

Using Genome-wide CRISPR screens to dissect the molecular mechanisms underlying therapy resistance and sensitivity in HER2-positive gastric cancer

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Abstract

Gastric cancer (GC) represents a significant global health challenge, ranking as the fifth most common cancer worldwide. Due to the absence of significant symptoms in early stages and the lack of screening programs it is commonly diagnosed at an advance stage, where effective treatment options are scarce [1, 2]. GC has a high complexity and can be classified from a morphological, molecular and genetic standpoint. Genetic analysis have revealed specific gene sets defining the primary molecular pathways in GC [3]. However, despite the identification of several targetable genomic alterations in GC, only one, human epidermal growth factor receptor 2 (HER2), has demonstrated predictive value [3, 4].

In GC, HER2 is overexpressed in approximately 20% of patients [5, 6]. HER2-positive GC patients can undergo treatment with HER2 inhibitors, such as trastuzumab. Unfortunately, not all HER2-positive patients respond to trastuzumab. Studies indicate that only 47% of patients benefit from trastuzumab, and a majority of initial responders eventually develop resistance [4, 7].

This project aims to uncover the biology behind the lack of efficacy of anti-HER2 therapies in HER2-positive GC. We hypothesize that there might be genes whose loss may enhance (or hinder) the efficacy of anti HER2 therapy. To test this hypothesis, we have performed a genome-wide CRISPR screen. This project's main goal is to validate two of the top hits genetically, RIC8A and RAB14. In parallel, we also did a pharmacological validation of RIC8A and PTEN. We used two cell lines as models: NCI-N87 cell line to mimic the patients that respond to the treatment and MKN74 to mimic the patients that have primary resistance to the treatment and do not respond.

Based on our findings, we successfully validated the candidate gene RAB14 in two-dimensional cell cultures, demonstrating its synergy with HER2 inhibition not only in the NCI-N87 cell line, which was used in the initial screen and is HER2-positive, but also in the HER2-negative MKN74 cell line. Although RIC8A exhibited some promising results during pharmacological validation, it did not achieve similar success in genetic validation, indicating the need for additional studies in both cell lines. Similarly, PTEN showed encouraging results in pharmacological validation regarding its antagonistic effect on HER2 inhibition.

In summary, this project has contributed to a better understanding of why anti-HER2 therapies may be ineffective in HER2-positive gastric cancer patients and has led to the

identification of three potential biomarkers (PTEN, RAB14 and RIC8A) whose expression could be used to predict response to anti-HER2 therapy in gastric cancer.

Resumo

O cancro gástrico (CG) representa um grande desafio à saúde global, sendo o quinto cancro mais comum globalmente. Devido à ausência de sintomas significativos nos estadios iniciais e à falta de programas de rastreio, é frequentemente diagnosticado em estadio avançado, onde as opções de tratamento eficazes são escassas [1, 2]. O CG possui alta complexidade e pode ser classificado sob aspetos morfológicos, moleculares e genéticos. Análises genéticas revelaram conjuntos específicos de genes que definem as principais vias moleculares no CG [3]. No entanto, apesar da identificação de várias alterações genómicas que podem ser alvos no CG, apenas uma, o recetor 2 do fator de crescimento epidérmico humano (HER2), demonstrou valor preditivo [3, 4].

Em CG, o HER2 está sobre expresso em aproximadamente 20% dos pacientes [5, 6]. Pacientes com CG positivo para HER2 podem ser tratados com inibidores de HER2, como o trastuzumab. Infelizmente, nem todos os pacientes HER2-positivos respondem ao tratamento. Estudos indicam que apenas 47% dos pacientes beneficiam do trastuzumab, e a maioria dos que inicialmente respondem eventualmente desenvolvem resistência [4, 7].

Este projeto tem como objetivo desvendar a biologia por trás da falta de eficácia das terapias anti-HER2 em pacientes com CG HER2-positivo. A nossa hipótese é de que podem haver genes cuja perda possa aumentar (ou diminuir) a eficácia da terapia anti-HER2. Para testar essa hipótese, realizamos um CRISPR screen em todo o genoma. O objetivo principal deste projeto é validar geneticamente dois dos principais alvos encontrados, RIC8A e RAB14. Paralelamente, também realizamos uma validação farmacológica do RIC8A e PTEN. Como modelos utilizamos duas linhas celulares: a linha NCI-N87 para mimetizar pacientes que respondem ao tratamento, e a MKN74 para mimetizar pacientes que apresentam resistência primária ao tratamento e não respondem.

Com base em nossos resultados, validamos com sucesso o gene candidato RAB14 em culturas celulares bidimensionais, demonstrando a presença de sinergia com a inibição de HER2 não apenas na linha NCI-N87, que foi usada no screen inicial e é HER2-positiva, mas também na linha MKN74, que é HER2-negativa. Embora o RIC8A tenha mostrado alguns resultados promissores durante a validação farmacológica, não obteve o mesmo sucesso na validação genética, indicando a necessidade de estudos adicionais em ambas as linhas celulares. Da mesma forma, o PTEN mostrou resultados encorajadores na validação farmacológica em relação ao seu efeito antagónico na inibição de HER2.

Resumindo, este projeto contribuiu para uma melhor compreensão do porque é que as terapias anti-HER2 podem ser ineficazes em pacientes com cancro gástrico HER2-positivo e levou à identificação de três potenciais biomarcadores (PTEN, RAB14 e RIC8A), cuja expressão poderia ser usada para prever a resposta à terapia anti-HER2 no cancro gástrico

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Abbreviations Index

ACRG	Asian Cancer Research Group
ATP	Adenosine Triphosphate
AJCC	American Joint Committee on Cancer
BC	Breast Cancer
BSA	Bovine Serum Albumin
BSC	Best Supportive Care
c-Cbl	Casitas B lineage lymphoma
CIN	Chromosomally Unstable
CG	Cancro Gástrico
ChT	Chemotherapy
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
DNA	Deoxyribonucleic Acid
dMMR	Deficient DNA Mismatch Repair
DMSO	Dimethyl sulfoxide
DRC	Drug Response Curve
EBV	Epstein-Barr Virus
EGF	Epidermal Growth Factor
EMR	Endoscopic Mucosal Resection
EGFR	Epidermal Growth Factor Receptor
EGFRi	Epidermal Growth Factor Receptor inhibitor
VEGF	Vascular Endothelial Growth Factor
EMT	Epithelial-Mesenchymal Transition
ERBB	Erythroblastic Leukemia Viral Oncogene Homologue
FDA	U.S. Food and Drug Administration

