UNIVERSITÀ
DEGLI STUDI
FIRENZE

DOTTORATO DI RICERCA IN
SCIENZE BIOMEDICHE

CICLO XXXVI

Coordinatore Prof. Fabrizio Chiti

Molecular features of chemoresistance in Gastric Adenocarcinoma

Settore Scientifico Disciplinare **MED/04**

Dottorando

Dott. Giampaolo Versienti

Supervisore

Prof.ssa Laura Papucci

Anni 2020 – 2023

Summary

1. Introduction

1.1 Gastric Cancer Epidemiology

Gastric Cancer (GC) is one of the main malignancies in the world. Even though we are now observing a decline in incidence in most Western countries *(Song H et al., 2015)*, according to Globocan 2020, GC is the sixth tumor form for new cases (representing 5.6% of the total) and the fourth for mortality (representing 7.7% among all cancers (in 2020 caused over 700.000 cancer-related deaths) (fig.1) *(Sung H et al., 2021)*.

Incidence of GC is straightly correlated to aging and sex: it increases with age and is generally two to three times more present in men than in women *(Luo G et al., 2017)*. Moreover, GC shows a considerable geographic variation with the highest incidence value is found in Eastern Asia (32.1 per 100.000 in men, 13,2 per 100.000 in women), while the regions with lowest incidence are North America (5,6 per 100.000 in men, 2,8 per 100.000 in women) and Africa (about 5 per 100.000 in men and 3-4 per 100.000 for women) *(Ferlay J et al., 2021)*.

Japan has the highest number of male cases diagnosed per year, but the 5-year survival rate it's around 90%, compared to 10-30% in Europe. In fact, while in Japan there is a strong mass screening policy starting from the age of 25 in males, in Western Countries the disease is spotted when the symptoms become evident, and the tumor is in advanced stages. This late diagnosis causes a worse prognosis for patient: in USA the average 5-year survival is 68,8% for pre-metastatic diagnosis, whereas it falls to 5,3% in metastatic patients. Endoscopy therefore represents the most sensitive and reliable screening method for diagnosis, but it is extremely expensive as mass screening test, and it's recommended only in regions with a high incidence rate, such as Japan *(Sitarz R et al., 2018)*.

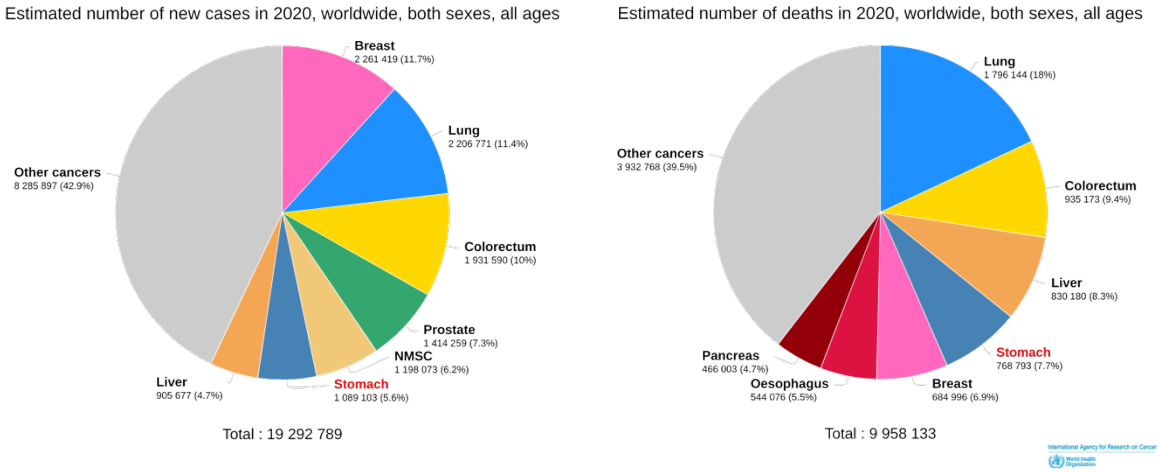
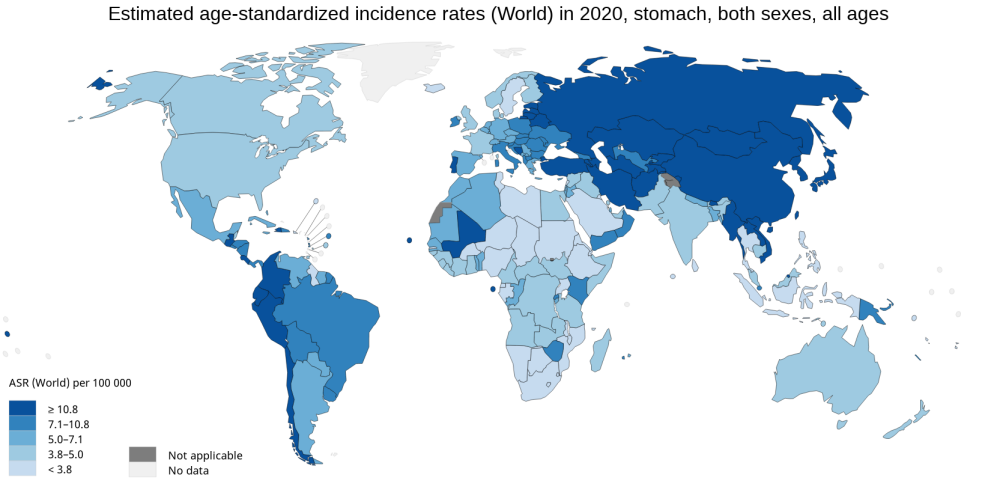


Figure 1: incidence and mortality of tumor (updated 2020) (*Sung et al. 2021*)



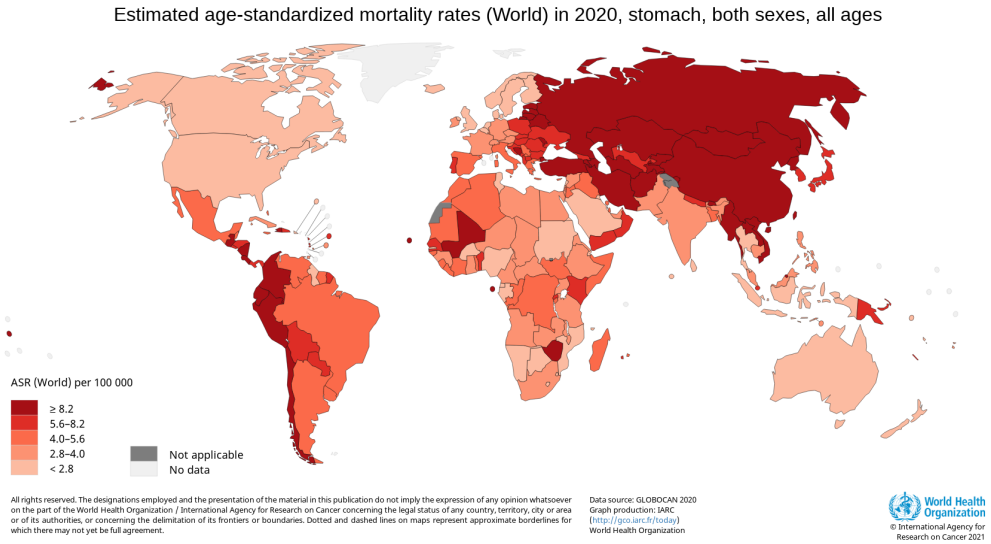


Figure 2: Worldwide gastric cancer incidence and mortality rates (age-adjusted according to the world standard population, per 100 000) in males and females in 2020 (*Morgan E et al., 2021*).

1.2 Gastric Cancer Etiology

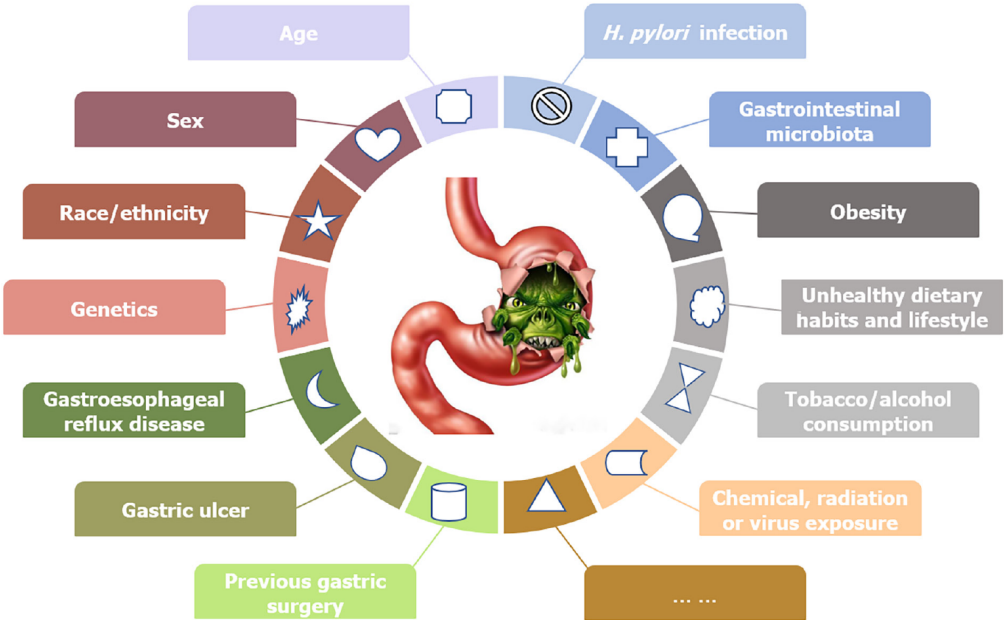


Figure 3: Summarization of GC risk factors (*Yang WJ et al., 2023*).

GC Etiology, in the same way of other tumor, is a combination of environmental and genetic factors. Among these, infective agents, lifestyle and diet are certainly the most known.

1.2.1 Diet and Life-style Risk Factors

Diet-related factors have a notable influence on gastric tumor growth. A first possible intervention to reduce the risk of developing this form of cancer is certainly maintain a balanced and healthy diet: among the foods positively associated with the risk of GC we find sausages, bacon and more generally processed red meat; in fact, they contain substances produced at high temperatures during cooking such as heterocyclic aromatic amines, polycyclic aromatic hydrocarbons, recognized as carcinogenic; various compounds found in smoked meat have also been linked to gastric cancer, such as N-nitrous nitrogen compounds and again polycyclic aromatic hydrocarbons *(Bouvard V et al., 2015)*. Furthermore, these foods contain high levels of salt, another risk factor linked to gastric cancer *(Nomura AM et al., 2003)*.

Other risk factors linked to diet may include a high-calorie diet and the consumption of incorrectly refrigerated foods which positively correlate with the development of GC *(Zhang Z et al. 2013)*.

On the other hand, regarding foods negatively associated with the development of GC, we certainly find vegetables, in particular dark green and yellow ones; the reason is their high content of beta-carotene, vitamins C and E and folic acid. The protective role of beta-carotene in GC is now well established: it is an excellent antioxidant and radical scavenger, as well as having immunomodulatory effects useful in carcinogenesis prevention. Vitamin C, being an antioxidant, can counteract the formation of N-nitrous nitrogen compounds in the stomach; however, it is only active in the aqueous phase. Vitamin E, on the other hand, being a fat-soluble vitamin, is also active in a lipidic environment: it also manages to prevent the process of intragastric nitration. Finally, Folic acid is an important intermediate for the biosynthesis of nucleic acids, and its lack has been correlated with tumor genesis *(Nomura AM et al., 2003)*.

Talking about lifestyle, smoking and alcohol, like as for many other malignancies, are the main risk factor GC occurrence. It was observed that in smokers the risk of developing gastric cancer was approximately 1.53, with a higher incidence in men than in women; Has also been demonstrated that tobacco smoking increases the risk of gastric cancer at the *cardia* level and is thought to be implicated in the increased incidence of this type of stomach cancer in developing countries. (WCRF/AICR, 2018 *(Ladeiras-Lopes R et al., 2008).*

Alcohol consumption is also positively correlated GC: opinions on moderate consumption are conflicting, while those on high consumption indicate a significant increase in the risk of GC occurrence; opinions on the quantity of alcohol are also under debate (12). Finally, obesity is a very important factor for tumor development, especially for non-asian males; in obese people, a greater production of Tumor Necrosis factor-alpha (TNF-alpha), Interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) can lead to greater inflammation at stomach level *(Tsugane S and Sasazuki S; Lyn XJ et al. 2014)*.

1.2.2 Infective factors: *Helicobacter Pylori* and *Epstein-Barr Virus*

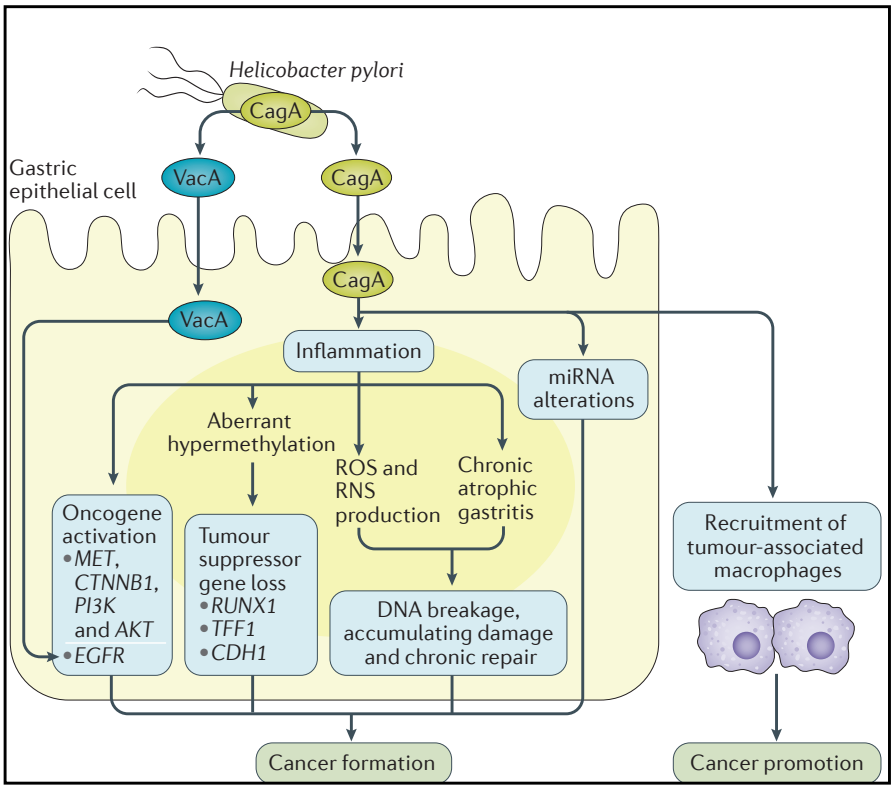


Figure 4: the effect of Helicobacter pylori infection on gastric epithelium. Action of Helicobacter can lead to Gastric Adenocarcinoma (*Ajani JA et al., 2017*).

Helicobacter pylori is a gram negative, microaerophilic bacterium *(Matsunaga S et al., 2018)* whose only natural reservoir is the human stomach *(Alipour M, 2021)*. In this environment it can survive thanks to the urease enzyme and the stomach protective mucus *(Correa P et al., 2006)*. In 1982 Warren and Marshall demonstrated the correlation between *H. pylori* and gastritis *(Warren J et al., 1983)* and in 1994 the International Agency for Research on Cancer (IARC) officially classified this pathogen as a Class I Carcinogen for "Non-Cardias" stomach cancer, confirming it in 2009 *(IARC Working group, 2012)*.

An untreated *H. pylori* infection can affect the patient lifelong. It has been estimated that almost 50% of the world population is affected by this infection, and approximately 15% of it develops gastric ulcers *(Bravo D et al., 2018)*. Many studies indicate that the 2-3% of people infected with this bacterium will develop gastric adenocarcinoma, and 0.1% will develop MALT (mucosal-associated lymphoid tissue) lymphoma *(Diaz P et al., 2018; Bagheri N et al., 2018)*. The initial response of the organism can vary depending on the virulence of the *H. pylori* strain: strains with low virulence produce weak inflammation and generally not progress towards

gastric atrophy or adenocarcinoma; on the other side, highly virulent strains have a closer correlation with more severe gastric diseases, such as adenocarcinoma, duodenal and gastric ulcers, multifocal atrophic gastritis.

The main factor that plays a role in virulence between various strains is the Cytotoxin Associated Gene A (CagA). Bacteria that express these gene are related to a higher inflammation rate and a higher risk of peptic ulcer and GC due to a facilitation to penetrate in the cell. Once in cytoplasm, CagA is phosphorylated and activated, determining damages to cytoskeleton and so inflammation. The more CagA is phosphorylated, the more is the inflammation. There is a different geographical distribution related to CagA: strains found in South-East Asia express an isoform that undergoes intense phosphorylation compared to the Western ones *(Correa P et al., 2006)*.

Another infectious agent related to GC is the Epstein-Barr Virus; the exact mechanism of EBV induction of tumorigenesis in stomach is not yet known *(Cho J et al., 2016)*, but has been observed that approximately 5-10% of GC are associated with this virus *(Boysen T et al., 2009)*: at genre level it is twice as common in males as in females and it has been hypothesized that co-presence of EBV and H. pylori may increase the incidence of gastric cancer *(Murphy G et al., 2009; Singh S et al., 2017)*.

1.2.3 Genetic factors

Although most GC have a sporadic frequency, approximately 10% of GC cases are related to hereditary factors; among this percentage, the 1-3% can be associated with three hereditary syndromes: HDGC (Hereditary Diffuse Gastric Cancer), GAPPS (Gastric Adenocarcinoma and Proximal Polyposis of the Stomach) and FIGC (Familial Intestinal Gastric Cancer) *(Gullo I et al., 2018)*.

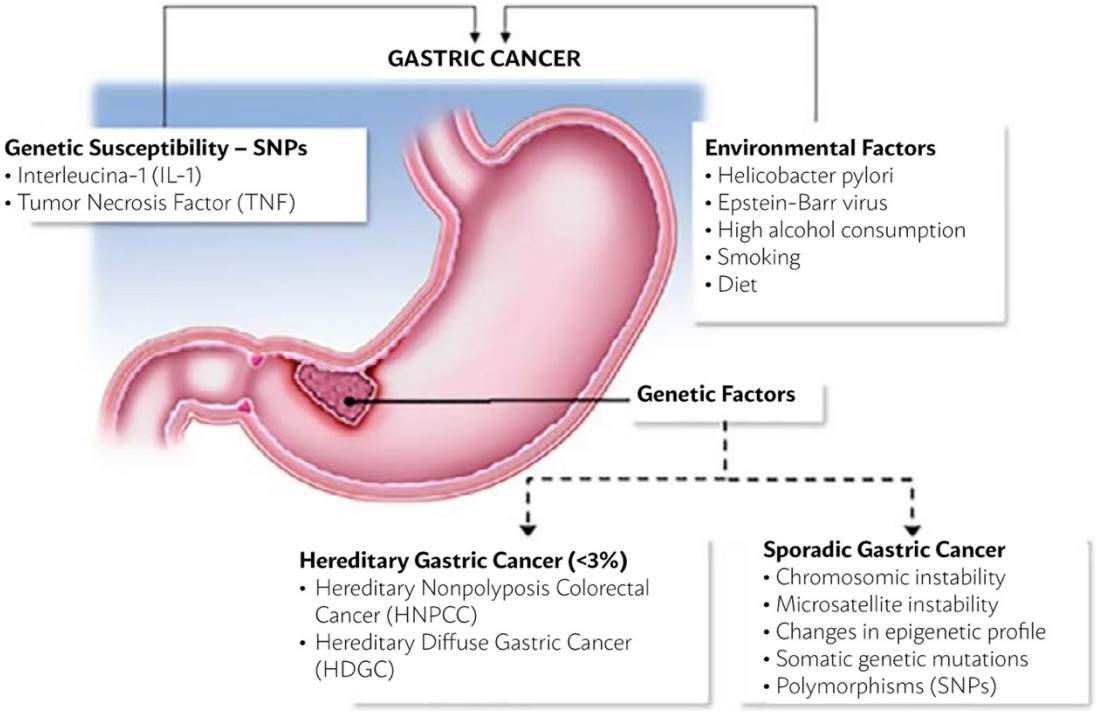


Figure 5: resume of risk factors of GC including genetics factors and associated diseases (*Ramos MFKP et al., 2018*)

HDGC is an autosomal dominant ereditary syndrome characterized by an approximately 80% lifetime risk of developing diffuse GC. This syndrome is related in approximately 50% of cases to the germline mutation of a gene, CDH1, which codes for E-cadherin, important for cell cohesion. In patients with this mutation there is also a risk of developing lobular breast carcinoma and colon cancer. Furthermore, mutations in a second adherents junction protein, α-E-catenin (CTNNA1) have also been observed in a small minority of HDGC cases *(Blair VR et al., 2020)*. GAPPS is a rare dominant autosomal syndrome characterized by characterized by mutation in the 1B region of the APC promoter that codes for β-catenin; is associated with polyposis of the fundic gland of the gastric body, with a high risk of developing gastric adenocarcinoma *(Vogelar IP et al., 2015)*. FIGC, an autosomal dominant hereditary syndrome characterized by IL12RB1 mutation is associated with onset of CG in multiple individuals of the same family *(Vogelar IP et al., 2015)*.

Gastric cancer is also associated with other hereditary tumor syndromes.

Lynch syndrome (LS) or non-polyposis colorectal cancer (HNPCC), an autosomal dominant disease responsible for 2-3% of colorectal cancer cases. It is characterized by the germline mutation of one of the genes responsible for correcting errors in the DNA duplication system, resulting in a change in the length of the repeated

sequences of tumor DNA called MSI (microsatellite instability). MSI is associated with the lack of expression of MLH1, MSH2, MSH6 and/or PMS2 proteins *(Syngal S et al., 2015)*.

Familial Adenomatous Polyposis (FAP) is an autosomal dominant inherited predisposition to adenomatous polyps caused by germline mutations in the APC gene. The risk of FAP patients developing CG is less than 1% and an environmental component is required to transform polyps into adenocarcinomas *(Nakamura K et al., 2017)*.

Peutz-Jeghers Syndrome (PJS) is a rare autosomal dominant inherited condition characterized by hamartomatous gastrointestinal polyposis and melanin patches on the lips and buccal mucosa. Patients affected by PJS have an 89% risk of developing CG and appear to be more predisposed to developing tumors of the intestine, pancreas, breast, testis, ovary and lung *(van Lier MG et al., 2010)*.

Juvenile Polyposis Syndrome (JPS) is caused by a mutation of SMAD4 or BMPR1A genes and manifests with the formation of numerous polyps in the colon or stomach at a young age; the incidence in those with this gastric cancer syndrome stands at around 21% *(Howe JR et al., 2004)*.

Li-Fraumeni Syndrome (LFS) is an autosomal dominant disease caused by mutation of the TP53 gene. Differently from other syndromes, patients do not present site-specific tumors, but multiple primary tumors with divergent phenotypes: GC is detected in 1.8-4.9% of LFS-positive individuals *(Masciari S et al., 2011)*.

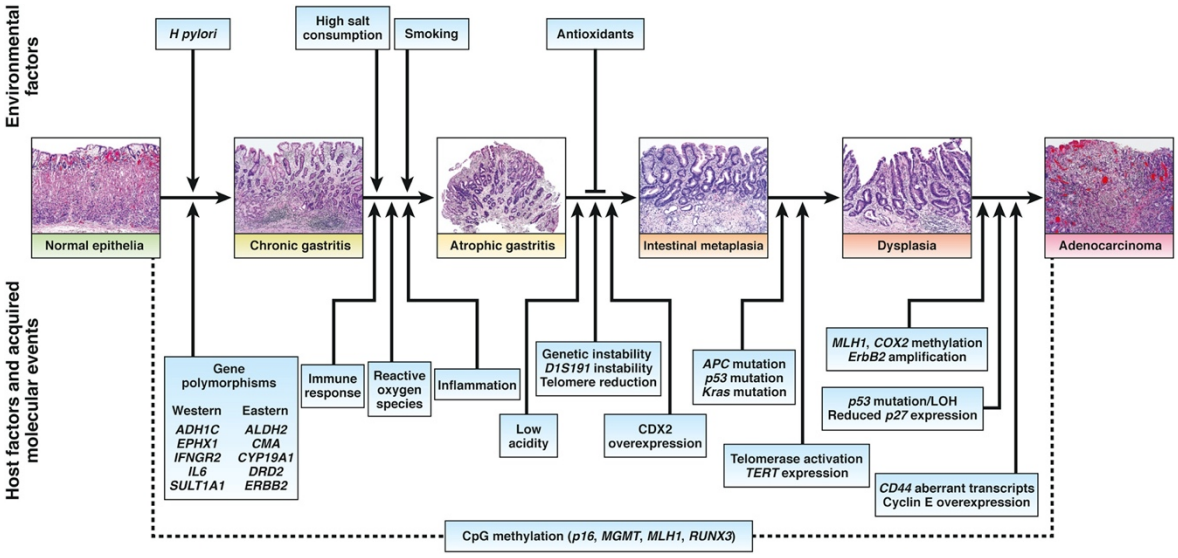


Figure 6: progression of GC, from normal tissue to adenocarcinoma phenotype; environmental and genetic factors intervention during aberrant transformation is enlighten (*Tan P et al., 2015*).

1.3 Gastric Cancer classifications

Gastric Cancer is a term commonly used referring to Gastric Adenocarcinoma, which represent the 95% of every stomach malignancy. The remaining are less common tumor that affect other gastric structures: lymphoma, gastrointestinal stromal and neuroendocrine tumor.

Due to the heterogeneity of gastric adenocarcinoma, with different phenotypes, there are many classification systems.

Based on histological criteria and starting from the '60 we find the Laurén *(Laurén P, 1965)*, Nakamura *(Nakamura K, 1968)*, Ming *(Ming SC, 1977)*, Goseki *(Goseki N et al., 1992)* and Carneiro *(Carneiro S et al., 1995)* classifications. In the 2000' Solcia *(Solcia E et al., 2009)*, the World Health Organization *(Bosman FT, t al. 2010)*, and the Japanese Gastric Cancer Association *(Japanese GCA, 2011)* proposed other classifications.

The most used is the Laurén classification, which distinguish Gastric Adenocarcinoma in two different main types: Diffuse and Intestinal. The diffuse type appears as loosely adherent cells, alone or in small groups, which grow in a highly infiltrating form. The Intestinal type is characterized by the formation of clearly visible papillary or glandular structures with adherent cells. Gastric tumors that cannot been classified in these two categories are defined as *Mixed* or *Indeterminate.* The first one occurs in young patients, mainly females, and has a hereditary component. Due to its aggressiveness, characterized by metastasizing through peritoneal surfaces, diffuse GC is a prognostic factor. On the other side, Intestinal GC occurs mostly in elderly male patients and its strongly associated with intestinal metaplasia and infection by *Helicobacter Pylori*.

Another largely used classification is the WHO, that combines specific histological aspects that can be observed in the entire Gastrointestinal tract. Indeed, according to these criteria the 95% of cases of GC can be classified as tubular, mucinous and papillary adenocarcinoma (which correspond both to the intestinal type according to Lauren classification), poorly cohesive adenocarcinoma (the Diffuse type) and mixed. The other 5% of cases are rare histological variants.

The main limit of every classification based just on histology is an inter-observer variability that often leads to difficult of reproducibility. For this reason, through the years many groups have proposed different criteria of classification based on molecular aspects.

Tan et al., used the gene expression signature to classify GC in G-INT and G-DIF subgroups *(Tan P. et al., 2015)*; Lei et al., further analyzing gene expression, identified the mesenchymal, the proliferative and the metabolic subtypes *(Lei Z et al., 2013)*.

The Cancer Genome Atlas study in 2014 elaborated a molecular bases classification that divides GC in four groups (fig.7):

1) Epstein-Barr virus-positive GCs with high DNA hypermethylation, JAK2 and PD-L1 amplification and PIK3CA mutations;
2) microsatellite instable GCs with DNA hypermethylation and MLH1 silencing;
3) stable GCs with frequent CDH1 and RHOA mutations and correlated with diffuse morphology;
4) chromosomally instable GCs with high TP53 mutations, tyrosine kinase receptors amplification and intestinal morphology *(Cancer Genome Atlas Research Network, 2014)*.

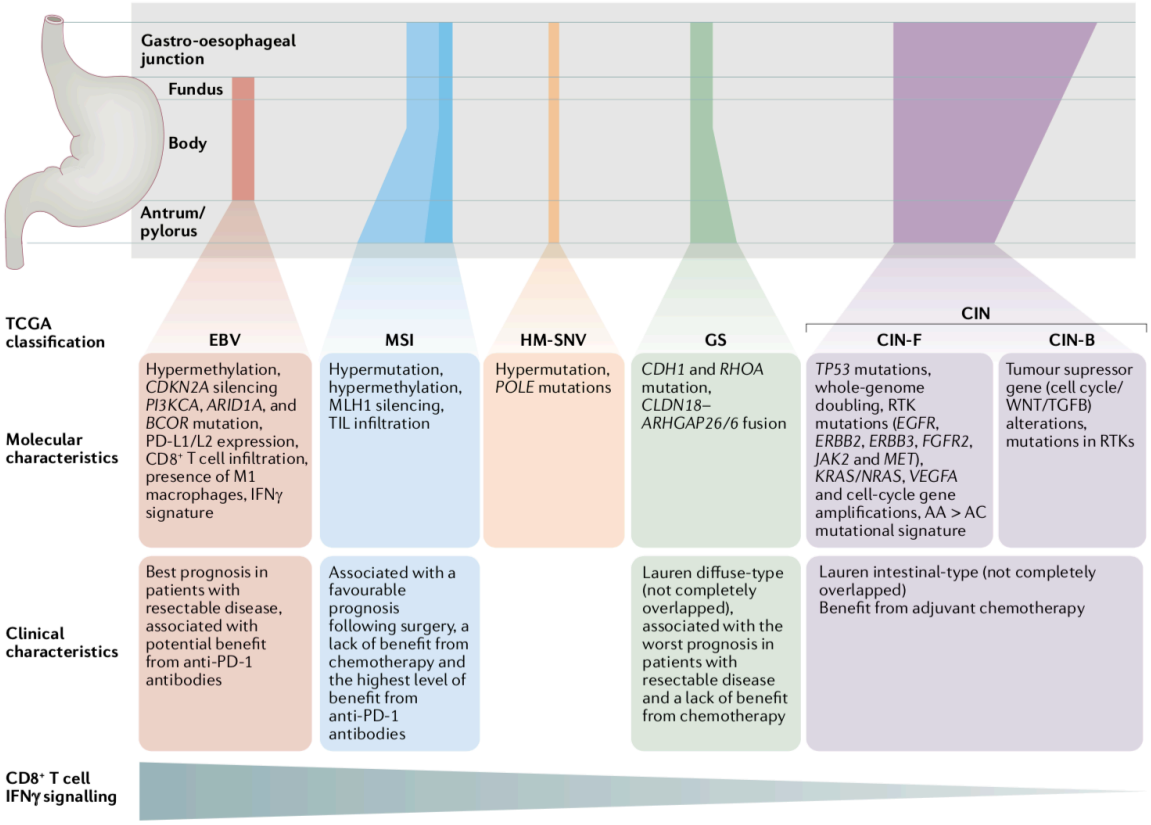


Figure 7: clinical and molecular characteristics of GC according to TCGA classification; subtypes of Gastric and Gastro-Esophageal Junction cancer by anatomical distribution (*Nakamura Y et al., 2021*)

The last molecular classification was elaborated in 2015 by The Asian Cancer Research Group (ACRG) *(Cristescu R et al. 2015)* defining four molecular subtypes with different prognosis:

1) microsatellite instable GCs, with an intestinal morphology and the best prognosis;
2) microsatellite stable with epithelial-to-mesenchymal signature GCs (MSS/EMT), with a diffuse morphology and the worst prognosis.
3) and 4) GC with microsatellite stability and no epithelial-to-mesenchymal signature and TP53-active (MSS/TP53+) or inactive (MSS/TP53–), and with an intermediate prognosis *(Cristescu R et al., 2015)*.

Laurén [14], 1965	Carneiro [18], 1995	WHO [20], 2010	Japanese classification [21], 2011	Nakamura [15], 1968	Goseki [17], 1992	Solcia [19], 2009
Intestinal	Glandular	Papillary Tubular	Papillary Tubular 1 (well differentiated) Tubular 2 (moderately differentiated)	Differentiated type	I. Good tubular differentiation, mucin poor	Cohesive, ordinary subtype Cohesive, tubular subtype Cohesive, ordinary subtype
		Mucinous	Mucinous	Undifferentiated type	II. Good tubular differentiation, mucin rich	Mucinous, muconodular subtype Mucinous, infiltrative subtype
Diffuse	Isolated cell	Poorly cohesive, SRC phenotype	SRC carcinoma	Undifferentiated type	IV. Poor tubular differentiation, mucin rich	Diffuse, ordinary subtype
		Poorly cohesive, other cell types	Poorly differentiated, non-solid type		III. Poor tubular differentiation, mucin poor	Diffuse, low-grade desmoplastic subtype
Mixed	Mixed	Mixed				
Indeter-minate	Solid	Undifferentiated	Poorly differentiated, solid type	Undifferentiated type	III. Poor tubular differentiation, mucin poor	Anaplastic
	Rare variants	Rare variants				High lymphoid response

SRC, signet ring cell.

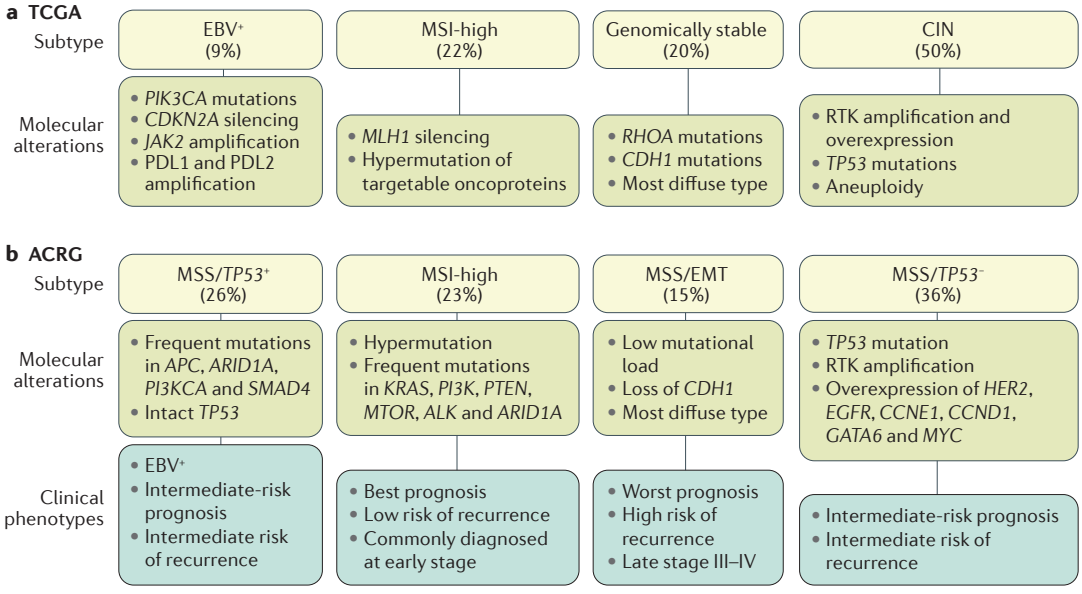


Figure 8: Overview of the heterogeneity classification of GC with histo-pathological (above) and mole Molecular classification of GC elaborated by TCGA and ACRG (*Gullo I et al., 2018; Ajani JA et al., 2017*)

1.4 Therapeutic Options

Regarding therapy, the only approach to eradicate GC is the complete resection by surgery with lymph node dissection *(Weleji EP et al., 2017)*. But in more than half of surgically removed GC, patients show peritoneal, locoregional or distance recurrence. Because of this, it has become evident that surgery alone is no more the standard treatment.

