound CM-272 induced a down-regulation of genes involved in mithocondrial metabolism for adenosine tri-phosphate (ATP) production. Metabolic dysregulation is an emerging hallmark of cancer, mainly through the high glucose and the glycolysis derivate pyruvate consumption to enter the Krebs cycle and produce ATP via electron transport chain (ETC). Even though the known Warburg effect indicates that cancer cells prefer an aerobic glycolysis more than mitochondrial oxidation of pyruvate, (TCA) and ETC play a pivotal role in carcinogenesis and cancer progression [146]. In fact, mitochondria supply energy, provide building blocks for new cells, and control redox homeostasis, oncogenic signalling, innate immunity, and apoptosis [146]. Moreover, new ETC small inhibitors are under investigation as anti-cancer therapies [147]. Valine, leucine and isoleucine are branched-chain amino acids (BCAAs) constituting approximately 35% of all essential amino acids that regulate protein metabolism via multiple pathways, including mTOR [148,149]. Carbon elements in valine and leucine are derived from pyruvate, while the isoleucine carbons are derived from threonine [150]. Leucine is a well known mTOR agonist, promoting protein translation, growth and cell survival [151]; alterations of levels of BCAA have been observed in serum and plasma of patients with gastrointestinal malignancies [152–154]. BCAA are also used as indirect nitrogen sources for nucleotide and non-essential amino acids biosynthesis via glutamate-glutamine axis, further catabolized to produce acetyl-CoA to use in the TCA for energy production [155]. The acetyl-CoA levels have an impact on the epigenetic changes of cells. It can influence diverse cellular processes, such as gene expression, cell-cycle progression and DNA repair [155]. The potential to epigenetically reprogram

and restore metabolic homeostasis is then an attractive option for cancer therapy, given the possibility to act simultaneously on multiple altered pathways.

9. Conclusions and future perspectives

Epigenetic mechanisms are a hallmark involved in all steps of malignant transformation. In this study, the rationale to target epigenetic mechanisms in gastric cancer has been demonstrated through a translational and pre-clinical approach. The tested compound demonstrated to have a potent effect on inhibition of gastric cancer cells in-vitro, remodelling gene expression and phenotype of treated cells. Further steps of the study will be to evaluate the potential of the compound in pre-clinical setting by in-vivo experimentation. In this direction, authorization for in-vivo experimentation of Italian Ministry of Health has been obtained, and the first preliminary studies to selected the right cell lines have been conducted.

10. References

1. Soybel DI. Anatomy and physiology of the stomach. *Surg Clin North Am*. 2005;85(5):875-894, v. doi:10.1016/j.suc.2005.05.009
2. Brandstaeter S, Fuchs SL, Aydin RC, Cyron CJ. Mechanics of the stomach: A review of an emerging field of biomechanics. *GAMM Mitteilungen*. 2019;42(3):1-17. doi:10.1002/gamm.201900001
3. Bornschein J, Malfertheiner P. Gastric carcinogenesis. *Langenbeck's Arch Surg*. 2011;396(6):729-742. doi:10.1007/s00423-011-0810-y
4. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. February 2021. doi:10.3322/caac.21660
5. Morgan E, Arnold M, Camargo MC, et al. The current and future incidence and mortality of gastric cancer in 185 countries, 2020-40: A population-based modelling study. *EClinicalMedicine*. 2022;47:101404. doi:10.1016/j.eclinm.2022.101404
6. Rawla P, Barsouk A. Epidemiology of gastric cancer: global trends, risk factors and prevention. *Prz Gastroenterol*. 2019;14(1):26-38. doi:10.5114/pg.2018.80001
7. Arnold M, Park JY, Camargo MC, Lunet N, Forman D, Soerjomataram I. Is gastric cancer becoming a rare disease? A global assessment of predicted incidence trends to 2035. *Gut*. 2020;69(5):823-829. doi:10.1136/gutjnl-2019-320234
8. Soerjomataram I, Bray F. Planning for tomorrow: global cancer incidence and the role of prevention 2020-2070. *Nat Rev Clin Oncol*. 2021;18(10):663-672. doi:10.1038/s41571-021-00514-z
9. Jun JK, Choi KS, Lee H-Y, et al. Effectiveness of the Korean National Cancer Screening Program in Reducing Gastric Cancer Mortality. *Gastroenterology*. 2017;152(6):1319-1328.e7. doi:10.1053/j.gastro.2017.01.029
10. Hamashima C, Ogoshi K, Okamoto M, Shabana M, Kishimoto T, Fukao A. A community-based, case-control study evaluating mortality reduction from gastric cancer by endoscopic screening in Japan. *PLoS One*. 2013;8(11):e79088. doi:10.1371/journal.pone.0079088
11. Taja-Chayeb L, Vidal-Millán S, Trejo-Becerril C, et al. Hereditary diffuse gastric cancer (HDGC). An overview. *Clin Res Hepatol Gastroenterol*. 2022;46(4):101820.

doi:10.1016/j.clinre.2021.101820

12. Kobielak A, Fuchs E. Alpha-catenin: at the junction of intercellular adhesion and actin dynamics. *Nat Rev Mol Cell Biol*. 2004;5(8):614-625. doi:10.1038/nrm1433

13. Kaurah P, MacMillan A, Boyd N, et al. Founder and recurrent CDH1 mutations in families with hereditary diffuse gastric cancer. *JAMA*. 2007;297(21):2360-2372. doi:10.1001/jama.297.21.2360

14. van Roy F. Beyond E-cadherin: roles of other cadherin superfamily members in cancer. *Nat Rev Cancer*. 2014;14(2):121-134. doi:10.1038/nrc3647