FIGC	Familial Intestinal Gastric Cancer
FISH	Fluorescent in-situ Hybridization
GC	Gastric Cancer
gRNA	Guide RNA
GS	Genomically Stable
HDGC	Hereditary Diffuse Gastric Cancer
HDR	Homology Directed Repair
HGF	Hepatocyte Growth Factor
HER2	Human Epidermal Growth Factor Receptor 2
HER2i	Human Epidermal Growth Factor Receptor 2 inhibitor
HER3	Human Epidermal Growth Factor Receptor 3
HER4	Human Epidermal Growth Factor Receptor 4
IHC	Immunohistochemistry
KO	Knock-out
Lap	Lapatinib
MAPK	Mitogen-Activated Protein Kinase
mBC	Metastatic Breast Cancer
mNSCLC	Metastatic Non-small cell lung cancer
MEK	Mitogen-activated protein kinase kinase
MLH1	Tumor Suppressor MutL Homolog 1
MSI	Microsatellite Unstable
MSI-H	Microsatellite Instability-High
MSS	Microsatellite-Stable
mBC	Metastatic Breast Cancer
mOS	Median Overall Survival
mTOR	Mammalian/mechanistic Target of Rapamycin

NHEJ	Non-Homologous End Joining
OS	Overall Survival
ON	Overnight
PAM	Protospacer Adjacent Motif
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline Tween
PCR	Polymerase chain reaction
PD-L1	Programmed Cell Death Ligand-1
PD-1	Programmed Cell Death Protein 1
PD-L2	Programmed Cell Death Ligand-2
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PTEN	Phosphatase and Tensin Homolog
PFS	Progression-free Survival
PVDF	Polyvinylidene Fluoride Membrane
RAS	Rat sarcoma
RAF	Rapidly Accelerated Fibrosarcoma
RIC8A	Resistance to inhibitors of cholinesterase-8A
RIPA	Radioimmunoprecipitation assay
RT	Room Temperature
SMAD4	Mothers against decapentaplegic homolog 4
TAE	Tris-acetate-EDTA
TALENs	Transcription Activator-like Effector Nucleases
TCGA	The Cancer Genome Atlas
TKI	Tyrosine Kinase Inhibitor
TNM	Tumor, Node, Metastasis
UNT	Untreated

WHO	World Health Organization
WT	Wild type
YAP	Yes-associated protein 1
ZFNs	Zinc-finger nucleases

I. INTRODUCTION

1. Gastric Cancer

1.1 Epidemiology and Risk Factors

Gastric cancer (GC) is a complex and multifaceted disease influenced by a diverse number of factors, encompassing both environmental and genetic elements. In 2022, GC was ranked as the fifth most prevalent cancer globally, with around 9 million new cases and standing as the fifth leading cause of cancer-related mortality [8] (**Figure 1**).

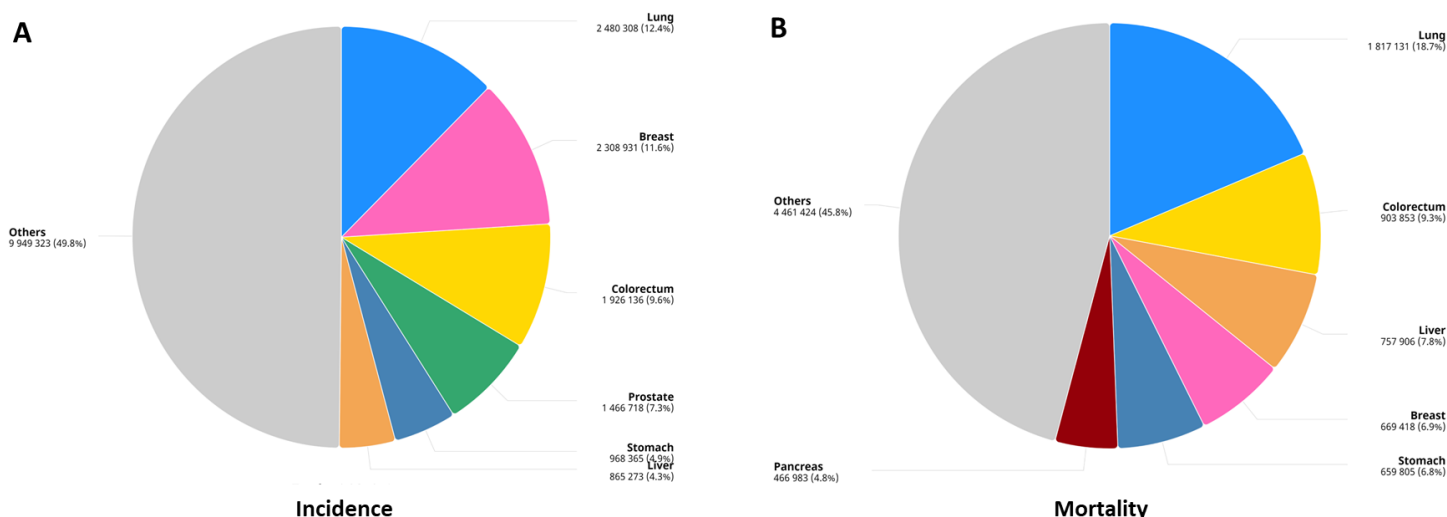


Figure 1. Pie charts representing the percentage of cancer-related incidence (A) and mortality (B) in both sexes, worldwide, in 2022. Gastric Cancer is the fifth most prevalent cancer globally and the fifth most deadly. Adapted from [1].

The incidence of gastric cancer exhibits significant geographical variability, with the highest rates observed in Eastern Asia and the lowest rates in Southern Asia in 2022 [8] (**Figure 2**).

In recent decades, there has been a noteworthy decline in the incidence and mortality rates of GC in various regions around the world. This positive trend has been attributed to economic progress and improved practices in food preservation, particularly advancements in refrigeration technologies. However, despite historical data indicating a consistent decrease in incidence across all age groups [9], recent research has brought attention to a concerning pattern of increasing incidence rates among individuals under the age of fifty [10]. Aiming to unravel the complexities and identify significant risk factors associated with the increased susceptibility to GC numerous studies have been conducted in recent years.