Through the years many studies have demonstrated the efficacy of the combination of surgery with a chemotherapeutic regimen: perioperative, coadjutant chemotherapy and chemoradiation are largely used and improve the outcome of GC resection with extended lymph node dissection *(Lazăr DC et a., 2016)*.

However, chemotherapy is used as elective therapy especially in patients with metastasis or when surgery is not possible. In fact, chemotherapy can reduce tumor mass growth before surgery, hit the cells that initiate the metastatic process and prevent possible local recurrence.

Target therapy, which is a successful approach in many tumors *(Lee Y et al., 2018)*, is unfortunately not a first line therapy in GC; in fact, only the epidermal growth factor receptor 2 (HER2) positive GC can be treated with the monoclonal antibody Trastuzumab *(Bang YJ et al., 2010)*.

According to International Guidelines, a target therapy-approach is recommended as first line treatment only for HER2 positive patients. In these cases, the therapy consists in a combination of Trastuzumab with fluoropyrimidines (5-fluorouracil or capecitabine) and platinum compounds (cisplatin or oxaliplatin); this combined approach has demonstrated a survival benefit compared to traditional chemotherapy (as shown by the ToGA trial). Unfortunately, the amount of HER2 patients is just a minor percentage of total GC cases (almost the 20%) *(Bang YJ et al., 2010)*.

The introduction of taxanes in the first line regimen has improved the efficacy of chemotherapy; these class of compounds, which have a toxic activity on microtubules, can be added to therapy in DCF (Docetaxel-Cisplatin-Fluorouracil) or FLOT (Fluorouracil-Oxaliplatin-Docetaxel) regimen *(Van Cutsem et al., 2006).*

As reported in 2017 by Al-Batran et al., the FLOT therapy demonstrated an improvement of progression-free and overall survival, compared to other perioperative regimes, which includes ECF (Epirubicin-Cisplatin-Fluorouracil) and ECX (Epirubicin-Cisplatin-Capecitabin) and young patients showed a pathology complete regression. On the other hand, the excellent efficacy of the FLOT treatment

is accompanied by important side effects which reduce the number of patients eligible for this type of therapy *(Al Batran SE et al., 2019)*.

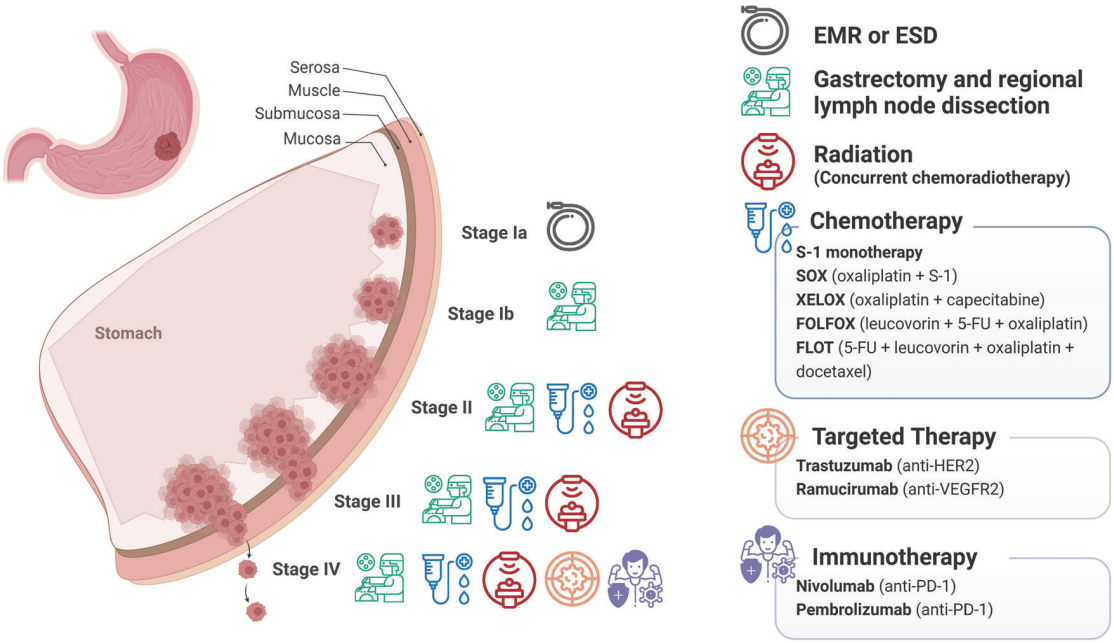


Figure 9: Current therapies for Gastric Adenocarcinoma based on staging. Therapeutic interventions for GC at different stages illustrated by icons. The mainly used regimens of chemotherapy, targeted therapy, and immunotherapy are listed (*Lei ZN et al., 2022*).

1.5 Chemoresistance in Gastric Cancer

Due to the paucity of target therapies in use in GC, one of the main issues for treatment is represented by the onset of side effects due to the use of traditional chemotherapeutics. Moreover, in many cases the occurrence of chemoresistance limits the effectiveness of pharmacologic treatment and in the worst scenario chemotherapy is no longer capable to inhibit tumor progression and tissues invasion *(Russi S et al., 2019)*. We can classify drug resistance in two different types: inherent and acquired. In the first case tumor cells already possess the ability to survive in presence of drugs. On the other side, acquired (or secondary) chemoresistance is induced by the exposure of cancer cells to drugs, that can induce expression of specifical proteins that help tumor growth.

In GC, chemoresistance has been studied for its link with the high expression of the multi-Drug efflux pumps. These proteins belong to the super-family of ATP-

Binding Cassette, also known as ABC carrier: they are ATP-dependent transporters able to extrude toxic agents from the cytoplasm. Their role in tumors has been described in several malignancies: ovarian cancer *(Szotek PP et al., 2006)*, colorectal cancer *(Haraguchi N et al., 2005)*, glioma cell lines *(Kondo T et al., 2004)* and *retinoblastoma (Seigel GM et al., 2005)*.

The ABCB1, or MDR1 (Multi-Drug Resistance 1), has been reported in 1993 as over-expressed in primary GC *(Wallner J et al., 1993)*; its expression and role has been further investigated and today we know that a high expression of this carrier correlates with a bad outcome for the patient *(de Oliveira J et al., 2014)*. Other carrier proteins have been studied for their ability to induce chemoresistance. The ATP7A and ATP7b (ATP-dependent transporter) have been described as biomarker for the platinum-compounds resistance *(Ly I YQ et al., 2019)*.

Chemoresistance occurrence has been also correlated to the alteration of enzymes involved in drug detoxification. The overexpression of enzymes involved in Glucuronidation or those related to the P450 cytochrome can inactivate drugs *(Daigo S et al., 2002)*.

One of the main mechanisms of action of anticancer drugs is the damaging of cell crucial component (such as DNA and cytoskeleton) which determines cell death. Survival to programmed cell death is another mechanism of resistance to drug treatment. Therefore, the imbalance between pro-apoptotic and anti-apoptotic signals determines the survival of cancer cells even in the presence of strong death signals.

P53 is the regulatory protein responsible for the inhibition of proliferation and induction of apoptosis. Known as the "Genome Guardian", this protein is activated by DNA damage signals; its correct activity can result in cell cycle arrest, DNA repair or apoptosis. Mutations in P53 have been described in several works as an important step of chemoresistance acquisition *(Surget S et al., 2016; Alvaro-Ortiz E et al., 2021; Yashiro M et al., 2009)*, also in Gastric Adenocarcinoma (*Marin JJ et al., 2020);* moreover, an overexpression of BCL2, impairment of BAX and activation of AKT/PI3K pathway, that regulate the correct activation of apoptosis, have been correlated to chemoresistance in GC.

Therefore, re-establishing correct functioning of the apoptotic cascade (from its activation to the execution of proteolytic cuts by Caspase) is one of the strategies that scientific research continues to investigate.

1.5.1 Cancer Stem Cells and Chemoresistance

As we previously exposed, chemoresistance is an event participated by many components of the tumor mass and one of the main peculiarities of cancer is certainly its cell population heterogeneity. Indeed, within the tumor lesion we find different types of cells: mutated cells, infiltrating cells, and fibroblasts. It is now a common belief that in every tumor there exists a nucleus of cells capable of ensuring continuous proliferation and at the same time maintaining a high clonogenic potential of the tumor itself. This core of cells, known as CSCs (Cancer Stem Cells) or TIC (Tumor Initiating cells) has the same role as the normal progenitor cells present in normal tissues, i.e., to regenerate the tissue (in the case of cancer, the tumor mass) when necessary.

The first identification of CSC dates back to 1998, when Bonnet and Dick isolated a leukemia cells subpopulation with high expression of the surface marker CD34 but not CD38; they established that the $CD34^{+}/CD38^{-}$ subpopulation can induce tumor genesis in NOD/SCID mice (*Bonnet D et al., 1997).*

The first evidence of stem-like cells in solid tumors followed in 2002 with the identification of a clonogenic, sphere-forming population isolated from adult human brain gliomas. Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers *in vitro*; cancer stem cells isolated from adult human gliomas were shown to induce tumors that resembled the parent one when grafted into intracranial nude mouse models *(Ignatova TN et al., 2002).*

The presence of stem-like cancer cells has also been correlated with the acquisition of resistance to pharmacological treatment, also thanks to the expression of specific molecules capable of guaranteeing their survival in the presence of toxic substances. Among these, the previously cited carriers of the ABC family (MDR-1 above all) and the Aldehyde Dehydrogenase enzymes identified in various models of malignant tumors, deserve mention.

The Aldehyde dehydrogenases (ALDHs) are a group of enzymes that catalyze the oxidation of aldehyde: nineteen genes encoding for ALDHs have been identified in human genome and they participate in many biological processes including the detoxification of exogenously and endogenously generated aldehydes *(Marchitti SA et al., 2008)*. Through the years ALDH has been described as one of the main markers of a stem-like population within the tumor mass, responsible for the maintenance of the proliferative machine *(Yasuda K et al., 2013; Clark DW et al., 2016)*.

Other mechanisms attributed to a stem cell phenotype and related to chemoresistance are the overexpression of DNA repair mechanisms *(Yuan M et al. 2014)*, the inhibition of proteins involved in apoptosis such as PARP (poly-ADP-ribose polymerase) *(Venere M et al., 2014)* and the acquisition of an epithelial-mesenchymal transition (EMT) phenotype *(Thiery JP, 2002)*.

As stated above, it emerges that chemoresistance is an extremely heterogeneous process in which proliferating tumor cells and the stem compartment play a pivotal role in the progression of the disease.
Also, the tumor microenvironment, now known as an active protagonist of cancer progression, also participates in the acquisition of drug resistance. Therefore, in the search for solutions to overcome chemoresistance, the molecules that participate in the modification of the microenvironment itself cannot be ignored.

1.6 Potential marker of Chemoresistance: the case of Carbonic Anhydrase

Carbonic anhydrases (CA) are a family of catalytic zinc-containing metalloenzymes, they are involved in the reversible hydration-dehydration reaction of carbon dioxide (CO_2) generating bicarbonate ions (HCO_3-) and protons (H+). The molecules involved in this reaction play fundamental physiological and biological roles, showing relevant differences in tissue distribution, subcellular localization, and biological function *(Parkkila S et al., 2000).*
Carbonic anhydrases are classified into eight genetically distinct classes named with Greek letters. The members of each class possess different transcripts and protein isoforms, characterized by different biochemical properties and specific tissue and subcellular localizations *(Capasso C and Supuran CT, 2016)*. The CAs present animal cells belong to the α class, which includes sixteen different isoforms: five cytosolic (CAI, CAII, CAIII, CAVII and CAXIII), five membrane bound (CAIV, CAIX, CAXII, CAXIV and CAXV), two mitochondrial (CAVA and CAVB), only one secreted isoform (CAIV). The remaining isoforms lack a catalytic site (CAVIII, CAX and CAXI) *(Supuran CT, 2008)*.
Carbonic anhydrase IX (CAIX) is an isoform that belongs to the recently identified carbonic anhydrase-α family. CAIX is considered an important tumor marker, since

its expression levels are increased in tumor cell lines and in many surgical tumor samples *(Zavada J et al, 1993)*. Is a homodimer transmembrane glycoprotein, made up of 414 amino acid residues with a molecular weight of 49.5 KDa. Its structure can be divided into different domains: the extracellular portion includes a catalytic domain (near the membrane), a proteoglycan-like domain, and an N-terminal signal peptide. The latter represents a unique feature of CAIX, absent in the remaining human isoforms *(Supuran CT, 2008)*.

While expression of CAIX in healthy tissues concerns almost exclusively stomach and the epithelial tissues of the duodenum, jejunum, ileum and small intestine in many solid tumors, a notable increase in the expression levels of this specific CA isoform has been detected, making it a new tumor market and therapeutic target *(Chen J et al., 2005)*. In GC, higher levels of CAIX are observed in healthy mucosa compared to tumor tissue, contradicting trends found in most solid tumors. However, histochemical analyzes highlighted a significant increase in CAIX in the invaded margins of GC, linking this enzyme to cancer progression and low post-operative outcome in a similar way of other cancer where overexpression of CAIX is correlated with chemoresistance and poor prognosis *(Chen J et al., 2005; Liao SY et al., 2009)*.

CAIX increased levels in various tumors are linked to the hypoxia, typical conditions in the various regions of solid tumors. Hypoxia is a key element in cancer development and is caused by poor vascularization of the growing tumor mass. Cell proliferation in the tumor mass is much faster than in the vasculature, so cells further inside the mass are found in an oxygen-deficient environment *(Folkman J et al., 2000)*.

Low oxygen levels cause necrosis events which are often detected through histological examinations *(Brahimi-Horn MC et al., 2007)* so tumor cells need to adapt to this condition to survive and proliferate. As the main adaptation mechanism in hypoxic conditions, cells activate hypoxia-inducible factor-1 (HIF-1), a heterodimer made up of α and β subunits. In hypoxic the two sub-units can interact and so HIF-1 become an activated heterodimer. conditions sub-HIF-1α is stabilized and can interact with sub-HIF-1β forming the active heterodimer *(Doe MR et al., 2011)*. In its activated form HIF-1 regulates the transcription of genes involved in glycolysis, angiogenesis, pH regulation and in many processes of tumor progression: EMT and invasion above all *(Ullah MS et al., 2006)*. CAIX is one of the genes regulated by HIF-1: the overexpression of CAIX in hypoxic conditions is linked to its catalytic function of hydration and dehydration of CO_2 to HCO_3- in the presence of H_2O *(Li Y et al.,*

2011). This reaction is necessary for the regulation of tumor microenvironment, characterized by a very low pH. In fact, CAIX activity leads to the acidification of the tumor microenvironment, a process coupled to the metabolic switch of tumor cells from oxidative to glycolytic resulting in lactic fermentations and production of H+ ions. This change is forced in tumor cells because the absence of oxygen limits oxidative phosphorylation *(Gillies RJ et al., 2015)*. Protons accumulation in the cytoplasm affects intracellular pH, representing a danger for the cell. The acids produced by metabolism can react with HCO_3- leading to the production of H_2O_2 and CO_2 which is permeable to the membrane, representing a way of elimination of metabolic acids from the cell. Through this mechanism, CAIX creates an efflux of CO_2, contributing to the maintenance of an alkaline intracellular pH and simultaneously to the acidification of the tumor microenvironment *(Swietach P et al., 2019)*. So, Hypoxia and extracellular acidity represent two closely linked elements of tumor microenvironment both capable to influence each other in the regulation of many tumors' molecular aspects *(Andreucci E et al., 2017)*.

CAIX driven acidification of microenvironment is very important for its consequence on chemotherapy. In fact, the majority of anti-tumor drugs are transported via active transport or passive diffusion through plasma membrane; since these transport mechanisms are pH sensitive, the cytotoxic activity of the drugs depends on the pHi (intracellular pH) and pHe (extracellular pH) *(Stuubs et al., 2000)*.

Over the years, various strategies of altering pHi and pHe have been tested to identify new therapies for the treatment of solid tumors *(Thiry A et al., 2006)*. Has been described an increasing efficacy of drugs with weak basic nature (Mitoxantrone, Paclitaxel and Topetecan) at the increase of pHe *(Robertson N et al., 2004)*. Furthermore, the slowing of tumor growth induced by the weak base doxorubicin is improved by the increase in tumor pHe, after chronic ingestion of a sodium bicarbonate-based solution *(Raghunand N et al., 1999)*. So, therapies targeted on CAIX can improve anti-tumor drugs activity and at the same time decrease the acidity of the tumor microenvironment associated with cancer progression.

1.6.1 CAIX inhibition and effects on cancer progression

CAIX inhibition is a current strategy to overcome chemoresistance; there are two possible approaches to inhibit CAIX: the first involves small molecules with an

inhibitory function, and the second the use of specific antibodies. The latter approach is based on the concept of antibody-mediated cellular cytotoxicity (ADCC), an example is the drug cG250, a possible antitumor agent in immunotherapy *(Surfus JE et al., 1996)*.

The specific inhibition of CAIX using small inhibitory molecules is a currently explored research topic *(Supuran CT, 2008)*. A typical CA-α inhibitor has three main components: a zinc binding group (ZBG), a linker region which can be a heterocycle or a benzene ring and finally a variable tail *(Pinard MA et al., 2015)*. The zinc-binding group has the function of interacting with the catalytic site preventing the catalysis of the reaction *(Wilkinson BL et al., 2006)*. Based on the orientation of the zinc bond, two main groups of ZBG can be distinguished: those that form a direct bond with zinc (such as sulfonamides and bisulphites) and those that interact with the bond between zinc and water (such as cyanates and formates) *(Supuran CT, 2015)*. Among the "classic" CAIX inhibitors, sulphonamides and their isosteres (sulphonamides/sulphamates) are the most studied group of inhibitors *(Supuran CT, 2008)*: these molecules bind directly to the zinc of the catalytic site, forming tetrahedral adducts and preventing catalysis. In this class of inhibitors, we find: acetozolamide, brinzolamide, dorzolamide and methazolamide *(McKenna R and Supuran CT, 2014)*. These molecules are currently classified as first- and second-generation inhibitors, since they form very strong and compact interactions within the catalytic site, often non-specific for the various CA isoforms, causing adverse effects. For this reason, inhibitory molecules with greater specificity for the target isoforms have been developed in recent years. SLC-0111 particularly has been described as an efficient inhibitor for the CAIX and CAXII (another CA isoform associated with solid tumors), with important anti-cancer effects in animal models methazolamide *(McKenna R and Supuran CT, 2014)*. Furthermore, the anti-tumor effect of this compound has also been reported on tumor stem cell population. SLC-0111 is a sulphonamide derivative belonging to a cogeneric series of 4-ureido-substituted-benzenesulphonamides, which act as specific inhibitors for CAIX. The specificity of this compound is linked both to the linker region and to the addition of the 4-fluorophenyl that can interact with the hydrophobic region of the catalytic site of the enzyme. The compound from which SLC-0111 derives was identified by the Supuran group by evaluating pharmacokinetic aspects (absorption, distribution, metabolism, and excretion) of a series of ureido-substituted compounds with inhibitory activity on human CAIX. In fact, SLC-0111 was selected for its excellent pharmacokinetic and antitumor profile demonstrated in preclinical

phases conducted on various models. This compound was synthesized by SignalChem Lifescience Corporation (SLC, British Columbia, Canada). SLC-0111 has completed phase I clinical trials for the treatment of solid tumors that overexpress CAIX. In 2017, SLC-0111 entered an ongoing phase Ib/II clinical trial, to evaluate its efficacy in combination with standard chemotherapeutic agents in the treatment of pancreatic cancer *(McDonald et al., 2020).*

So, a further the study of the effects of SLC-0111 activity can help to better understand the role of CAIX in GC progression end eventually its possible role as a target for therapy.

1.7 Natural compounds as Therapeutic Agents – The activity of Polyphenols in human diseases

The search for molecules that can interfere with tumor progression does not only concern synthetic drugs. Indeed, since the 1990s, nutraceuticals have become one of the fields in which to seek new therapeutic possibilities.

"Nutraceuticals" was coined in 1989 by Stephen Felice to indicate all products contained in foods capable of having beneficial effects on health. Compared to traditional drugs, nutraceutical compounds have some advantages: mainly the almost total absence of significant side effects and their easy availability *(Sauer S et al., 2017).*

The main resource of these compounds is undoubtedly food. The daily introduction of food involves the intake of substances naturally produced by it. Plants represent the main source of compounds with pharmacological activity and a diet rich in vegetables exposes the body to their effects.

Polyphenols are the most interesting class of compounds due to their diffusion and their ability to have positive effects in physiological and pathological conditions.

The name "Polyphenols" is referred to their chemical structure: they are natural products with a specific structure including hydroxyl groups and aromatic rings *(Manach C et al., 2004).*

There are four principal classes of polyphenols:

- Phenolic acids, that include caffeic acid
- Flavonoids include flavones, flavanols, flavanols, flavanones, isoflavones, proanthocyanidins, and anthocyanins; flavonoids are particularly abundant

in food: catechin (tea, fruits), oleuropein and oleocanthal (olive) hesperetin (citrus fruits), cyanidin (red fruits and berries), daidzein and genistein (soybean), proanthocyanidins (apple, grape, cocoa), and quercetin (onion, tea, apples)

- Lignans, polyphenols derived from phenylalanine
- Stilibenes, widely used in cosmesis

The main interest regarding polyphenols is for cancer therapy and prevention; It has been seen that diet is closely implicated in tumor prevention, especially in reducing the onset of cancer. It has been estimated that approximately two thirds of cancers in human could be prevented with lifestyle and diet modifications *(Choudhari et al., 2020)*.

Specifically, a diet rich in fruit and vegetables is directly linked to a lower risk of developing various cancers *(Law BY et al., 2016)*: more than 200 studies have linked polyphenols consumption with a reduction of tumor incidence, particularly of the gastro-intestinal tract, were the incidence seemed to be half reduced *(Petra S et al., 2021; Talero E et al., 2012).*

There are many mechanisms by which polyphenols exerts their preventive action: they regulate apoptosis, autophagia and cell cycle arrest. They can inhibit invasion and angiogenesis and have a natural anti-inflammatory activity *(Petra S et al., 2021).* Regulation of apoptosis is one of the main processes in which polyphenols shoe their function. For example, Curcumin is a strong antioxidant and has been described for inducing apoptosis in tumor cell lines: in glioma cells (U-87 MG) regulates caspase-3 and Bcl2; in mesothelioma cells (MM-F1 and MM-B1) induces an over expression of Bax/Bcl2 and moreover determines the cleavage of PARP and the nuclear translocation of p53 *(Masuelli L et al., 2017).* Quercetin, another polyphenol, has shown its efficacy on HeLa cells (cervical carcinoma) inducing apoptosis and reducing tumor mass growth *(Vidya Priyadarsini R et al., 2010)* and also in osteosarcoma model *(Badziul et al., 2014)*. Genistein has proven effectiveness in many tumors, such as prostate, breast and lung cancer, leukemia, and melanoma *(Li HQ et al., 2012)* through various mechanisms, including the upregulation of Bax and downregulation of Bcl-2 (particularly in breast cancer breast) *(Hung JY et al., 2009).*

The biological effect of these compounds has been studied also in combination with conventional therapy. In fact, many works described a synergism between polyphenols and chemotherapeutics when administered together as a therapy. The previously cited curcumin is able to re-establish sensitivity to Oxaliplatin (one of

the most used drugs in cancer-therapy) in chemo-resistant cells of ovarian cancer, inducing apoptosis via NF-kB *(Nessa MU et al., 2012).* Always in association with platinum-compounds, also quercetin has shown efficacy in ovarian cancer and in nasopharyngeal carcinoma, interfering with tumor cells cycle and modulating apoptosis *(Daker M et al., 2012).*

There are many other polyphenols studied for their anti-tumor applications, but in these work we have focused our attention on two compounds that derive from Extra Vergin Olive Oil and Soybean.

1.7.1 Oleocanthal- A Mediterranean Polyphenol against Cancer

Extra Virgin Olive Oil (EVOO) is one of the main foods and the first source of fat in the Mediterranean diet. It has been widely documented that a high intake of olive oil is correlated with a lower risk of developing chronical diseases including cancer, cardiovascular disease, and neurodegenerative diseases *(Scarmeas et al., 2009; Ruiz-Canela M et al., 2011; Pelucchi C et al., 2010).*

The beneficial effects of EVOO were attributed to the high levels of monounsaturated fatty acids (like as linoleic acid) and of Minor Polar Compounds (MPCs) that include polyphenols *(Romani A et al., 2019).*

Among the various polyphenols described in EVOO, including tyrosol and hydroxytyrosol, in 1992 *Montedoro et al.* discovered a molecule responsible for the sensation of oral irritation by olive oil. In 1993, the same group revealed the molecular structure and in 2005 Bauchamp et al. named it Oleocanthal (OC) *(Beauchamp et al., 2005).*

This molecule, It has an excellent anti-inflammatory and antioxidant action (was described as similar to ibuprofen, the main NSAID *(Iacono A et al., 2010),* making its use in diseases such as tumors, neurodegenerative and osteoarticular diseases very interesting and worthy of further investigation. As previously described, oleocanthal is associated with the typical sensation of prickle produced by EVOO. A molecular study by Peyrot de Ganchons et al. correlated this typical sensation to the activation of transient receptor potential cation channel type A1 (TRPA1) *(Peyrot des Gachons C et al., 2011).*

Consumption of OC in Mediterranean Diet is associated with a slightly lower incidence of breast and colon cancers, compared to other populations. Many studies

support this thesis, and everyone agrees that the consumption of EVOO enriched with OC, could be a possible approach in tumor prevention *(Goren L et al., 2019).*
Moreover, OC activity has been studied also in breast cancer with promising results. OC has proven to be an excellent inhibitor of MET tyrosine kinases (c-MET), controlling breast cancer progression and recurrence; blocking these proteins leads to a reduction in their activity, growth, migration, and invasion of breast tumor cells, induces G1 arrest of the cell cycle and apoptosis; in *in vivo* mouse models it has demonstrated an inhibition of carcinogenesis. Furthermore, the combination of this polyphenol with innovative drugs, such as Lapatinib, allows the administered dose to be reduced, thus decreasing chemoresistance and side effects occurrence, while maintaining its therapeutic activity *(Khanfar MA et al., 2015; Tajmim A et al., 2019; Siddque AB et al., 2019).*
OC has also shown a strong anti-proliferative activity, blocking mTOR signaling in a metastatic cell line of breast cancer, the MDA-BD-231 *(Khanfar MA et al., 2015)* and in 2015 *Le Gendre et al* demonstrated the selective activity of OC against tumor: the treatment induced cell death only in cancer cells while no effect on normal cells. The mechanism was mediated by the inhibition of Acid sphingomyelinase inducing permeabilization of Liposomal Membrane *(Le Gendre O et al., 2015).*
Other studies evaluated the effects of OC on hepatocarcinoma; OC has been observed able to inhibit tumor growth and metastases formation through inhibition of STAT3 pathway (both *in vitro* and *in vivo* models); these results were obtained in various hepatocarcinoma cell lines, inhibiting tumor migration and invasion *(Pei T et al., 2016).*

1.7.2 The role of Genistein, a soy derived isoflavone, in cancer

We previously exposed the differences of Gastric Adenocarcinoma incidence in different countries. Asia is the first continent for GC diffusion, so in literature there is a great abundance of publication regarding correlation between diet and GC.
Eastern Countries (Japan especially) are particularly known for their typical diet which includes rice, fresh fish (rich in omega-3 and omega-6), a lot of vegetables and soy.
Talking about polyphenols, soy (and other leguminous) has a rich amount of Genistein, an Isoflavone. The effects of soy-rich diet have been mainly studied for the protein contents; the high quality of soy proteins and the low contents of fat

make this food favorable compared to meat-derived proteins. Furthermore, Genistein is a hormone-like compound, with a 17beta estradiol-like structure and acts as a phytoestrogen, so its presence in the cells can obviously causes a biological effect.

Genistein has been studied for its function as antioxidant compound like other polyphenols, but some studies highlighted its role as a helping agent in chronic disease.

The curative potential of Genistein has been investigated in several studies. The earliest publications discussed its role as a hormone-like compounds: indeed, the presence of estrogenic receptors that can bind Genistein in the key for its activity.

First evidence about Genistein role in cancer have been found in breast cancer cells, due to the high presence of hER in this kind of tumor. *Morito et al* in 2001 studied the effects of many Isoflavones on MCF7 breast cancer cells with a focus on the affinity of these compounds for receptors hER-alfa and hER-beta, finding that the binding affinities of genistein and Equol (one of its metabolites) are comparable to the binding affinity of 17 β-estradiol resulting in growth stimulation of MCF7 *(Morito K et al., 2001).*

These initial data suggested a positive effect of Genistein on tumor cells, but later studies showed different results.

In 2009-2010 three different groups highlighted effects of Genistein in brain, cervix and prostate. A meta-analysis evaluated the effect of High consumption of soy-based food identifying five cohort studies and 8 case-control studies using databases form South Korea, China and Japan; their results showed that Genistein and daidzein were associated with a lower risk of prostate cancer, suggesting that soy food consumption could lower the risk of prostate cancer *(Huang YW et al., 2009).*

In 2009, a Korean research group investigated the effect of Genistein on the growth of cervical cancer cells, HeLa and CaSki discovering an inhibition of cell growth mediated by Genistein with a modulation of AKT and various mitogen-activated protein kinases mediated pathway *(Kim SH et al., 2009).*

In the third study the authors evaluated the action of many flavonoids (apigenin, (-)-epigallocatechin, (-)-epigallocatechin-3-gallate (EGCG), and Genistein) administered as treatment on human glioblastoma T98G and U87MG cells; these compounds did not induce cell death in normal astrocytes but were found increment of ROS production and apoptosis induction with phosphorylation of p38 mitogen-activated protein kinase (MAPK) *(Das A et al., 2010).*

The activity of genistein in cancer was also discussed for its correlation with stem compartment. Specifically, genistein seemed to be efficient in reducing the CSC population in breast cancer models (MCF7) through the modulation of Hedgehog signaling, which is responsible for the correct process of self-renewal *(Fan P et al., 2013).* The inhibiting effect of genistein on Hedgehog pathway has been also observed in a prostate cancer cell line (PCa9 cells) treated with the polyphenols accompanied by significant reduction of proliferation and re-generation of tumor mass *in vitro* and *in vivo* in mice models *(Zhang L et al., 2012).*

Furthermore, a study conducted on GC cell lines MGC-803 and SGC-7901, showed that genistein is efficient in re-sensitizing drug-resistant cells to chemotherapy and to inhibit stem-like population through the downregulation of the carrier ABCG2.

So, the activity of genistein in GC therapy, also considering the relationship between chemoresistance and stem cells, deserve attention.

2. Aim of the study

The need to understand the mechanisms that contribute to chemoresistance is one of the fundamental steps for the development of new strategies to combat tumor progression. The aim of this research was to investigate some molecular characteristics of Gastric Adenocarcinoma.

This study was carried out investigating two aspects: the first is the detection of crucial molecules that maintain tumor cellular homeostasis and progression and the understanding of their role in the pathological context of gastric cancer. In this case our attention was focused on Carbonic Anhydrase 9 (CAIX), an enzyme responsible for the de-acidification of the intracellular compartment and acidification of the external microenvironment of the tumor, identified in recent years as one of the main drivers of cancerous progression towards chemo-resistant and more aggressive phenotypes. We aimed to evaluate the activity of SLC-0111, a synthetic CAIX inhibitor, on our experimental model of resistant gastric adenocarcinoma. The evaluation of its activity was carried out either as stand-alone treatment or in combination with the current chemotherapy regimens used in gastric adenocarcinoma, in order to overcome chemoresistance.

The second aim of this work was the evaluation of the effects of two polyphenols, Oleocanthal and Genistein, in our experimental model. It is now known that many natural compounds such as polyphenols are able to exert favorable activities both in contrasting tumor progression and in reducing the side effects that traditional therapies entail. The evaluation of the efficacy of these two polyphenols was also carried out in combination with chemotherapeutics on models of chemo-resistant gastric adenocarcinoma. We evaluated their effect to understand their possible use in clinical practice as adjuvants to traditional chemotherapy.

Ultimately, in this work, we tried to characterize a relationship between chemoresistance and stem cell capacity found in some solid tumors. Indeed, if it is true that the presence of stem cells in the mass of tumors represents the proliferative reserve of the tumor itself, we need more data to establish a clear correlation between the onset of chemoresistance (which, as already mentioned, is one of the main causes of the failure to eradicate the tumor) and the presence of a stem population in gastric cancer. So, one of the aims of this work was to evaluate the presence or absence of a stem population within gastric cancer cell lines in conditions of chemoresistance.

3. Materials and methods

3.1 Gastric Cancer cell lines and Chemotherapeutic agents

The ACC-201 cells (also known as 23132/87) were purchased from DSMZ (Leibniz Institute, DSMZ-German collection of and Cell Cultures GmbH). Cells were maintained in RMPI 1640 medium (Euroclone) supplemented with 10% FBS (Euroclone), 1% L-glutamine (Euroclone) and 1% Penicillin-Streptomycin (Euroclone).

AGS cells were provided by ATCC (CRL-1739TM) and maintained in F12K medium (Corning) with a supplement of 10% FBS (Euroclone), 1% L-glutamine (Euroclone) and 1% Penicillin-Streptomycin (Euroclone).

5-Fluorouracil (5-FU, F6627), Paclitaxel (TAX, T7402), and Cisplatin (CIS, P4394) were purchased from Sigma-Aldrich (Sigma-Aldrich,) while Oxaliplatin (HY-17371) and Docetaxel (HY–B0011) from MedChemExpress (distributed by DBA).

The AGS chemo-resistant cell lines (5FUr, CISr and TAXr) were selected and established in our laboratory by Peri S. and colleagues *(Peri S et al. 2021)*, following the "high-level laboratory models" reported by McDermott and colleagues *(McDermott et al. 2014)*, so for the treatment and establishment of ACC-201 resistant cells and the AGS FLOTr we followed the same protocol.

Briefly, ACC-201 cells seeded at low density were treated for 48 h with 3 μM of 5-fluorouracil (5FU), 2 μM cisplatin (CIS) or 1 nM paclitaxel (TAX), in order to obtain respectively 5FU-resistant (5Fur), CIS-resistant (CISr) and TAX-resistant (TAXr) ACC-201 cells. For the FLOT treatment, the drugs were administered simultaneously in the same plate: we started administering 3 μM of 5FU, 2 μM of Oxaliplatin (OXA) and 1 nM of Docetaxel (DOCE).

After the treatment, the plates were washed with PBS (Dulbecco Phosphate Buffer without calcium and magnesium, Euroclone), and the cells were cultured in drug-free fresh complete media. We maintained the cells in drug-free media for 2-3 days in order to reach sub-confluence. Then we detached the cells with trypsin-EDTA (Euroclone) and plated them in new petri dishes (60 mm) at low confluence.

This procedure was repeated for about 9 months and during this period we increased chemotherapeutics concentration until we reached 25 μM for 5FUr, 15 μM for CISr, and 10 nM for TAXr; for the FLOT treatment on ACC-201 cells were finally maintained at 10 μM for 5FU, 5 μM for OXA and 2 nM for DOCE. The FLOT

treatment for AGS cells achieved concentrations of 10 μM for 5FU, 1 μM for OXA and 2 nM for DOCE.

3.2 Patients' enrollment and retrospective analysis

Twenty-six patients undergoing pre-operative therapy followed by gastrectomy at the Azienda Ospedaliera Universitaria Careggi (AOUC) of Florence were classified into two groups: Responders and Non-Responders. The classification was carried out through the evaluation of the Tumor Regression Grade (TRG) parameter, established by pathologists following the Mandard score. Patients with TRG 1-2 were classified as Responders and those with TRG 3-5 as Non-responders.
Sections of Formalin-fixed Paraffin-embedded (FFPE) were used following approval from the Local Ethics Committee of the AOUC of Florence according to the Declaration of Helsinki and informed consent was obtained for each enrolled patient.