15. Hellström PM. Spotlight on gastroenterology - the Nobel Prize Laureates in Physiology or Medicine 2005: John Robin Warren and Barry James Marshall. *Scand J Gastroenterol*. 2005;40(12):1383-1385. doi:10.1080/00365520500390402

16. Mukaisho K-I, Nakayama T, Hagiwara T, Hattori T, Sugihara H. Two distinct etiologies of gastric cardia adenocarcinoma: interactions among pH, Helicobacter pylori, and bile acids. *Front Microbiol*. 2015;6:412. doi:10.3389/fmicb.2015.00412

17. Plummer M, Franceschi S, Vignat J, Forman D, de Martel C. Global burden of gastric cancer attributable to Helicobacter pylori. *Int J cancer*. 2015;136(2):487-490. doi:10.1002/ijc.28999

18. Personal habits and indoor combustions. Volume 100 E. A review of human carcinogens. *IARC Monogr Eval Carcinog risks to humans*. 2012;100(Pt E):1-538.

19. Ajani JA, Lee J, Sano T, Janjigian YY, Fan D, Song S. Gastric adenocarcinoma. *Nat Rev Dis Prim*. 2017;3:17036. doi:10.1038/nrdp.2017.36

20. Wang F, Meng W, Wang B, Qiao L. Helicobacter pylori-induced gastric inflammation and gastric cancer. *Cancer Lett*. 2014;345(2):196-202. doi:10.1016/j.canlet.2013.08.016

21. Chang C-C, Kuo W-S, Chen Y-C, Perng C-L, Lin H-J, Ou Y-H. Fragmentation of CagA Reduces Hummingbird Phenotype Induction by Helicobactor pylori. *PLoS One*. 2016;11(3):e0150061. doi:10.1371/journal.pone.0150061

22. Rassow J, Meinecke M. Helicobacter pylori VacA: a new perspective on an invasive chloride channel. *Microbes Infect*. 2012;14(12):1026-1033. doi:10.1016/j.micinf.2012.07.002

23. Lamb A, Chen L-F. Role of the Helicobacter pylori-induced inflammatory response in the development of gastric cancer. *J Cell Biochem*. 2013;114(3):491-497. doi:10.1002/jcb.24389

24. Linz B, Balloux F, Moodley Y, et al. An African origin for the intimate association between

humans and Helicobacter pylori. *Nature*. 2007;445(7130):915-918. doi:10.1038/nature05562

25. Hooi JKY, Lai WY, Ng WK, et al. Global Prevalence of Helicobacter pylori Infection: Systematic Review and Meta-Analysis. *Gastroenterology*. 2017;153(2):420-429. doi:10.1053/j.gastro.2017.04.022

26. Hansson LE, Nyrén O, Hsing AW, et al. The risk of stomach cancer in patients with gastric or duodenal ulcer disease. *N Engl J Med*. 1996;335(4):242-249. doi:10.1056/NEJM199607253350404

27. Lu Z, Li X, Xu Y, et al. microRNA-17 functions as an oncogene by downregulating Smad3 expression in hepatocellular carcinoma. *Cell Death Dis*. 2019;10(10):723. doi:10.1038/s41419-019-1960-z

28. Singh S, Jha HC. Status of Epstein-Barr Virus Coinfection with Helicobacter pylori in Gastric Cancer. *J Oncol*. 2017;2017:3456264. doi:10.1155/2017/3456264

29. Wu AH, Tseng C-C, Bernstein L. Hiatal hernia, reflux symptoms, body size, and risk of esophageal and gastric adenocarcinoma. *Cancer*. 2003;98(5):940-948. doi:10.1002/cncr.11568

30. Abrams JA, Gonsalves L, Neugut AI. Diverging trends in the incidence of reflux-related and Helicobacter pylori-related gastric cardia cancer. *J Clin Gastroenterol*. 2013;47(4):322-327. doi:10.1097/MCG.0b013e318260177a

31. Tsugane S, Sasazuki S. Diet and the risk of gastric cancer: review of epidemiological evidence. *Gastric cancer Off J Int Gastric Cancer Assoc Japanese Gastric Cancer Assoc*. 2007;10(2):75-83. doi:10.1007/s10120-007-0420-0

32. Tramacere I, Negri E, Pelucchi C, et al. A meta-analysis on alcohol drinking and gastric cancer risk. *Ann Oncol Off J Eur Soc Med Oncol*. 2012;23(1):28-36. doi:10.1093/annonc/mdr135

33. Van Cutsem E, Sagaert X, Topal B, Haustermans K, Prenen H. Gastric cancer. *Lancet*. 2016;388(10060):2654-2664. doi:10.1016/S0140-6736(16)30354-3

34. Siewert JR, Stein HJ. Classification of adenocarcinoma of the oesophagogastric junction. *Br J Surg*. 1998;85(11):1457-1459. doi:10.1046/j.1365-2168.1998.00940.x

35. Amin MB, Greene FL, Edge SB, et al. The Eighth Edition AJCC Cancer Staging Manual: Continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging. *CA Cancer J Clin*. 2017;67(2):93-99. doi:10.3322/caac.21388

36. Lauwers G, Carneiro F, Graham DY. WHO classification of tumours of the digestive system -

3rd chapter. *WHO Classif cation tumours Dig Syst 4th ed, Lyon, IARC*. 2010:44-58.