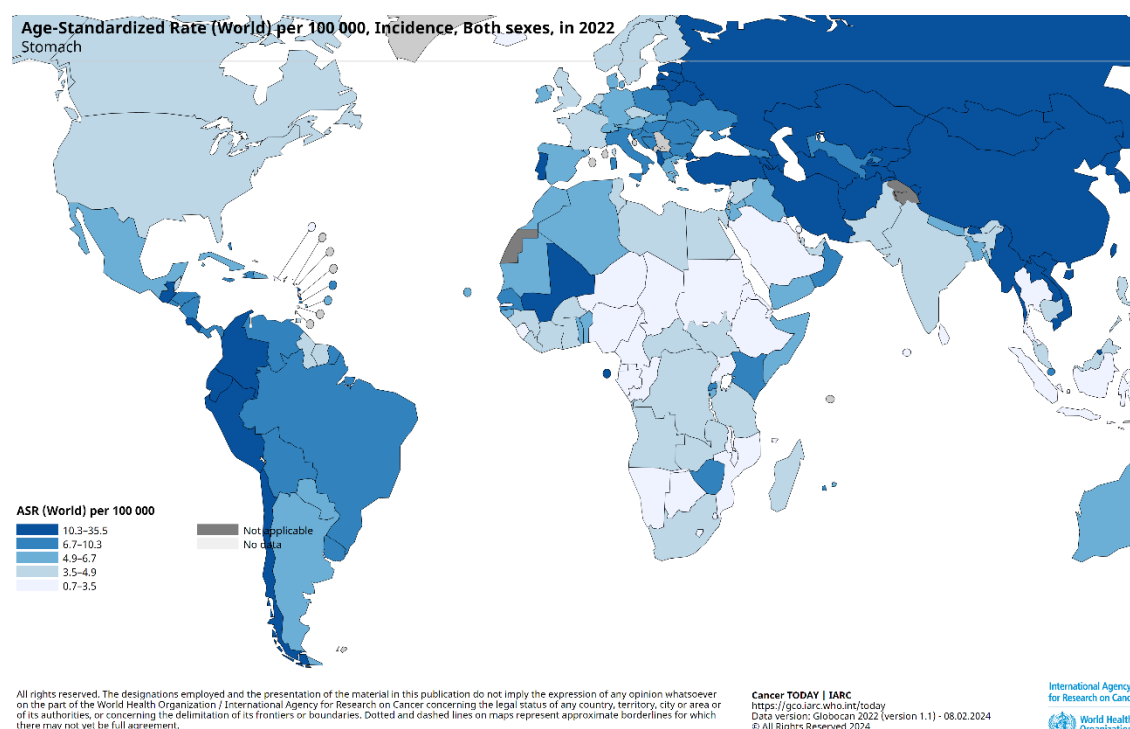


Figure 2. Map with the global distribution of incidence rates of GC, in both sexes, in 2022. Source: GLOBOCAN 2022. The incidence of gastric cancer exhibits significant geographical variability, with the highest rates observed in Eastern Asia and the lowest rates.

Among the factors with substantial impact are family history, dietary patterns, smoking, alcohol consumption, as well as infections with *Helicobacter pylori* and the *Epstein-Barr virus* (EBV). Notably, family history stands out as one of the most crucial risk factors [11]. However, only a small fraction of GC cases, approximately 10%, exhibit familial aggregation, and within this subset, genetic defects account for only 1%-3% of cases [12]. Familial or hereditary GC can be categorized into three syndromes: hereditary diffuse gastric cancer (HDGC), familial intestinal gastric cancer (FIGC), and polyposis forms [13]. HDGC, associated with alterations in CDH1 leading to changes in the adhesion molecule E-cadherin 1, is the most recognizable syndrome [14, 15]. Individuals with a family history of HDGC face a threefold increased risk of developing GC compared to those without such a history [16].

Dietary habits also show a significant correlation with GC risk. Evidence from several studies suggests that the consumption of broiled and charbroiled animal meats, salt-preserved foods, smoked foods, and pickled vegetables may elevate the risk of GC [11, 17, 18]. Lifestyle choices, such as smoking and alcohol consumption, also increase the risk of developing these type of tumours. A prospective cohort study in China revealed that smokers faced an elevated risk of GC compared to non-smokers, even after adjusting for

alcohol and other confounding factors. Among non-drinkers, a similar trend was observed, with smokers exhibiting an approximately 80% increased risk of developing GC [19].

Helicobacter pylori, a Gram-negative bacterium, is prevalent in approximately half of the world's population. While most infections are asymptomatic, chronic infections can induce gastric inflammation, leading to the destruction of normal gastric glands and their replacement by intestinal-type epithelium, resulting in atrophy of the gastric mucosa. This alteration in the gastric tissue microenvironment promotes epithelial-mesenchymal transition, contributing to GC progression, cell proliferation, and survival [20, 21].

In addition to *H. pylori*, the EBV is also associated with GC development. Approximately 10% of GC cases are reported to be EBV-positive, although there is insufficient evidence to establish a specific etiological role of EBV in GC development [22].

1.1 Classification

Anatomically, gastric tumours can be categorized into true gastric cancer (non-cardia) and gastroesophageal junction cancers (cardia), each exhibiting distinctions in terms of incidence, geographical distribution, etiology, clinical course, and treatment options [13].

Various pathological classifications have been proposed over time to assess tumour behaviour in gastric cancer. The Lauren classification, established in 1965, remains the most widely utilized system. This classification identifies two main histological subtypes: intestinal and diffuse, with an indeterminate type later included for infrequent histology [23].

Studies consistently indicate that the intestinal subtype is the most common, followed by the diffuse subtype, and finally the indeterminate type [11]. Intestinal carcinoma is characterized by visible glands and cohesive tumour cell organization, while the diffuse subtype exhibits poorly cohesive cells diffusely infiltrating the gastric wall, with minimal gland formation, often presenting small, round cells and signet ring cell formation [11]. Data suggest that diffuse-type gastric carcinoma originates from normal gastric mucosa and is more prevalent in females and young individuals, while the intestinal type is associated with chronic atrophic gastritis, intestinal metaplasia, and *H. pylori* infection [24, 25]. Mixed gastric cancers present a poorer prognosis compared to the other two types, potentially due to cumulative adverse effects of the two components within a single tumour [3].

Another classification that can be used is World Health Organization (WHO) classification. In contrast to the straightforward and robust Lauren scheme, the WHO classification encompasses five main histopathological cancer entities. Its advantage lies in

its alignment of histological cancer classifications in other parts of the gut, promoting harmonization in classification. The WHO classification, 5th edition (2019) [26] categorizes gastric adenocarcinoma into tubular, papillary, mucinous, poorly cohesive (including signet ring cell carcinoma), and mixed subtypes, along with other rare subtypes. The tubular adenocarcinoma is identified as the most common subtype, followed by papillary and mucinous types. Proximately 10% of gastric cancers are signet ring cell carcinomas and within this one's almost 50% of the tumour is composed of signet ring cells [11].