3.3 Immunohistochemistry

The immunohistochemistry experiments were realized with the collaboration of Professor Daniela Massi research group (Department of Health Sciences, University of Florence); the tumor samples were analyzed to assess CAIX expression, and the analysis was performed in the Ventana Discovery Ultra Immunostainer. Representative FFPE tumor sections 3 μm thick were deparaffinized in EZ prep (950-102; Ventana), and antigen retrieval was obtained through the incubation with cell-conditioning solution 1 (CC1) (950-124; Ventana). Sections were then incubated with a mouse monoclonal anti-CAIX primary antibody (dilution 1:80, Abcam ab107257).
The signal was developed with UltraMap anti-mouse HRP and visualized using Chromomap DAB (760-159, ready to use, Ventana Medical Systems). Then, the sections were counterstained with Hematoxylin II (790–2208, ready to use, Ventana Medical Systems). Negative control was obtained through the substitution of the primary antibody with a Mouse IgG1 kappa Isotype Control Clone P3.6.2.8.1, dilution 1:80 (Invitrogen, Catalog # 14-4714-82).

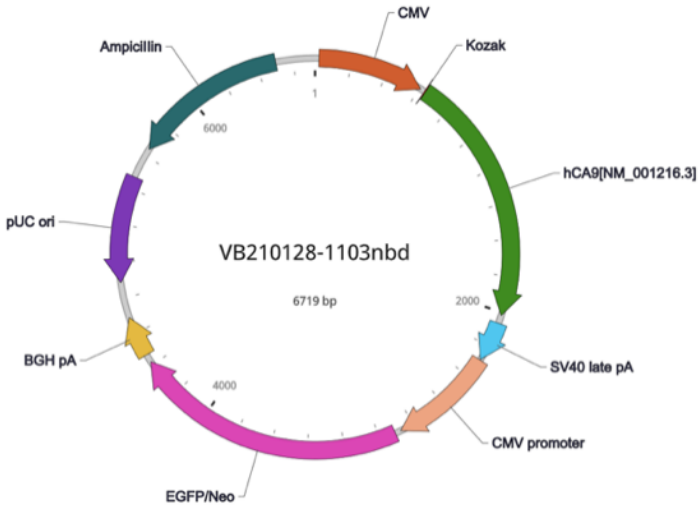
3.4 Other treatments

The SLC-0111 (CAIX inhibitor) was kindly provided by Professor Claudiu T. Supuran NEUROFARBA Department, University of Florence, Italy).
OCF (Olea Extract Fraction) was selected by Peri et al and was quantitatively and qualitatively evaluated for its content in bioactive compounds using HP-1260 liquid chromatography with the equipment of a DAD detector *(Peri S et al., 2022)*
Genistein was purchased by Sigma-Aldrich (G6649); the powder was resuspended in DMSO as indicated by Sigma-Aldrich and stored at the concentration of 200 mM at -20°C.

3.5 IC50 assay – Viability Assay with MTT

In order to test the sensitivity of cells to chemotherapy we performed MTT viability assays. Thanks to this procedure was possible to calculate the IC50 (Inhibiting Concentration) for every treatment we used to select resistant cells.
Cells were plated at a density of 5x10^3 (AGS) or 7x10^3 (ACC-201) in a 96 multi-well plate. After 24h culture media were removed and replaced by fresh media in which cells were treated with serial dilutions of the drugs. After 72h the plate was incubated for 90 minutes at 37°C in the dark with 0,5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in medium without phenol red. Then, MTT was removed from the plate and cells were lysed in DMSO (100 μl per well). After the lysis we recorded absorbance values at 595 nm wavelength with an automatic plate reader; to optimize the analysis we subtracted the absorbance values of *blank* (consisting of only 100 μl of DMSO) to independent measurement of each sample. The obtained data were expressed as a percentage of Mean +/- Standard Deviation with respect to treated-untreated controls.
Then, the data were transferred to GraphPad Prism to assess IC50 curves and calculate the IC50.
The MTT was also performed for the avaluation of cell viability after treatment with the studied compounds: SLC-0111, OCF and genistein.

3.6 Overexpression and silencing of CAIX



For the overexpression and silencing of CAIX we used the Lipofectamine, a common transfection reagent. Thanks to this methodology it was possible to modulate the levels of CAIX in the AGS and ACC-201 lines with the aim to evaluate the role of CAIX in supporting chemo-resistance and therefore in the therapeutic response. For the CAIX overexpression condition, AGS cells were transfected via Lipofectamine 3000 (ThermoFisherScientific) containing an empty vector or with a plasmid (fig.28) for CAIX overexpression (pRP-EGFP/Neo-CMV > hCA9).
In this plasmid the CAIX ORF is in frame with the fluorescent protein Enhanced green fluorescent protein (eGFP) and a gentamicin resistance gene. Following selection using gentamicin,cell population characterized by overexpression of CAIX (CAIX+) was isolated using FACS Melody exploiting eGFP fluorescence.
A similar approach was used for CAIX silencing in both WT and FLOTr AGS and ACC-201 cell lines. Cells were transfected using Lipofectamine 3000 transfection reagent (ThermoFisherm Scientific), containing MISSION siRNA Universal Negative Control or MISSION esiRNA Human CA9 (Sigma-Aldrich) following the manufacturer's instructions. The kit used for MISSION® esiRNA silencing consists of a heterogeneous mixture of siRNA endoribonuclease preparations, all capable of recognizing and selectively silencing the same mRNA sequence. Both controls and CAIX-silenced samples were tested for CAIX expression levels by Western blot analysis, and the silenced cells were used for further testing.

3.7 GC-MS (Gas Chromatography Mass Spectrometry)

A further characterization of metabolism of our cells was performed by the evaluat the production of Tricarboxylic Acid Cycle metabolities. Cells were starved (cultured in FBS-free and Glutamine-free medium) for 6h. Then, they were prepared for GC-MS analysis on an Intuvo9000/5977 B MSD (Agilent Technologies, Santa Clara, CA, USA) via selected ion monitoring (SIM) mode MS.
Cells were washed with a solution of NaCl 0,9% at 4°C. and then with a solution of 80% cold Methanol (-20°C) in water with 1 μg/ml of Norvaline as internal standard. The samples were sonicated in ice for 5 seconds for 3 times (with an interval of 5 seconds) at 70% amplitude. Then they were centrifugated (14000 rpm) for 10 minutes at 4°C. After centrifugation the supernatants were collcted and lyophilized. We resuspended the pellet remained after centrifugation in 50 μl of NaOH 200 mM denatured them at 96 ◦C for 15 min, centrifuged at 14000 rpm for 5 minutes. These samples were used as standard of protein content for the normalization.

3.8 Seahorse technology

We used the Seahorse technology to evaluate the metabolic profile of ACC-201 and AGS cells, WT and Chemo-resistant. The adherent cells were plated under sterile conditions in their own growth medium and at concentration (3 x 10^4 per well). The same day, the "XF Sensor Cartidge" plate is hydrated with the appropriate "XF Calibration Buffer" and incubated overnight in the absence of CO_2 at 37°C. The following day we proceed with the preparation of the specific soil for the test; this medium containing, 100 mL of Seahorse XF DMEM at pH 7.4 with 10 mM glucose XF, 1 mM pyruvate XF and 2 mM glutamine. At this point, the cells are removed from the incubator and then examined using an optical microscope to verify their confluence and morphology. The growth medium is then removed from the plate and replaced with the specific one for the assay, which has been previously prepared. Subsequently, the plate is incubated in CO_2-free conditions at 37 °C for approximately 60 minutes before the experiment.
During this incubation we proceed with the reconstitution of the inhibitors supplied with the kit: oligomycin and rotenone/antimycin A. Following the kit instructions,

prepare 3 ml of solution for each drug so that the concentration of these solutions is 15 μM for oligomycin and 5 μM for rotenone/antimycin A.
Subsequently, 20 μl of oligomycin is then loaded into port A, while 22 μl of rotenone/antimycin A into port B of all the wells, even those that correspond to the control, so that the final concentrations are 1.5 μM per oligomycin and 0.5 μM for rotenone/antimycin A.
Oligomycin released from port A is an inhibitor of oxidative phosphorylation. Specifically, it acts on complex V of the mitochondrial respiratory chain, the so-called ATPase. It binds to a 23kDa polypeptide of the Fo subunit and blocks the ATP synthesis of the Fo/F1 ATPase. The addition of oligomycin reduces the amount of mitochondrial respiration to zero, since the energy necessary to power the respiratory chain is no longer generated. Finally, Port B releases a solution of respiration inhibitors, rotenone and antimycin. Rotenone inhibits the transfer of electrons at complex I (NADH dehydrogenase) level of the electron transport chain, while antimycin blocks the complex III of respiratory chain, between cytochrome b and cytochrome c1, preventing oxidation of both NADH and succinate, and so inhibiting OxPhos process.
Before starting the assay, the medium is removed again from the plate with the cells and replaced with 180 μl of fresh medium equilibrated to a temperature of 37 °C.
Subsequently, the "XF Sensor Cartridge" plate is inserted into the "XF Cell Culture Plate" where the cells were plated, and the two together are placed in the appropriate machine at least 20 minutes before to allow calibration.
The Seahorse XFe96 analyzer (Seahorse Bioscience) can record two parameters that show the metabolic status of the samples: the OCR (Oxygen Consumption Rate measured in pmoles O_2 consumed/min) and the ECAR (Extra Cellular Acidification Rate, measured in mpH/min). Depending on the desired analysis, many kits provided by the Agilent company can be used. For our experiment we used the Agilent Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies) to measure mitochondrial function and the Seahorse XF Glycolysis Stress Test Kit or the Glycolytic Rate Assay Kit (Agilent Technologies) that measures the glycolytic function. Briefly, we plated AGS and ACC-201 cells at the concentration of 3x10^4 cells in quintuplicate into Seahorse XFe96 microplates. Cells were then resuspended in Seahorse assay buffer and the following drugs were added: 1 μM oligomycin, 1 μM FCCP (carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone), 0.5 μM rotenone/antimycin A for Mito Stress Assay, 10 mM glucose, 1 μM oligomycin and 50 mM 2-DG for Glycolysis Stress Assay, and 0.5 μM rotenone/antimycin A and 50

mM 2-DG for the Glycolytic Rate Assays. Every experiment was performed at 37°C and normalized via cell protein content measure with a Pierce BCA Protein Assay Kit (ThermoFisher Scientific).

3.9 Lactate Production

The D-lactate assay kit provided by BioVision gives a quick and easy way to accurately measure D-lactate in a variety of biological samples such as serum, plasma, cells, culture and fermentation media. In the D-Lactate Assay Kit, D-lactate is specifically oxidized by D-lactate dehydrogenase and proportionally generates readable color at the wavelength of λ max = 450 nm. The kit detects D-lactate in samples such as serum, plasma, cells, culture and fermentation media. The useful concentration range in samples is 0.01 mM - 10 mM D-lactate.
A reaction mix was then prepared using 46 μl of D-Lactate Assay buffer, 2 μl of D-Lactate Substate Mix and 2 μl of D-Lactate Enzyme Mix and to obtain a standard curve, having resuspended known quantities of a standard of D-lactate in an additional 50 μl of Assay buffer in different wells of a 96 multiwell. For the experiment we plated the cells in medium culture and after 24h
To measure the lactate produced by the various cell cultures, as per the protocol illustrated in the kit, the conditioned medium of the cells is taken 24 hours after having plated them to ensure they have a confluence of 80-90% such as to condition the medium. The cells that conditioned the medium at the time of sampling are then counted. In a 96 multiwell, 50 μl of conditioned medium have been incubated for each well in addition to 50 μl of the previously prepared mix, therefore for a total of 100 μl for each well for 30 minutes at room temperature. We proceed with the reading of the spectrophotometer at λ = 450 nm and interpolate the concentration of lactate (nM) in each conditioned medium on a previously prepared standard curve. Finally, the data obtained were normalized on the number of cells that conditioned the medium *(Andreucci et al. 2022)*

3.10 Flow Cytometry analysis

3.10.1 Expression of CAIX

To evaluate the expression of CAIX in GC cells we performed a staining in flow cytometry.
GC cells in number of 2x10^5, detached with Accutase (EuroClone), and collected in tubes for flow cytometry were washed with PBS, centrifugated at 1200 r.p.m. for 5 minutes and then were stained for 1h at 4°C in the dark with anti-CAIX antibody conjugated with Alexa Fluor 488 (sc-365,900 AF488; Santa Cruz Biotechnology).
After the stain, cells were washed in PBS and analyzed with BD FACS Canto II (BD Biosciences). We also isolated the CAIX-high and CAIX-low-expressing cell populations within AGS WT cells by FACS sorting after the CAIX staining, thanks to the FACS Melody (BD Bioscience).
The analysis of CAIX expression was performed on in AGS and ACC-201 WT and resistant cells.

3.10.2 Double staining Annexin V-PI in Flow cytometry– Evaluation of viability/apoptosis

The analysis of viable and apoptotic cells after treatment has been performed via flow cytometry by annexin V-PI double staining. For the experiments, we used the Annexin V-APC conjugated provided by Immunotools, Germany.
For the assay, the cells were harvested with Accutase and centrifugated at 1200 r.p.m. for 5 minutes; then they were resuspended in Annexin Binding buffer, a solution made of 100 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), 140mM sodium chloride, 25mM calcium chloride supplemented with 2 μl of Annexin V-APC and 1 μl of PI 100 μg/ml. Cells were incubated for 15 minutes in the dark at room temperature. Subsequently, 400 μl of the Annexin Binding buffer were added to each sample and we proceeded with the flow cytometric analysis performed by BD FACS Canto II flow cytometer. The positivity to Annexin V and/or PI determines the cell distribution on the plot, distinguishing and measuring the percentage of viable cells (annexin V negative/PI negative), early apoptosis (annexin V positive/PI negative), late apoptosis (annexin V and PI positive) and necrosis (annexin V negative and PI positive).

The avaluation of cell viability and death was performed on AGS and ACC-201 WT and resistant cells after treatment with SLC-0111, OCF and genistein.

3.10.3 ROS Stress evaluation in Flow cytometry – DCFDA assay

ROS assessment has been performed after treatment of 72h with OCF or 48h Genistein. AGS and ACC-201 cells were plated in 60 mm petri dishes and treated with the compounds. At the relative time points the cells were detached using Trypsin-EDTA centrifugated at 1200 r.p.m. for 5 minutes and then resuspended in medium pre-warmed at 37°C supplemented with DCFDA 10 μM (Sigma Aldrich). This probe, which extended name is dichlorodihydrofluorescein diacetate, exerts fluorescence in presence of ROS.
The cells were incubated in the dark for 30 minutes at 37°C. To optimize the experiment a positive control was prepared adding H_2O_2 20 mM during incubation with DCFDA. After incubation, the cells were analyzed through BD FACS Canto II flow cytometer. The DCFDA signal was detected in the FITC channel.
This assay was performed for the evaluation of ROS production after treatment with OCF and genistein.

3.10.4 Intracellular staining of p53

We evaluated the expression of p53 in flow cytometry by performing intracellular staining of AGS and ACC 201 WT and Drug-Resistant cells after treatment.
Cells treated for 72h with OCF or 48 with Genistein were detached with Accutase (EuroClone) and centrifugated at 1200 r.p.m. for 5 minutes. Cell pellets were washed with PBS w/o Calcium and Magnesium for two times, centrifugated and then resuspended in Paraformaldehyde (PFA) 4% in PBS and incubated 10 minutes at 4°C. Then they were centrifugated at 1200 r.p.m. for 5 minutes and resuspended in a permeabilization buffer, made of PBS with 0.25% TritonTM X-100 (Sigma Aldrich). The permeabilization process lasted 5 minutes.
At this point, cells were centrifugated again and then incubated overnight at 4°C with a 2 μg/μl anti-p53 primary mouse antibody (1:100, Santa Cruz Biotechnology) prepared in PBS 0.1% TritonTM X-100 0.2% BSA.
The day after the cells were washed twice with PBS and then incubated with the secondary antibody: an anti-mouse FITC-conjugated (1:200, Merck Millipore) prepared in PBS 0.1% TritonTM X-100 0.2% BSA. The incubation with the secondary

antibody lasted 1h at room temperature in the dark. Then, the cells were washed with PBS, resuspended in PBS, and subjected to analysis through BD FACS Canto II.
The staining and quantification of p53 expression was performed on AGS and ACC-201 WT and resistant cells after treatment with OCF and genistein.

3.11 Western Blot Analysis

For the western blot analysis, the cells were firstly incubated with the lysis buffer, the RIPA (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA) supplemented with a protease inhibitor solution (Sigma-Aldrich); the incubation lasted 30 minutes on ice. The resulting lysates were centrifugated at 14.000 rpm for 15 minutes. The supernatants containing the protein mixture were collected and evaluated for their protein concentration with the Bradford method. The SDS page was performed using pre-cast gel provided by Thermofisher.
Western Blot experiments were performed for the evaluation of CAIX expression in AGS and ACC-201 cells; the primary antibody was a mouse anti-CAIX (sc-365900, Santa Cruz Biotechnology) and a mouse anti-vinculin (sc-73614, Santa Cruz Biotechnology). The secondary antibody used is a goat anti-mouse IgG
For the expression of p21, Rb and pRb after treatment with OCF on AGS WT and resistant cells the primary antibodies used were a rabbit anti-p21 (1:1000, Cell Signaling Technology, distributed by Euroclone), a rabbit anti- phospho Rb (1:1000, Cell Signaling Technology) and a rabbit anti Rb (1:1000, Cell Signaling Technology). The rabbit anti-vinculin (1:1000, Cell Signaling Technology) was used as housekeeper gene to assess the equal amounts of protein loaded in each lane.

3.12 RNA extraction and qRT-PCR

WT and chemo-resistant AGS cells were cultured in 100 mm petri dishes in complete medium. Before the reaching of confluence, they were lysed in 500 μl of TRI reagent (Sigma-Aldrich). Following manufacturer's instructions RNA was extracted and solubilized in RNase/DNase-free water; then was doses using a

NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermofisher Scientific). We use 1 μg of RNA for the retro transcription reaction to the cDNA using the iScript™ cDNA Synthesis Kit (Biorad) and following the manufacturers' instructions. The cDNA obtained after reaction was diluted 1:5 in RNase/DNase-free water. The PCR reaction in Real time was performed using a CFX96 Touch Real-Time PCR Detection System (Biorad) using the primers listed in table 3. 1 μl 4 μM of forward and reverse primers mix, 2 μl of diluted cDNA, 2 μl of water and 5 μl of Sso Advanced Universal SYBR Green Supermix (Biorad) were dispensed into a 96 well plate with each experimental point in triplicate. Real time was performed with the following steps:

- 1) 2 min at 95 ◦C,
- 2) 15 s at 95 ◦C,
- 3) 30 s at 60 ◦C,
- 4) repeat from step 2, 39 more times
- 5) and then temperature increase from 55 ◦C to 95 ◦C, increasing 0.5 ◦C per second.

The data were then analyzed using the CFX Maestro software (Biorad)

	forward		reverse
18S	5'- CGGCTACCACATCCAAGGAA -3'		5'- GCTGGAATTACCGCGGCT -3'
AKR1B1	5'- CCAACTTCAACCATCTCCAGGTG -3'		5'- GTCACCACGATGCCTTTGGACT -3'
AKR1B10	5'- CCAAGTCTGTGACACCAGCA -3'		5'- CGTTACAGGCCCTCCAGTTT -3'
AKR1C1	5'- TGCATAATGCCTGGGCTATCTT -3'		5'- AGGCCATGACAGTGTTTGAG -3'
AKR1C2	5'- GACCAGCCTTGGAAAGGTCA -3'		5'- AGACATGCAATCACGGAAGT -3'
AKR1C3	5'- ATGCCTGTCCTGGGATTTGG -3'		5'- GGCGGAACCCAGCTTCTATT -3'
GPX2	5'- CCCTTGCAACCAATTTGGAC -3'		5'- TCCTTCAGGTAGGCGAAGAC -3'

Table 1: description and sequence of primers used in qRT-PCR for the evaluation of antioxidant enzymes expression.

3.13 In cell western – evaluation of CAIX intracellular content

We plated 2x10^4 cells per well in a 96-well plate; then the cells were fixed and permeabilized respectively with PFA 4% in PBS and 0,1% Triton X-100. We used Immobilion Block FL (Fluorescent Blocker, Millipore, Sigma-Aldrich, Milan Italy), as a blocking solution for 1h and then performed two staining: with an anti-CAIX primary antibody (Abcam, distributed by Prodotti Gianni) and with anti-tubulin primary antibody (Cell Signaling, EuroClone), both at 1:100 concentration.

After incubation, the plate was washed with PBS and stained with the secondary antibodies (respectively a goat antirabbit for CAIX and a goat anti-mouse for tubulin) both at 1:1000 concentration (ThermoFisherScientific)).
After the incubation with secondary antibodies, the plate was washed with PBS 0,1% Tween and then analyzed through the Odissey Scanner Imager (LI-COR).
The in-cell western analysis was performed for the evaluation of CAIX expression in AGS and ACC-201 WT and resistant cells.

3.14 AldeRed assay – Evaluation of Aldheyde dehydrogenase activity

We evaluated the activity of Aldheyde dehydrogenase through AldeRed assay (588-A ALDERED ALDH Detection Assay for Cancer Stem Cells, SCR150, Sigma-Aldrich). The AldeRed reagent is a red-shifted fluorescent substrate for aldehyde dehydrogenase (ALDH) that allows to identify and isolate cells by flow cytometry. The emitted fluorescence is detectable by PE channel.
For our experiments, WT and chemo-resistant ACC-201 cells were cultured in complete medium (RPMI) in 60 mm petri dishes. They were monitored and once 70% confluence was reached, they were detached in Accutase (Euroclone). The cells were resuspended in fresh medium and centrifugated for 5 minutes at 1200 r.p.m. The labeling with AldeRed followed the preparation of the buffer (supplemented with verapamil to avoid the extrusion of the AldeRed probe) according to manufacturer's instructions. Cells were counted and resuspended in fresh medium in number of 3 x 10^5. The cell suspension was split in in two: one half served as a control for the optimization of the assay and was transferred to a new tube containing the specific ALDH1 inhibitor diethylamino benzaldehyde (DEAB). Cells were then incubated for 1 h at 37°C in darkness. Then, cells were centrifugated (1200 r.p.m. for 5 min); the supernatants were discarded, and cells pellets were resuspended in 500 μl of cold AldeRed assay buffer. Samples were stored on ice and then subjected to analysis through BD FACS Canto II.

3.15 *In vitro* limiting dilution assay

We evaluated the ability of a GC cell line to regenerate tumor mass in extra limiting dilution condition. WT and chemo-resistant ACC-201 were seeded in conditioned medium made with: DMEM/F12 supplemented with B27, 20 μg/ml insulin, 20 ng/ml FGF-2, and 20 ng/ml EGF in ultra-low attachment 96-multiwell (all provided by ThermoFisher Scientific). Cells were plated at different doses (from 1 up to 50 cells per well).

Colony formation was evaluated by visual inspection. After 14 days, for each dilution series, we counted positive wells that showed sphere formation. Data obtained were analyzed and displayed using the Extreme limiting dilution assay (ELDA) software http://bioinf.wehi.edu.au/software/elda/ *(Hu and Smyth 2009).*

3.16 Statistical Analysis

The results obtained in this study are obtained from at least three independent experiments and are expressed as Mean +/- Standard Deviation. The statistical analysis is performed through GraphPad Prism software; we performed multiple comparison tests as specified in each figure legend. The statistical significance was accepted at $p < 0,05$. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$; **** $p < 0,0001$.

4. Results

4.1 Establishment of Chemo-resistant GC cell lines

Our in vitro Gastric Cancer model consist in two different Gastric Adenocarcinoma cell lines, the AGS and the ACC-201. These cells are characterized for deriving from primary tumors.

AGS gastric cancer cell line was derived from a primary tumor resected from a female Caucasian gastric adenocarcinoma patient (54 years old) who did not receive prior therapy. According to Lauren Classification the tumor had a mixed phenotype *(Laurén, 1965)*. Genetically, these cells are negative for EBV infection *(Zhao J et al., 2013)*, and without microsatellite instability *(Yao Y et al., 2004)*.

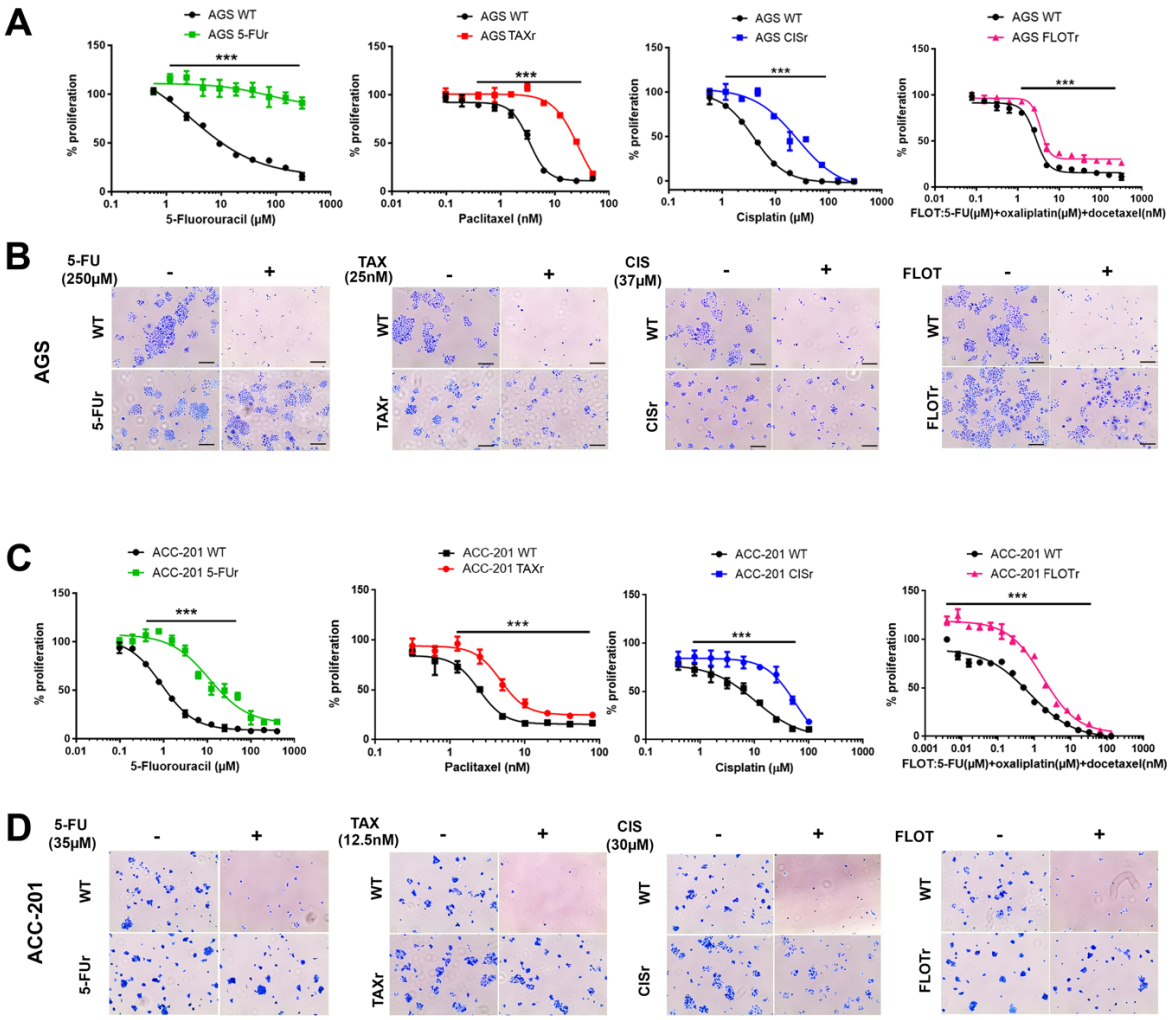
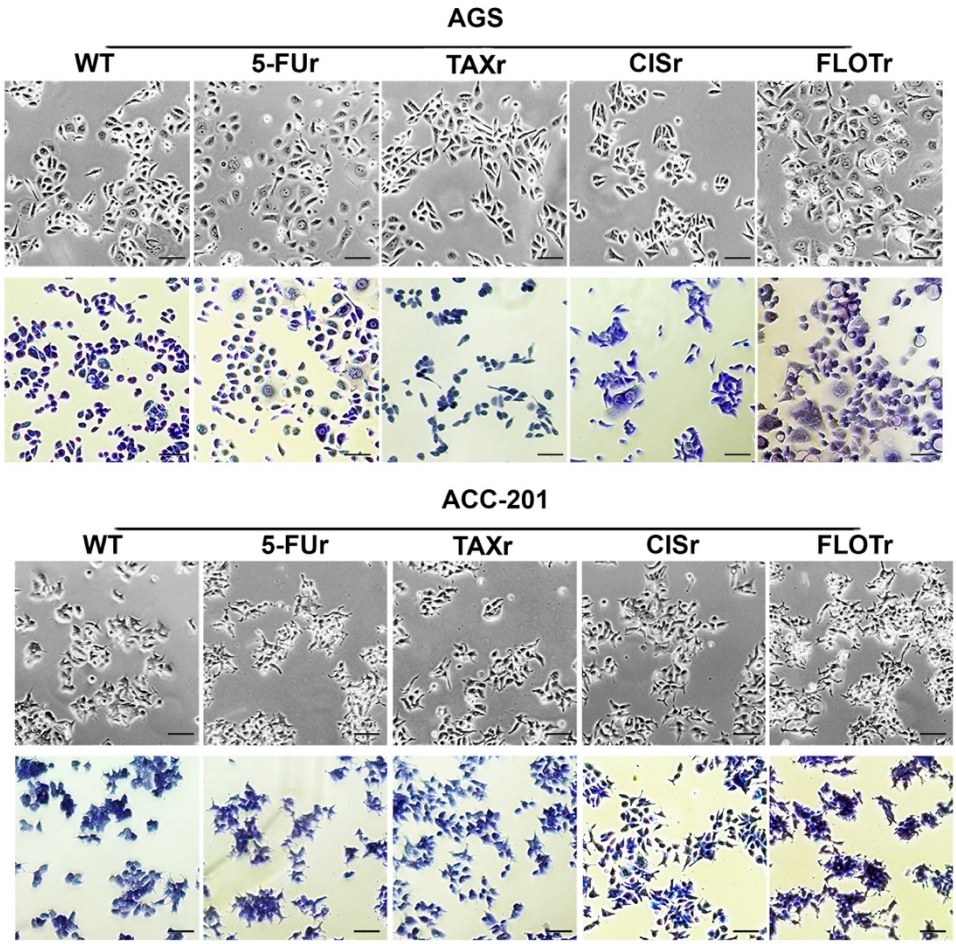


Figure 10: Growth curves and representative pictures of AGS WT, 5Fur, CISr, TAXr and FLOTr. (A-B) and of ACC-201 WT, 5FUr, Cisr, TAXr and FLOTr (C-D): Statistical analysis performed by two-way ANOVA (*Andreucci et al., 2023*)

A



B

AGS		ACC-201	
	IC50		IC50
5FUr	250 µM	**5FUr**	35 µM
TAXr	25 nM	**TAXr**	12,5 nM
CISr	37 µM	**CISr**	30 µM
FLOTr		**FLOTr**	
5fu	15 µM	5fu	11,5 µM
doce	2,4 nM	doce	2 nM
oxa	1,5 µM	oxa	5 µM

Figure 11: representative photos and hematoxylin/eosin staining of GC cells AGS and ACC-201 (wild type and chemoresistance) (A); table reporting the actual IC50 of ACC201 and AGS chemo-resistant cells lines.

The ACC 201 (also known as 23132/87) were established from the primary tumor of a 72-year-old man with gastric adenocarcinoma in 1987 *(Vollmers HP et al., 1993).* They have a hypertetraploid karyotype and microsatellite instability and are EBV-negative.

These two cell lines have been used to establish four chemo-resistant subpopulations. The resistant AGS cells were previously obtained in our laboratory

(Peri S et al., 2021) so for the establishing of ACC-201 chemo-resistant cells we followed the same method.
We subjected ACC 201 cells to chronic exposure of the three main chemotherapeutic drugs for the treatment of GC: 5-fluorouracil (5FU), cisplatin (CIS), paclitaxel (TAX) and FLOT regimen which consist in a combination of drugs (5FU, oxaliplatin and docetaxel).
After 9 months of chronic treatment, we evaluated the IC50 for every treatment.
The graphs in figure 10 show the survival curves of ACC-201 WT cells and of AGS cells (in black) that have been subjected to chronic treatment with the different therapeutic regimens (green for 5FU, blue for CIS, red for TAX and violet for FLOT treatment).
It is possible to observe a clear distance between the two curves which represents the acquisition of resistance to chronic treatment.
The table in figure 11 is a resume of the IC50 of all resistant cells selected by chronic administration of chemotherapeutic agents.

4.2 CAIX expression in GC tissues is increased in non-responder patients compared to responder patients subjected to perioperative FLOT/FOLFOX treatment

We evaluated the expression of CAIX in 23 responder and non-responder GC patients treated with perioperative FLOT/FOLFOX regimen (16 patients treated with FLOT and 7 with FOLFOX).
All the patients were grouped basing on the TRG value as we described in Materials and Methods. Between the two groups we observed a significative difference in the expression of CAIX. Non-responder patients showed a significative increasing in percentage and score of CAIX. All the results are shown in the figure below.