37. Nagtegaal ID, Odze RD, Klimstra D, et al. The 2019 WHO classification of tumours of the digestive system. *Histopathology*. 2020;76(2):182-188. doi:10.1111/his.13975

38. LAUREN P. THE TWO HISTOLOGICAL MAIN TYPES OF GASTRIC CARCINOMA: DIFFUSE AND SO-CALLED INTESTINAL-TYPE CARCINOMA. AN ATTEMPT AT A HISTO-CLINICAL CLASSIFICATION. *Acta Pathol Microbiol Scand*. 1965;64:31-49. doi:10.1111/apm.1965.64.1.31

39. Smyth EC, Nilsson M, Grabsch HI, van Grieken NC, Lordick F. Gastric cancer. *Lancet (London, England)*. 2020;396(10251):635-648. doi:10.1016/S0140-6736(20)31288-5

40. Tan IB, Ivanova T, Lim KH, et al. Intrinsic subtypes of gastric cancer, based on gene expression pattern, predict survival and respond differently to chemotherapy. *Gastroenterology*. 2011;141(2):476-485, 485.e1-11. doi:10.1053/j.gastro.2011.04.042

41. Lei Z, Tan IB, Das K, et al. Identification of molecular subtypes of gastric cancer with different responses to PI3-kinase inhibitors and 5-fluorouracil. *Gastroenterology*. 2013;145(3):554-565. doi:10.1053/j.gastro.2013.05.010

42. Comprehensive molecular characterization of gastric adenocarcinoma. *Nature*. 2014;513(7517):202-209. doi:10.1038/nature13480

43. Cristescu R, Lee J, Nebozhyn M, et al. Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes. *Nat Med*. 2015;21(5):449-456. doi:10.1038/nm.3850

44. Lordick F, Carneiro F, Cascinu S, et al. Gastric cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. *Ann Oncol Off J Eur Soc Med Oncol*. 2022;33(10):1005-1020. doi:10.1016/j.annonc.2022.07.004

45. Maspero M, Sposito C, Benedetti A, et al. Impact of Surgical Margins on Overall Survival after Gastrectomy for Gastric Cancer: A Validation of Japanese Gastric Cancer Association Guidelines on a Western Series. *Ann Surg Oncol*. 2022;29(5):3096-3108. doi:10.1245/s10434-021-11010-0

46. De Manzoni G, Marrelli D, Baiocchi GL, et al. The Italian Research Group for Gastric Cancer (GIRCG) guidelines for gastric cancer staging and treatment: 2015. *Gastric cancer Off J Int Gastric Cancer Assoc Japanese Gastric Cancer Assoc*. 2017;20(1):20-30. doi:10.1007/s10120-016-0615-3

47. Allum W, Lordick F, Alsina M, et al. ECCO essential requirements for quality cancer care: Oesophageal and gastric cancer. *Crit Rev Oncol Hematol*. 2018;122:179-193. doi:10.1016/j.critrevonc.2017.12.019

48. Al-Batran S-E, Homann N, Pauligk C, et al. Perioperative chemotherapy with fluorouracil plus leucovorin, oxaliplatin, and docetaxel versus fluorouracil or capecitabine plus cisplatin and epirubicin for locally advanced, resectable gastric or gastro-oesophageal junction adenocarcinoma (FLOT4): a r. *Lancet (London, England)*. 2019;393(10184):1948-1957. doi:10.1016/S0140-6736(18)32557-1

49. Cats A, Jansen EPM, van Grieken NCT, et al. Chemotherapy versus chemoradiotherapy after surgery and preoperative chemotherapy for resectable gastric cancer (CRITICS): an international, open-label, randomised phase 3 trial. *Lancet Oncol*. 2018;19(5):616-628. doi:10.1016/S1470-2045(18)30132-3

50. Lee J, Lim DH, Kim S, et al. Phase III trial comparing capecitabine plus cisplatin versus capecitabine plus cisplatin with concurrent capecitabine radiotherapy in completely resected gastric cancer with D2 lymph node dissection: the ARTIST trial. *J Clin Oncol Off J Am Soc Clin Oncol*. 2012;30(3):268-273. doi:10.1200/JCO.2011.39.1953

51. Park SH, Lim DH, Sohn TS, et al. A randomized phase III trial comparing adjuvant single-agent S1, S-1 with oxaliplatin, and postoperative chemoradiation with S-1 and oxaliplatin in patients with node-positive gastric cancer after D2 resection: the ARTIST 2 trial(☆). *Ann Oncol Off J Eur Soc Med Oncol*. 2021;32(3):368-374. doi:10.1016/j.annonc.2020.11.017

52. Macdonald JS, Smalley SR, Benedetti J, et al. Chemoradiotherapy after surgery compared with surgery alone for adenocarcinoma of the stomach or gastroesophageal junction. *N Engl J Med*. 2001;345(10):725-730. doi:10.1056/NEJMoa010187

53. Al-Batran S-E, Lorenzen S, Homann N, et al. 1429P Pathological regression in patients with microsatellite instability (MSI) receiving perioperative atezolizumab in combination with FLOT vs. FLOT alone for resectable esophagogastric adenocarcinoma: Results from the DANTE trial of the German Gastric . *Ann Oncol*. 2021;32:S1069. doi:https://doi.org/10.1016/j.annonc.2021.08.1538

54. Al-Batran S-E, Hartmann JT, Probst S, et al. Phase III trial in metastatic gastroesophageal adenocarcinoma with fluorouracil, leucovorin plus either oxaliplatin or cisplatin: a study of the Arbeitsgemeinschaft Internistische Onkologie. *J Clin Oncol Off J Am Soc Clin Oncol*. 2008;26(9):1435-1442. doi:10.1200/JCO.2007.13.9378