Nevertheless, new methods of classification for GC are required and advances in genomic technology and high-throughput analysis have led to a molecular classification of gastric cancer [27].

The Cancer Genome Atlas (TCGA) consortium proposed a groundbreaking molecular classification in 2014, dividing gastric cancer into four subtypes: EBV-positive, microsatellite-unstable (MSI), genomically stable (GS), and chromosomally unstable (CIN) [28].

EBV-positive gastric cancer is distinguished by its localization in the gastric fundus or body, absence of intestinal metaplasia, and higher incidence among male patients. It is characterized by elevated EBV burden, DNA hypermethylation, mutations in specific genes, overexpression of programmed death ligand-1/2 (PD-L1/2), and amplification of certain genes [29].

Microsatellite instability is defined by genomic instability arising from an impaired DNA mismatch repair system [28]. Individuals diagnosed with this subtype exhibit an advanced onset at a median age of 72 years, with a considerable prevalence among female patients. The primary cause of this subtype is largely attributed to promoter methylation, inducing transcriptional silencing of the DNA mismatch repair gene MLH1, consequently giving rise to a type of genomic instability recognized as microsatellite instability [29].

The genomically stable subtype is more frequently diagnosed in younger patients, characterized by a low somatic copy-number aberration [29]. Considering the therapeutic implications, a molecular classification system also takes into account the HER2 (*ERBB2*) status of gastric cancers [13].

Chromosomal instability is characterized by aberrations such as DNA aneuploidy, structural alterations, and mutations in proto-oncogenes and tumour suppressor genes [28]. This instability is associated with responsiveness to cisplatin-based chemotherapy and unfavourable survival outcomes, predominantly found in gastroesophageal junction/cardia tumours with intestinal histology [29, 30].

Another molecular classification by the Asian Cancer Research Group (ACRG) introduces four molecular subtypes: MSI, microsatellite-stable (MSS)/TP53 active, MSS/TP53 inactive, and MSS/mesenchymal-type [31]. Differences between the TCGA and ACRG classifications include the absence of a specific EBV-associated group in the ACRG classification, which is clearly defined in the TCGA study.

1.2 Gastric Cancer Stages

Staging cancer is very important for characterization of cancer extension in the body, such as the size of tumour, where it has spread and how far it has spread from the primary location. Knowing the disease stage is crucial for treatment selection.

There are numerous systems available for cancer staging but the most used worldwide for stomach is the TNM staging system.

In stomach staging the layers in the stomach wall are very important and have a major role in the identification of the stage; in more advanced GC the cancer cells spread from the mucosa to other layers. Beside the layers of the stomach wall, nearby lymph nodes are also very important for the staging, as described in the **Table 1**.

Table 1. Summary of stomach cancer stages and characteristics. AJCC classification which was last updated in 2018.

Adapted from [32].

Stage	Characteristics
0 (In Situ)	Abnormal cells are found in the mucosa. These cells may become cancer and spread
IA	Cancer has formed in the mucosa and may have spread to the submucosa
IB	• Cancer has formed in the mucosa and may have spread to the submucosa and has spread to 1 or 2 nearby lymph nodes • Cancer has formed in the mucosa and has spread to the muscle layer
IIA	• Cancer may have spread to the submucosa and has spread to 3 to 6 nearby lymph nodes • Cancer has spread to the muscle layer and to 1 or 2 nearby lymph nodes • Cancer has spread to the submucosa
IIB	• Cancer may have spread to the submucosa and has spread to 7 to 15 nearby lymph nodes • Cancer has spread to the muscle layer and to 3 to 6 nearby lymph nodes • Cancer has spread to the subserosa and to 1 or 2 lymph nodes • Cancer has spread to the serosa
IIIA	• Cancer has spread to the muscle layer and to 7 to 15 nearby lymph nodes • Cancer has spread to the subserosa and to 3 to 6 nearby lymph nodes • Cancer has spread to the serosa and to 1 to 6 nearby lymph nodes • Cancer has spread to nearby organs, such as the spleen, colon, liver, diaphragm, pancreas, abdomen wall, adrenal gland, kidney, or small intestine, or to the back of the abdomen
IIIB	• Cancer may have spread to the submucosa or to the muscle layer and has spread to 16 or more nearby lymph nodes • Cancer has spread to the subserosa or to the serosa and has spread to 7 to 15 nearby lymph nodes • Cancer has spread to nearby organs such as the spleen, colon, liver, diaphragm, pancreas, abdomen wall, adrenal gland, kidney, or small intestine, or to the back of the abdomen. And has also spread to 1 to 6 nearby lymph nodes
IIIC	• Cancer has spread to the subserosa or to the serosa and to 16 or more nearby lymph nodes • Cancer has spread to nearby organs such as the spleen, colon, liver, diaphragm, pancreas, abdomen wall, adrenal gland, kidney, or small intestine, or to the back of the abdomen. And has also spread to 7 or more nearby lymph nodes
IV	• Cancer has spread to other parts of the body, such as lungs, liver, distant lymph nodes, and the tissue that lines the abdomen wall

1.3 Available treatments

Despite the high incidence of GC, the majority of patients are diagnosed at an advanced stage. This, coupled with the frequent development of therapy resistance and limited therapeutic options (largely associated with the intratumoral and intertumoral heterogeneity characteristic of GC), contributes to the often observed poor prognosis in these patients. [33].

Gastric cancer treatment typically involves a combination of surgery, chemotherapy, and radiation therapy, with the choice of treatment depending on factors such as cancer stage, tumour location, patient health, and specific genetic markers. For early-diagnosed GC, surgical resection is often the preferred treatment over systematic chemotherapy, serving as the primary approach for resectable GC [33, 34]. However, studies suggest that when combined with perioperative or adjuvant chemotherapy, there is a reduced risk of recurrence and improved long-term survival [33, 35, 36]. In cases where patients undergo primary surgery but have a stage II or stage III tumour, adjuvant chemotherapy is recommended, as evidence suggests it may enhance prognosis when compared to surgery alone [33] (**Table 2**).

In Western countries, around 50% of the patients present a locally advanced or metastatic gastric cancer when diagnosed [37]. Treatment options become more limited in these cases, and metastatic GC is often associated with an incurable situation [37]. Systematic chemotherapy becomes the gold standard treatment for these patients, with a median overall survival (OS) not exceeding 12 months [37, 38].