A

		Responder (TRG 2)	Non-responder (TRG 3-5)
Number of patients		7	16
Age		57-77 (average: 68,57)	49-79 (average: 67,17)
Sex	Male	4	10
	Female	3	6

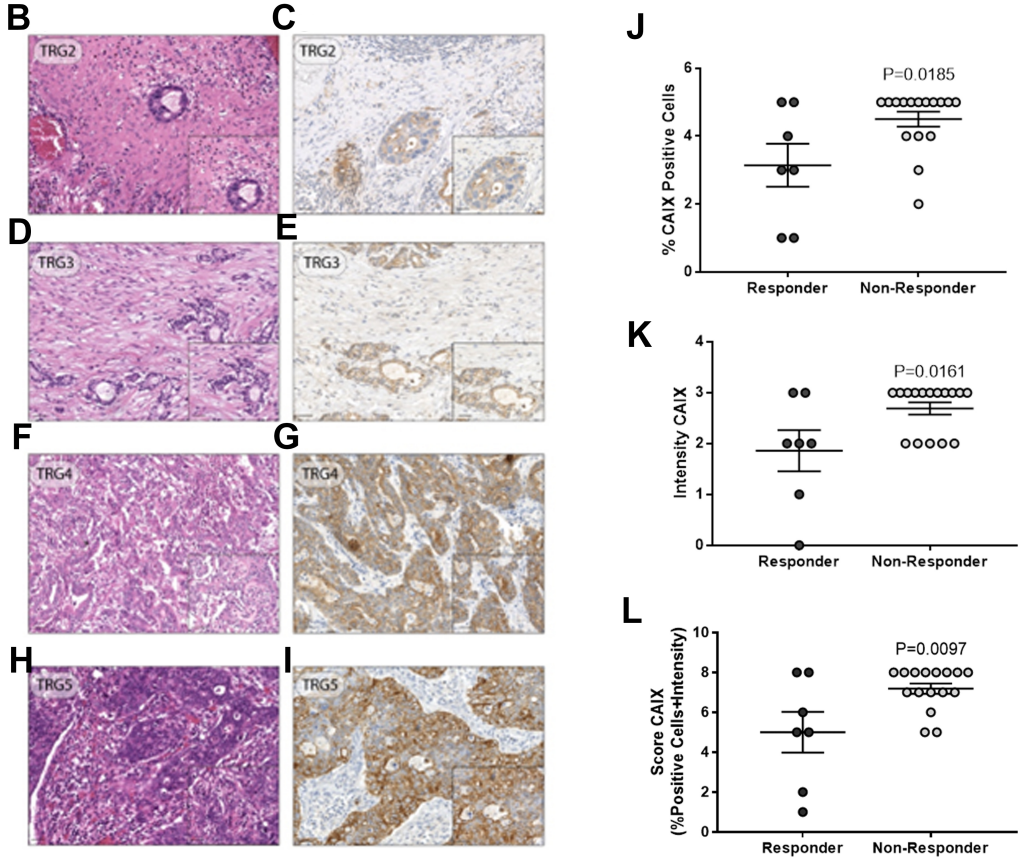


Figure 12: Expression of CAIX in responder and non-responder patients with different TRG. (A) table reporting responder and non-responder patients to FLOT/FOLFOX treatment. (B-I) Representative images of TRG2: (B) Hematoxylin/Eosin staining and (C) CAIX IHC staining. Representative images of TRG3 (D) Hematoxylin/Eosin staining and (E) CAIX IHC staining. Representative images of TRG4 (F) Hematoxylin/Eosin staining and (G) CAIX IHC staining. Representative images of TRG5 (H) Hematoxylin/Eosin staining and (I) CAIX IHC staining. (Magnification ×20, inset ×40; scale bar 100 μm, 50 μm, respectively). (J-L) Percentage of positive (J), intensity (K) and score (L) of CAIX staining in samples of responder and non-responder GC patients with gastric adenocarcinoma and subjected to gastrectomy following the perioperative FLOT/FOLFOX treatment (Statistical significance obtained with T-test)

4.2.1 Metabolic Profile of AGS and ACC 201 cells – Chemo-resistant Gastric Cancer cell line shows a more glycolytic metabolism compared to the wild-type

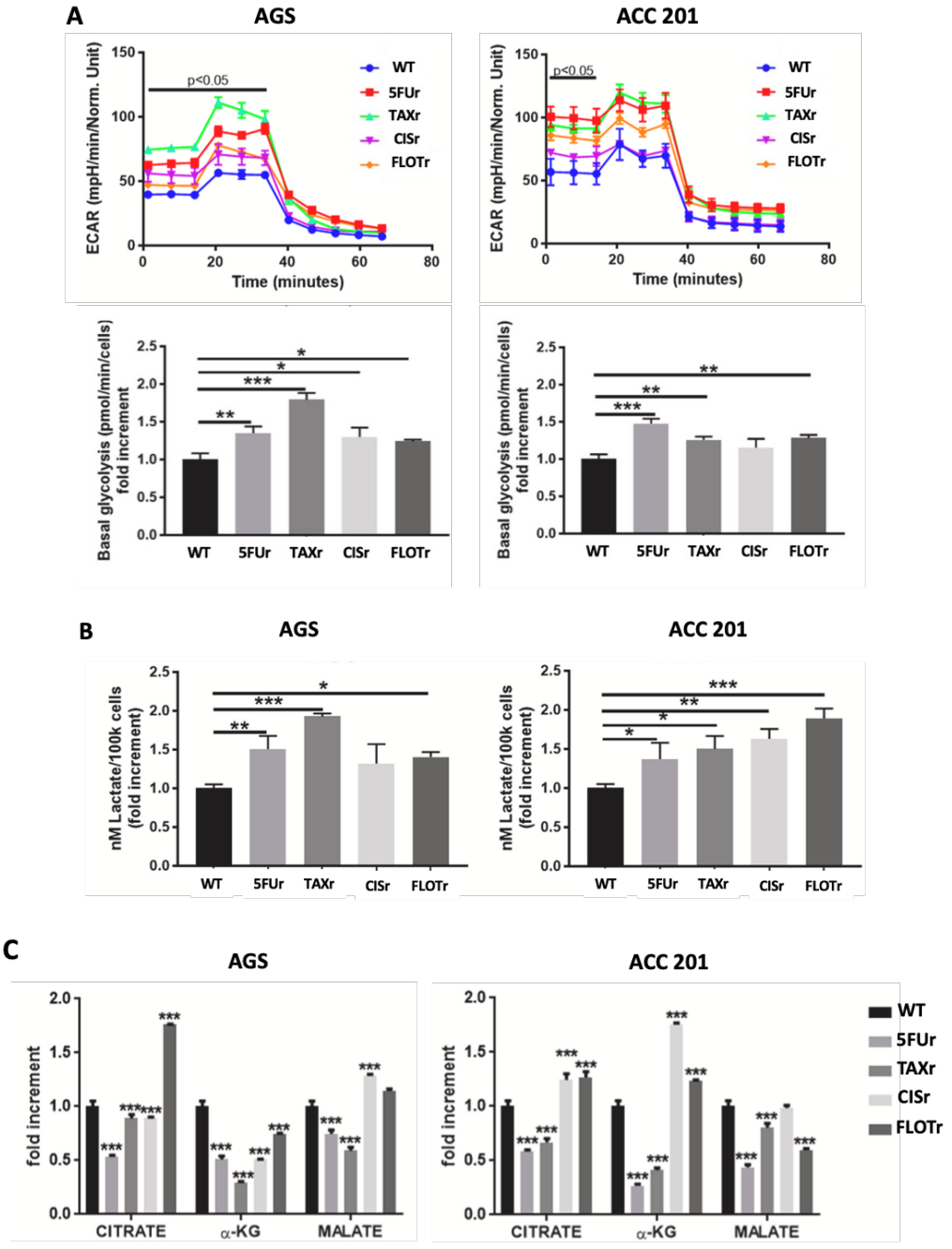


Figure 13: Metabolic profile of WT and Chemo-resistant GC cells; Glyco Rate Assay was performed by Seahorse Analyzer XFe96 (A), lactate production evaluated by colorimetric assay (B) and quantification of production of alfa-ketoglutarate, citrate and malate (C) (Statistic: One-way ANOVA for A and B, Two-Way ANOVA for C; WT vs drug-resistant cells).

In the figure 13 are shown the graphs obtained by the Seahorse analysis of ECAR (Extra Cellular Acidification Rate) for ACC-201 and AGS cells, WT and Resistant. There is also a quantification of the content of TCA metabolites.
The first experiment performed by Seahorse Analyzer XFe96 shows a different metabolic profile between resistant and WT cells, both in AGS and ACC-201. The resistant ones seem to have a more glycolytic metabolism (A); this evidence is confirmed by the increasing in Lactate production (that is a result of high consumption of glucose in tumor) showed in figure (B). These data are accompanied by a reduction in the production of TCA metabolites (Citrate, alfa-Ketoglutarate and Malate) in the Resistant cells compared to the wild type.

4.2.2 Chemo-resistant GC Cells shows an increment of CAIX expression compared to the WT control

Given the boosted glycolytic metabolism of chemo-resistant AGS and ACC201 accompanied by increased lactic acid production and release in the extracellular milieu, we tested whether the expression of carbonic anhydrase IX (CAIX), a key regulator of intracellular and extracellular pH, is varied in chemo-resistant, high-glycolytic cells compared to the WT cells.

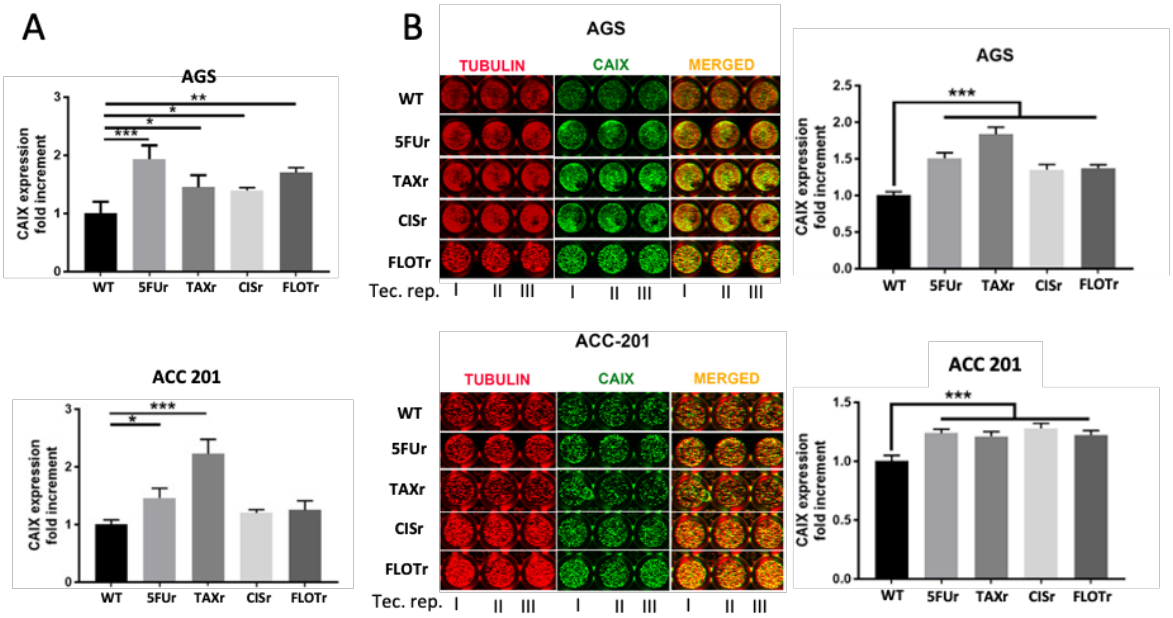


Figure 14: Expression level of CAIX in WT and Chemo-resistant AGS and ACC 201. Graphs of membrane staining performed in Flow Cytometry (A) and in *in-cell* western(B). All data normalized on tubulin (Statistic: One-way ANOVA; WT vs drug-resistant cells).

We observed that chemo-resistant cells are indeed characterized by an increased expression of CAIX compared to the WT cells. As can be observed by the graphs above (fig.14A) the flow cytometry experiments revealed an increase of CAIX expression on cell surface of drug-resistant cells compared to the WT control in both AGS and ACC-201 cell lines. In the AGS cells every drug-resistant cell shows significant difference in the expression of the protein with the 5FUr cells shows an almost doubled expression of CAIX - compared to the WT tested in flow cytometry. In the ACC-201 group, no significant differences in CAIX cell surface expression were observed among WT and CISr or FLOTr cells, but a strong increase of CAIX expression was again observed in 5FUr and in especially in the TAXr cells, which showed a doubled expression of CAIX compared to the ACC-201 WT.

When looking at the total expression of CAIX (thus analyzing the whole cell lysate) we obtained similar results of flow cytometry: in both AGS and ACC-201 drug-resistant cells there is an increased expression of CAIX compared to the control WT, with statistical significance.

In order to further highlight the relationship between CAIX and chemo resistance in GC cell lines, we sorted the CAIX-high- and the CAIX-low-expressing cells within the AGS WT population. AGS WT cells were thus sorted based on the expression levels of CAIX, so we obtained: CAIX-low-expressing cells=25% of AGS WT with the lowest expression of CAIX; CAIX-high-expressing cells= 25% of the AGS WT with the highest expression of CAIX. These two subpopulations were subsequently subjected to FLOT treatment, to make a comparison in terms of response to chemotherapeutic agents. The data obtained by flow cytometry reported in figure 15, indicate that the CAIX-high subpopulation has a greater resistance than the CAIX-low subpopulation, as showed by the decrease in cleaved caspase 3/7 levels - which is a reporter of apoptosis - detected in the CAIX-high after the treatment with chemotherapeutics for 72 hours. Particularly, CAIX-low AGS cells treated with TAX shows the highest value of apoptosis but in every treatment a significant difference between CAIX-low and CAIX-high is noticeable.

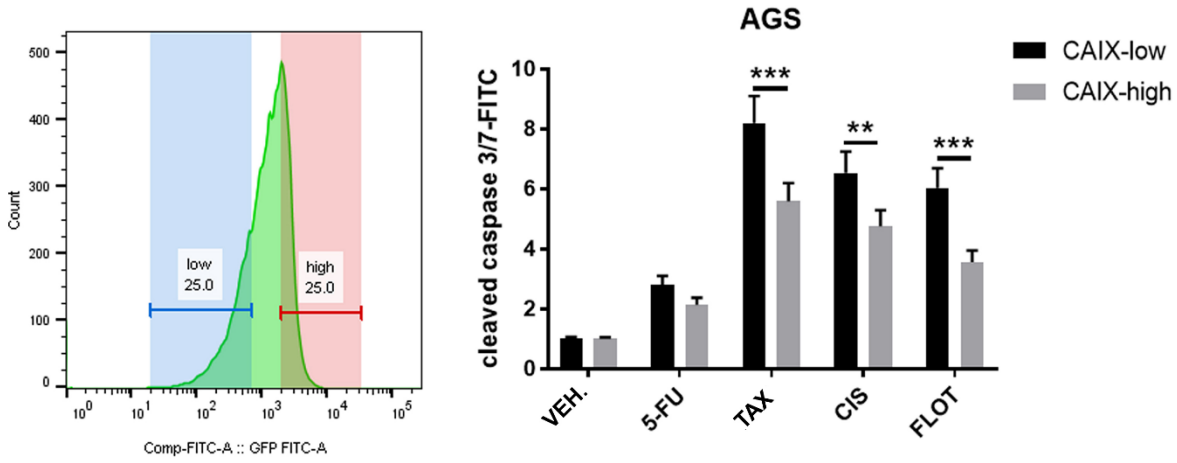


Figure 15: (on the left) AGS WT sorting of the 25% low-CAIX and high-CAIX expressing cells; (on the right): Apoptosis level detected with Cleaved Caspase 3/7 in flow cytometry in CAIX-lo and CAIX-high AGS cells after treatment with 5FU, TAX, CIS and FLOT for 72h (Statistic: Two-way ANOVA).

In a similar way, when we induced the expression of CAIX in AGS WT cells, via transfection with lipofectamine containing the pRP-EGFP/Neo-CMV>hCA9 plasmid, the cells with overexpression of CAIX (CAIX+) showed a reduction in terms of therapeutic response after treatments with chemotherapeutics. In every treatment group there is a correlation between the increased expression of CAIX and the increasing of cell proliferation, as reported by graphs in figure 16.

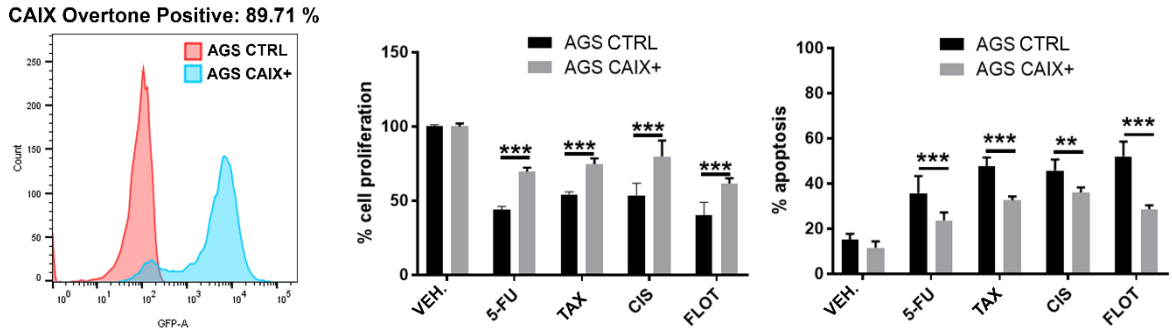


Figure 16: (on the left): flow cytometer plot and overtone value of CAIX expression in control (CTRL) and CAIX+ AGS cells; (in the middle): graph representation of data obtained by MTT assays and (on the right) flow cytometry evaluation of apoptosis of CTRL and CAIX+ AGS cells after 72h of treatment with 5FU, TAX, CIS and FLOT. (Statistic: Two-way ANOVA).

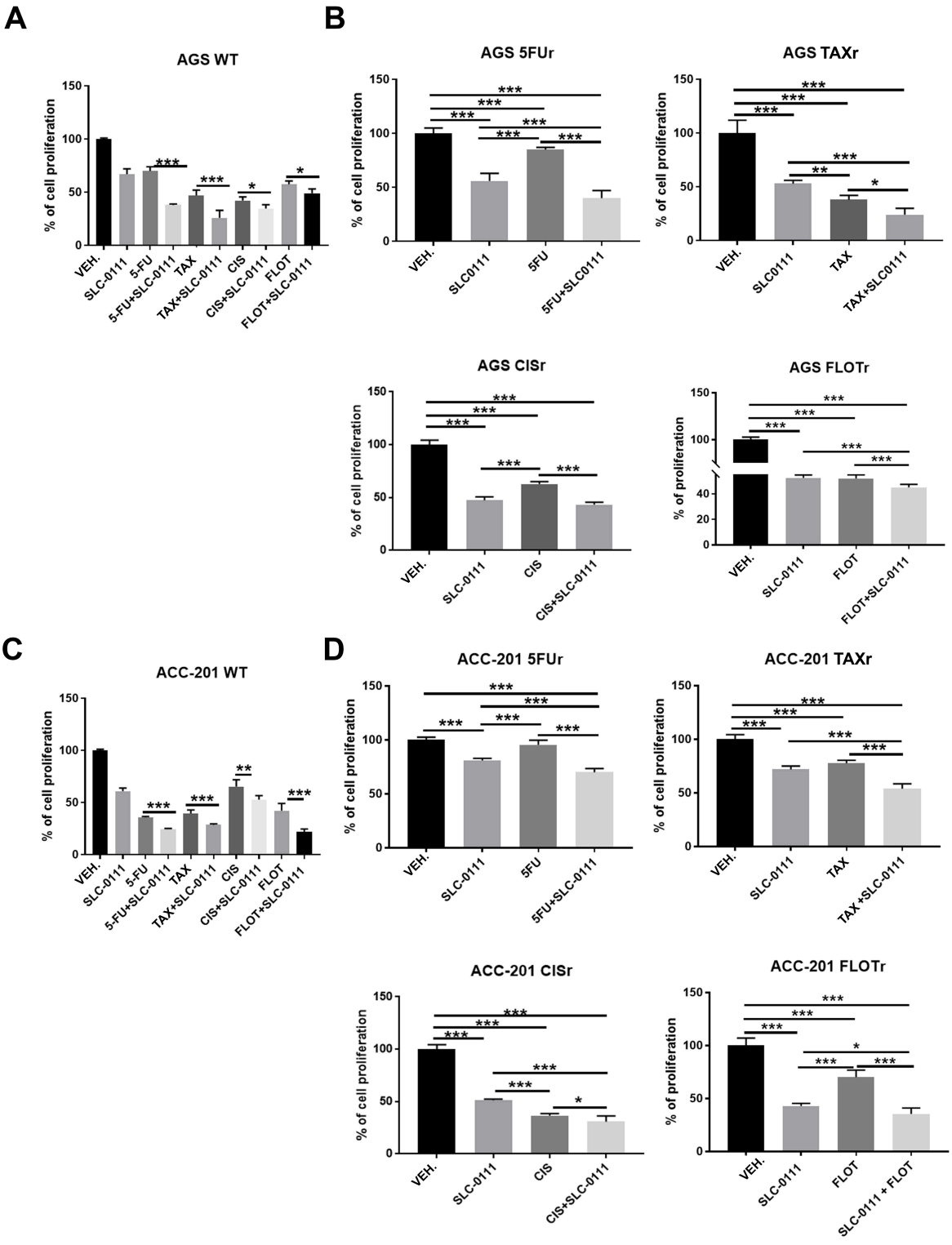
4.2.3 The CAIX inhibitor SLC-0111 sensitizes chemo-resistant cells with a better response to therapy.

SLC-0111 is a CAIX inhibitor that already showed its efficacy in *in vitro* model of melanoma, breast cancer, and colorectal carcinoma *(Andreucci et al., 2019; Peppicelli et al., 2020).* This drug has successfully passed the phase I clinical trial (NCT03450018) showing that is safe and well tolerated when administered to patients with advanced stage of cancer *(McDonald et al., 2020).* Nowadays, SLC-0111 is under phase Ib/II clinical trials (NCT03450018) for the treatment of CAIX-positive metastatic pancreatic cancer in combination with capecitabine.

So, after the observation of CAIX expression in our GC model we decided to test his efficacy on AGS and ACC 201 resistant and WT and to evaluate its possible ability to re-sensitize the cells to the therapy. We observed that SLC-0111 alone is able to reduce of almost 50% the proliferation of ACC 201 and AGS WT cells. The combination of SLC-0111 with the drugs used for treatment (5FU, CIS, TAX and FLOT) induced a better response to the drug; a significative decrease of proliferation has been observed in both lines when subjected to combinatory treatment with respect to single chemotherapy administration (50% in AGS and 30% in ACC 201).

An interesting data emerged after the administration of SLC-011 as single treatment to AGS and ACC 201 5FUr, CISr, TAXr and FLOTr: in every AGS resistant cells. SLC-0111 induced a reduction in proliferation of about 50%; moreover, in the combined treatment SLC-0111 was able to reduce AGS 5FUr, CISr, TAXr and FLOTr cells proliferation, demonstrating an improvement of anti-proliferative effect of the chemotherapeutic drugs.

Regarding ACC 201 resistant cells, single treatment with SLC-0111 induced a reduction of 30% of proliferation rate in 5FUr and TAXr. In CISr and FLOTr has been observed a reduction of 50%. Also in ACC 201, in the combined treatment SLC-0111 showed its capacity to improve to all the drugs.


A
AGS WT
% of cell proliferation
VEH.
SLC-0111
5-FU
5-FU+SLC-0111
TAX
TAX+SLC-0111
CIS
CIS+SLC-0111
FLOT
FLOT+SLC-0111
B
AGS 5FUr
AGS TAXr
AGS CISr
AGS FLOTr
% of proliferation
C
ACC-201 WT
D
ACC-201 5FUr
ACC-201 TAXr
ACC-201 CISr
ACC-201 FLOTr
SLC-0111 + FLOT

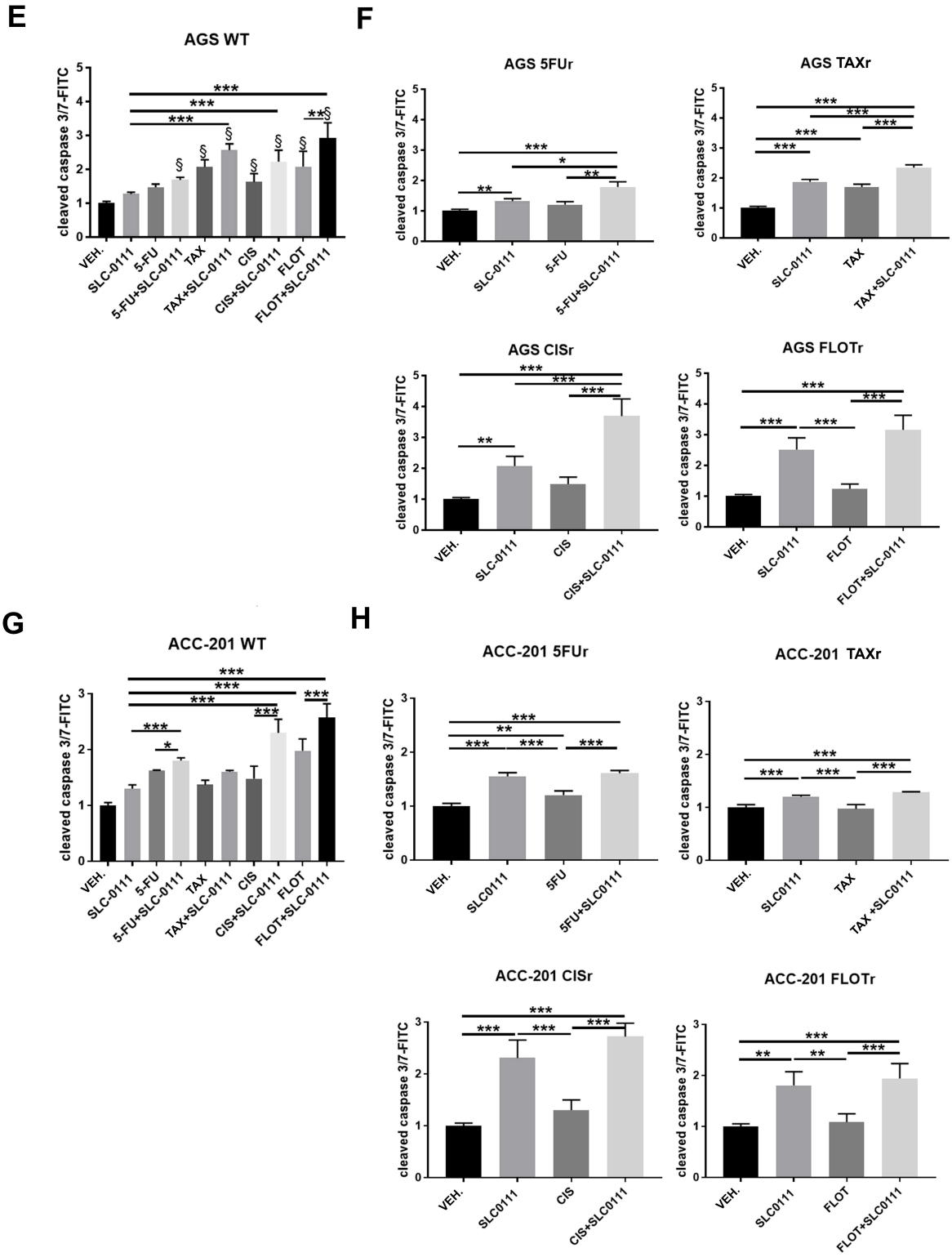
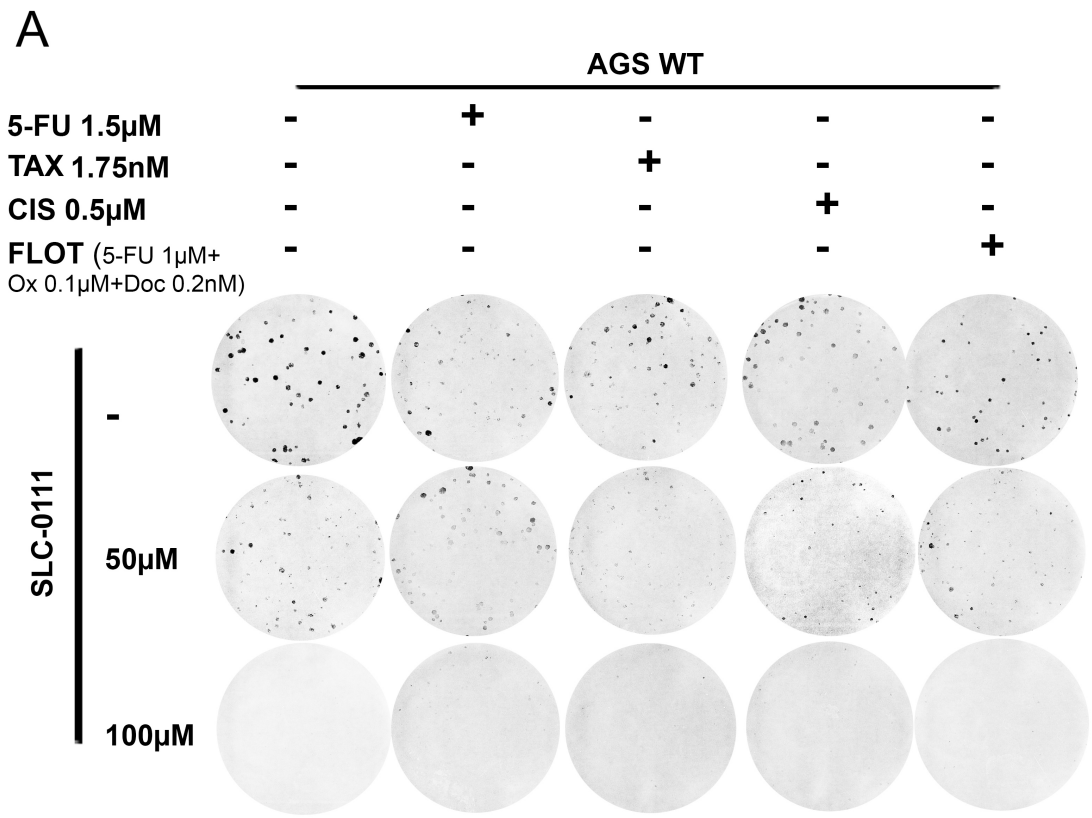
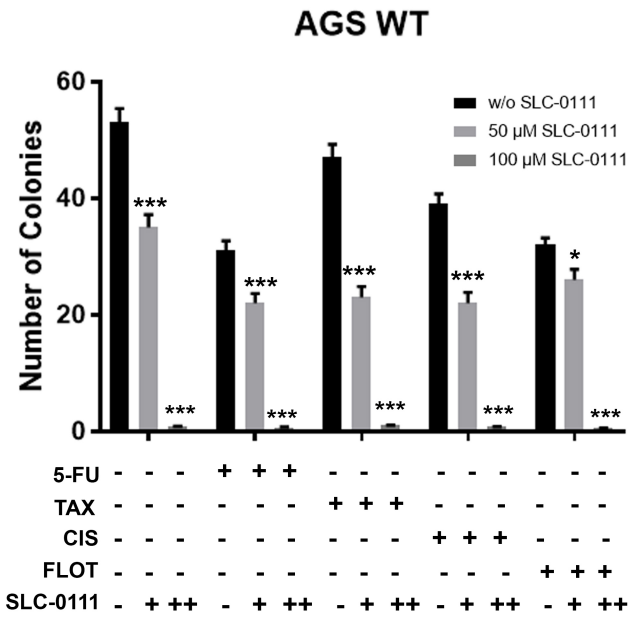
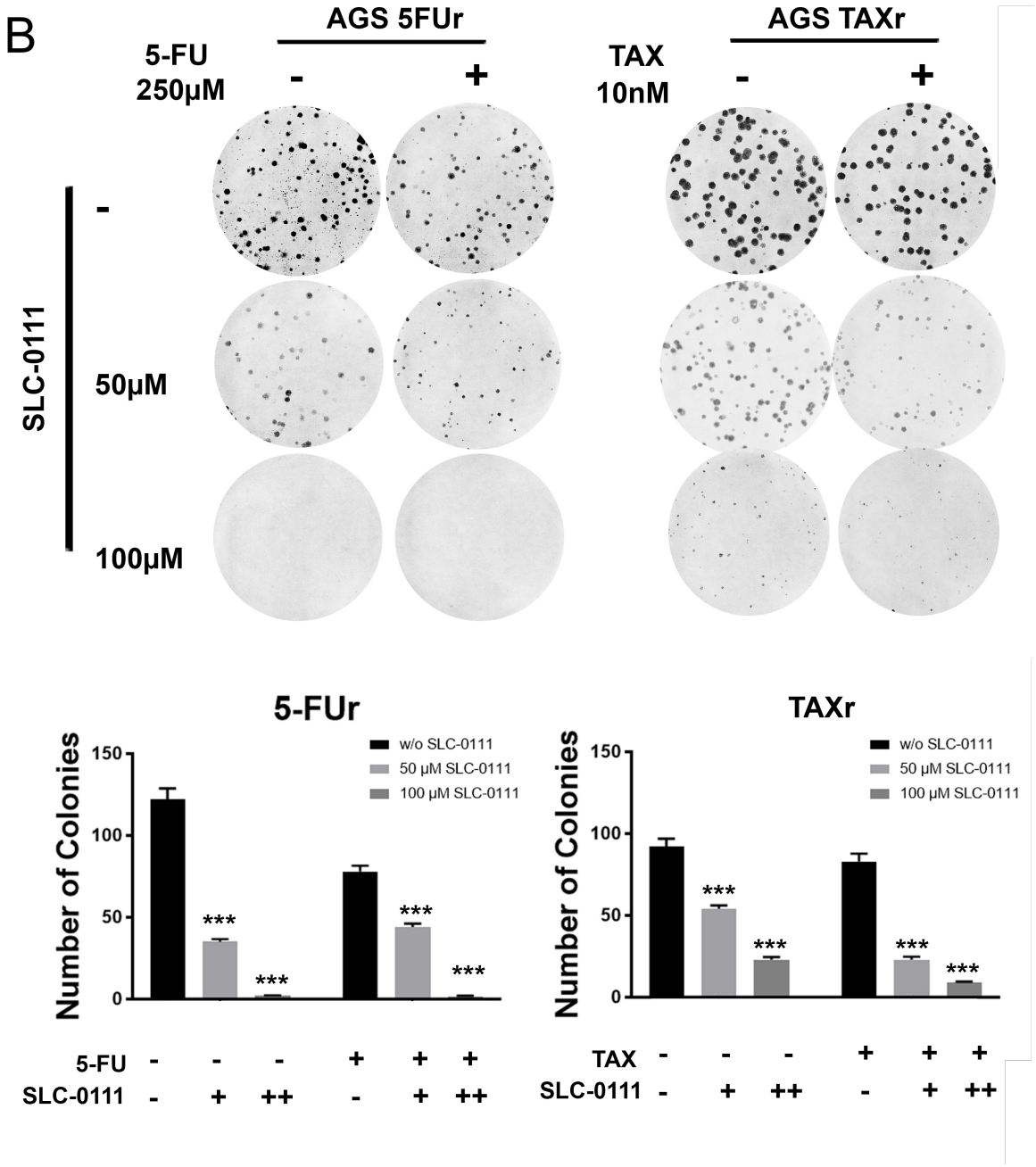


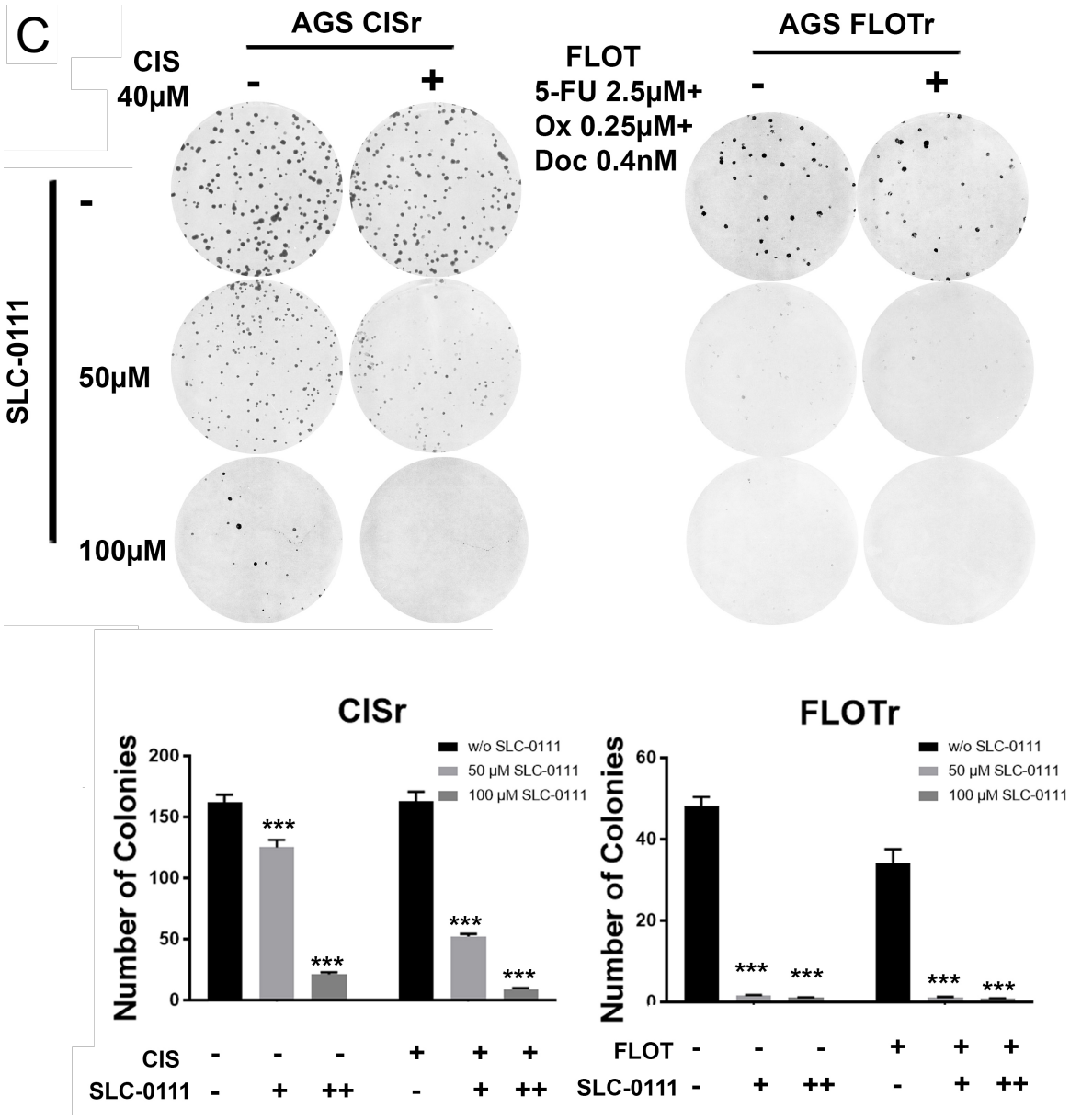
Figure 17: (A-D) Graphs that show the cytostatic and cytotoxic effects of SLC-0111 on GC cells; MTT assay of WT, 5-FUr, PTXr, CISr, and FLOTr AGS (A and B) and ACC-201 (C and D) cells after 72h of treatment with the respective IC50 dose of 5-FU, TAX, CIS, and FLOT administered as single treatments or in combination with 100 μM SLC-0111 (Statistical analysis performed by two-way ANOVA test); (E-H) Cleaved caspase 3/7 flow cytometer assay performed on WT, 5-FUr, PTXr, CISr, and FLOTr AGS (E and F) and ACC 201 (G and H) cells, after 72h of treatment with the respective IC50 dose of 5-FU, TAX, CIS, and FLOT administered as single treatments or in combination with 100 μM SLC-0111 (Statistic: two-way ANOVA test).