55. Koizumi W, Narahara H, Hara T, et al. S-1 plus cisplatin versus S-1 alone for first-line treatment of advanced gastric cancer (SPIRITS trial): a phase III trial. *Lancet Oncol*. 2008;9(3):215-221. doi:10.1016/S1470-2045(08)70035-4

56. Van Cutsem E, Moiseyenko VM, Tjulandin S, et al. Phase III study of docetaxel and cisplatin plus fluorouracil compared with cisplatin and fluorouracil as first-line therapy for advanced gastric cancer: a report of the V325 Study Group. *J Clin Oncol Off J Am Soc Clin Oncol*. 2006;24(31):4991-4997. doi:10.1200/JCO.2006.06.8429

57. Yamada Y, Boku N, Mizusawa J, et al. Docetaxel plus cisplatin and S-1 versus cisplatin and S-1 in patients with advanced gastric cancer (JCOG1013): an open-label, phase 3, randomised controlled trial. *lancet Gastroenterol Hepatol*. 2019;4(7):501-510. doi:10.1016/S2468-1253(19)30083-4

58. Guimbaud R, Louvet C, Ries P, et al. Prospective, randomized, multicenter, phase III study of fluorouracil, leucovorin, and irinotecan versus epirubicin, cisplatin, and capecitabine in advanced gastric adenocarcinoma: a French intergroup (Fédération Francophone de Cancérologie Digestive, Fé. *J Clin Oncol Off J Am Soc Clin Oncol*. 2014;32(31):3520-3526. doi:10.1200/JCO.2013.54.1011

59. Bang Y-J, Van Cutsem E, Feyereislova A, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet (London, England)*. 2010;376(9742):687-697. doi:10.1016/S0140-6736(10)61121-X

60. Janjigian YY, Shitara K, Moehler M, et al. First-line nivolumab plus chemotherapy versus chemotherapy alone for advanced gastric, gastro-oesophageal junction, and oesophageal adenocarcinoma (CheckMate 649): a randomised, open-label, phase 3 trial. *Lancet (London, England)*. 2021;398(10294):27-40. doi:10.1016/S0140-6736(21)00797-2

61. Shitara K, Van Cutsem E, Bang Y-J, et al. Efficacy and Safety of Pembrolizumab or Pembrolizumab Plus Chemotherapy vs Chemotherapy Alone for Patients With First-line, Advanced Gastric Cancer: The KEYNOTE-062 Phase 3 Randomized Clinical Trial. *JAMA Oncol*. 2020;6(10):1571-1580. doi:10.1001/jamaoncol.2020.3370

62. Sun J-M, Shen L, Shah MA, et al. Pembrolizumab plus chemotherapy versus chemotherapy alone for first-line treatment of advanced oesophageal cancer (KEYNOTE-590): a randomised, placebo-controlled, phase 3 study. *Lancet (London, England)*. 2021;398(10302):759-771. doi:10.1016/S0140-6736(21)01234-4

63. Pietrantonio F, Randon G, Di Bartolomeo M, et al. Predictive role of microsatellite instability for PD-1 blockade in patients with advanced gastric cancer: a meta-analysis of randomized clinical trials. *ESMO open*. 2021;6(1):100036. doi:10.1016/j.esmoop.2020.100036

64. Fujitani K, Yang H-K, Mizusawa J, et al. Gastrectomy plus chemotherapy versus chemotherapy alone for advanced gastric cancer with a single non-curable factor (REGATTA): a phase 3, randomised controlled trial. *Lancet Oncol*. 2016;17(3):309-318. doi:10.1016/S1470-2045(15)00553-7

65. Al-Batran S-E, Homann N, Pauligk C, et al. Effect of Neoadjuvant Chemotherapy Followed by Surgical Resection on Survival in Patients With Limited Metastatic Gastric or Gastroesophageal Junction Cancer: The AIO-FLOT3 Trial. *JAMA Oncol*. 2017;3(9):1237-1244. doi:10.1001/jamaoncol.2017.0515

66. Rau B, Lang H, Königsrainer A, et al. 1376O The effect of hyperthermic intraperitoneal chemotherapy (HIPEC) upon cytoreductive surgery (CRS) in gastric cancer (GC) with synchronous peritoneal metastasis (PM): A randomized multicentre phase III trial (GASTRIPEC-I-trial). *Ann Oncol*. 2021;32:S1040. doi:https://doi.org/10.1016/j.annonc.2021.08.1485

67. Eveno C, Jouvin I, Pocard M. PIPAC EstoK 01: Pressurized IntraPeritoneal Aerosol Chemotherapy with cisplatin and doxorubicin (PIPAC C/D) in gastric peritoneal metastasis: a randomized and multicenter phase II study. *Pleura and peritoneum*. 2018;3(2):20180116. doi:10.1515/pp-2018-0116

68. Wagner AD, Syn NL, Moehler M, et al. Chemotherapy for advanced gastric cancer. *Cochrane database Syst Rev*. 2017;8(8):CD004064. doi:10.1002/14651858.CD004064.pub4

69. Wilke H, Muro K, Van Cutsem E, et al. Ramucirumab plus paclitaxel versus placebo plus paclitaxel in patients with previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (RAINBOW): a double-blind, randomised phase 3 trial. *Lancet Oncol*. 2014;15(11):1224-1235. doi:10.1016/S1470-2045(14)70420-6

70. Fuchs CS, Tomasek J, Yong CJ, et al. Ramucirumab monotherapy for previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (REGARD): an international, randomised, multicentre, placebo-controlled, phase 3 trial. *Lancet (London, England)*. 2014;383(9911):31-39. doi:10.1016/S0140-6736(13)61719-5