A new era in GC treatment is emerging with the introduction of targeted therapies for advanced gastric cancer. Currently trastuzumab with chemotherapy is the standard therapy for HER2-positive gastric cancer. Besides this, an anti-vascular endothelial growth factor receptor 2 inhibitor, such as, ramucirumab, has been already approved as a second-line treatment. Another promising procedure is immunotherapy using checkpoint inhibitors, like, pembrolizumab which was approved as a second-line for MSI-H tumours [39, 40] (APPENDIXES, **Table 13**).

Table 2. Summary of treatments for GC per stage. Adapted from [41].

Stage or condition	Treatment
In situ	• Surgery • Endoscopic mucosal resection (EMR)
Stage I	• Surgical resection • EMR for selected patients with stage IA • Postoperative chemoradiation therapy or perioperative chemotherapy for patients with IB stage
Stage II and III	• Surgical resection • Perioperative chemotherapy • Postoperative (adjuvant) chemoradiation therapy • Postoperative (adjuvant) chemotherapy
Stage IV, inoperable and recurrent GC	
First-line palliative systemic therapy for patients with HER2-negative tumours	• Palliative chemotherapy with or without immunotherapy
First-line palliative systemic therapy for patients with HER2-positive tumours	• Immunotherapy with chemotherapy ◦ Nivolumab with chemotherapy → regardless of PD-L1 score ◦ Trastuzumab with chemotherapy
Second-line palliative systemic therapy	For patients who develop disease progression after first-line palliative chemotherapy there is no standard treatment • Palliative chemotherapy • Ramucirumab with or without chemotherapy • Pembrolizumab for patients with dMMR or MSI-H tumours • Trastuzumab deruxtecan for patients with HER2-positive tumours
Third-line palliative systemic therapy	• Trifluridine and tipiracil

2. HER2-positive gastric cancer

2.1 HER2 and ERBB family

The epidermal growth factor (EGF) family integrates four structurally related members – EGFR (*ErbB1*), HER2 (*ErbB2*), HER3 (*ErbB3*) and HER4 (*ErbB4*), [42]. These receptors are a single chain transmembrane glycoproteins constituted by an extracellular ligand-bind ectodomain , a transmembrane domain, a short juxtamembrane section, a tyrosine kinase domain and a tyrosine-containing C-terminal tail [42]. They have a crucial role in the development of normal cells and when dysregulated can lead to uncontrolled proliferation, invasion and unchecked cell survival which are strongly linked to the development of cancer [43, 44].

HER2, in particular, is a transmembrane tyrosine kinase receptor protein encoded by the *ERBB2* gene, a proto-oncogene located on chromosome region 17q21 [24, 45] and, as mentioned above, it is part of the EGF family. Similar to its family members, HER2 is a protein type I transmembrane growth factor receptor that activates intracellular signalling pathways in response to extracellular signals [46]. However, in contrast to the other ERBB receptors, HER2 activation is independent of a ligand, being heterodimerization with EGFR and HER3 the primary way of activation, or homodimerization when in high concentrations [47]. HER2 homo and heterodimerization is illustrated in **Figure 3**.

In the presence of HER2 overexpression the cell tends to follow a malignant transformation [48], which interferes with many cell mechanisms. This overexpression can stem from gene amplification, resulting in constitutive activation of the HER2 signalling network [47]. However, this is not the only explanation, as somatic mutations can also cause HER2 overexpression. In such cases it is almost always detected in the absence of *ERBB2* amplification [47], making it one of the most prevalent mechanisms leading to overexpression. The mutations can occur not only in *ERBB2* but also in genes that regulate HER2 expression, which is the case of its negative regulator *c-Cbl* [49].

Moreover, HER2 overexpression can result in an overabundance of the heterodimer HER2-HER3, a potent heterodimer, leading to the increased activation of PI3K/AKT signalling pathways [49], as represented in **Figure 3**. When activated, AKT influences numerous cell mechanics important for cancer cells, such as cell proliferation and survival, cell size, response to nutrient availability, glucose metabolism, epithelial-mesenchymal transition (EMT), cell invasiveness, genome stability, and angiogenesis [50]. The overactivation of AKT can also lead to higher expression of hypoxia-inducible factor 1a which results in more angiogenesis and tumorigenic potential [49].

Even though heterodimerization is the principal process that leads to activation of the PI3K/AKT signalling pathway, HER2 can also activate that pathway by itself, as shown experimentally in RN6 fibroblasts which lack EGFR expression [51]. Besides this, HER2 overexpression can also activate SRC kinases triggering RAS/RAF and PI3K/AKT pathways contributing to increased cell proliferation and tumorigenesis because of loss of polarity and disruption of cell adhesions related to SRC kinases [49].