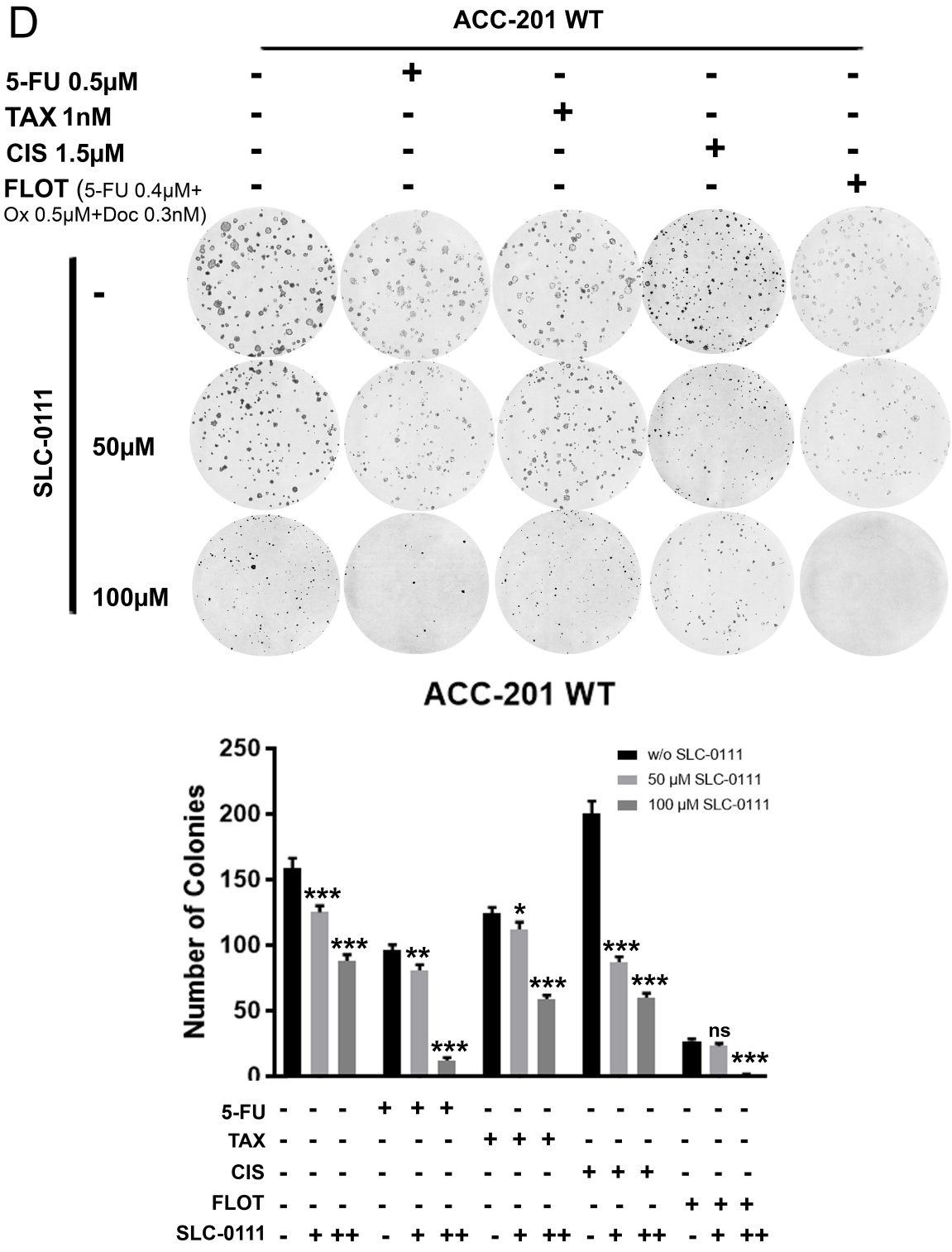
In accordance with these results, colony formation assays were performed to evaluate the proliferative capacity of single cells treated with SLC-0111 and/or 5FU, TAX, CIS and FLOT and therefore having an indication on the power of the treated cells to lead to recrudescence after treatment. The colony formation assay confirmed the ability of SLC-0111 to improve response to the chemotherapeutic treatment and at the same time to restore sensitivity to chemotherapeutics in chemo-resistant cell lines.

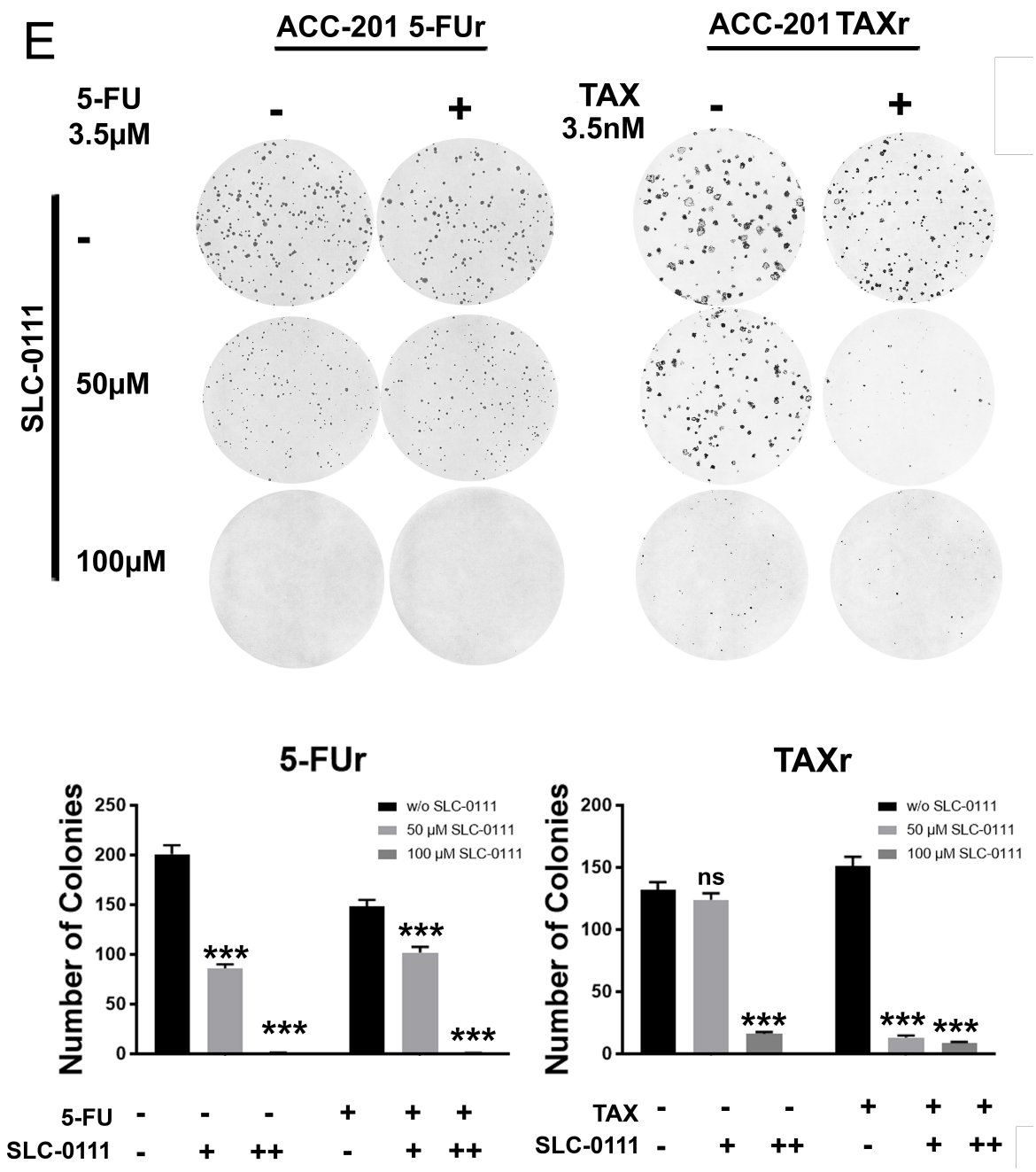





B
AGS 5FUr
AGS TAXr
5-FU
250μM
TAX
10nM
-
+
SLC-0111
50μM
100μM
5-FUr
TAXr
Number of Colonies
w/o SLC-0111
50 μM SLC-0111
100 μM SLC-0111
***
5-FU
TAX
SLC-0111
++


C
AGS CISr
CIS
40μM
-
+
SLC-0111
-
50μM
100μM
AGS FLOTr
FLOT
5-FU 2.5μM+
Ox 0.25μM+
Doc 0.4nM
-
+
CISr
Number of Colonies
200
150
100
50
0
w/o SLC-0111
50 μM SLC-0111
100 μM SLC-0111
***
***
***
***
***
***
CIS - - - + + +
SLC-0111 - + ++ - + ++
FLOTr
Number of Colonies
60
40
20
0
w/o SLC-0111
50 μM SLC-0111
100 μM SLC-0111
***
***
***
***
FLOT - - - + + +
SLC-0111 - + ++ - + ++


D
ACC-201 WT
5-FU 0.5μM
TAX 1nM
CIS 1.5μM
FLOT (5-FU 0.4μM+ Ox 0.5μM+Doc 0.3nM)
SLC-0111
50μM
100μM
ACC-201 WT
Number of Colonies
w/o SLC-0111
50 μM SLC-0111
100 μM SLC-0111
ns
5-FU
TAX
CIS
FLOT
SLC-0111


E
ACC-201 5-FUr
ACC-201 TAXr
5-FU
3.5µM
TAX
3.5nM
SLC-0111
50µM
100µM
5-FUr
TAXr
Number of Colonies
w/o SLC-0111
50 µM SLC-0111
100 µM SLC-0111
ns
5-FU
TAX
SLC-0111

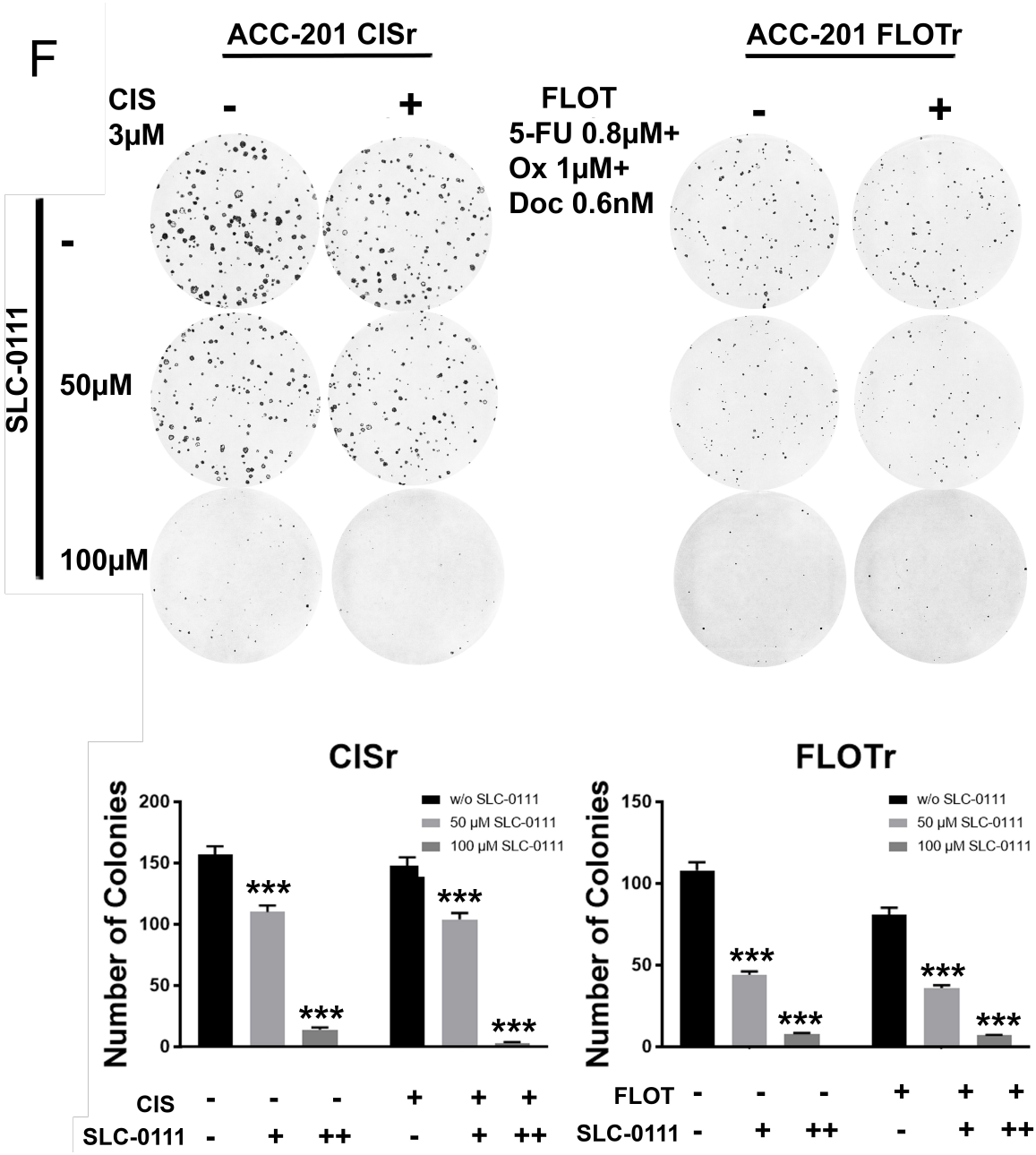


Figure 18: Colony formation assay of AGS WT (A) and ACC-201 WT (D) cells treated with SLC-0111 alone and in a combined therapy with chemotherapeutic drugs. Colony formation assay of chemo-resistant AGS (B, C) and ACC-201 (E,F) cells. The colony formation assay was performed after treatment with SLC (alone and in combination with chemotherapeutic drugs) at two different concentrations: 50 µM (+) and 100 µM (++) (Statistic: Two-way ANOVA).

To verify and confirm the crucial role of CAIX in supporting chemoresistance, we silenced CAIX via siRNA in AGS and ACC-201 WT and FLOTr cells. Once verified the correct silencing we tested them for the response to SLC-0111 treatment. The outcome of silencing was evaluated by detecting the expression levels of total CAIX, via Western blot. We detected a 75-80% decrease in total CAIX expression levels in AGS WT and FLOTr, a halving in ACC 201 WT cells and a notable decrease (about 85%) in ACC 201 FLOTr.

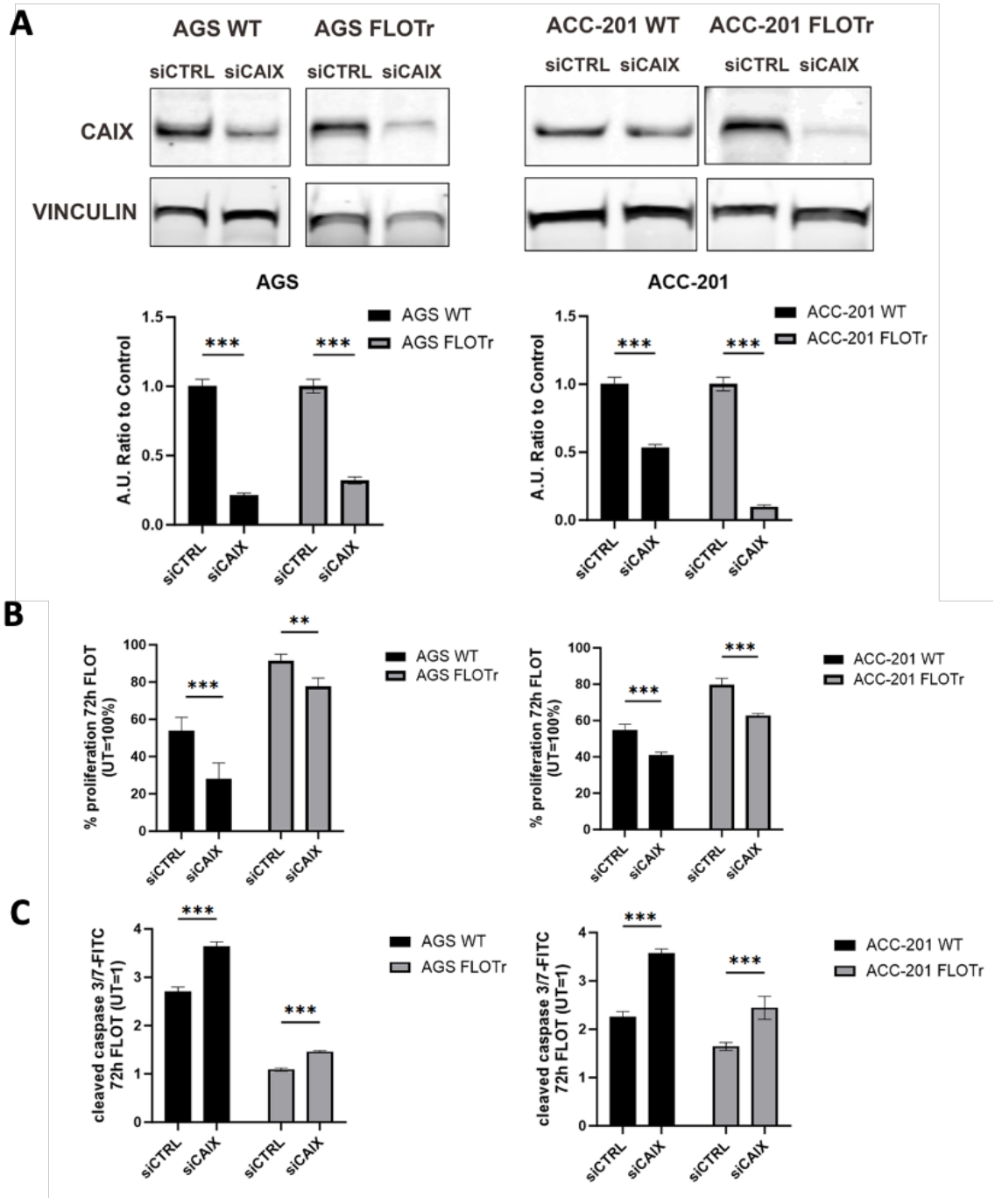


Figure 19: Expression and quantification of CAIX expression of of AGS and ACC cells WT and FLOTr. (A) Western blot quantification by densitometry. (B); MTT/viability assay of AGS and ACC 201 WT and FLOTr subjected to FLOT treatment fo 72h after CAIX silencing; (C) quantification of cleaved caspase 3/7 performed in flow cytometry of AGS and ACC WT treated with FLOT for 72h after CAIX silencing. (Statistic: One-way ANOVA).

Once we ascertained the effectiveness of silencing, we performed an MTT assay on AGS and ACC 201 WT and FLOTr cells after 72h of FLOT treatment. We observed that the downregulation of CAIX determines an improvement in the therapeutic response in both sensitive and drug-resistant cells. Specifically, not-silenced AGS WT cells (siCTRL) showed both in the MTT assay a decrease in viability of 50%

when treated with the FLOT regimen whereas the silenced ones (siCAIX) showed a 75% decrease. In AGS FLOTr cells the viability went from 90% in siCTRL to 80% in siCAIX. Similarly, a slight viability reduction was observed in WT ACC 201 and FLOTr siCAIX cells when treated with the FLOT regimen (respectively from 55% to 40% and from 80% to 60%).
These data were supported by the detection of a significant increase in caspase 3 and 7 activity in the same cells. This evidence indicated a decrease in chemoresistance in GC cells with a down-regulation of CAIX, supporting the relationship between CAIX and chemoresistance in GC.

As introduced in section 1, the study of natural compounds, specifically Polyphenols and their activity in cancer is a current topic in scientific research. Many studies involving different models of malignant tumor treaed with Polyphenols, have showe promising results relatively to their possible usage in clinical practice.
One of the main target of this research field is the evaluation of the potential of polyphenols to exert toxic effect on malignant cells, to sensitize cells to chemotherapy and/or possbly re-establish a sensitivity in resistant tumors. In this work, we focused our experiments on the evaluation of two compounds that have shown their ability to interfere with tumor progression in several cancer models: Oleocanthal and Genistein. The first one is on of the many product of Olea Euopaea, that was described as an efficient anti-inflammatory, anti-angiogenic and anti-proliferative agent ; Genistein, an isoflavone that can be found abundant in soybean, demonstrated its efficacy on cancer cells inhibiting cell cycle progression and inducing apoptosis.
In this work, will be exposed the results obtained treating our GC model (the AGS and ACC-201 cells) with this two molecules in condition of normal response to anti-cancer therapy or chemoresistance.

4.3 Effects of Oleocanthal-Enriched Extract in WT and Chemo-resistant Gastric Cancer cell lines

4.3.1 Composition and characterization of Oleocanthal-Enriched Evo Oil extract.

For this work, we used a solution of Oleocanthal-enriched extract defined Olea Extract Fraction (OCF), resuspended in Ethanol (EtOH) 70%. This OCF was obtained by the selection of EVO oils from Leccino, Frantoio and Moraiolo cultivarrs, all obtained from two phase mills. The blend of the oils used for the extration has a total MPC content of 856 mg/kg (HPLC-DAD-MS).

Olea Extract Fraction (OCF)

hydroxytyrosol	12.38
tyrosol	8.29
elenolic acid	76.32
10-hydroxyoleocanthal	287.62
oleocanthal	189.16
oleuropein aglycone	95.44
ligstroside	23.29
secoiridoidic derivatives	171.99
Total	**864.50**

Table 2: OCF composition obtained by HPLC-DAD-MS

4.3.2 OCF administration induces a reduction of viability in AGS WT and chemo-resistant 5FUr, CISr and TAXr cells.

A preliminary assessment of the toxicity of OCF on healthy, non-tumor cells was first carried out; we treated NHDF cells (Neonatal Human Dermal Fibroblasts) and HMVEC (Human Microvascular Endothelial Cells). On both these lines, no marked toxicity of the compound was observed except at the 120 μM concentration. After this initial evaluation we treated AGS, WT and chemo-resistant GC tumor cells.

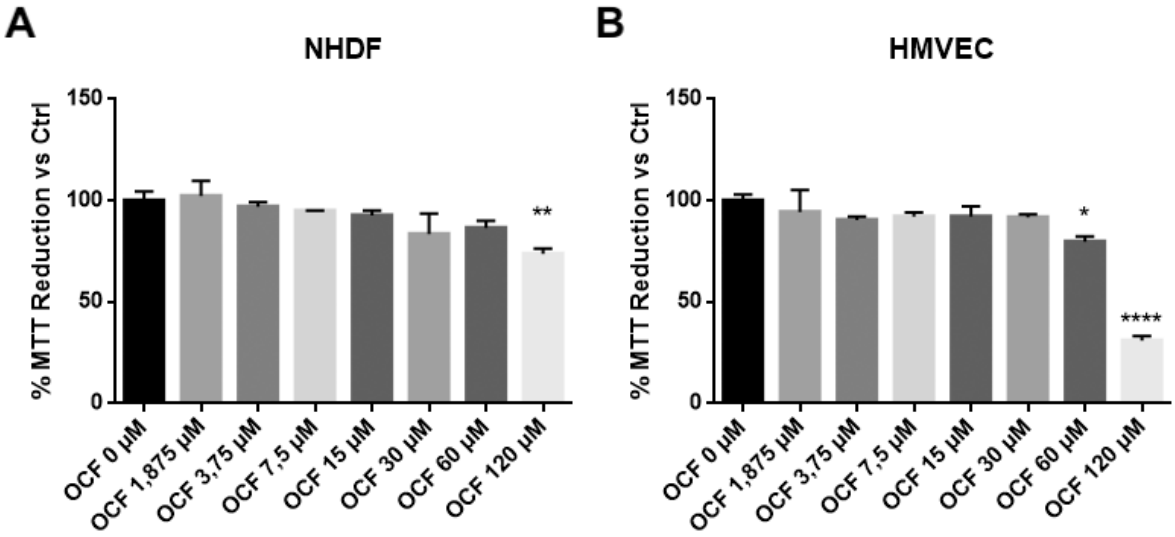


Figure 20: graphic representation of the results of MTT assay on normal cells. NHDF (Neonatal Human Dermal Fibroblasts) (A) and HMVEC (Human Microvascular Endothelial Cells) (B) are non-transformed cell cultures and were used to test their vitality using an OCF dose-dependent treatment through a MTT assay (Statistic: One-way ANOVA).

We tested on AGS WT, 5FUr, CISr and TAXr cells serial dilutions of OCF starting from 240 μM to 3,7 μM treating cells for 72h. No effect on viability or proliferation was observed until the 60 μM dose. At 60 μM OCF the reduction in cell viability was approximately 25% at, 50% at 120 μM and by approximately 75-80% at 240 μM of OCF in all cell lines but CISr.

The AGS CISr cells, on the other hand, seemed to be unsensitive to the OCF treatment until the 120 μM dose in which we observed a slightly but not significant decrease in cell viability. At 240 μM dose the reduction of viability was about the 50%, lower compared with the other cell lines, but notable (fig.21).

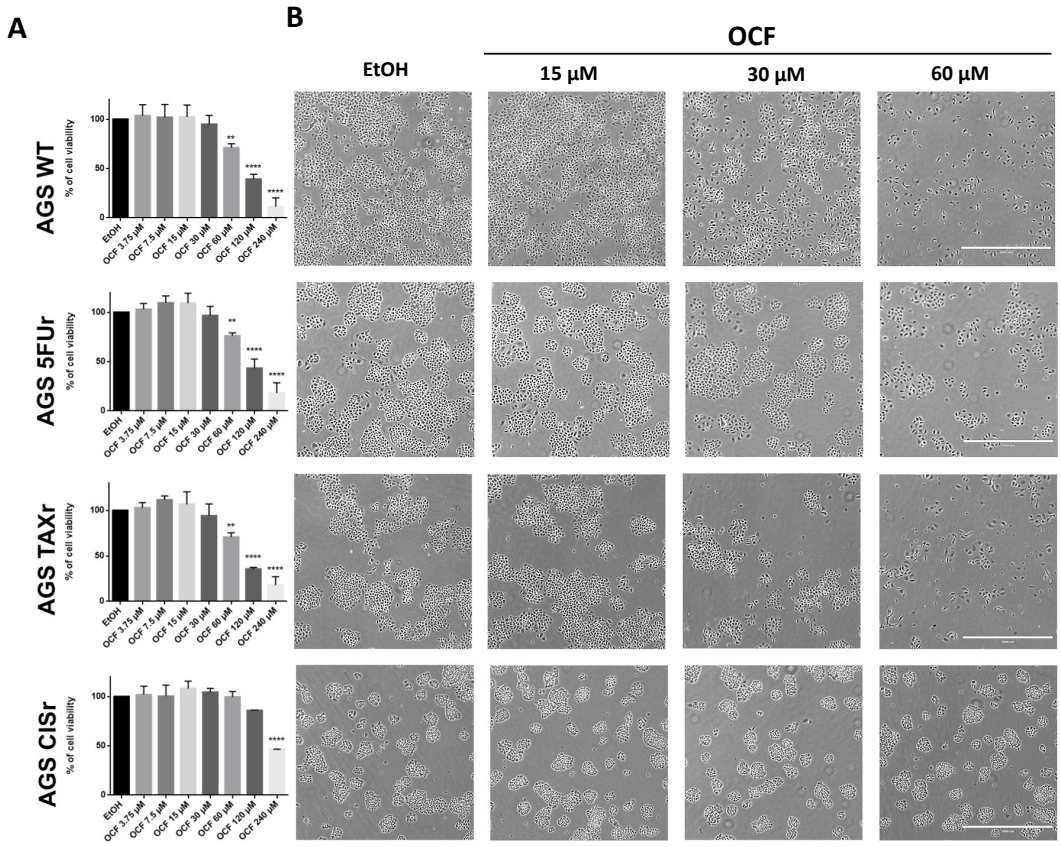


Figure 21: (A) Graph representation of results obtained by MTT assay after 72h of treatment with OCF in AGS WT, 5FUr, CISr and TAXr. (B) representative pictures of OCF effect when administered to AGS cells, WT and chemoreistant. (Statistic: One-way ANOVA).

4.3.3 Treatment with OCF affects the progression of cell cycle in WT, 5FUr and TAXr AGS cells but not in AGS CISr.

4.3.3.1 Western-blot Analysis - OCF unbalances the equilibrium between p21 and pRb in WT, 5FUr and TAXr but not in CISr AGS cells

To better understand the molecular mechanism of OCF activity on GC cells we evaluated the expression of two protein involved in the correct progression of cell cycle and survival: Rb (phosphorylated and not) and p21.

The expression of both these transcription factor has been assessed by western blot analysis.

In a similar way to the viability assay, also the results obtained after western blot experiments suggested a difference in response to 60 μM OCF treatment within our cell lines cohort. In fact, a strong decrease of pRb expression, about 50%, was observed in WT, 5FUr and TAXr AGS cells whereas the CISr AGS cells showed no

differences between control and treatment. Similarly, while in WT, 5FUr and TAXr AGS cells treated with 60 μM OCF the decrease in pRb expression is accompanied by an increase in p21 expression, in AGS CISr cells this effect disappears and p21 expression stands at almost the same value between treated and control.

4.3.3.2 Flow Cytometry-Increase of p53 expression induced by OCF treatment in WT, 5FUr and TAXr but not in CISr AGS cells.

A further investigation about the effect of OCF treatment on WT and drug resistant AGS cells has been performed in flow cytometry. P53 plays a crucial role in regulation of cell cycle, apoptosis, and genomic stability so we evaluated its expression after 60 μM OCF treatment through an intracellular staining.
The data obtained confirmed that OCF administered to WT, 5FUr and TAXr resulted in an overexpression of p53. In the three cell lines the increment in protein expression stands at similar values (25-30% in WT and 25% in 5FUr and TAXr AGS) after a 60 μM OCF treatment.
In the CISr AGS cells we observed no difference in p53 expression comparing treated cells with control. This data confirmed the first observation provided by the western blot analysis of pRb and p21.
Due to the strong correlation occurring between p53 overexpression and apoptosis induction we decided to evaluate the status of cell viability/cell death in our GC cell lines after treatment with OCF.
We performed a double staining with Annexin V and PI in treated and control WT, 5FUr, CISr and TAXr and obtained the quantification of Apoptosis (late and early), necrosis and live cells.
Even in this case we found similar results for WT, 5FUr and TAXr but different for CISr, as shown in figure 21.
The data obtained by flow cytometry analysis of Apoptosis revealed that OCF at 15 and 30 μM was unable to enhance cell death (reported as "dead" in the graphs in fig.) in comparison to the vehicle treatment (10- 20% attested values for WT, 5FUr and TAXr AGS); the 60 μM concentration determined an halving in live cells in AGS WT, a 25% of death cells in 5FUr AGS and an highest number of the dead cells compared to the live ones in TAXr. This behavior common to three cell lines is not respected in the CISr AGS cells, in which we did not observe any significant increases in cell death even at the 60 μM concentration (fig. 21 E-F)

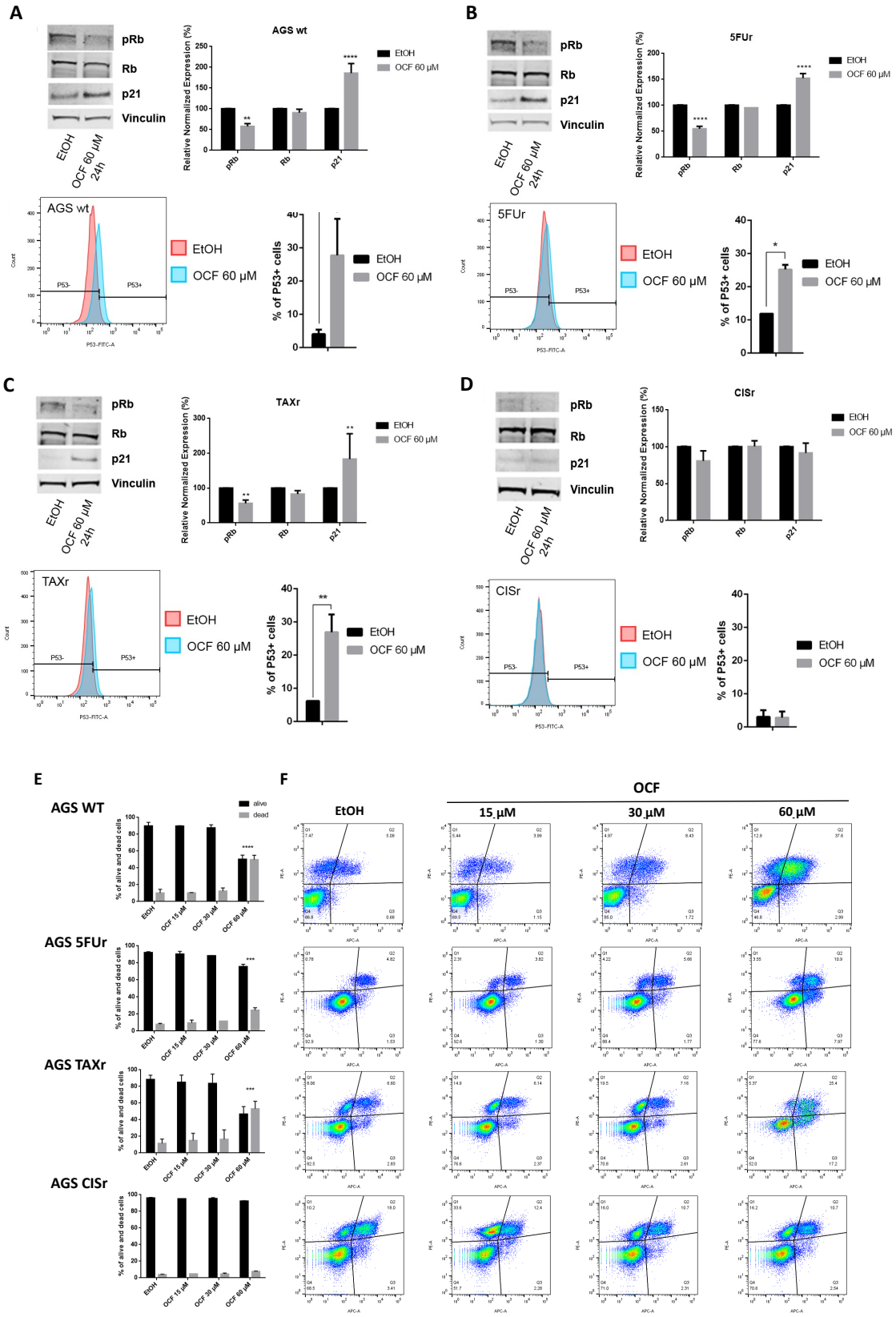


Figure 21: Effect of OCF treatment in GC Cells. (A-D) Western blot and flow cytometry analysis of OCF 60 µM treatment effect on expression of p21, Rb and pRb (evaluated in western blot, densitometry, and graph above) and p53 (evaluated in flow-cytometry, plot and graphs below); the data are normalized of vinculin expression. (E and F) Flow cytometry anlysis and graphs of the expression of p53 after treatment at 3 doses of OCF performed by double staining with Annexin V/PI (Statistic: Two-way ANOVA).