71. Chao J, Fuchs CS, Shitara K, et al. Assessment of Pembrolizumab Therapy for the Treatment of Microsatellite Instability-High Gastric or Gastroesophageal Junction Cancer Among

Patients in the KEYNOTE-059, KEYNOTE-061, and KEYNOTE-062 Clinical Trials. *JAMA Oncol*. 2021;7(6):895-902. doi:10.1001/jamaoncol.2021.0275

72. Marabelle A, Le DT, Ascierto PA, et al. Efficacy of Pembrolizumab in Patients With Noncolorectal High Microsatellite Instability/Mismatch Repair-Deficient Cancer: Results From the Phase II KEYNOTE-158 Study. *J Clin Oncol Off J Am Soc Clin Oncol*. 2020;38(1):1-10. doi:10.1200/JCO.19.02105

73. Shitara K, Bang Y-J, Iwasa S, et al. Trastuzumab Deruxtecan in Previously Treated HER2-Positive Gastric Cancer. *N Engl J Med*. May 2020. doi:10.1056/NEJMoa2004413

74. Shitara K, Doi T, Dvorkin M, et al. Trifluridine/tipiracil versus placebo in patients with heavily pretreated metastatic gastric cancer (TAGS): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol*. 2018;19(11):1437-1448. doi:10.1016/S1470-2045(18)30739-3

75. Goldberg AD, Allis CD, Bernstein E. Epigenetics: a landscape takes shape. *Cell*. 2007;128(4):635-638. doi:10.1016/j.cell.2007.02.006

76. Replogle JM, De Jager PL. Epigenomics in translational research. *Transl Res*. 2015;165(1):7-11. doi:10.1016/j.trsl.2014.09.011

77. Esteller M. Non-coding RNAs in human disease. *Nat Rev Genet*. 2011;12(12):861-874. doi:10.1038/nrg3074

78. Goll MG, Bestor TH. Eukaryotic cytosine methyltransferases. *Annu Rev Biochem*. 2005;74:481-514. doi:10.1146/annurev.biochem.74.010904.153721

79. Stricker SH, Köferle A, Beck S. From profiles to function in epigenomics. *Nat Rev Genet*. 2017;18(1):51-66. doi:10.1038/nrg.2016.138

80. Parbin S, Kar S, Shilpi A, et al. Histone deacetylases: a saga of perturbed acetylation homeostasis in cancer. *J Histochem Cytochem Off J Histochem Soc*. 2014;62(1):11-33. doi:10.1369/0022155413506582

81. Singh BN, Zhang G, Hwa YL, Li J, Dowdy SC, Jiang S-W. Nonhistone protein acetylation as cancer therapy targets. *Expert Rev Anticancer Ther*. 2010;10(6):935-954. doi:10.1586/era.10.62

82. Canale M, Casadei-Gardini A, Ulivi P, et al. Epigenetic Mechanisms in Gastric Cancer: Potential New Therapeutic Opportunities. *Int J Mol Sci*. 2020;21(15). doi:10.3390/ijms21155500

83. Li Y, Liang J, Hou P. Hypermethylation in gastric cancer. *Clin Chim Acta*. 2015;448:124-132. doi:10.1016/j.cca.2015.07.001

84. Yoda Y, Takeshima H, Niwa T, et al. Integrated analysis of cancer-related pathways affected by genetic and epigenetic alterations in gastric cancer. *Gastric cancer Off J Int Gastric Cancer Assoc Japanese Gastric Cancer Assoc*. 2015;18(1):65-76. doi:10.1007/s10120-014-0348-0

85. Fattahi S, Nikbakhsh N, Taheri H, et al. Prevalence of multiple infections and the risk of gastric adenocarcinoma development at earlier age. *Diagn Microbiol Infect Dis*. 2018;92(1):62-68. doi:10.1016/j.diagmicrobio.2018.04.015

86. Muhammad JS, Eladl MA, Khoder G. Helicobacter pylori-induced DNA Methylation as an Epigenetic Modulator of Gastric Cancer: Recent Outcomes and Future Direction. *Pathog (Basel, Switzerland)*. 2019;8(1). doi:10.3390/pathogens8010023

87. Peng Y, Wu Q, Wang L, Wang H, Yin F. A DNA methylation signature to improve survival prediction of gastric cancer. *Clin Epigenetics*. 2020;12(1):1-16. doi:10.1186/s13148-020-0807-x

88. Bai Y, Wei C, Zhong Y, et al. Development and validation of a prognostic nomogram for gastric cancer based on DNA methylation-driven differentially expressed genes. *Int J Biol Sci*. 2020;16(7):1153-1165. doi:10.7150/ijbs.41587

89. Padmanabhan N, Ushijima T, Tan P. How to stomach an epigenetic insult: the gastric cancer epigenome. *Nat Rev Gastroenterol Hepatol*. 2017;14(8):467-478. doi:10.1038/nrgastro.2017.53

90. Matsusaka K, Kaneda A, Nagae G, et al. Classification of Epstein-Barr virus-positive gastric cancers by definition of DNA methylation epigenotypes. *Cancer Res*. 2011;71(23):7187-7197. doi:10.1158/0008-5472.CAN-11-1349

91. Urabe M, Matsusaka K, Ushiku T, et al. Adenocarcinoma of the stomach and esophagogastric junction with low DNA methylation show poor prognoses. *Gastric cancer Off J Int Gastric Cancer Assoc Japanese Gastric Cancer Assoc*. 2023;26(1):95-107. doi:10.1007/s10120-022-01344-3