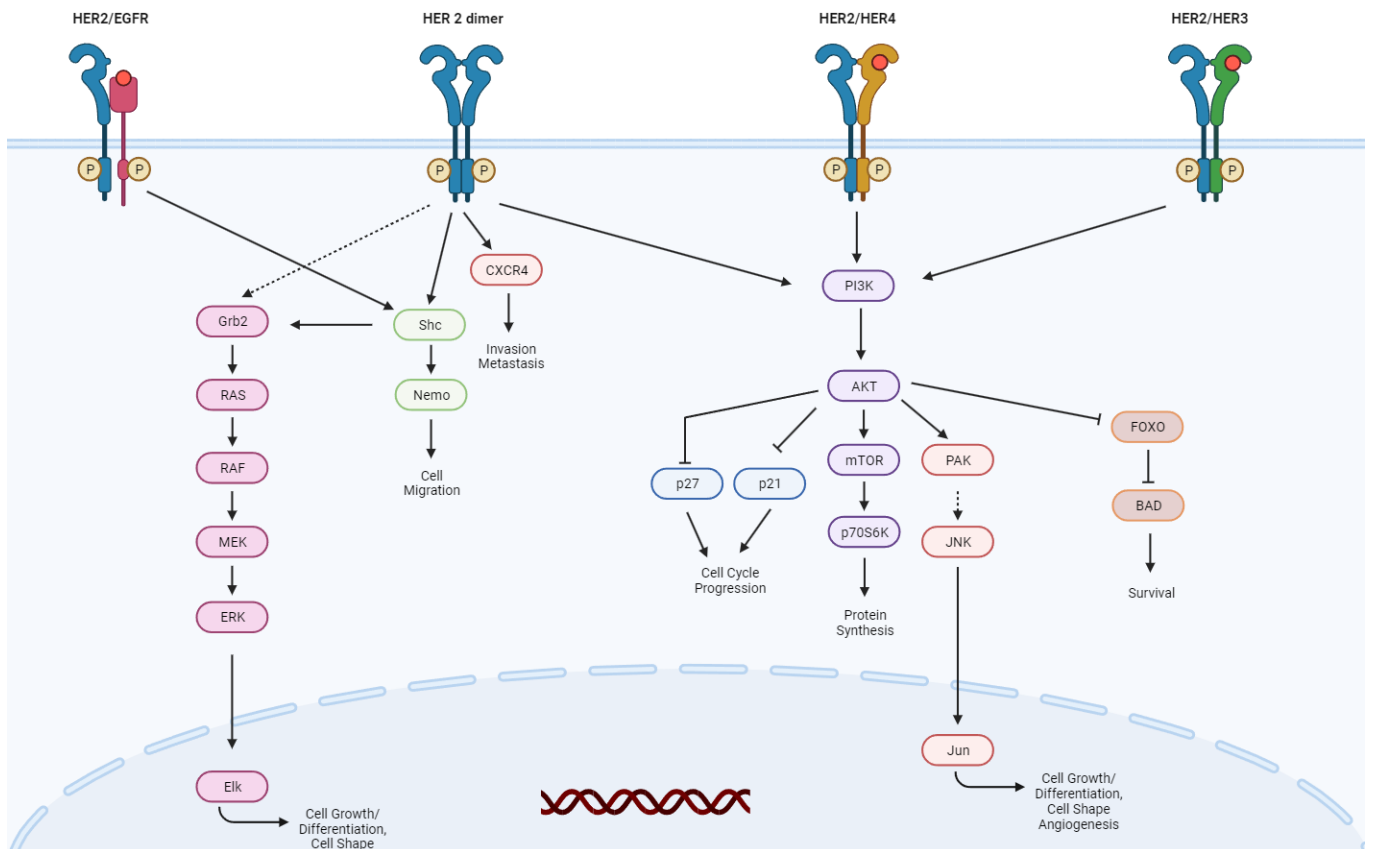


Figure 3. Overview of HER2 dimerization partners and the downstream pathways. Adapted from [4, 5]. Created with BioRender.com

2.2 HER2-positive tumours

Approximately 15% to 30% of invasive breast cancers exhibit HER2 overexpression, [52] which is considered an early event in breast tumorigenesis and significantly impacts the patient's prognosis [53]. Overexpression is often attributed to an increased number of *ERBB2* gene copies [52]. When compared to non-HER2 overexpression BC, HER2-overexpression is associated with shorter disease-free and overall survival [52] and more aggressive behaviour [54]. This aggressiveness is attributed to the correlation between HER2 amplification and increased tumour-cell multiplication and invasion, resulting in focal progression and distant metastases. [53, 54]. Signalling transduction mediated by activation of PI3K/AKT and Ras/Raf/MEK/MAKP pathways [55] is also responsible for these adverse

biological characteristics. Additionally, HER2-positive breast cancer shows increased sensitivity to certain cytotoxic chemotherapeutic agents and resistance to hormonal agents [56].

In ovarian cancer, HER2 overexpression is present in about 18%-43% of the cases, and similarly to BC, HER2 overexpression reflects a HER2 amplification [57]. In bladder cancer the survival rate of patients with HER2-positive tumours is significantly lower when compared to the HER2-negative tumours [57]. This is also observed in colorectal cancer, however, unlike BC, HER2 positivity seems to be a late event in the carcinogenesis of this neoplasia. The overexpression in this type of cancer is also controversial, with a big range that can span from 0 to 83% [58].

In GC approximately 20% of the patients exhibit HER2 overexpression [5, 6], which correlates with poorer outcomes and more aggressive disease [52, 59]. Contrary to the well-established prognostic value of HER2 positivity in BC, the prognostic significance in GC is more controversial, with inconsistent findings [60, 61]. HER2 overexpression varies across primary sites, being more frequent in tumours that are located in the gastroesophageal junction [5, 62]. Additionally, it is more common in the intestinal type compared to the diffuse type [62].

2.3 HER2 testing and therapy

For patients with inoperable, locally advanced, recurrent or metastatic GC, where therapy with trastuzumab is possible, the recommendation is to conduct testing for HER2 status [7]. This test assesses HER2-positivity by quantifying the HER2 cell surface receptors using immunohistochemistry (IHC) and/or the number of HER2 gene copy numbers utilizing fluorescent in-situ hybridization (FISH) [7]. The primary approach is IHC but, when deemed necessary, is complemented with FISH[6].

Hofmann *et al.* [63] proposed guidelines for the evaluation of HER2 in samples of GC, which was optimized by Rüschoff *et al* [64]. According to Hofmann, a sample is considered positive when the score for IHC is 3+ or 2+, but in case of 2+ confirmation by FISH is necessary [7, 24, 52, 63, 65]. Additionally, to be a 3+ score it is necessary the presence of at least five clustered positive cells in a biopsy tissue or a minimum of 10% positive neoplastic cells in a surgical resection, with the stipulation that IHC staining reveals intense, complete, basolateral, or lateral membranous activity [64].

Quantification of HER2 is also necessary for BC to determine eligibility for trastuzumab. However, there are some differences between these two neoplasia. In BC the

distribution of HER2 is primarily circumferential instead of basolateral and lateral as in GC [65]. Besides this, the intratumoral heterogeneity is common in GC and constitutes a problem for HER2 testing due to potential score variations in different tumour areas [60, 62, 64]. Additionally, BC doesn't show alteration in the incidence of HER2 expression in different anatomic locations contrary to GC in which HER2 expression is more frequent in the proximal stomach when compared to the distal stomach [65].

The first anti-HER2 monoclonal antibody, developed in 1990, was trastuzumab. This antibody binds to the extracellular domain of HER2 inhibiting the homodimerization of this receptor and consequently preventing the signalling mediated by HER2 [5, 66]. This therapy was initially approved for BC and, when used in combination with chemotherapy improved the survival rate of patients with HER2-positive metastatic BC [67]. Later on, it was also discovered that it enhances the outcome of patients with early-stage HER2-positive BC [68].