4.3.4 Evaluation of colony formation capacity - OCF decrease capacity to initiate colony growth in WT, 5FUr and TAXr AGS cells

In cancer research, the clonogenic assay is useful for having an initial evaluation of the tumor model ability to initiate a new tumorigenic process on a different adhesion substrate.

We evaluated the clonogenic potential of WT and drug resistant AGS after treatment with vehicle, 15, 30 and 60 μM OCF.

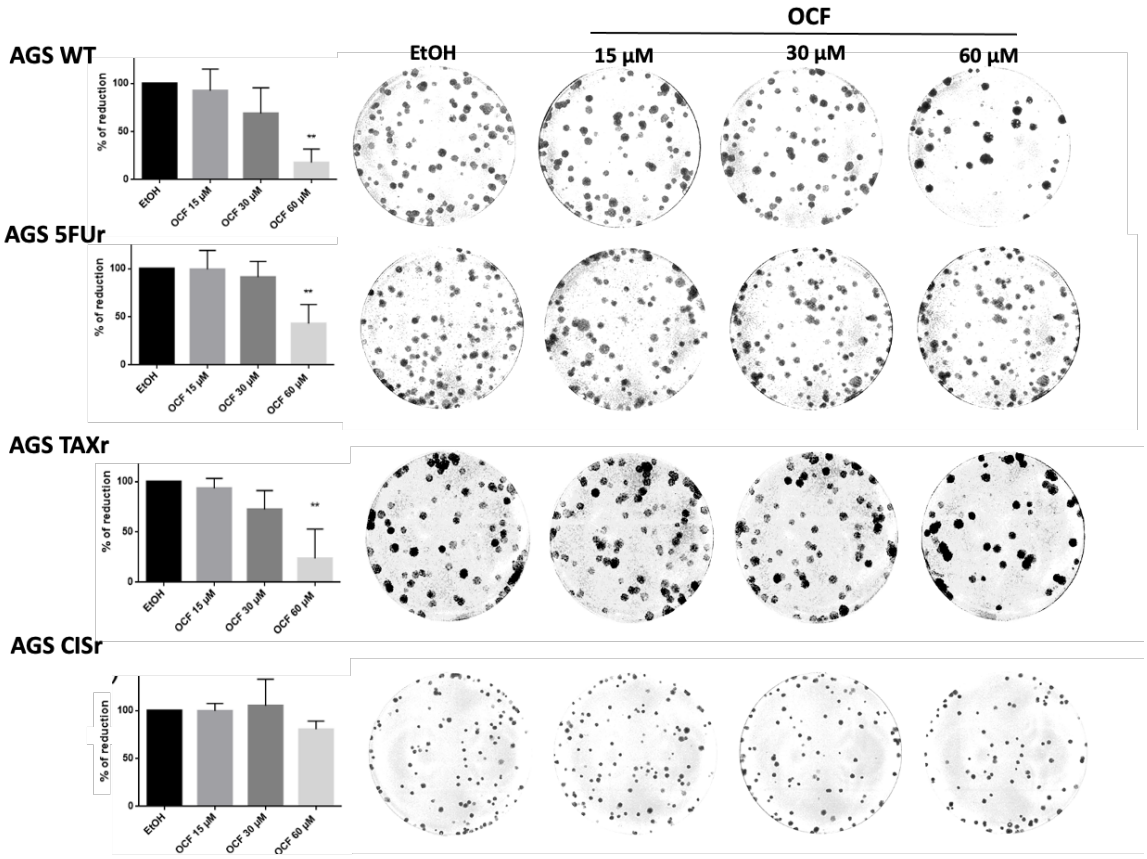


Figure 22: Clonogenic assay of WT, 5FUr, CISr and TAXr AGS cells after 72h of treatment with 3 doses of OCF (Statistic: One-way ANOVA).

Also, in this case we observed that treatment with OCF (as the doses increased) resulted in a reduction in the ability to give rise to new colonies. This finding was observed in AGS WT, 5FUr and TAXr cells; the AGS CISr cells, on the other hand, confirming that they were less sensitive to OCF treatment, maintained their ability to originate colonies comparable to vehicle.

4.3.5 Pro-Oxidant Activity of the OCF on GC Cells

As reported in literature by Cusimano et al., oleocanthal can induce toxicity in colorectal and hepatocellular cancer cells. So, we tried to verify whether the toxicity observed after OCF treatment in our GC cell model could be related to an increased ROS production *(Cusimano et al., 2017)*. Indeed, the over-expression of p53 with OCF treatment could be a first suggestion of a relation between oxidative stress and cell cycle arrest, due to the implication of p53 in stress-response, including ROS-response *(Srinivas US et al., 2019)*

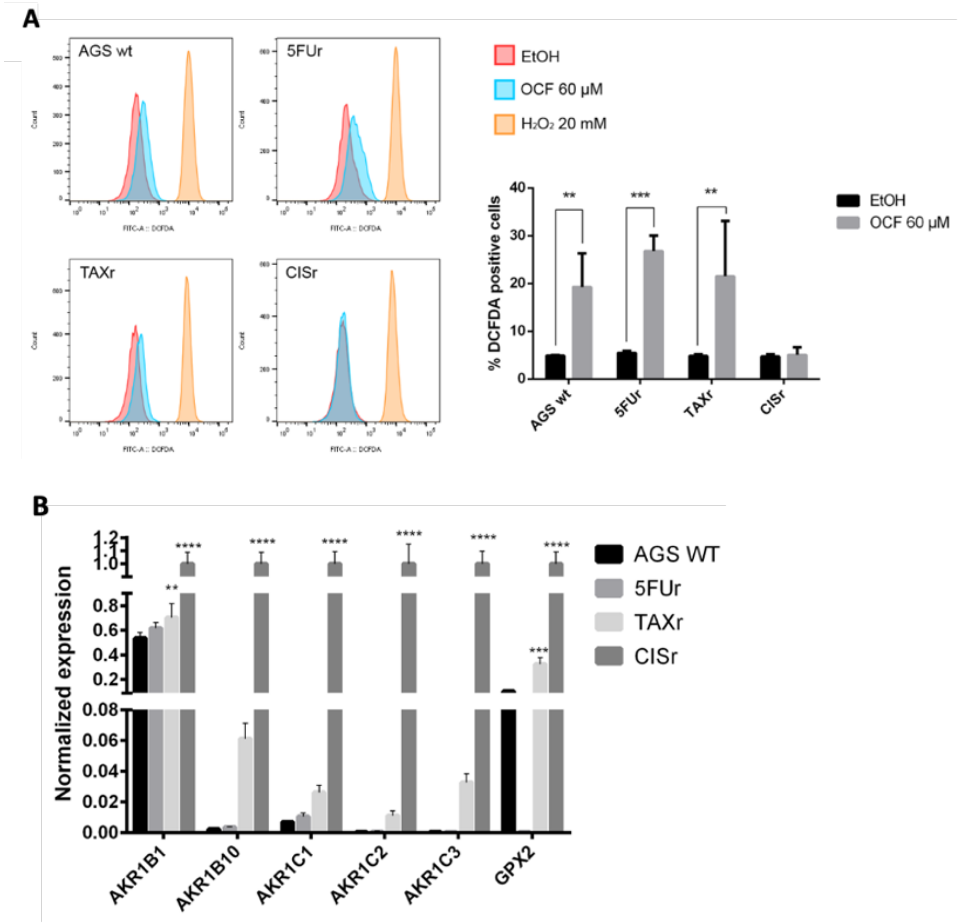


Figure 23: ROS production induced by OCF treatment and evaluation of anti-oxydant enzymes expression. (A) flow-cytometry plot and graphs of ROS level (detected by DCFDA) in WT, 5FUr, CISr and TAXr AGS cellsafter 72h treatment with 60 µM OCF). (B) Real time PCR data analysis of anti—oxydant anzimes in WT, 5FUr, CISr and TAXr AGS cells (Statistic: Two-way ANOVA).

We performed an evaluation of oxidative stress through a staining with DCFDA in flow-cytometry. We verified that the dose 60 μM of OCF was able to increase the ROS production in all the AGS cell lines with the exception of the CISr AGS. This data suggested a possible mechanism of detoxification present in CISr AGS that was not evident in other experiments. This hypothesis, accompanied by the observation

of the effect of OCF treatment on WT, 5FUr and TAXr AGS cells led us to further investigate.
So, we assessed by qPCR the expression of different antioxidant genes, such as AKR1B, AKR1B10, AKR1C1, AKR1C2, AKR1C3 and GPX2. The results of the qPCR showed that CISr AGS cells expressed a higher level of these genes involved in ROS-response compared to the other cell lines. This increased expression could be the reason of the very low ROS production induced by OCF in CISr AGS cells.

4.3.6 Evaluation of combined therapy with OCF plus 5-Fluorouracil, Cisplatin and Paclitaxel on WT and resistant AGS cells

As described in the introduction of this work, the re-sensitization to chemotherapy in drug-resistant cells is one of the keys to overcome chemoresistance in many tumors. For this reason, we tested a possible potentiation effect of the OCF in the toxicity of 5-fluorouracil, cisplatin or Paclitaxel on WT and resistant AGS cells.
In AGS cells the administration of 60 μM OCF enhanced the effect of all 3 drugs, reducing their number after 72h of treatment: Particularly, there is a reduction of approximately 80-85% of the cell population when OCF was administered with 5-fluorouracil and Paclitaxel and of approximately 70% with cisplatin (Fig.24). The same effect was observed when 5FUr, TAXr and CISr AGS cells were treated with OCF added to their respective drug (Fig.24).
Overall, OCF treatment induced an increase of drug toxicity of approximately 43% in WT AGS cells treated with 5-Fluorouracil of 25% in AGS cells treated with paclitaxel, and of 30% in 5FUr cells treated with 5-fluorouracil; an increased toxicity of 22% was also observed in CISr cells treated with cisplatin and of 27% in TAXr cells treated with paclitaxel.

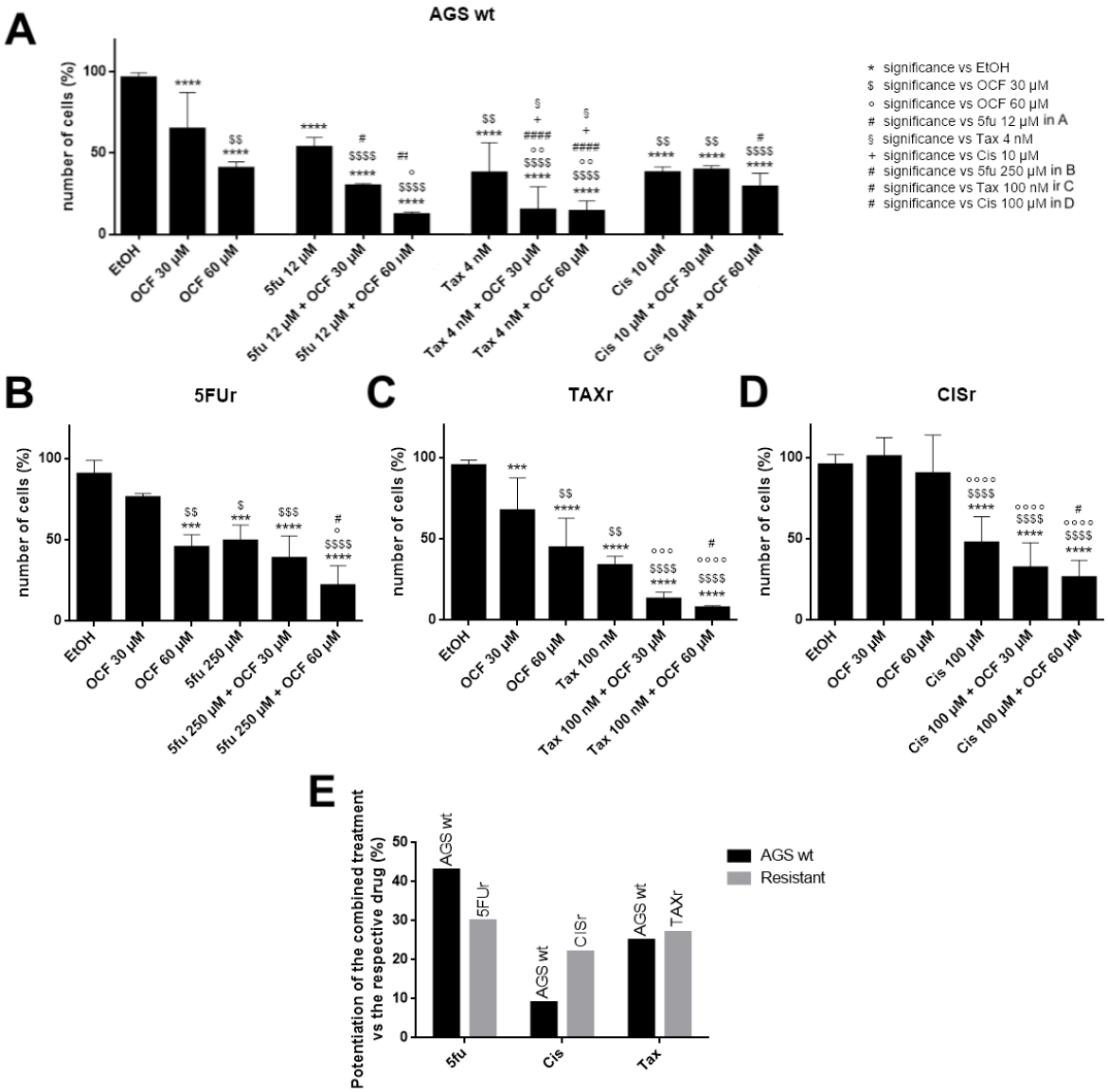


Figure 24: effects of the combined administration of chemotherapeutic drugs with OCF after 72h of treatment. AGS cells were treated with vehicle and 2 concentrations of OCF (30 and 60 µM) for 72h. AGS wild type were treated with their IC50 of 5FU, CIS and TAX, alone or in combination with OCF (A). 5FUr (B), CISr (C), and TAXr (D) GAS cells were treated with OCF alone and in combitation with their IC50 dose. Graphic representation of the increased efficacy of chemotherapeutic drugs administered with OCF compared to chemotherapy alone in 5FUr, CISr and TAXr AGS cells (E) (Statistic: Two-way ANOVA)..

According to literature evidence, Genistein has demonstrated promising results in counteract tumor progression in *in vitro* model of hepatocellular, breast, colorectal and in some gastric cancer cell lines. *(Spagnuolo et al., 2015)*

In this work we decided to evaluate the effect of Genistein in our GC cell lines, specifically the ACC 201 cells.

4.4 Effects of genistein in WT and Chemo-resistant Gastric Cancer cell lines

4.4.1 Genistein toxicity on GC cell lines – Genistein induces reduction of viability in WT, 5FUr, CISr, TAXr and FLOTr ACC 201 cells.

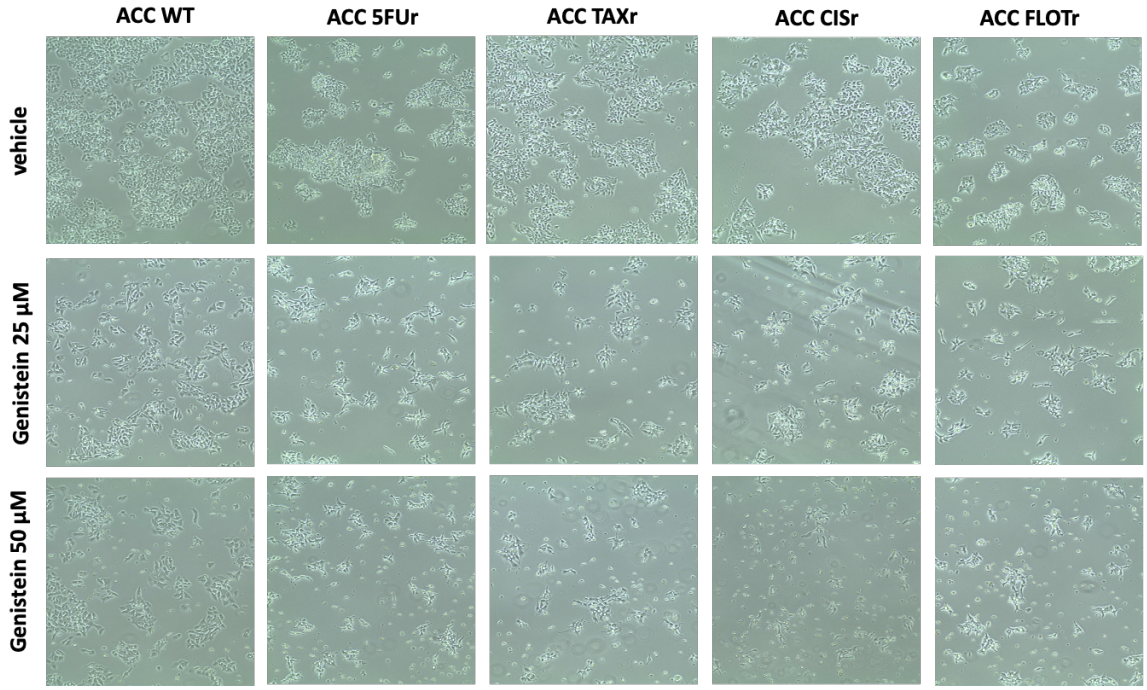


Fig 25: representative photos of WT, 5FUr, TAXr CISr and FLOTr ACC-201 cells after treatment with genistiein at 25 µM and 50 µM doses.

For a primary evaluation of Genistein toxicity we treated ACC 201WT and drug-resistant cells with two different concentrations of Genistein: 25 μM and 50 μM for 48h.

A first microscope evaluation (figure 25) showed a reduction of cell number in all the resistant cell lines, particularly in CISr, TAXr and FLOTr cells at the 25 μM dose whereas both WT and 5FUr ACC 201 cells seemed to be less sensitive compared to the other resistant cells. At the dose 50 μM the reduction of viability was more evident in the cell plate for the CISr ACC-201; TAXr and FLOTr ACC-201were similar while 5FUr and WT cells showed signs of distress.

We proceeded by MTT assay to test cell viability after treatment. A preliminary assessment of the toxicity of genistein on healthy, non-tumor cells was performed; we treated WI-38 cells (a Fethal fibroblast cell line. On these cells, no marked toxicity

of the compound was observed at 25 μM dose while we found a decrease of cell viability at 50 μM.

The viability assay conducted on GC cells, WT and chemoesistant, revealed a sensitivity of all the cell lines to the treatment. Particularly, at the 25 μM dose of genistein every cell showed a derease in viability of about 20%, with the highest value observed in TAXr ACC (about 35%); with the 50 μM treatment we observed a more marked decrease of viability and even in this case the TAXr ACC demonstrated a higher sensibility with a viability reduction of about 45%.

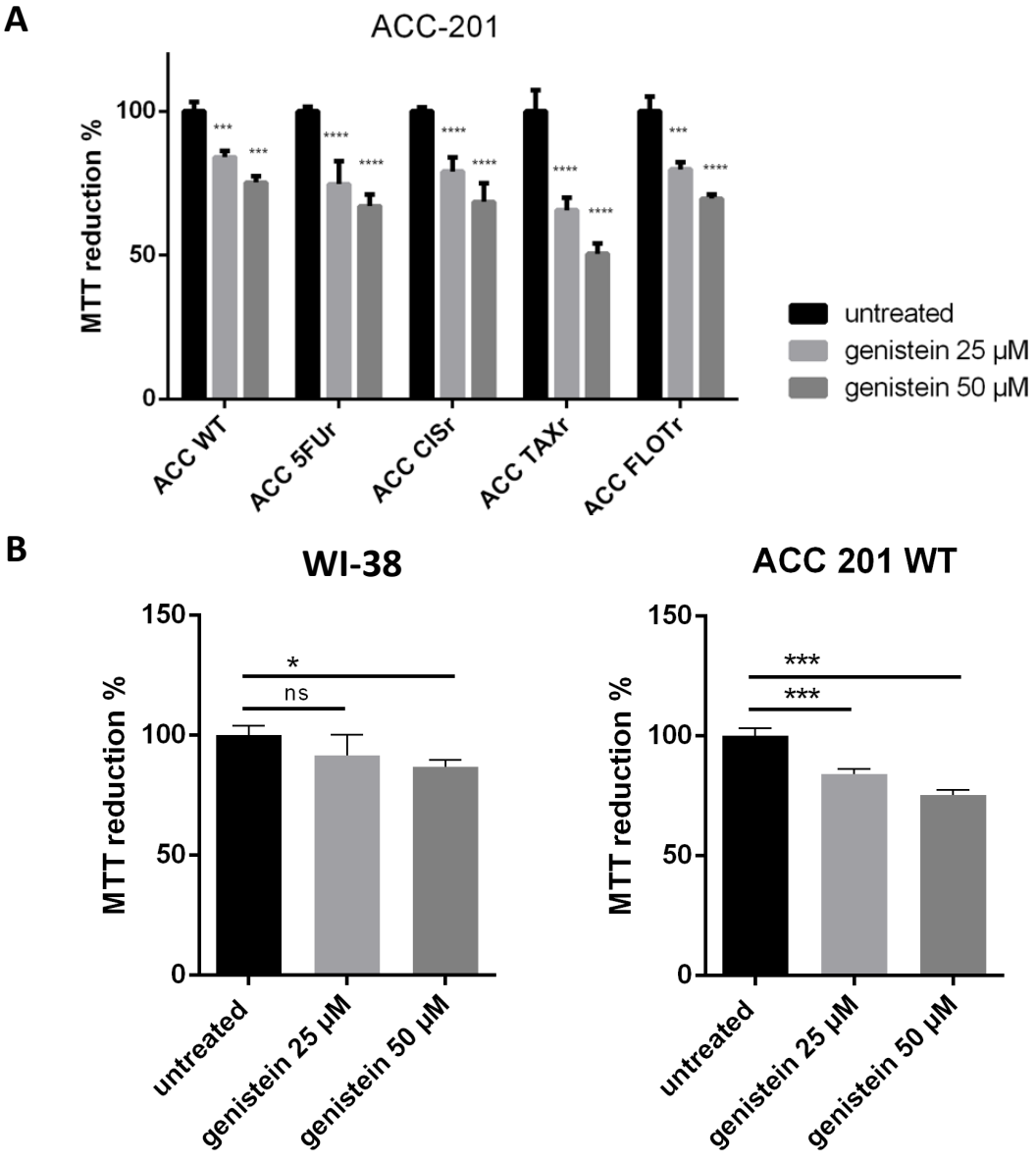


Fig 26: evaluation of genistein effect on GC cell line (ACC 201) and fetal fibroblast (WI-38); results of viability assay of WT, 5FUr, CISr, TAXr and FLOTr ACC 201 cells after treatment with 25 µM and 50 µM of Genistein for 48h (A); results of MTT asay performed of WI-38 fetal fibroblast compared to ACC 201 WT treated with 25 µM and 50 µM for 48h (B) (Statistic: One-way ANOVA).

4.4.2 Genistein induces overexpression of p53 in WT, 5FUr, CISr, TAXr and FLOTr ACC-201 cells.

Once we observed the effect of genistein on WT and chemo-resistant ACC 201 cells we wanted to explore the mechanism by which the polyphenol induced decrease of proliferation.

Due to its correlation with the inhibition of cell cycle, we naturally evaluated the expression of p53, using the same approach used for the evaluation of OCF activity (as described in relative section).

The evaluation of p53 was performed by flow cytometry.

The experiments revealed an important increase of p53 expression level in all the resistant cell lines, as we expected by the MTT assay and by microscope observation. The highest value was found in TAXr ACC cells that showed an expression of p53 of about 60% while 5FUr, CISr and FLOTr ACC are at similar values (around 50%). Regarding WT cells, in this case, the percentage of p53 expression is of about 40%. All this value in treated samples were compared with a DMSO-treated control (genistein is resuspended in DMSO) and in every cell line was determined a statistical significance by one-way ANOVA test (figure 27).

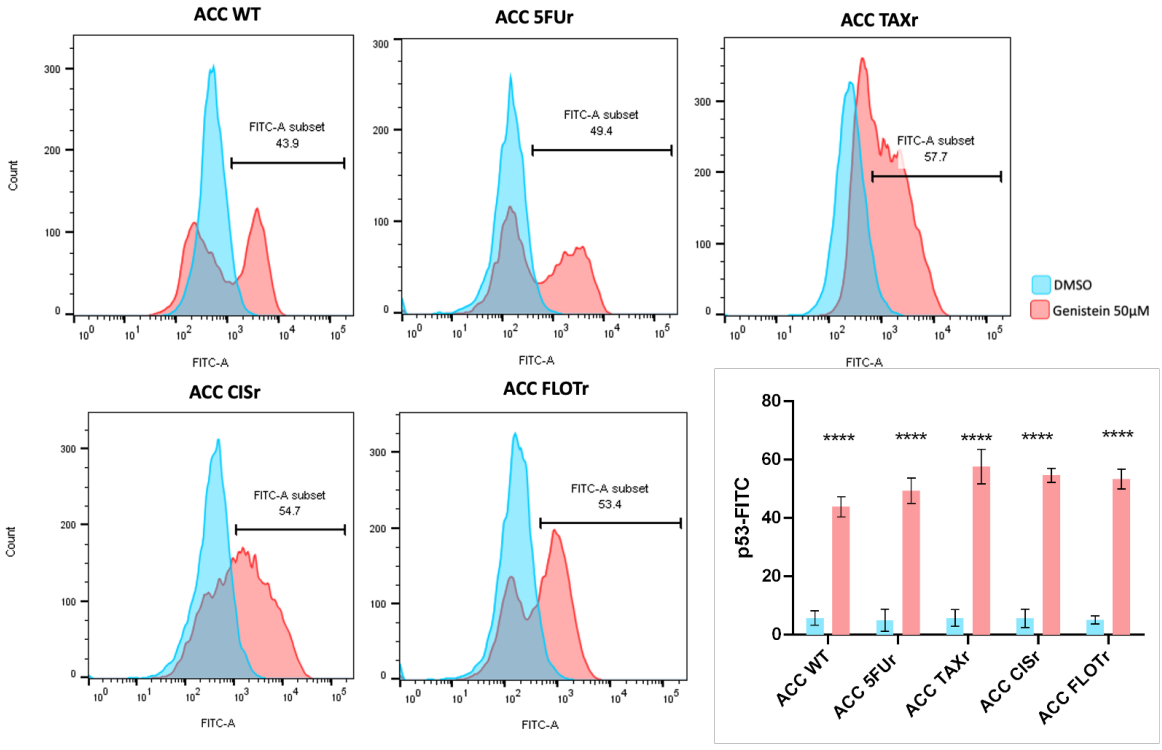


Figure 27: Effect of genistein treatment in GC Cells; flow cytometry plots and graph show the differential expression of p53 after the administration of genistein 50 µM for 48h; the DMSO was administered as control (Statistic: One-way ANOVA).

High expression of p53 is not only related to the cell cycle arrest but is one of the first step of apoptosis: in presence of irreversible damage p53 leads the process of activation of genes involved in programmed cell death.

So, in order to understand if genistein, through p53 overexpression, induced just an inhibition of proliferation or cell death, we performed a double staining with Annexin V/PI. This assay provided an indication of the status of cell death in our cell lines.

As can be observed by the graphs in figure the "live" population in every treated sample is sensibly reduced; in particular, the percentage of viable cells in TAX ACC the half in samples treated with genistein 50 μM with respect to the untreated control; in addition in TAX cells we observed the highest value in late apoptotic cells whereas in 5FUr, CISr and FLOTr, the values of non-live cells are similar. Even ACC WT showed an increment in mortality (about 40%). In every cell line the untreated samples have similar values of vitality. The percentage of necrotic cells in every sample, after treatment with genistein is higher than in the untread ones but is similar for all cell lines, except for the 5FUr cells.

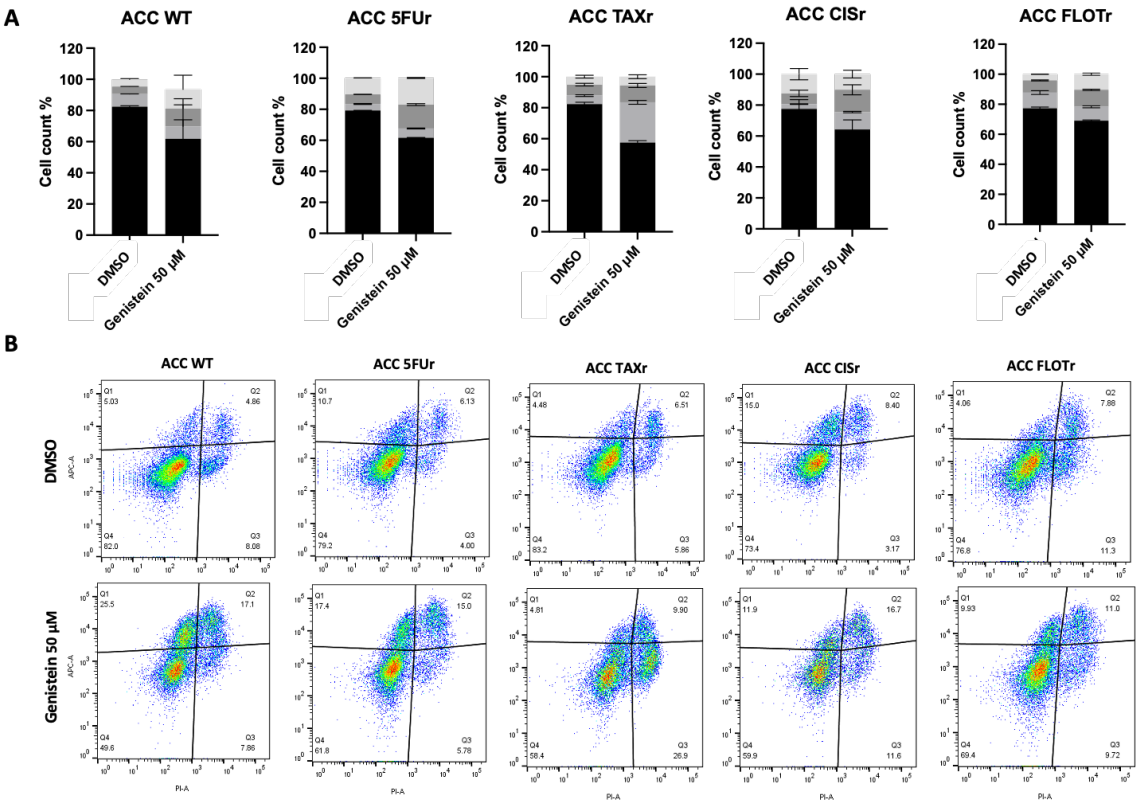


Figure 28: Evaluation of Apoptosis and cell viability after treatment with 50 µM genistein for 48h. Representative plot obtained by flow cytometry (after Annexin V/PI double staining of WT, 5FUr, CISr, TAXr and FLOTr ACC 201 cells; administered as control (A). Graphs reportin cell count divided in "Live", "Early apoptosis", "Late apoptosis" and "Necrosis" (B).

4.4.3 Pro-Oxidant Activity of genistein on GC Cells

Genistein, like as many other polyphenols is a natural antioxidant: in healthy cells it can reduce the damage induced by the high production of ROS. Even OCF (as previously described) was firstly studied for its natural antioxidant activity. But many studies in literature showed that sometimes, polyphenols can induce the opposite effect. In fact, in many tumor models, many of these compounds has been described for their ability to induce an overproduction of ROS *(Navaneetha Krishnan S et al., 2019; León-González AJ et al., 2015; Bi Y et al., 2016; Zhang Q et al., 2015)*.
About Genistein, its main toxicity mechanism in cancer was described in section 1 of this work. In accordance with several publications demonstrating a pro-oxidant effect of genistein in colorectal and small cells lung cancer *(Alorda-Clara M et al., 2022; Chan L et al., 2022)* we wanted to evaluate the effect of genistein on ROS production in our GC model.
We verified the pro-oxidative effect of genistein treatment (at 50 μM): this dose was able to significantly increase ROS production in WT, CISr and FLOTr but not in 5FUr and TAXr ACC cells. This data confirmed the high sensibility of CISr ACC to treatment (shown by Annexin V/PI and p53 evaluation) but was apparently discording with previous data concerning TAXr. Also, in WT we observed a ROS production increase, lower compared to CISr and FLOTr but significant.

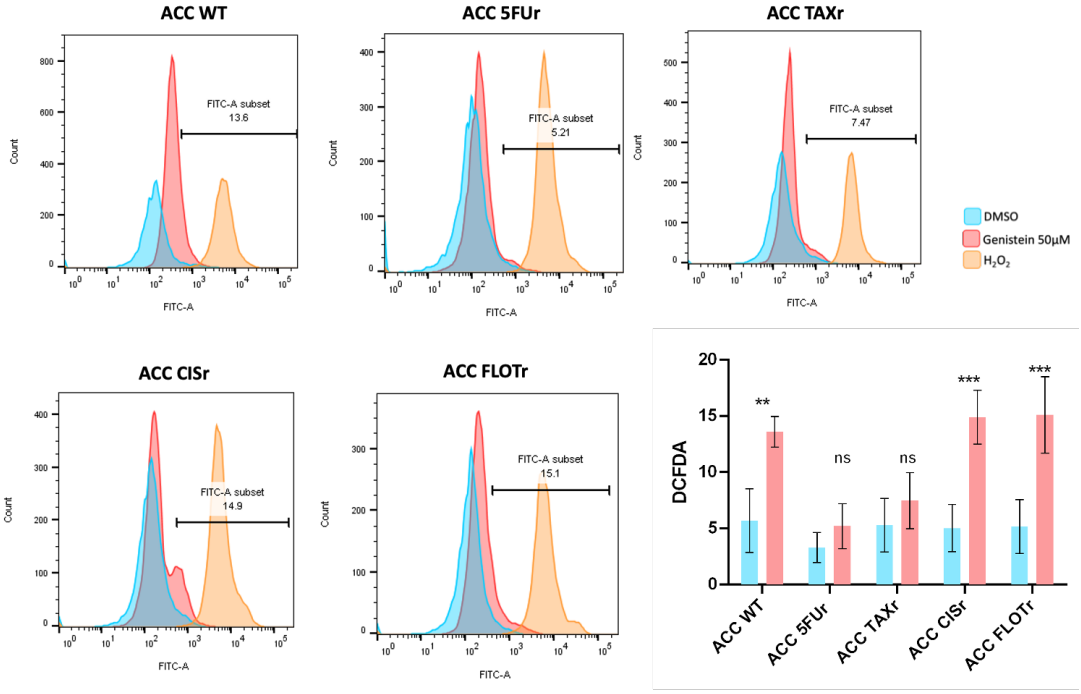


Figure 29: ROS production induced by OCF treatment; flow-cytometry plot and graphs of ROS level (detected by DCFDA) in WT, 5FUr, CISr, TAXr and FLOTr AGS cells after 48h treatment with 50 µM genistein) (Statistic: One-wat ANOVA).

4.4.4 Evaluation of combined therapy with genistein plus 5-Fluorouracil, Cisplatin, Paclitaxel and FLOT regiment on WT and resistant ACC cells

In order to confirm the efficacy of genistein and to evaluate its efficacy as treatment on chemo-resistant cells we assessed the effect of a combined therapy made by administration of genistein in combination with evert chemotherapeutic treatment.