92. Yang X, Han H, De Carvalho DD, Lay FD, Jones PA, Liang G. Gene body methylation can alter gene expression and is a therapeutic target in cancer. *Cancer Cell*. 2014;26(4):577-590. doi:10.1016/j.ccr.2014.07.028

93. Kim Y, Wen X, Jeong S, Cho N-Y, Kim WH, Kang GH. Combinatory low methylation statuses of SAT-α and L1 are associated with shortened survival time in patients with advanced gastric cancer. *Gastric cancer Off J Int Gastric Cancer Assoc Japanese Gastric Cancer Assoc*. 2019;22(1):37-47. doi:10.1007/s10120-018-0852-8

94. Corso G, Carvalho J, Marrelli D, et al. Somatic mutations and deletions of the E-cadherin gene predict poor survival of patients with gastric cancer. *J Clin Oncol Off J Am Soc Clin Oncol*. 2013;31(7):868-875. doi:10.1200/JCO.2012.44.4612

95. Shenoy S. CDH1 (E-Cadherin) Mutation and Gastric Cancer: Genetics, Molecular Mechanisms and Guidelines for Management. *Cancer Manag Res*. 2019;11:10477-10486. doi:10.2147/CMAR.S208818

96. van der Post RS, Vogelaar IP, Carneiro F, et al. Hereditary diffuse gastric cancer: Updated clinical guidelines with an emphasis on germline CDH1 mutation carriers. *J Med Genet*. 2015;52(6):361-374. doi:10.1136/jmedgenet-2015-103094

97. Graziano F, Arduini F, Ruzzo A, et al. Combined analysis of E-cadherin gene (CDH1) promoter hypermethylation and E-cadherin protein expression in patients with gastric cancer: implications for treatment with demethylating drugs. *Ann Oncol Off J Eur Soc Med Oncol*. 2004;15(3):489-492. doi:10.1093/annonc/mdh108

98. Balgkouranidou I, Matthaios D, Karayiannakis A, et al. Prognostic role of APC and RASSF1A promoter methylation status in cell free circulating DNA of operable gastric cancer patients. *Mutat Res*. 2015;778:46-51. doi:10.1016/j.mrfmmm.2015.05.002

99. Li QL, Ito K, Sakakura C, et al. Causal relationship between the loss of RUNX3 expression and gastric cancer. *Cell*. 2002;109(1):113-124. doi:10.1016/S0092-8674(02)00690-6

100. Dong C-X, Deng D-J, Pan K-F, et al. Promoter methylation of p16 associated with Helicobacter pylori infection in precancerous gastric lesions: a population-based study. *Int J cancer*. 2009;124(2):434-439. doi:10.1002/ijc.23891

101. Fang JY, Zhu SS, Xiao SD, et al. Studies on the hypomethylation of c-myc, c-Ha-ras oncogenes and histopathological changes in human gastric carcinoma. *J Gastroenterol Hepatol*. 1996;11(11):1079-1082. doi:10.1111/j.1440-1746.1996.tb00040.x

102. Rando OJ. Combinatorial complexity in chromatin structure and function: revisiting the histone code. *Curr Opin Genet Dev*. 2012;22(2):148-155. doi:10.1016/j.gde.2012.02.013

103. Park YS, Jin MY, Kim YJ, Yook JH, Kim BS, Jang SJ. The global histone modification pattern

correlates with cancer recurrence and overall survival in gastric adenocarcinoma. *Ann Surg Oncol*. 2008;15(7):1968-1976. doi:10.1245/s10434-008-9927-9

104. Wang Z, Tang F, Qi G, et al. KDM5B is overexpressed in gastric cancer and is required for gastric cancer cell proliferation and metastasis. *Am J Cancer Res*. 2015;5(1):87-100. http://www.ncbi.nlm.nih.gov/pubmed/25628922%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4300698.

105. Hu CE, Liu YC, Zhang HD, Huang GJ. JMJD2A predicts prognosis and regulates cell growth in human gastric cancer. *Biochem Biophys Res Commun*. 2014;449(1):1-7. doi:10.1016/j.bbrc.2014.04.126

106. Estève P-O, Chin HG, Smallwood A, et al. Direct interaction between DNMT1 and G9a coordinates DNA and histone methylation during replication. *Genes Dev*. 2006;20(22):3089-3103. doi:10.1101/gad.1463706

107. Calcagno DQ, Gigek CO, Chen ES, Burbano RR, Smith M de AC. DNA and histone methylation in gastric carcinogenesis. *World J Gastroenterol*. 2013;19(8):1182-1192. doi:10.3748/wjg.v19.i8.1182

108. Schizas D, Mastoraki A, Naar L, et al. Histone Deacetylases (HDACs) in gastric cancer: An update of their emerging prognostic and therapeutic role. *Curr Med Chem*. July 2019. doi:10.2174/0929867326666190712160842

109. Bouyahya A, Mechchate H, Oumeslakht L, et al. The Role of Epigenetic Modifications in Human Cancers and the Use of Natural Compounds as Epidrugs: Mechanistic Pathways and Pharmacodynamic Actions. *Biomolecules*. 2022;12(3). doi:10.3390/biom12030367

110. Abdelfatah E, Kerner Z, Nanda N, Ahuja N. Epigenetic therapy in gastrointestinal cancer: the right combination. *Therap Adv Gastroenterol*. 2016;9(4):560-579. doi:10.1177/1756283X16644247

111. Nervi C, De Marinis E, Codacci-Pisanelli G. Epigenetic treatment of solid tumours: a review of clinical trials. *Clin Epigenetics*. 2015;7:127. doi:10.1186/s13148-015-0157-2