Taking into account the benefits of trastuzumab for patients with HER2-positive BC an open-label phase III trial named "*Trastuzumab for Gastric Cancer (ToGA)*" [4] was conducted and aimed to demonstrate the clinical benefits of trastuzumab combined with chemotherapy for patients with HER2-positive advanced GC compared to chemotherapy alone. The analysis of this study showed a benefit when trastuzumab was combined with chemotherapy in patients with locally advanced, recurrent and/or metastatic gastric or gastro–esophageal junction tumours that overexpress HER2. Following these findings, in 2010 Trastuzumab was approved in combination with chemotherapy as a first line of treatment for patients with metastatic adenocarcinoma of the stomach or gastro–esophageal junction whose tumours were HER2-positive [69]. It is therefore highly recommended for all patients with GC to have their tumour tested for HER2 at the time of diagnosis [64]. Despite the proven benefits of trastuzumab, there are no definitive biomarkers to predict patient responses, and that is mainly due to the high number of signalling pathways involved in the tumorigenesis of GC [60]. Regarding the benefits of trastuzumab for HER2-positive patients, some studies have shown that despite the decreased OS associated with patients with HER2 overexpression compared to HER2-negative patients, the administration of anti-HER2 therapy can result in the HER2-positive patients achieving a similar OS similar to their HER2-negative counterparts [59].

Pertuzumab (another monoclonal antibody for HER2) binds to the extracellular domain of the receptor that disrupts the HER2 dimerization with HER3 [66], and has a synergistic effect when combined with trastuzumab in BC, leading to apoptosis *in vitro* [62, 70]. Based on this finding, a study was conducted to evaluate the efficacy and safety of pertuzumab in combination with trastuzumab and chemotherapy as a first-line of treatment

for HER2-positive metastatic gastric or gastro-oesophageal junction cancer. The conclusions were that although the study failed the first end-point of the OS, it also demonstrated some evidence of treatment activity and an acceptable safety profile [71].

Lapatinib, a reversible dual Tyrosine Kinase Inhibitor (TKI) that selectively targets and inhibits HER2 and EGFR proved to have some effectiveness in clinical trials. The TKI is homologous to ATP so they compete for the ATP-binding domain of protein kinases preventing their phosphorylation [72]. Clinical trials such as LOGiC and TyTAN [73, 74] evaluated the efficacy of lapatinib when combined with chemotherapy in patients with HER2-positive gastric tumours. Unfortunately, the results were not very positive and did not show any improvement in the OS, which could suggest that there is some drug resistance or presence of an alternative pathway. However, this drug was approved by FDA in 2007 for metastatic HER2-positive BC [72] for those that have received prior therapy including an anthracycline, a taxane and trastuzumab [75, 76].

Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) has also been studied for GC, and is a mechanism of immune response where effector cells bearing Fc receptors can identify and eliminate target cells linked to antibodies, which can express antigens derived from tumours or pathogens in their surface [77]. In recent years, several clinical trials have been studying the effect of antibody-drug conjugates such as Trastuzumab deruxtecan as a second line of treatment for patients with HER2-positive gastric or gastro-oesophageal junction cancer who had received a prior trastuzumab-based treatment, leading to its approval in 2024 by EMA and FDA [78, 79]. Trastuzumab deruxtecan is a combination of monoclonal antibody against HER2 and a topoisomerase I inhibitor connected by a cleaved linker; after internalisation from the tumour cells the linker is cleaved leading to a cytotoxic reaction inducing an anti-tumour reaction not only in these cells, but also in tumour cells next to HER2 expressing cells [80].

2.4 Resistance mechanisms and possible solutions

Given that most of patients develop resistance against anti HER2 therapy, it is expected that the tumour cells have acquired some characteristics that allow them to do this. According to the literature, there are multiple mechanisms identified that can contribute to resistance against anti-HER2 therapy. Of the patients that can benefit from anti-HER2 therapy more than 50% of them exhibit primary resistance, and even the ones that initially respond to the treatment eventually develop a secondary resistance [81, 82].

One of the main opponents of an efficient anti HER2 therapy is the intratumoral heterogeneity, with the range of HER2 expression within the tumour varying from 45% to

79% in IHC (in BC only around 40% of tumours show heterogeneity) [6, 83, 84]. Moreover, heterogeneity exists not only within the primary tumour but also between the primary tumour and the metastatic site, as demonstrated in the GASTHER1 study [85]. This study evaluated the HER2 status in metastatic sites of patients with GC whose primary tumour in a first biopsy was HER2-negative but when reassessed the metastatic site turned out to be HER2-positive. Another study also investigated the differences in HER2 expression between the primary tumour and metastatic site revealing it in approximately 10% of the patients [86].

Loss of HER2 positivity or/and acquired HER2 mutations is also one of the primary reason for the failure of anti HER2 therapy [87]. Recent studies have been evaluating the loss of HER2 positivity. One of them is GASTHER3 [88] where the authors assessed the HER2 expression in patients with advanced GC who had received trastuzumab as the first-line of treatment. It was verified that, during the treatment, many patients developed loss of HER2 expression and/or loss of HER2 overexpression, and this was more frequent in patients with an initial IHC score of 2+. Thus, it is crucial to test HER2 status before starting a second-line of anti-HER2 therapy. *De novo* mutations have been observed *in vitro* in cell lines from GC with overexpression of HER2 and are more frequent in the central portion of the kinase domain in the active form of the protein [6, 83].

Furthermore, alterations in the HER2 downstream pathway can induce acquired resistance. Activation of the HER2 receptor triggers several intracellular pathways, such as the PI3K/AKT and RAS/RAF/MEK pathways, as mentioned before. Anti-HER2 therapy aims to block this signalling. However, if there are any somatic mutations in the PI3K pathway, that might lead to a constitutive activation of that pathway thereby conferring resistance to anti-HER2 therapy [89]. Regarding to this, a study has evaluated the rate of response to trastuzumab in gastric adenocarcinoma tumours with and without PTEN loss and they observed that the tumours with PTEN loss did not respond to the anti HER2 therapy, suggesting that the loss of this protein could be associated with trastuzumab resistance [83]. Besides this, other studies described that acquired resistance to trastuzumab is related to the persistent activation of MAPK/ERK and PI3K/mTOR pathways mediated by Src [90], which suggests that the resistance to trastuzumab can also be associated with the activation of alternative pathways.

Although HER2 doesn't have an activating ligand, when heregulin interacts with HER3, it induces the formation of an heterodimer of this tyrosine receptor with HER2 and consequent activation of the PI3K pathway [91], which is associated with a shorter time to treatment failure for trastuzumab therapy. As such, it was found that higher levels of

heregulin in the tumour are associated with larger levels of resistance to trastuzumab in gastric and breast cancers [92].