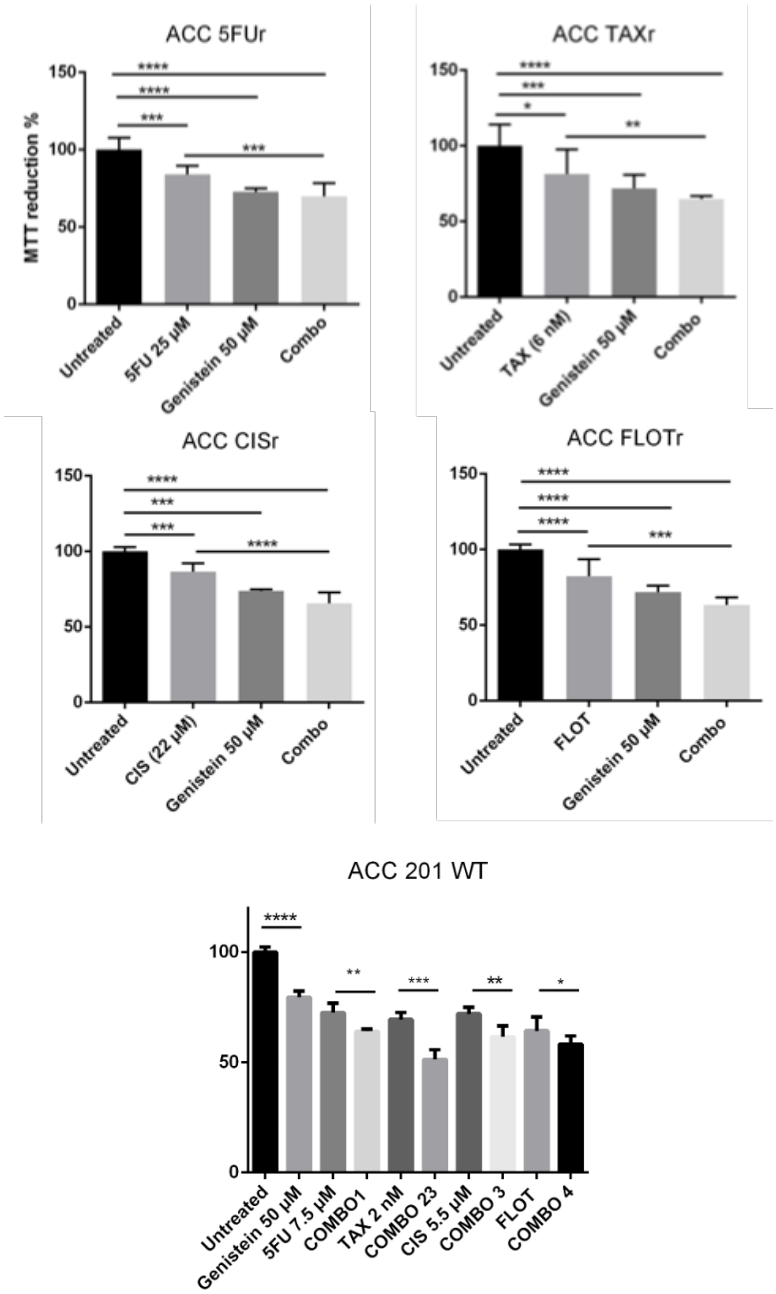


Figure 30: effects of the combined administration of chemotherapeutic drugs with genistein after 48h of treatment. ACC cells were treated with vehicle and 1 concentration of genistein (50 µM) for 48h. ACC wild type were treated with their IC50 of 5FU, CIS and TAX, alone or in combination with genistein. 5FUr, CISr, TAXr and FLOTr ACC cells were treated with genistein alone and in combination with their IC50 dose (Statistic: Two-way ANOVA).

In ACC cells the administration of 50 μM genistein enhanced the effect of the 4 chemotherapeutic treatments, reducing their number after 48 h. Particularly, there is a reduction of approximately 40% of the cell population when genistein was administered with 5-fluorouracil, paclitaxel and cisplatin. Regarding the FLOT treatment, combined therapy induced a lower decrease in cell viability but still significant.
The drug-resistant ACC-201 cells showed a decrease in viability after treatment with their respective drug in combination with genistein 50 μM. The observed viability reduction was about 45% (Fig.30).

When we introduced the topic of chemoresistance in malignant tumor we defined a relation between the acquisition of a non-responsive phenotype to therapy and the presence of stem-like cells, so called CSCs or TICs. This population with clonogenic characteristics has been described in literature as a driving factor for cancer growth and diffusion and, in the worst scenario for recrudescence.
So, identifying stem-like cells is a current goal for scientific research, in order to establish new approach for targeting this population further understand its function.
In this work we tried to identify a stem-like population in our GC model (particularly the ACC-201 cells). There are many methods to identify CSC, the most used is the identification of surface marker. But some tissues, and stomach is one of this, demonstrates high heterogeneity so we decided for a functional approach.
In ACC-201 WT and chemo resistant we evaluated the activity of Aldehyde Dehydrogenase through AldeRed assay and the capacity to regenerate a proliferative tumor core, using the ELDA method.

4.5 Evaluation of stem-like properties of WT and drug-resistant cell lines – AldeRed Assay and limited-dilution assay

The evaluation of ALDH was performed in flow cytometry using the AldeRed method: this probe is processed in cytoplasm by ALDH and become fluorescent emitting light detectable in the PE channel by flow cytometry.

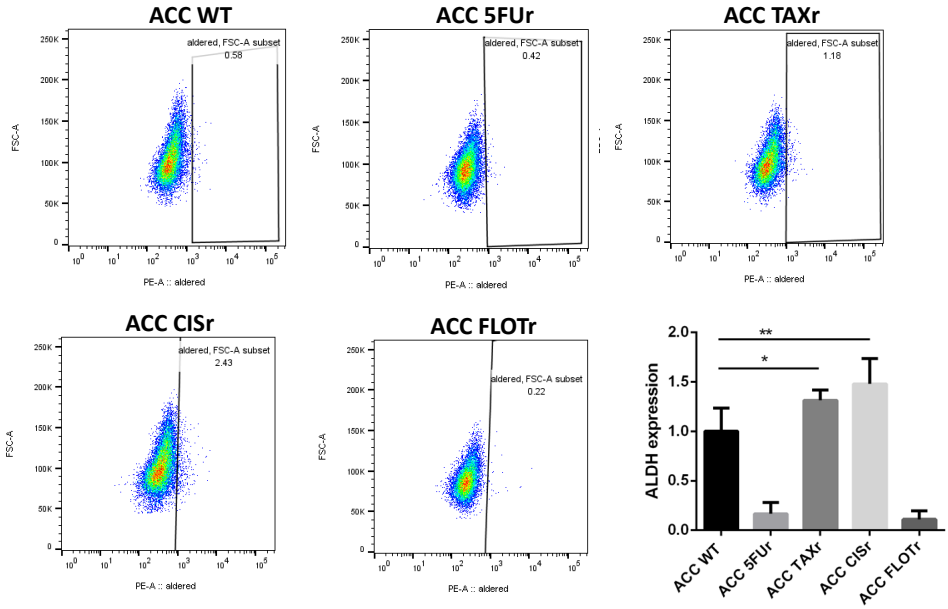
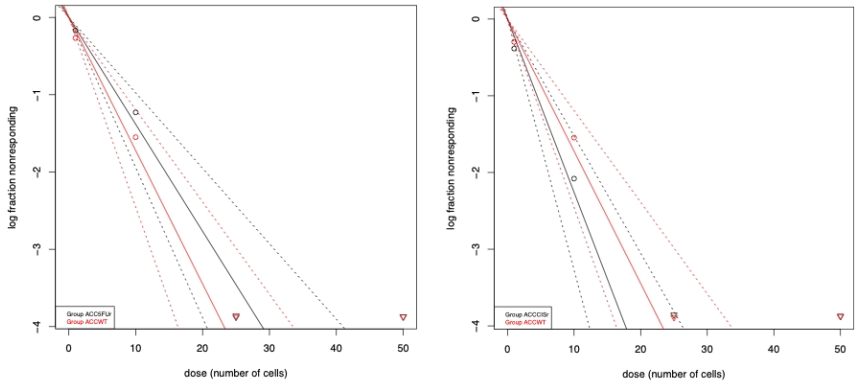


Figure 31: representative flow cytometry plot and graph representation of ALDH activity (detected through AldeRed assay) in WT, 5FUr, CISr and TAXr ACC-201 cells (Statistic: One-way ANOVA).

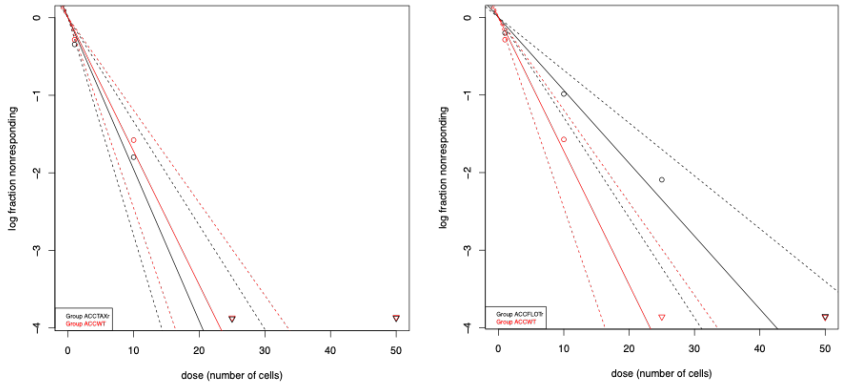
The results of the experiments showed low values of AldeRed signal in all the analyzed cells. This evidence suggested that in every cell line the ALDH activity of was detectable but not very high. The only differences observed regarded the WT, CISr and TAXr ACC-201 cells. Indeed, in all these cells the activity of ALDH was higher compared to 5FUr and FLOTr. As shown in chart in figure the value of AldeRed signal was double in CISr, and greater of almost 50% in TAXr with respect to WT.

This little differences between WT and two of the resistant cells needed a further investigation. In order to confirm a potential stem-like activity of the chemo-resistant ACC-201 cells we performed an Extreme Limited dilution assay in order to evaluate the minimum number of cells able to origin a new tumor cluster. Cells were seeded in a conditioned medium, as described in Materials and methods, and after two weeks we evaluate the presence of colonies.



	ACC WT	ACC 5FUr
TIC (%)	17,24%	13,83%
95% C.I.	1/8,38-1/1,08	1/10,25-1/5.15

	ACC WT	ACC CISr
TIC (%)	17,24%	22,57%
95% C.I.	1/8,38-1/1,08	1/6,55-1/3,06



	ACC WT	ACC TAXr
TIC (%)	17,24%	19,57%
95% C.I.	1/8,38-1/1,08	1/7,46-1/3,56

	ACC WT	ACC FLOTr
TIC (%)	17,24%	9,43%
95% C.I.	1/8,38-1/1,08	1/14,69-1/9,43

Figure 32: Limiting dilution assay of WT, 5FUr, CISr, TAXr and FLOTr ACC-201 cells; table reporting the TIC (tumor initiating cell); graph output of ELDA Software for the evaluation of TIC (Statistic: Two-way ANOVA).

After the count of positive wells, the data were processed by ELDA software according to instructions. The tables in figure report the calculated TIC (Tumor Initiating Cells) and it's the output of the ELDA software analysis. The graphs above are a graphic representation of the tendency of the cells to regenerate in extreme diluted conditions. As we can see, the highest percentage values in TIC match with the lines more near to zero in the graph. The meaning is that cells with highest TIC values are able to regenerate a tumor mass better than others, in our model, CISr and TAXr reported the highest value (22,57% and 19,57% respectively); WT ACC cells shows a value of 17,24%, lower than CISr and TAXr but higher than the other two drug-resistant cell lines.

A deeper evaluation of the stem cell potential and regenerative capacity of chemo-resistant ACC-201 cells is currently the subject of our studies.

5. Discussion

Gastric Cancer (GC) represents one of the main causes of cancer-related death (fifth cancer in terms of number of deaths in a year). Its lethality and incidence make it an interesting object of study for scientific research.

Among the most peculiar aspects of this pathology there is certainly the phenomenon of chemoresistance, a mechanism through which tumor cells acquire the ability to survive the chemotherapeutic agents used in conventional therapy. This condition in GC occurs periodically in patients undergoing therapy: after an initial reduction in tumor mass, in some cases, a resumption of growth is observed. The acquisition of chemoresistance is one of the first steps towards a more aggressive tumor phenotype capable of disseminating in the organism.

The acquisition of these characteristics is due to many factors including modifications at the cellular genome level, microenvironmental modifications and the presence of cells capable of providing a proliferative reserve to the mass.

In this research work, we investigated these aspects that occur in many solid tumors, in a chemo-resistant GC model.

We used two lines of primary gastric adenocarcinoma taken from Caucasian patients, AGS and ACC-201 cells. The selection of chemo-resistant populations from these primary lines was carried out through the chronic administration of the main chemotherapeutic agents, in particular: 5-Fluorouracil (selecting the 5FUr cells), Cisplatin (obtaining the CISr cells) paclitaxel (obtaining the TAXr cells) and the FLOT treatment (obtaining FLOTr) which currently represents the gold standard for the treatment of HER2-negative gastric cancer *(Al Batran SE er al., 2019).*

Once we obtained the chemo-resistant cells we moved on to the evaluation of their characteristics. The evaluation of the metabolism of AGS and ACC-201 cells highlighted differences between the resistant populations and the wild type. From the evaluation of the glycolytic rate, performed through the Seahorse Analyzer, a boosted glycolytic activity emerged in chemo-resistant cells accompanied by an increased lactate production. This metabolic reprogramming in drug-resistant cells was confirmed by GC-MS analysis: the quantification of the metabolites of the 3C acid cycle showed a lower amount of alpha-ketoglutarate, citrate, and malate in AGS and ACC-201, therefore demonstrating a decrease in their mitochondrial activity with respect to WT ones.

The metabolic peculiarities of the tumor cells are closely related to the microenvironment in which the cells themselves are located. This relationship is also mediated by the activity of the enzymes that modify the microenvironment inside and outside the cell. Among these, Carbonic Anhydrase 9 (CAIX) has emerged in recent years as one of the main players in tumor progression due to its ability to modify the tumor microenvironment and influence the outcome of the disease *(Liao SY et al., 2009; Chen J et al., 2009; Robertson N et al; 2004).* In this work, we evaluated the expression of CAIX in our wild-type and chemo-resistant cell lines and, in parallel, we carried out a retrospective study on patients subjected to FLOT/FOLFOX chemotherapy. From this study it has emerged that patients classified as non-responders (TRG=3/5) were characterized by a greater expression of CAIX compared to responder patients (with TRG=2), thus highlighting a correlation between the lack of response to therapy and the increase in enzyme expression.

The validation of these observations was also carried out in parallel with our *in vitro* model. Notably, all our chemo-resistant cell lines that showed a consistent glycolytic phenotype, showed an increase in CAIX expression compared to the corresponding wild-type ones.

We then investigated the correlation between CAIX and chemoresistance in our *in vitro* model using a CAIX inhibitor, that is currently employed in clinical study, known as SLC-0111 *(Supuran CT et al., 2015)* to treat our chemo-resistant GC cells. From our experiments, it has been observed that the administration of SLC-0111 is able to impair the proliferation and the viability of both wild-type and chemo-resistant cells by inducing the activation of caspases 3 and 7 and by inhibiting the ability of the cells to form colonies after the treatment. Again, the correlation between chemoresistance and CAIX expression was confirmed thanks to silencing experiments that showed similar results, which resulted in a significant increase in the cellular response to therapy following CAIX silencing compared to control cells. Moreover, the results of our experiments showed not only the relation occurring between CAIX and chemoresistance but also highlighted the possibility of re-sensitizing chemo-resistant cells to therapy thanks to the combination with other effective drugs. Taking this into account, from this point of view, even the search for such molecules in nature can be fruitful. Many natural compounds are studied for their activity in chronic diseases including cancer. Among these, polyphenols are certainly the most investigated.

In this work, we evaluated the effect of Oleocanthal and Genistein, two different polyphenols, on our *in vitro* model of GC, in conditions of chemoresistance and not. Specifically, after a first evaluation of the toxicity of these two compounds, we demonstrated that both could exert an anti-proliferative and a pro-apoptotic effect on WT and drug-resistant AGS and ACC-201 cells. The data obtained by the evaluation of p53 expression revealed that the administration of oleocanthal and genistein induced its overexpression; due to the correlation between p53, cell cycle arrest and induction of apoptosis we evaluated the possible induction of cell death, and we discovered an increased of apoptotic and necrotic cells after treatment with the two compounds. Moreover, both polyphenols exhibited an efficient reduction of cell viability when administered in combination with the canonical chemotherapeutic drugs. This result is particularly interesting for the chemoresistance cells, in which we observed a significant decrease in viability, thus indicating a possible restoration of drug response or suggesting a considerable sensitivity to the polyphenols' treatment.

Regarding oleocanthal, the only cell line that seemed to be less sensitive to the treatment was the CISr AGS cells. In particular, the experiments conducted showed that this cell line better tolerated the treatment with oleocanthal, hence not observing any overexpression of p53, p21 or pRb. In addition, the evaluation of ROS stress performed by DCFDA assay showed an increase in ROS production after treatment with oleocanthal in every AGS cell line except for CISr. Indeed, after the evaluation of detoxification enzymes expression by qPCR we observed that these enzymes were significantly more expressed in CISr AGS than in the other cell lines.

Regarding genistein, we followed a similar approach: the evaluation of p53 showed overexpression of the protein in ACC-201 cell lines after treatment with genistein. Particularly, CISr and TAXr ACC-201 seemed more sensitive to genistein. Therefore, we proceeded to the assessment of ROS production with the same approach used for oleocanthal. In this case, we observed an increased production of ROS in CISr but not in the TAXr. Speculating on this result we can hypothesize that genistein may manifest its toxic effect on TAXr cells (confirmed by viability assays and p53 quantification) in a different way from the induction of oxidative stress. Anyway, both oleocanthal and genistein showed efficacy in combined treatment on GC cells and, more interestingly, in drug-resistant cells. A further study on the activity of these natural compounds is now the subject of our research activity.

As we previously discussed, chemoresistance is correlated in many solid tumors with the presence of a stem-like population called Cancer Stem Cells (CSCs). The presence of this subpopulation was demonstrated in many studies and correlated with the acquisition of characteristics that can lead to a more aggressive phenotype. The stem-like profile is also related to typical characteristics of the tumor microenvironment previously discussed such as hypoxia. It is well known that this condition is linked to several metabolic modifications within the tumor mass, characterized by the presence of high glycolytic cells. As previously disclosed, we demonstrated the presence of a significantly higher expression of CAIX – one of the main players of this metabolic and microenvironmental switching – in our chemo-resistant GC models rather than in WT ones. We were questioning if a correlation between what we observed in our cell lines and the presence of CSCs could occur. Therefore, we tried to identify a stem-like population in ACC-201 cell lines, that showed an increased expression of CAIX.

We evaluated the activity of Aldehyde Dehydrogenase (ALDH), which is considered a stem population marker, and we observed that CISr and TAXr ACC-201 showed higher activity of these enzymes compared to the other drug-resistant and WT ones. Moreover, the ELDA assay was performed to evaluate the percentage of tumor-initiating cells (TIC) in every ACC-201 cell line; the obtained results demonstrated that chemo-resistant CISr and TAXr ACC-201 cells (that also showed high expression of CAIX) had a higher ability to regenerate a tumor mass than the WT ones. This evidence could establish a relationship between the acquisition of chemoresistance in GC cells and the presence of a population with a stem cell phenotype capable of providing a proliferative reserve to the tumor mass and responsible for the progression of the disease.

Surprisingly, drug-resistant CISr and TAXr ACC-201 cells proved to be the most sensitive to genistein treatment in our experiments. This data could also be in concordance with previous studies that showed the efficacy of genistein in inhibiting a key pathway of stem-signaling, the Hedgehog path, in breast and prostate cancer *(Fan P et al., 2013; Zhang L et al., 2012).* Genistein could exert its activity with more efficiency on stem-like cells (which we identified within CISr and TAXr ACC-201 cell lines).

In conclusion, this work exhibits the identification of a chemoresistance marker in an *in vitro* model of gastric cancer, which was also validated and highlighted in GC patients' biopsies. Furthermore, we have established a close relationship between microenvironmental and metabolic modifications and the acquisition of

chemoresistance in GC cell lines. We also demonstrated the effectiveness of three compounds in re-sensitizing chemo-resistant cells to conventional therapy and highlighted the presence of a stem-like population in a GC cell line. A deeper characterization of this population (currently ongoing) could possibly lead to a further step forward in the development of targeted therapies in the treatment of chemo-resistant gastric adenocarcinoma.

6. Bibliography

Ajani JA, Lee J, Sano T, Janjigian YY, Fan D, Song S. Gastric adenocarcinoma. Nat Rev Dis Primers. 2017 Jun 1;3:17036. doi: 10.1038/nrdp.2017.36. PMID: 28569272.

Al-Batran SE, Homann N, Pauligk C, Goetze TO, Meiler J, Kasper S, Kopp HG, Mayer F, Haag GM, Luley K, Lindig U, Schmiegel W, Pohl M, Stoehlmacher J, Folprecht G, Probst S, Prasnikar N, Fischbach W, Mahlberg R, Trojan J, Koenigsmann M, Martens UM, Thuss-Patience P, Egger M, Block A, Heinemann V, Illerhaus G, Moehler M, Schenk M, Kullmann F, Behringer DM, Heike M, Pink D, Teschendorf C, Löhr C, Bernhard H, Schuch G, Rethwisch V, von Weikersthal LF, Hartmann JT, Kneba M, Daum S, Schulmann K, Weniger J, Belle S, Gaiser T, Oduncu FS, Güntner M, Hozaeel W, Reichart A, Jäger E, Kraus T, Mönig S, Bechstein WO, Schuler M, Schmalenberg H, Hofheinz RD; FLOT4-AIO Investigators. 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 randomised, phase 2/3 trial. Lancet. 2019 May 11;393(10184):1948-1957. doi: 10.1016/S0140-6736(18)32557-1. Epub 2019 Apr 11. PMID: 30982686.

Alipour M. Molecular Mechanism of Helicobacter pylori-Induced Gastric Cancer. J Gastrointest Cancer. 2021 Mar;52(1):23-30. doi: 10.1007/s12029-020-00518-5. Epub 2020 Sep 14. PMID: 32926335; PMCID: PMC7487264.

Alorda-Clara M, Torrens-Mas M, Morla-Barcelo PM, Roca P, Sastre-Serra J, Pons DG, Oliver J. High Concentrations of Genistein Decrease Cell Viability Depending on Oxidative Stress and Inflammation in Colon Cancer Cell Lines. Int J Mol Sci. 2022 Jul 7;23(14):7526. doi: 10.3390/ijms23147526. PMID: 35886874; PMCID: PMC9323408.

Alvarado-Ortiz E, de la Cruz-López KG, Becerril-Rico J, Sarabia-Sánchez MA, Ortiz-Sánchez E, García-Carrancá A. Mutant p53 Gain-of-Function: Role in Cancer Development, Progression, and Therapeutic Approaches. Front Cell Dev Biol. 2021 Feb 11;8:607670. doi: 10.3389/fcell.2020.607670. PMID: 33644030; PMCID: PMC7905058.

Andreucci E, Peppicelli S, Carta F, Brisotto G, Biscontin E, Ruzzolini J, Bianchini F, Biagioni A, Supuran
CT, Calorini L. Carbonic anhydrase IX inhibition affects viability of cancer cells adapted to extracellular acidosis. J Mol Med (Berl). 2017 Dec;95(12):1341-1353. doi: 10.1007/s00109-017-1590-9. Epub 2017 Sep 19. PMID: 28929255.

Andreucci E, Ruzzolini J, Bianchini F, Versienti G, Biagioni A, Lulli M, Guasti D, Nardini P, Serratì S, Margheri F, Laurenzana A, Nediani C, Peppicelli S, Calorini L. miR-214-Enriched Extracellular Vesicles Released by Acid-Adapted Melanoma Cells Promote Inflammatory Macrophage-Dependent Tumor Trans-Endothelial Migration. Cancers (Basel). 2022 Oct 18;14(20):5090. doi: 10.3390/cancers14205090. PMID: 36291876; PMCID: PMC9599952.

Andreucci E, Ruzzolini J, Peppicelli S, Bianchini F, Laurenzana A, Carta F, Supuran CT, Calorini L. The carbonic anhydrase IX inhibitor SLC-0111 sensitises cancer cells to conventional chemotherapy. J Enzyme Inhib Med Chem. 2019 Dec;34(1):117-123. doi: 10.1080/14756366.2018.1532419. PMID: 30362384; PMCID: PMC6211231.

Bądziul D, Jakubowicz-Gil J, Paduch R, Głowniak K, Gawron A. Combined treatment with quercetin and imperatorin as a potent strategy for killing HeLa and Hep-2 cells. Mol Cell Biochem. 2014 Jul;392(1-2):213-27. doi: 10.1007/s11010-014-2032-4. Epub 2014 Mar 30. PMID: 24682729; PMCID: PMC4148393.

Bagheri N, Azadegan-Dehkordi F, Rafieian-Kopaei M, Rahimian G, Asadi-Samani M, Shirzad H. Clinical relevance of Helicobacter pylori virulence factors in Iranian patients with gastrointestinal diseases. Microb Pathog. 2016 Nov;100:154-162. doi: 10.1016/j.micpath.2016.09.016. Epub 2016 Sep 22. PMID: 27666510.

Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Rüschoff J, Kang YK; ToGA Trial Investigators. 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. 2010 Aug 28;376(9742):687-97. doi: 10.1016/S0140-6736(10)61121-X. Epub 2010 Aug 19. Erratum in: Lancet. 2010 Oct 16;376(9749):1302. PMID: 20728210.

Beauchamp GK, Keast RS, Morel D, Lin J, Pika J, Han Q, Lee CH, Smith AB, Breslin PA. Phytochemistry: ibuprofen-like activity in extra-virgin olive oil. Nature. 2005 Sep 1;437(7055):45-6. doi: 10.1038/437045a. PMID: 16136122.

Bi Y, Shen C, Li C, Liu Y, Gao D, Shi C, Peng F, Liu Z, Zhao B, Zheng Z, Wang X, Hou X, Liu H, Wu J, Zou H, Wang K, Zhong C, Zhang J, Shi C, Zhao S. Inhibition of autophagy induced by quercetin at a late stage enhances cytotoxic effects on glioma cells. Tumour Biol. 2016 Mar;37(3):3549-60. doi: 10.1007/s13277-015-4125-4. Epub 2015 Oct 10. PMID: 26454746.

Blair VR, McLeod M, Carneiro F, Coit DG, D'Addario JL, van Dieren JM, Harris KL, Hoogerbrugge N, Oliveira C, van der Post RS, Arnold J, Benusiglio PR, Bisseling TM, Boussioutas A, Cats A, Charlton A, Schreiber KEC, Davis JL, Pietro MD, Fitzgerald RC, Ford JM, Gamet K, Gullo I, Hardwick RH, Huntsman DG, Kaurah P, Kupfer SS, Latchford A, Mansfield PF, Nakajima T, Parry S, Rossaak J, Sugimura H, Svrcek M, Tischkowitz M, Ushijima T, Yamada H, Yang HK, Claydon A, Figueiredo J, Paringatai K, Seruca R, Bougen-Zhukov N, Brew T, Busija S, Carneiro P, DeGregorio L, Fisher H, Gardner E, Godwin TD, Holm KN, Humar B, Lintott CJ, Monroe EC, Muller MD, Norero E, Nouri Y, Paredes J, Sanches JM, Schulpen E, Ribeiro AS, Sporle A, Whitworth J, Zhang L, Reeve AE, Guilford P. Hereditary diffuse gastric cancer: updated clinical practice guidelines. Lancet Oncol. 2020 Aug;21(8):e386-e397. doi: 10.1016/S1470-2045(20)30219-9. PMID: 32758476; PMCID: PMC7116190.

Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997 Jul;3(7):730-7. doi: 10.1038/nm0797-730. PMID: 9212098.

Bouvard V, Loomis D, Guyton KZ, Grosse Y, Ghissassi FE, Benbrahim-Tallaa L, Guha N, Mattock H, Straif K; International Agency for Research on Cancer Monograph Working Group. Carcinogenicity of consumption of red and processed meat. Lancet Oncol. 2015 Dec;16(16):1599-600. doi: 10.1016/S1470-2045(15)00444-1. Epub 2015 Oct 29. PMID: 26514947.

Boysen T, Mohammadi M, Melbye M, Hamilton-Dutoit S, Vainer B, Hansen AV, Wohlfahrt J, Friborg J. EBV-associated gastric carcinoma in high- and low-incidence areas for nasopharyngeal carcinoma. Br J Cancer. 2009 Aug 4;101(3):530-3. doi: 10.1038/sj.bjc.6605168. Epub 2009 Jul 14. PMID: 19603026; PMCID: PMC2720225.

Brahimi-Horn MC, Chiche J, Pouysségur J. Hypoxia and cancer. J Mol Med (Berl). 2007 Dec;85(12):1301-7. doi: 10.1007/s00109-007-0281-3. Epub 2007 Nov 20. PMID: 18026916.

Bravo D, Hoare A, Soto C, Valenzuela MA, Quest AF. *Helicobacter pylori* in human health and disease: Mechanisms for local gastric and systemic effects. World J Gastroenterol. 2018 Jul 28;24(28):3071-3089. doi: 10.3748/wjg.v24.i28.3071. PMID: 30065554; PMCID: PMC6064966.

Cancer Genome Atlas Research Network. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014 Sep 11;513(7517):202-9. doi: 10.1038/nature13480. Epub 2014 Jul 23. PMID: 25079317; PMCID: PMC4170219.

Capasso C, Supuran CT. An Overview of the Carbonic Anhydrases from Two Pathogens of the Oral Cavity: Streptococcus mutans and Porphyromonas gingivalis. Curr Top Med Chem. 2016;16(21):2359-68. doi: 10.2174/1568026616666160413135522. PMID: 27072694.

Carneiro F, Seixas M, Sobrinho-Simões M. New elements for an updated classification of the carcinomas of the stomach. Pathol Res Pract. 1995 Jul;191(6):571-84. doi: 10.1016/S0344-0338(11)80878-2. PMID: 7479380.

Chan L, Pang Y, Wang Y, Zhu D, Taledaohan A, Jia Y, Zhao L, Wang W. Genistein-induced mitochondrial dysfunction and FOXO3a/PUMA expression in non-small lung cancer cells. Pharm Biol. 2022 Dec;60(1):1876-1883. doi: 10.1080/13880209.2022.2123933. PMID: 36200643; PMCID: PMC9553144.

Chen J, Röcken C, Hoffmann J, Krüger S, Lendeckel U, Rocco A, Pastorekova S, Malfertheiner P, Ebert MP. Expression of carbonic anhydrase 9 at the invasion front of gastric cancers. Gut. 2005 Jul;54(7):920-7. doi: 10.1136/gut.2004.047340. PMID: 15951534; PMCID: PMC1774603.

Cho J, Kang MS, Kim KM. Epstein-Barr Virus-Associated Gastric Carcinoma and Specific Features of the Accompanying Immune Response. J Gastric Cancer. 2016 Mar;16(1):1-7. doi: 10.5230/jgc.2016.16.1.1. Epub 2016 Mar 31. PMID: 27104020; PMCID: PMC4834615.

Choudhari AS, Mandave PC, Deshpande M, Ranjekar P, Prakash O. Phytochemicals in Cancer Treatment: From Preclinical Studies to Clinical Practice. Front Pharmacol. 2020 Jan 28;10:1614. doi: 10.3389/fphar.2019.01614. Erratum in: Front Pharmacol. 2020 Feb 28;11:175. PMID: 32116665; PMCID: PMC7025531.

Clark DW, Palle K. Aldehyde dehydrogenases in cancer stem cells: potential as therapeutic targets. Ann Transl Med. 2016 Dec;4(24):518. doi: 10.21037/atm.2016.11.82. PMID: 28149880; PMCID: PMC5233526.

Correa P, Piazuelo MB, Camargo MC. Etiopathogenesis of gastric cancer. Scand J Surg. 2006;95(4):218-24. doi: 10.1177/145749690609500402. PMID: 17249268.

Cristescu R, Lee J, Nebozhyn M, Kim KM, Ting JC, Wong SS, Liu J, Yue YG, Wang J, Yu K, Ye XS, Do IG, Liu S, Gong L, Fu J, Jin JG, Choi MG, Sohn TS, Lee JH, Bae JM, Kim ST, Park SH, Sohn I, Jung SH, Tan P, Chen R, Hardwick J, Kang WK, Ayers M, Hongyue D, Reinhard C, Loboda A, Kim S, Aggarwal A. Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes. Nat Med. 2015 May;21(5):449-56. doi: 10.1038/nm.3850. Epub 2015 Apr 20. PMID: 25894828.

Daigo S, Takahashi Y, Fujieda M, Ariyoshi N, Yamazaki H, Koizumi W, Tanabe S, Saigenji K, Nagayama S, Ikeda K, Nishioka Y, Kamataki T. A novel mutant allele of the CYP2A6 gene (CYP2A6*11) found in a cancer patient who showed poor metabolic phenotype towards tegafur. Pharmacogenetics. 2002 Jun;12(4):299-306. doi: 10.1097/00008571-200206000-00005. PMID: 12042667.

Daker M, Ahmad M, Khoo AS. Quercetin-induced inhibition and synergistic activity with cisplatin - a chemotherapeutic strategy for nasopharyngeal carcinoma cells. Cancer Cell Int. 2012 Jul 18;12(1):34. doi: 10.1186/1475-2867-12-34. PMID: 22809533; PMCID: PMC3460759.

Das A, Banik NL, Ray SK. Flavonoids activated caspases for apoptosis in human glioblastoma T98G and U87MG cells but not in human normal astrocytes. Cancer. 2010 Jan 1;116(1):164-76. doi: 10.1002/cncr.24699. PMID: 19894226; PMCID: PMC3159962.

de Oliveira J, Felipe AV, Neto RA, Oshima CT, de Souza Silva M, Forones NM. Association between ABCB1 immunohistochemical expression and overall survival in gastric cancer patients. Asian Pac J Cancer Prev. 2014;15(16):6935-8. doi: 10.7314/apjcp.2014.15.16.6935. PMID: 25169549.

Díaz P, Valenzuela Valderrama M, Bravo J, Quest AFG. *Helicobacter pylori* and Gastric Cancer: Adaptive Cellular Mechanisms Involved in Disease Progression. Front Microbiol. 2018 Jan 22;9:5. doi: 10.3389/fmicb.2018.00005. PMID: 29403459; PMCID: PMC5786524.

Doe MR, Ascano JM, Kaur M, Cole MD. Myc posttranscriptionally induces HIF1 protein and target gene expression in normal and cancer cells. Cancer Res. 2012 Feb 15;72(4):949-57. doi: 10.1158/0008-5472.CAN-11-2371. Epub 2011 Dec 20. PMID: 22186139; PMCID: PMC3288382.

Fan P, Fan S, Wang H, Mao J, Shi Y, Ibrahim MM, Ma W, Yu X, Hou Z, Wang B, Li L. Genistein decreases the breast cancer stem-like cell population through Hedgehog pathway. Stem Cell Res Ther. 2013;4(6):146. doi: 10.1186/scrt357. PMID: 24331293; PMCID: PMC4054948.

Ferlay J, Colombet M, Soerjomataram I, Parkin DM, Piñeros M, Znaor A, Bray F. Cancer statistics for the year 2020: An overview. Int J Cancer. 2021 Apr 5. doi: 10.1002/ijc.33588. Epub ahead of print. PMID: 33818764.

Folkman J, Hahnfeldt P, Hlatky L. Cancer: looking outside the genome. Nat Rev Mol Cell Biol. 2000 Oct;1(1):76-9. doi: 10.1038/35036100. PMID: 11413493.

Gillies RJ, Gatenby RA. Metabolism and its sequelae in cancer evolution and therapy. Cancer J. 2015 Mar-Apr;21(2):88-96. doi: 10.1097/PPO.0000000000000102. PMID: 25815848; PMCID: PMC4446699.

Goren L, Zhang G, Kaushik S, Breslin PAS, Du YN, Foster DA. (-)-Oleocanthal and (-)-oleocanthal-rich olive oils induce lysosomal membrane permeabilization in cancer cells. PLoS One. 2019 Aug 14;14(8):e0216024. doi: 10.1371/journal.pone.0216024. PMID: 31412041; PMCID: PMC6693737.

Goseki N, Takizawa T, Koike M. Differences in the mode of the extension of gastric cancer classified by histological type: new histological classification of gastric carcinoma. Gut. 1992 May;33(5):606-12. doi: 10.1136/gut.33.5.606. PMID: 1377153; PMCID: PMC1379287.

Gullo I, Carneiro F, Oliveira C, Almeida GM. Heterogeneity in Gastric Cancer: From Pure Morphology to Molecular Classifications. Pathobiology. 2018;85(1-2):50-63. doi: 10.1159/000473881. Epub 2017 Jun 16. PMID: 28618420.