112. Morel D, Jeffery D, Aspeslagh S, Almouzni G, Postel-Vinay S. Combining epigenetic drugs with other therapies for solid tumours - past lessons and future promise. *Nat Rev Clin Oncol*. 2020;17(2):91-107. doi:10.1038/s41571-019-0267-4

113. Zouridis H, Deng N, Ivanova T, et al. Methylation subtypes and large-scale epigenetic alterations in gastric cancer. *Sci Transl Med*. 2012;4(156):156ra140.

doi:10.1126/scitranslmed.3004504

114. Yang W, Mok MTS, Li MSM, et al. Epigenetic silencing of GDF1 disrupts SMAD signaling to reinforce gastric cancer development. *Oncogene*. 2016;35(16):2133-2144. doi:10.1038/onc.2015.276

115. Schneider BJ, Shah MA, Klute K, et al. Phase I study of epigenetic priming with azacitidine prior to standard neoadjuvant chemotherapy for patients with resectable gastric and esophageal adenocarcinoma: Evidence of tumor hypomethylation as an indicator of major histopathologic response. *Clin Cancer Res*. 2017;23(11):2673-2680. doi:10.1158/1078-0432.CCR-16-1896

116. Regel I, Merkl L, Friedrich T, et al. Pan-histone deacetylase inhibitor panobinostat sensitizes gastric cancer cells to anthracyclines via induction of CITED2. *Gastroenterology*. 2012;143(1):99-109.e10. doi:10.1053/j.gastro.2012.03.035

117. Hernández-Rosas F, Hernández-Oliveras A, Flores-Peredo L, et al. Histone deacetylase inhibitors induce the expression of tumor suppressor genes Per1 and Per2 in human gastric cancer cells. *Oncol Lett*. 2018;16(2):1981-1990. doi:10.3892/ol.2018.8851

118. Dong J, Zheng N, Wang X, et al. A novel HDAC6 inhibitor exerts an anti-cancer effect by triggering cell cycle arrest and apoptosis in gastric cancer. *Eur J Pharmacol*. 2018;828(December 2017):67-79. doi:10.1016/j.ejphar.2018.03.026

119. Yoo C, Ryu M-H, Na Y-S, Ryoo B-Y, Lee C-W, Kang Y-K. Vorinostat in combination with capecitabine plus cisplatin as a first-line chemotherapy for patients with metastatic or unresectable gastric cancer: phase II study and biomarker analysis. *Br J Cancer*. 2016;114(11):1185-1190. doi:10.1038/bjc.2016.125

120. Covre A, Coral S, Nicolay H, et al. Antitumor activity of epigenetic immunomodulation combined with CTLA-4 blockade in syngeneic mouse models. *Oncoimmunology*. 2015;4(8):e1019978. doi:10.1080/2162402X.2015.1019978

121. San José-Enériz E, Agirre X, Rabal O, et al. Discovery of first-in-class reversible dual small molecule inhibitors against G9a and DNMTs in hematological malignancies. *Nat Commun*. 2017;8:15424. doi:10.1038/ncomms15424

122. Barcena-Varela M, Caruso S, Llerena S, et al. Dual Targeting of Histone Methyltransferase G9a and DNA-Methyltransferase 1 for the Treatment of Experimental Hepatocellular Carcinoma. *Hepatology*. 2019;69(2):587-603. doi:10.1002/hep.30168

123. Colyn L, Bárcena-Varela M, Álvarez-Sola G, et al. Dual targeting of G9a and DNMT1 for the

treatment of experimental cholangiocarcinoma. *Hepatology*. November 2020. doi:10.1002/hep.31642

124. Bray NL, Pimentel H, Melsted P, Pachter L. Near-optimal probabilistic RNA-seq quantification. *Nat Biotechnol*. 2016;34(5):525-527. doi:10.1038/nbt.3519

125. Soneson C, Love MI, Robinson MD. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. *F1000Research*. 2015;4:1521. doi:10.12688/f1000research.7563.2

126. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15(12):550. doi:10.1186/s13059-014-0550-8

127. Waskom M. Seaborn: Statistical Data Visualization. *J Open Source Softw*. 2021;6(60):3021. doi:10.21105/joss.03021

128. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A*. 2005;102(43):15545-15550. doi:10.1073/pnas.0506580102

129. Fang Z, Liu X, Peltz G. GSEApy: a comprehensive package for performing gene set enrichment analysis in Python. *Bioinformatics*. 2023;39(1). doi:10.1093/bioinformatics/btac757

130. Du J, Johnson LM, Jacobsen SE, Patel DJ. DNA methylation pathways and their crosstalk with histone methylation. *Nat Rev Mol Cell Biol*. 2015;16(9):519-532. doi:10.1038/nrm4043

131. Ferry L, Fournier A, Tsusaka T, et al. Methylation of DNA Ligase 1 by G9a/GLP Recruits UHRF1 to Replicating DNA and Regulates DNA Methylation. *Mol Cell*. 2017;67(4):550-565.e5. doi:10.1016/j.molcel.2017.07.012

132. Zhang C, Wei S, Hu J, Xiong Z. Upregulated expression of G9a is correlated with poor prognosis of gastric cancer patients. *Medicine (Baltimore)*. 2019;98(48):e18212. doi:10.1097/MD.0000000000018212

133. Hu L, Zang M, Wang H-X, et al. G9A promotes gastric cancer metastasis by upregulating ITGB3 in a SET domain-independent manner. *Cell Death Dis*. 2018;9(3):278. doi:10.1038/s41419-018-0322-6