Haraguchi N, Utsunomiya T, Inoue H, Tanaka F, Mimori K, Barnard GF, Mori M. Characterization of a side population of cancer cells from human gastrointestinal system. Stem Cells. 2006 Mar;24(3):506-13. doi: 10.1634/stemcells.2005-0282. Epub 2005 Oct 20. PMID: 16239320.

Howe JR, Sayed MG, Ahmed AF, Ringold J, Larsen-Haidle J, Merg A, Mitros FA, Vaccaro CA, Petersen GM, Giardiello FM, Tinley ST, Aaltonen LA, Lynch HT. The prevalence of MADH4 and BMPR1A mutations in juvenile polyposis and absence of BMPR2, BMPR1B, and ACVR1 mutations. J Med Genet. 2004 Jul;41(7):484-91. doi: 10.1136/jmg.2004.018598. PMID: 15235019; PMCID: PMC1735829.

Hu Y, Smyth GK. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J Immunol Methods. 2009 Aug 15;347(1-2):70-8. doi: 10.1016/j.jim.2009.06.008. Epub 2009 Jun 28. PMID: 19567251.

Hung JY, Hsu YL, Li CT, Ko YC, Ni WC, Huang MS, Kuo PL. 6-Shogaol, an active constituent of dietary ginger, induces autophagy by inhibiting the AKT/mTOR pathway in human non-small cell lung cancer A549 cells. J Agric Food Chem. 2009 Oct 28;57(20):9809-16. doi: 10.1021/jf902315e. PMID: 19799425.

Hwang YW, Kim SY, Jee SH, Kim YN, Nam CM. Soy food consumption and risk of prostate cancer: a meta-analysis of observational studies. Nutr Cancer. 2009;61(5):598-606. doi: 10.1080/01635580902825639. PMID: 19838933.

Iacono A, Gómez R, Sperry J, Conde J, Bianco G, Meli R, Gómez-Reino JJ, Smith AB 3rd, Gualillo O. Effect of oleocanthal and its derivatives on inflammatory response induced by lipopolysaccharide in a murine chondrocyte cell line. Arthritis Rheum. 2010 Jun;62(6):1675-82. doi: 10.1002/art.27437. PMID: 20201078.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Biological agents. IARC Monogr Eval Carcinog Risks Hum. 2012;100(Pt B):1-441. PMID: 23189750; PMCID: PMC4781184.

Ignatova TN, Kukekov VG, Laywell ED, Suslov ON, Vrionis FD, Steindler DA. Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. Glia. 2002 Sep;39(3):193-206. doi: 10.1002/glia.10094. PMID: 12203386.

Japanese Gastric Cancer Association. Japanese classification of gastric carcinoma: 3rd English edition. Gastric Cancer. 2011 Jun;14(2):101-12. doi: 10.1007/s10120-011-0041-5. PMID: 21573743.

Khanfar MA, Bardaweel SK, Akl MR, El Sayed KA. Olive Oil-derived Oleocanthal as Potent Inhibitor of Mammalian Target of Rapamycin: Biological Evaluation and Molecular Modeling Studies. Phytother Res. 2015 Nov;29(11):1776-82. doi: 10.1002/ptr.5434. Epub 2015 Aug 7. PMID: 26248874; PMCID: PMC5051273.

Kim SH, Kim SH, Kim YB, Jeon YT, Lee SC, Song YS. Genistein inhibits cell growth by modulating various mitogen-activated protein kinases and AKT in cervical cancer cells. Ann N Y Acad Sci. 2009 Aug;1171:495-500. doi: 10.1111/j.1749-6632.2009.04899.x. PMID: 19723095.

Kondo T, Setoguchi T, Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. Proc Natl Acad Sci U S A. 2004 Jan 20;101(3):781-6. doi: 10.1073/pnas.0307618100. Epub 2004 Jan 7. PMID: 14711994; PMCID: PMC321758.

Ladeiras-Lopes R, Pereira AK, Nogueira A, Pinheiro-Torres T, Pinto I, Santos-Pereira R, Lunet N. Smoking and gastric cancer: systematic review and meta-analysis of cohort studies. Cancer Causes Control. 2008 Sep;19(7):689-701. doi: 10.1007/s10552-008-9132-y. Epub 2008 Feb 22. PMID: 18293090.

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. PMID: 14320675.

Law BY, Mok SW, Wu AG, Lam CW, Yu MX, Wong VK. New Potential Pharmacological Functions of Chinese Herbal Medicines via Regulation of Autophagy. Molecules. 2016 Mar 17;21(3):359. doi: 10.3390/molecules21030359. PMID: 26999089; PMCID: PMC6274228.

Lazăr DC, Tăban S, Cornianu M, Faur A, Goldiş A. New advances in targeted gastric cancer treatment. World J Gastroenterol. 2016 Aug 14;22(30):6776-99. doi: 10.3748/wjg.v22.i30.6776. PMID: 27570417; PMCID: PMC4974579.

Lee YT, Tan YJ, Oon CE. Molecular targeted therapy: Treating cancer with specificity. Eur J Pharmacol. 2018 Sep 5;834:188-196. doi: 10.1016/j.ejphar.2018.07.034. Epub 2018 Jul 20. PMID: 30031797.

LeGendre O, Breslin PA, Foster DA. (-)-Oleocanthal rapidly and selectively induces cancer cell death via lysosomal membrane permeabilization. Mol Cell Oncol. 2015;2(4):e1006077. doi: 10.1080/23723556.2015.1006077. PMID: 26380379; PMCID: PMC4568762.

Lei Z, Tan IB, Das K, Deng N, Zouridis H, Pattison S, Chua C, Feng Z, Guan YK, Ooi CH, Ivanova T, Zhang S, Lee M, Wu J, Ngo A, Manesh S, Tan E, Teh BT, So JB, Goh LK, Boussioutas A, Lim TK, Flotow H, Tan P, Rozen SG. Identification of molecular subtypes of gastric cancer with different responses to PI3-kinase inhibitors and 5-fluorouracil. Gastroenterology. 2013 Sep;145(3):554-65. doi: 10.1053/j.gastro.2013.05.010. Epub 2013 May 14. PMID: 23684942.

Lei ZN, Teng QX, Tian Q, Chen W, Xie Y, Wu K, Zeng Q, Zeng L, Pan Y, Chen ZS, He Y. Signaling pathways and therapeutic interventions in gastric cancer. Signal Transduct Target Ther. 2022 Oct 8;7(1):358. doi: 10.1038/s41392-022-01190-w. PMID: 36209270; PMCID: PMC9547882.

León-González AJ, Auger C, Schini-Kerth VB. Pro-oxidant activity of polyphenols and its implication on cancer chemoprevention and chemotherapy. Biochem Pharmacol. 2015 Dec 1;98(3):371-80. doi: 10.1016/j.bcp.2015.07.017. Epub 2015 Jul 20. PMID: 26206193.

Li HQ, Luo Y, Qiao CH. The mechanisms of anticancer agents by genistein and synthetic derivatives of isoflavone. Mini Rev Med Chem. 2012 Apr;12(4):350-62. doi: 10.2174/138955712799829258. PMID: 22303948.

Li Y, Yang Y, Lu M, Shen L. Predictive value of serum CEA, CA19-9 and CA72.4 in early diagnosis of recurrence after radical resection of gastric cancer. Hepatogastroenterology. 2011 Nov-Dec;58(112):2166-70. doi: 10.5754/hge11753. PMID: 22024091.

Li YQ, Yin JY, Liu ZQ, Li XP. Copper efflux transporters ATP7A and ATP7B: Novel biomarkers for platinum drug resistance and targets for therapy. IUBMB Life. 2018 Mar;70(3):183-191. doi: 10.1002/iub.1722. Epub 2018 Feb 2. PMID: 29394468.

Liao SY, Lerman MI, Stanbridge EJ. Expression of transmembrane carbonic anhydrases, CAIX and CAXII, in human development. BMC Dev Biol. 2009 Mar 16;9:22. doi: 10.1186/1471-213X-9-22. PMID: 19291313; PMCID: PMC2666674.

Lin XJ, Wang CP, Liu XD, Yan KK, Li S, Bao HH, Zhao LY, Liu X. Body mass index and risk of gastric cancer: a meta-analysis. Jpn J Clin Oncol. 2014 Sep;44(9):783-91. doi: 10.1093/jjco/hyu082. Epub 2014 Jun 20. PMID: 24951830.

Luo G, Zhang Y, Guo P, Wang L, Huang Y, Li K. Global patterns and trends in stomach cancer incidence: Age, period and birth cohort analysis. Int J Cancer. 2017 Oct 1;141(7):1333-1344. doi: 10.1002/ijc.30835. Epub 2017 Jun 27. PMID: 28614909.

Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr. 2004 May;79(5):727-47. doi: 10.1093/ajcn/79.5.727. PMID: 15113710.

Marchitti SA, Brocker C, Stagos D, Vasiliou V. Non-P450 aldehyde oxidizing enzymes: the aldehyde dehydrogenase superfamily. Expert Opin Drug Metab Toxicol. 2008 Jun;4(6):697-720. doi: 10.1517/17425255.4.6.697. PMID: 18611112; PMCID: PMC2658643.

Marin JJG, Perez-Silva L, Macias RIR, Asensio M, Peleteiro-Vigil A, Sanchez-Martin A, Cives-Losada C, Sanchon-Sanchez P, Sanchez De Blas B, Herraez E, Briz O, Lozano E. Molecular Bases of Mechanisms Accounting for Drug Resistance in Gastric Adenocarcinoma. Cancers (Basel). 2020 Jul 30;12(8):2116. doi: 10.3390/cancers12082116. PMID: 32751679; PMCID: PMC7463778.

Masciari S, Dewanwala A, Stoffel EM, Lauwers GY, Zheng H, Achatz MI, Riegert-Johnson D, Foretova L, Silva EM, Digianni L, Verselis SJ, Schneider K, Li FP, Fraumeni J, Garber JE, Syngal S. Gastric cancer in individuals with Li-Fraumeni syndrome. Genet Med. 2011 Jul;13(7):651-7. doi: 10.1097/GIM.0b013e31821628b6. PMID: 21552135; PMCID: PMC3595598.

Masuelli L, Benvenuto M, Di Stefano E, Mattera R, Fantini M, De Feudis G, De Smaele E, Tresoldi I, Giganti MG, Modesti A, Bei R. Curcumin blocks autophagy and activates apoptosis of malignant mesothelioma cell lines and increases the survival of mice intraperitoneally transplanted with a malignant mesothelioma cell line. Oncotarget. 2017 May 23;8(21):34405-34422. doi: 10.18632/oncotarget.14907. PMID: 28159921; PMCID: PMC5470978.

Matsunaga S, Nishiumi S, Tagawa R, Yoshida M. Alterations in metabolic pathways in gastric epithelial cells infected with Helicobacter pylori. Microb Pathog. 2018 Nov;124:122-129. doi: 10.1016/j.micpath.2018.08.033. Epub 2018 Aug 20. PMID: 30138760.

McDermott M, Eustace AJ, Busschots S, Breen L, Crown J, Clynes M, O'Donovan N, Stordal B. In vitro Development of Chemotherapy and Targeted Therapy Drug-Resistant Cancer Cell Lines: A Practical Guide with Case Studies. Front Oncol. 2014 Mar 6;4:40. doi: 10.3389/fonc.2014.00040. PMID: 24639951; PMCID: PMC3944788.

McDonald PC, Chia S, Bedard PL, Chu Q, Lyle M, Tang L, Singh M, Zhang Z, Supuran CT, Renouf DJ, Dedhar S. A Phase 1 Study of SLC-0111, a Novel Inhibitor of Carbonic Anhydrase IX, in Patients With Advanced Solid Tumors. Am J Clin Oncol. 2020 Jul;43(7):484-490. doi: 10.1097/COC.0000000000000691. PMID: 32251122; PMCID: PMC7323835.

McKenna R, Supuran CT. Carbonic anhydrase inhibitors drug design. Subcell Biochem. 2014;75:291-323. doi: 10.1007/978-94-007-7359-2_15. PMID: 24146385.

Ming SC. Gastric carcinoma. A pathobiological classification. Cancer. 1977 Jun;39(6):2475-85. doi: 10.1002/1097-0142(197706)39:6<2475::aid-cncr2820390626>3.0.co;2-l. PMID: 872047.

Morgan E, Arnold M, Camargo MC, Gini A, Kunzmann AT, Matsuda T, Meheus F, Verhoeven RHA, Vignat J, Laversanne M, Ferlay J, Soerjomataram I. The current and future incidence and mortality of gastric cancer in 185 countries, 2020-40: A population-based modelling study. EClinicalMedicine. 2022 Apr 21;47:101404. doi: 10.1016/j.eclinm.2022.101404. PMID: 35497064; PMCID: PMC9046108.

Morito K, Hirose T, Kinjo J, Hirakawa T, Okawa M, Nohara T, Ogawa S, Inoue S, Muramatsu M, Masamune Y. Interaction of phytoestrogens with estrogen receptors alpha and beta. Biol Pharm Bull. 2001 Apr;24(4):351-6. doi: 10.1248/bpb.24.351. PMID: 11305594.

Murphy G, Pfeiffer R, Camargo MC, Rabkin CS. Meta-analysis shows that prevalence of Epstein-Barr virus-positive gastric cancer differs based on sex and anatomic location. Gastroenterology. 2009 Sep;137(3):824-33. doi: 10.1053/j.gastro.2009.05.001. Epub 2009 May 13. Erratum in: Gastroenterology. 2011 Mar;140(3):1109. PMID: 19445939; PMCID: PMC3513767.

Nakamura K, Nonaka S, Nakajima T, Yachida T, Abe S, Sakamoto T, Suzuki H, Yoshinaga S, Oda I, Matsuda T, Sekine S, Kanemitsu Y, Katai H, Saito Y, Hirota S. Clinical outcomes of gastric polyps and neoplasms in patients with familial adenomatous polyposis. Endosc Int Open. 2017 Mar;5(3):E137-E145. doi: 10.1055/s-0042-119809. PMID: 28271094; PMCID: PMC5339024.

Nakamura K, Sugano H, Takagi K. Carcinoma of the stomach in incipient phase: its histogenesis and histological appearances. Gan. 1968 Jun;59(3):251-8. PMID: 5726267.

Nakamura Y, Kawazoe A, Lordick F, Janjigian YY, Shitara K. Biomarker-targeted therapies for advanced-stage gastric and gastro-oesophageal junction cancers: an emerging paradigm. Nat Rev Clin Oncol. 2021 Aug;18(8):473-487. doi: 10.1038/s41571-021-00492-2. Epub 2021 Mar 31. PMID: 33790428

NavaneethaKrishnan S, Rosales JL, Lee KY. ROS-Mediated Cancer Cell Killing through Dietary Phytochemicals. Oxid Med Cell Longev. 2019 May 14;2019:9051542. doi: 10.1155/2019/9051542. PMID: 31217841; PMCID: PMC6536988.

Nessa MU, Beale P, Chan C, Yu JQ, Huq F. Combinations of resveratrol, cisplatin and oxaliplatin applied to human ovarian cancer cells. Anticancer Res. 2012 Jan;32(1):53-9. PMID: 22213288.

Nomura AM, Hankin JH, Kolonel LN, Wilkens LR, Goodman MT, Stemmermann GN. Case-control study of diet and other risk factors for gastric cancer in Hawaii (United States). Cancer Causes Control. 2003 Aug;14(6):547-58. doi: 10.1023/a:1024887411846. PMID: 12948286.

Parkkila S, Parkkila AK, Saarnio J, Kivelä J, Karttunen TJ, Kaunisto K, Waheed A, Sly WS, Türeci O, Virtanen I, Rajaniemi H. Expression of the membrane-associated carbonic anhydrase isozyme XII in the human kidney and renal tumors. J Histochem Cytochem. 2000 Dec;48(12):1601-8. doi: 10.1177/002215540004801203. PMID: 11101628.

Patra S, Pradhan B, Nayak R, Behera C, Das S, Patra SK, Efferth T, Jena M, Bhutia SK. Dietary polyphenols in chemoprevention and synergistic effect in cancer: Clinical evidences and molecular mechanisms of action. Phytomedicine. 2021 Sep;90:153554. doi: 10.1016/j.phymed.2021.153554. Epub 2021 Aug 6. PMID: 34371479.

Pei T, Meng Q, Han J, Sun H, Li L, Song R, Sun B, Pan S, Liang D, Liu L. (-)-Oleocanthal inhibits growth and metastasis by blocking activation of STAT3 in human hepatocellular carcinoma. Oncotarget. 2016 Jul 12;7(28):43475-43491. doi: 10.18632/oncotarget.9782. PMID: 27259268; PMCID: PMC5190038.

Pelucchi C, Bosetti C, Negri E, Lipworth L, La Vecchia C. Olive oil and cancer risk: an update of epidemiological findings through 2010. Curr Pharm Des. 2011;17(8):805-12. doi: 10.2174/138161211795428920. PMID: 21443483.

Peppicelli S, Andreucci E, Ruzzolini J, Bianchini F, Nediani C, Supuran CT, Calorini L. The Carbonic Anhydrase IX inhibitor SLC-0111 as emerging agent against the mesenchymal stem cell-derived pro-survival effects on melanoma cells. J Enzyme Inhib Med Chem. 2020 Dec;35(1):1185-1193. doi: 10.1080/14756366.2020.1764549. PMID: 32396749; PMCID: PMC7269050.

Peri S, Biagioni A, Versienti G, Andreucci E, Staderini F, Barbato G, Giovannelli L, Coratti F, Schiavone N, Cianchi F, Papucci L, Magnelli L. Enhanced Vasculogenic Capacity Induced by 5-Fluorouracil Chemoresistance in a Gastric Cancer Cell Line. Int J Mol Sci. 2021 Jul 19;22(14):7698. doi: 10.3390/ijms22147698. PMID: 34299320; PMCID: PMC8303918.

Peri S, Ruzzolini J, Urciuoli S, Versienti G, Biagioni A, Andreucci E, Peppicelli S, Bianchini F, Bottari A, Calorini L, Nediani C, Magnelli L, Papucci L. An Oleocanthal-Enriched EVO Oil Extract Induces the ROS Production in Gastric Cancer Cells and Potentiates the Effect of Chemotherapy. Antioxidants (Basel). 2022 Sep 7;11(9):1762. doi: 10.3390/antiox11091762. PMID: 36139836; PMCID: PMC9495378.

Peyrot des Gachons C, Uchida K, Bryant B, Shima A, Sperry JB, Dankulich-Nagrudny L, Tominaga M, Smith AB 3rd, Beauchamp GK, Breslin PA. Unusual pungency from extra-virgin olive oil is attributable to restricted spatial expression of the receptor of oleocanthal. J Neurosci. 2011 Jan 19;31(3):999-1009. doi: 10.1523/JNEUROSCI.1374-10.2011. PMID: 21248124; PMCID: PMC3073417.

Pinard MA, Mahon B, McKenna R. Probing the surface of human carbonic anhydrase for clues towards the design of isoform specific inhibitors. Biomed Res Int. 2015;2015:453543. doi: 10.1155/2015/453543. Epub 2015 Feb 24. PMID: 25811028; PMCID: PMC4355338.

Raghunand N, He X, van Sluis R, Mahoney B, Baggett B, Taylor CW, Paine-Murrieta G, Roe D, Bhujwalla ZM, Gillies RJ. Enhancement of chemotherapy by manipulation of tumour pH. Br J Cancer. 1999 Jun;80(7):1005-11. doi: 10.1038/sj.bjc.6690455. PMID: 10362108; PMCID: PMC2363059.

Ramos MFKP, Ribeiro Júnior U, Viscondi JKY, Zilberstein B, Cecconello I, Eluf-Neto J. Risk factors associated with the development of gastric cancer - case-control study. Rev Assoc

Med Bras (1992). 2018 Jul;64(7):611-619. doi: 10.1590/1806-9282.64.07.611. PMID: 30365663.

Robertson N, Potter C, Harris AL. Role of carbonic anhydrase IX in human tumor cell growth, survival, and invasion. Cancer Res. 2004 Sep 1;64(17):6160-5. doi: 10.1158/0008-5472.CAN-03-2224. PMID: 15342400.

Romani A, Ieri F, Urciuoli S, Noce A, Marrone G, Nediani C, Bernini R. Health Effects of Phenolic Compounds Found in Extra-Virgin Olive Oil, By-Products, and Leaf of *Olea europaea* L. Nutrients. 2019 Aug 1;11(8):1776. doi: 10.3390/nu11081776. PMID: 31374907; PMCID: PMC6724211.

Ruiz-Canela M, Martínez-González MA. Olive oil in the primary prevention of cardiovascular disease. Maturitas. 2011 Mar;68(3):245-50. doi: 10.1016/j.maturitas.2010.12.002. Epub 2011 Jan 8. PMID: 21216542.

Russi S, Verma HK, Laurino S, Mazzone P, Storto G, Nardelli A, Zoppoli P, Calice G, La Rocca F, Sgambato A, Lucci V, Falco G, Ruggieri V. Adapting and Surviving: Intra and Extra-Cellular Remodeling in Drug-Resistant Gastric Cancer Cells. Int J Mol Sci. 2019 Jul 31;20(15):3736. doi: 10.3390/ijms20153736. PMID: 31370155; PMCID: PMC6695752.

Sauer S, Plauth A. Health-beneficial nutraceuticals-myth or reality? Appl Microbiol Biotechnol. 2017 Feb;101(3):951-961. doi: 10.1007/s00253-016-8068-5. Epub 2017 Jan 9. PMID: 28070666.

Scarmeas N, Luchsinger JA, Schupf N, Brickman AM, Cosentino S, Tang MX, Stern Y. Physical activity, diet, and risk of Alzheimer disease. JAMA. 2009 Aug 12;302(6):627-37. doi: 10.1001/jama.2009.1144. PMID: 19671904; PMCID: PMC2765045.

Seigel GM, Campbell LM, Narayan M, Gonzalez-Fernandez F. Cancer stem cell characteristics in retinoblastoma. Mol Vis. 2005 Sep 12;11:729-37. PMID: 16179903.

Siddique AB, Ayoub NM, Tajmim A, Meyer SA, Hill RA, El Sayed KA. (-)-Oleocanthal Prevents Breast Cancer Locoregional Recurrence After Primary Tumor Surgical Excision and Neoadjuvant Targeted Therapy in Orthotopic Nude Mouse Models. Cancers (Basel). 2019 May 8;11(5):637. doi: 10.3390/cancers11050637. PMID: 31072015; PMCID: PMC6562541.

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. Epub 2017 Mar 21. PMID: 28421114; PMCID: PMC5379099.

Sitarz R, Skierucha M, Mielko J, Offerhaus GJA, Maciejewski R, Polkowski WP. Gastric cancer: epidemiology, prevention, classification, and treatment. Cancer Manag Res. 2018 Feb 7;10:239-248. doi: 10.2147/CMAR.S149619. PMID: 29445300; PMCID: PMC5808709.

Solcia E, Klersy C, Mastracci L, Alberizzi P, Candusso ME, Diegoli M, Tava F, Riboni R, Manca R, Luinetti O. A combined histologic and molecular approach identifies three groups of gastric cancer with different prognosis. Virchows Arch. 2009 Sep;455(3):197-211. doi: 10.1007/s00428-009-0813-z. Epub 2009 Aug 12. PMID: 19672623.

Song H, Ekheden IG, Zheng Z, Ericsson J, Nyrén O, Ye W. Incidence of gastric cancer among patients with gastric precancerous lesions: observational cohort study in a low risk Western population. BMJ. 2015 Jul 27;351:h3867. doi: 10.1136/bmj.h3867. Erratum in: BMJ. 2015;351:h4134. PMID: 26215280; PMCID: PMC4516137.

Spagnuolo C, Russo GL, Orhan IE, Habtemariam S, Daglia M, Sureda A, Nabavi SF, Devi KP, Loizzo MR, Tundis R, Nabavi SM. Genistein and cancer: current status, challenges, and future directions. Adv Nutr. 2015 Jul 15;6(4):408-19. doi: 10.3945/an.114.008052. PMID: 26178025; PMCID: PMC4496735.

Srinivas US, Tan BWQ, Vellayappan BA, Jeyasekharan AD. ROS and the DNA damage response in cancer. Redox Biol. 2019 Jul;25:101084. doi: 10.1016/j.redox.2018.101084. Epub 2018 Dec 21. PMID: 30612957; PMCID: PMC6859528.

Stubbs M, McSheehy PM, Griffiths JR, Bashford CL. Causes and consequences of tumour acidity and implications for treatment. Mol Med Today. 2000 Jan;6(1):15-9. doi: 10.1016/s1357-4310(99)01615-9. PMID: 10637570.

Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021 May;71(3):209-249. doi: 10.3322/caac.21660. Epub 2021 Feb 4. PMID: 33538338.

Supuran CT, Winum JY. Designing carbonic anhydrase inhibitors for the treatment of breast cancer. Expert Opin Drug Discov. 2015 Jun;10(6):591-7. doi: 10.1517/17460441.2015.1038235. Epub 2015 Apr 20. PMID: 25891195.

Supuran CT. Carbonic anhydrases--an overview. Curr Pharm Des. 2008;14(7):603-14. doi: 10.2174/138161208783877884. PMID: 18336305.

Supuran CT. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. Nat Rev Drug Discov. 2008 Feb;7(2):168-81. doi: 10.1038/nrd2467. PMID: 18167490.

Surfus JE, Hank JA, Oosterwijk E, Welt S, Lindstrom MJ, Albertini MR, Schiller JH, Sondel PM. Anti-renal-cell carcinoma chimeric antibody G250 facilitates antibody-dependent cellular cytotoxicity with in vitro and in vivo interleukin-2-activated effectors. J Immunother Emphasis Tumor Immunol. 1996 May;19(3):184-91. doi: 10.1097/00002371-199605000-00003. PMID: 8811493.

Surget S, Khoury MP, Bourdon JC. Uncovering the role of p53 splice variants in human malignancy: a clinical perspective. Onco Targets Ther. 2013 Dec 19;7:57-68. doi: 10.2147/OTT.S53876. PMID: 24379683; PMCID: PMC3872270.

Swietach P. What is pH regulation, and why do cancer cells need it? Cancer Metastasis Rev. 2019 Jun;38(1-2):5-15. doi: 10.1007/s10555-018-09778-x. PMID: 30707328; PMCID: PMC6626545.

Syngal S, Brand RE, Church JM, Giardiello FM, Hampel HL, Burt RW; American College of Gastroenterology. ACG clinical guideline: Genetic testing and management of hereditary gastrointestinal cancer syndromes. Am J Gastroenterol. 2015 Feb;110(2):223-62; quiz 263. doi: 10.1038/ajg.2014.435. Epub 2015 Feb 3. PMID: 25645574; PMCID: PMC4695986.

Szotek PP, Pieretti-Vanmarcke R, Masiakos PT, Dinulescu DM, Connolly D, Foster R, Dombkowski D, Preffer F, Maclaughlin DT, Donahoe PK. Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian Inhibiting Substance responsiveness. Proc Natl Acad Sci U S A. 2006 Jul 25;103(30):11154-9. doi: 10.1073/pnas.0603672103. Epub 2006 Jul 18. PMID: 16849428; PMCID: PMC1544057.

Tajmim A, Siddique AB, El Sayed K. Optimization of Taste-Masked (-)-Oleocanthal Effervescent Formulation with Potent Breast Cancer Progression and Recurrence Suppressive Activities. Pharmaceutics. 2019 Oct 5;11(10):515. doi: 10.3390/pharmaceutics11100515. PMID: 31590382; PMCID: PMC6835910.

Talero E, Ávila-Roman J, Motilva V. Chemoprevention with phytonutrients and microalgae products in chronic inflammation and colon cancer. Curr Pharm Des. 2012;18(26):3939-65. doi: 10.2174/138161212802083725. PMID: 22632755.

Tan P, Yeoh KG. Genetics and Molecular Pathogenesis of Gastric Adenocarcinoma. Gastroenterology. 2015 Oct;149(5):1153-1162.e3. doi: 10.1053/j.gastro.2015.05.059. Epub 2015 Jun 12. PMID: 26073375.

Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer. 2002 Jun;2(6):442-54. doi: 10.1038/nrc822. PMID: 12189386.

Thiry A, Dogné JM, Masereel B, Supuran CT. Targeting tumor-associated carbonic anhydrase IX in cancer therapy. Trends Pharmacol Sci. 2006 Nov;27(11):566-73. doi: 10.1016/j.tips.2006.09.002. Epub 2006 Sep 25. PMID: 16996620.

Tsugane S, Sasazuki S. Diet and the risk of gastric cancer: review of epidemiological evidence. Gastric Cancer. 2007;10(2):75-83. doi: 10.1007/s10120-007-0420-0. Epub 2007 Jun 25. PMID: 17577615.

Ullah MS, Davies AJ, Halestrap AP. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism. J Biol Chem. 2006 Apr 7;281(14):9030-7. doi: 10.1074/jbc.M511397200. Epub 2006 Feb 1. PMID: 16452478.

Van Cutsem E, Moiseyenko VM, Tjulandin S, Majlis A, Constenla M, Boni C, Rodrigues A, Fodor M, Chao Y, Voznyi E, Risse ML, Ajani JA; V325 Study Group. 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. 2006 Nov 1;24(31):4991-7. doi: 10.1200/JCO.2006.06.8429. PMID: 17075117.

van Lier MG, Wagner A, Mathus-Vliegen EM, Kuipers EJ, Steyerberg EW, van Leerdam ME. High cancer risk in Peutz-Jeghers syndrome: a systematic review and surveillance

recommendations. Am J Gastroenterol. 2010 Jun;105(6):1258-64; author reply 1265. doi: 10.1038/ajg.2009.725. Epub 2010 Jan 5. PMID: 20051941.

Venere M, Hamerlik P, Wu Q, Rasmussen RD, Song LA, Vasanji A, Tenley N, Flavahan WA, Hjelmeland AB, Bartek J, Rich JN. Therapeutic targeting of constitutive PARP activation compromises stem cell phenotype and survival of glioblastoma-initiating cells. Cell Death Differ. 2014 Feb;21(2):258-69. doi: 10.1038/cdd.2013.136. Epub 2013 Oct 11. PMID: 24121277; PMCID: PMC3890948.

Vidya Priyadarsini R, Senthil Murugan R, Maitreyi S, Ramalingam K, Karunagaran D, Nagini S. The flavonoid quercetin induces cell cycle arrest and mitochondria-mediated apoptosis in human cervical cancer (HeLa) cells through p53 induction and NF-κB inhibition. Eur J Pharmacol. 2010 Dec 15;649(1-3):84-91. doi: 10.1016/j.ejphar.2010.09.020. Epub 2010 Sep 19. PMID: 20858478.

Vogelaar IP, van der Post RS, van de Vosse E, van Krieken JH, Hoogerbrugge N, Ligtenberg MJ, Gómez García E. Gastric cancer in three relatives of a patient with a biallelic IL12RB1 mutation. Fam Cancer. 2015 Mar;14(1):89-94. doi: 10.1007/s10689-014-9764-x. PMID: 25467645.

Vollmers HP, Stulle K, Dämmrich J, Pfaff M, Papadopoulos T, Betz C, Saal K, Müller-Hermelink HK. Characterization of four new gastric cancer cell lines. Virchows Arch B Cell Pathol Incl Mol Pathol. 1993;63(6):335-43. doi: 10.1007/BF02899281. PMID: 8100658.

Wallner J, Depisch D, Gsur A, Götzl M, Haider K, Pirker R. MDR1 gene expression and its clinical relevance in primary gastric carcinomas. Cancer. 1993 Feb 1;71(3):667-71. doi: 10.1002/1097-0142(19930201)71:3<667::aid-cncr2820710303>3.0.co;2-6. PMID: 8431845.

Warren JR, Marshall B. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet. 1983 Jun 4;1(8336):1273-5. PMID: 6134060.

Weledji EP. The principles of the surgical management of gastric cancer. Int J Surg Oncol (N Y). 2017 Aug;2(7):e11. doi: 10.1097/IJ9.0000000000000011. Epub 2017 Jul 12. PMID: 29177225; PMCID: PMC5673153.

Wilkinson BL, Bornaghi LF, Houston TA, Innocenti A, Vullo D, Supuran CT, Poulsen SA. Inhibition of membrane-associated carbonic anhydrase isozymes IX, XII and XIV with a library of glycoconjugate benzenesulfonamides. Bioorg Med Chem Lett. 2007 Feb 15;17(4):987-92. doi: 10.1016/j.bmcl.2006.11.046. Epub 2006 Nov 17. PMID: 17157501.

Yang WJ, Zhao HP, Yu Y, Wang JH, Guo L, Liu JY, Pu J, Lv J. Updates on global epidemiology, risk and prognostic factors of gastric cancer. World J Gastroenterol. 2023 Apr 28;29(16):2452-2468. doi: 10.3748/wjg.v29.i16.2452. PMID: 37179585; PMCID: PMC10167900.

Yao Y, Tao H, Kim JJ, Burkhead B, Carloni E, Gasbarrini A, Sepulveda AR. Alterations of DNA mismatch repair proteins and microsatellite instability levels in gastric cancer cell lines. Lab Invest. 2004 Jul;84(7):915-22. doi: 10.1038/labinvest.3700117. PMID: 15133479.

Yashiro M, Inoue T, Nishioka N, Matsuoka T, Boland CR, Hirakawa K. Allelic imbalance at p53 and microsatellite instability are predictive markers for resistance to chemotherapy in gastric carcinoma. Ann Surg Oncol. 2009 Oct;16(10):2926-35. doi: 10.1245/s10434-009-0590-6. Epub 2009 Jul 14. PMID: 19597886; PMCID: PMC2865194.

Yasuda K, Torigoe T, Morita R, Kuroda T, Takahashi A, Matsuzaki J, Kochin V, Asanuma H, Hasegawa T, Saito T, Hirohashi Y, Sato N. Ovarian cancer stem cells are enriched in side population and aldehyde dehydrogenase bright overlapping population. PLoS One. 2013 Aug 13;8(8):e68187. doi: 10.1371/journal.pone.0068187. PMID: 23967051; PMCID: PMC3742724.

Yuan M, Eberhart CG, Kai M. RNA binding protein RBM14 promotes radio-resistance in glioblastoma by regulating DNA repair and cell differentiation. Oncotarget. 2014 May 15;5(9):2820-6. doi: 10.18632/oncotarget.1924. PMID: 24811242; PMCID: PMC4058047.

Závada J, Závadová Z, Pastoreková S, Ciampor F, Pastorek J, Zelník V. Expression of MaTu-MN protein in human tumor cultures and in clinical specimens. Int J Cancer. 1993 May 8;54(2):268-74. doi: 10.1002/ijc.2910540218. PMID: 8486430.

Zhang L, Li L, Jiao M, Wu D, Wu K, Li X, Zhu G, Yang L, Wang X, Hsieh JT, He D. Genistein inhibits the stemness properties of prostate cancer cells through targeting Hedgehog-Gli1 pathway. Cancer Lett. 2012 Oct 1;323(1):48-57. doi: 10.1016/j.canlet.2012.03.037. Epub 2012 Apr 3. PMID: 22484470.

Zhang Q, Cheng G, Qiu H, Zhu L, Ren Z, Zhao W, Zhang T, Liu L. The p53-inducible gene 3 involved in flavonoid-induced cytotoxicity through the reactive oxygen species-mediated mitochondrial apoptotic pathway in human hepatoma cells. Food Funct. 2015 May;6(5):1518-25. doi: 10.1039/c5fo00142k. PMID: 25820747.

Zhang Z, Xu G, Ma M, Yang J, Liu X. Dietary fiber intake reduces risk for gastric cancer: a meta-analysis. Gastroenterology. 2013 Jul;145(1):113-120.e3. doi: 10.1053/j.gastro.2013.04.001. Epub 2013 Apr 5. PMID: 23567349.

Zhao J, Liang Q, Cheung KF, Kang W, Dong Y, Lung RW, Tong JH, To KF, Sung JJ, Yu J. Somatostatin receptor 1, a novel EBV-associated CpG hypermethylated gene, contributes to the pathogenesis of EBV-associated gastric cancer. Br J Cancer. 2013 Jun 25;108(12):2557-64. doi: 10.1038/bjc.2013.263. Epub 2013 May 30. PMID: 23722468; PMCID: PMC3694244.