134. Ma T, Li H, Sun M, Yuan Y, Sun L-P. DNMT1 overexpression predicting gastric carcinogenesis, subsequent progression and prognosis: a meta and bioinformatic analysis. *Oncotarget*. 2017;8(56):96396-96408. doi:10.18632/oncotarget.21480

135. Zhou L, Shang Y, Jin Z, et al. UHRF1 promotes proliferation of gastric cancer via mediating tumor suppressor gene hypermethylation. *Cancer Biol Ther*. 2015;16(8):1241-1251. doi:10.1080/15384047.2015.1056411

136. Babacan NA, Egilmez HR, Yücel B, et al. The prognostic value of UHRF-1 and p53 in gastric cancer. *Saudi J Gastroenterol Off J Saudi Gastroenterol Assoc*. 2016;22(1):25-29. doi:10.4103/1319-3767.173755

137. Moro H, Hattori N, Nakamura Y, et al. Epigenetic priming sensitizes gastric cancer cells to irinotecan and cisplatin by restoring multiple pathways. *Gastric Cancer*. September 2019. doi:10.1007/s10120-019-01010-1

138. Gettinger SN, Bazhenova LA, Langer CJ, et al. Activity and safety of brigatinib in ALK-rearranged non-small-cell lung cancer and other malignancies: a single-arm, open-label, phase 1/2 trial. *Lancet Oncol*. 2016;17(12):1683-1696. doi:10.1016/S1470-2045(16)30392-8

139. Schmitt CA, Wang B, Demaria M. Senescence and cancer - role and therapeutic opportunities. *Nat Rev Clin Oncol*. 2022;19(10):619-636. doi:10.1038/s41571-022-00668-4

140. Hernandez-Segura A, Nehme J, Demaria M. Hallmarks of Cellular Senescence. *Trends Cell Biol*. 2018;28(6):436-453. doi:10.1016/j.tcb.2018.02.001

141. Lee S, Schmitt CA. The dynamic nature of senescence in cancer. *Nat Cell Biol*. 2019;21(1):94-101. doi:10.1038/s41556-018-0249-2

142. Crouch J, Shvedova M, Thanapaul RJRS, Botchkarev V, Roh D. Epigenetic Regulation of Cellular Senescence. *Cells*. 2022;11(4). doi:10.3390/cells11040672

143. Stern DF. Keeping Tumors Out of the MAPK Fitness Zone. *Cancer Discov*. 2018;8(1):20-23. doi:10.1158/2159-8290.CD-17-1243

144. Braicu C, Buse M, Busuioc C, et al. A Comprehensive Review on MAPK: A Promising Therapeutic Target in Cancer. *Cancers (Basel)*. 2019;11(10). doi:10.3390/cancers11101618

145. Grabsch H, Sivakumar S, Gray S, Gabbert HE, Müller W. HER2 expression in gastric cancer: Rare, heterogeneous and of no prognostic value - conclusions from 924 cases of two independent series. *Cell Oncol Off J Int Soc Cell Oncol*. 2010;32(1-2):57-65. doi:10.3233/CLO-2009-0497

146. Zong W-X, Rabinowitz JD, White E. Mitochondria and Cancer. *Mol Cell*. 2016;61(5):667-676. doi:10.1016/j.molcel.2016.02.011

147. Urra FA, Weiss-López B, Araya-Maturana R. Determinants of Anti-Cancer Effect of Mitochondrial Electron Transport Chain Inhibitors: Bioenergetic Profile and Metabolic Flexibility of Cancer Cells. *Curr Pharm Des*. 2016;22(39):5998-6008. doi:10.2174/1381612822666160719122626

148. Harper AE, Miller RH, Block KP. Branched-chain amino acid metabolism. *Annu Rev Nutr*. 1984;4:409-454. doi:10.1146/annurev.nu.04.070184.002205

149. Dill KA. Dominant forces in protein folding. *Biochemistry*. 1990;29(31):7133-7155. doi:10.1021/bi00483a001

150. Amorim Franco TM, Blanchard JS. Bacterial Branched-Chain Amino Acid Biosynthesis: Structures, Mechanisms, and Drugability. *Biochemistry*. 2017;56(44):5849-5865. doi:10.1021/acs.biochem.7b00849

151. Ananieva EA, Powell JD, Hutson SM. Leucine Metabolism in T Cell Activation: mTOR Signaling and Beyond. *Adv Nutr*. 2016;7(4):798S-805S. doi:10.3945/an.115.011221

152. Nezami Ranjbar MR, Luo Y, Di Poto C, et al. GC-MS Based Plasma Metabolomics for Identification of Candidate Biomarkers for Hepatocellular Carcinoma in Egyptian Cohort. *PLoS One*. 2015;10(6):e0127299. doi:10.1371/journal.pone.0127299

153. Budhathoki S, Iwasaki M, Yamaji T, Yamamoto H, Kato Y, Tsugane S. Association of plasma concentrations of branched-chain amino acids with risk of colorectal adenoma in a large Japanese population. *Ann Oncol Off J Eur Soc Med Oncol*. 2017;28(4):818-823. doi:10.1093/annonc/mdw680

154. Mayers JR, Wu C, Clish CB, et al. Elevation of circulating branched-chain amino acids is an early event in human pancreatic adenocarcinoma development. *Nat Med*. 2014;20(10):1193-1198. doi:10.1038/nm.3686

155. Jung MK, Okekunle AP, Lee JE, Sung MK, Lim YJ. Role of Branched-chain Amino Acid Metabolism in Tumor Development and Progression. *J cancer Prev*. 2021;26(4):237-243. doi:10.15430/JCP.2021.26.4.237