Despite the clinical benefits of trastuzumab, resistance development remains a challenge. Understanding the underlying mechanisms and developing new stratification strategies are crucial. Recent studies explore alternative drugs and mechanisms of action. For example, afatinib, an irreversible inhibitor of EGFR, HER2, and HER4, is being studied for patients with HER2 mutations in various cancers [93].

Considering the dysregulation of HER2 downstream signalling as a key mechanism for trastuzumab resistance, combining HER2 and mTOR inhibitors might provide considerable benefits for patients with HER2-positive GC [62]. In addition, inhibitors of HSP90, an ATP-dependent chaperone, are also being explored in combination with trastuzumab for GC patients with HER2 amplification, showing significant synergy in recent studies [94]. These approaches represent promising avenues for overcoming resistance and improving outcomes for patients with HER2-positive GC.

3. CRISPR Cas9

CRISPR-Cas9 is a revolutionary gene-editing technology that utilizes a protein, Cas9, and a guide RNA (gRNA) to precisely target and modify specific genomic locations. Unlike earlier gene-editing technologies such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) [95], CRISPR-Cas9 is known for its simplicity, efficiency, and broad applicability [96].

This system developed naturally in bacteria and archaea as a defence mechanism against the infection of phage and plasmid [95]. Briefly, this technology enables one to do a specific cut in any genomic location that has a specific 20 nucleotides (nt) stretch followed by NGG, also called the PAM (Protospacer Adjacent Motif) [97, 98]. For this technology to work, only two factors are essential, the presence of the Cas9 protein and the presence of a so-called gRNA (i.e. a hybrid RNA molecule containing first the 20nt that are identical to the stretch that you want to target and a constant portion called tracrRNA) [96]. During the editing process the 5' -end of the gRNA recognizes the target region and the 3' -end binds to the Cas9 protein, spotting it as the target [96, 99] (**Figure 4**).

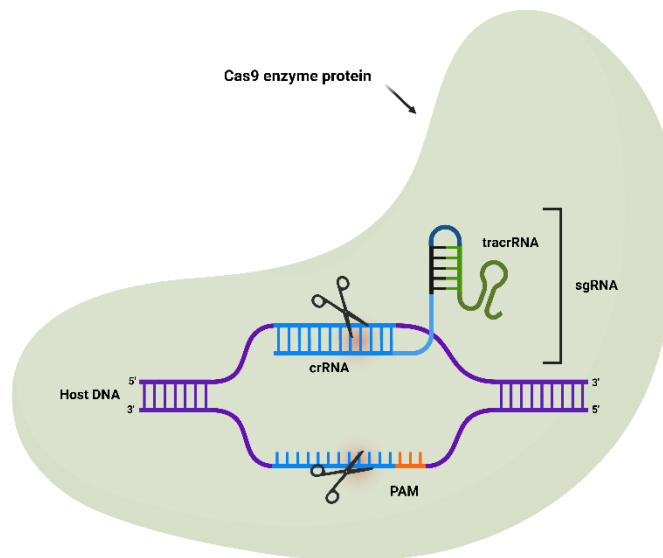


Figure 4. CRISPR-Cas9 system, with DNA sequence, Cas9 enzyme, PAM site and sgRNA. Adapted from [3]. Created with BioRender.com

After the cut has been made (which happens between nt 17 and 18 of the gRNA), cells will initiate a repair process. Most cells will do this by Non-Homologous End Joining (NHEJ). Unlike Homology Directed Repair (HDR), which uses homologous DNA templates to reconstruct broken DNA, NHEJ is error-prone and might lead to insertions and deletions (indels) at the target site [100]. However, not every mutation will lead to a full knockout (e.g.

a three nt deletion can result in the in-frame loss of only a single amino-acid, which may result in a relatively mild or even no phenotype).

CRISPR-Cas9's efficiency and flexibility have made it a powerful tool for performing screens in cancer research. Such screens can be used to identify genotype-specific vulnerabilities or to identify genes that can work in synergy with a drug or that might lead to drug resistance, and consequently understand the response mechanisms [100]. For a CRISPR-Cas9 screen a gRNA library is introduced into the cells that already express Cas9. The gRNA library can range from a few gRNA up to genome-wide library meaning that there is at least one gRNA for each gene of the genome, allowing us to evaluate the effect of each one of them in an unbiased way. This delivery is normally performed using lentivirus which is integrated into the DNA of the target cells. After selection and treatment with a drug it is possible to conclude that if a gRNA is depleted in the cell population it is because it confers sensitivity to the drug and, conversely, if it is enriched it confers resistance [101].

Cas9 can be used in its wild-type form to create knockouts in the genes of interest. However, modifications to the Cas9 protein can yield different functionalities. For instance, mutating the RuvC domain of Cas9 turns it into a nickase, preferentially inducing single-strand breaks and promoting the HDR repair process. [102]. Besides this, various modifications to Cas9 enable applications beyond traditional gene knockout, such as RNA modifications, epigenetic modifications, DNA base editing and gene activation are also possible [99].

II. RATIONAL AND AIMS

Given the current lack of understanding regarding the underlying principles that prevent the effectiveness of trastuzumab in HER2-positive patients, and consequently lead to its suboptimal clinical implementation, it is important to understand if there are any genes that upon loss of expression cause either resistance, or increase the sensitivity to HER2 inhibitors, and one way to do that is using CRISPR-Cas9 technology.

In this work, we aimed to uncover the biology behind the lack of efficacy of anti-HER2 therapies in HER2-positive GC patients. Using genome-wide CRISPR screens, we aim to identify genes whose inhibition either enhances the efficacy or causes resistance to anti-HER2 therapies. As such, the goal of this research project is to validate (a part of) the hits obtained in a previous CRISPR screen, in multiple GC cell lines (HER2-positive and HER2-negative) using a genetic and (when possible) a pharmacological approach.

To achieve our goal, the following tasks were pursued:

- I. Perform a database analysis to evaluate the relevance of the screen hits in GC
- II. Perform a pharmacological validation of the screen hits which have an inhibitor available.
- III. Perform a genetic validation of the top screen hits:
 - a. Generate gene knock-outs using CRISPR-Cas9 in multiple cell lines;
 - b. Perform drug-response assays with HER2 inhibitors in the KO cells

In this study, we expect our results to show that when a gene that increases sensitivity is knocked-out or inhibited, and the cells are treated with anti-HER2 therapy, the combination will have a synergistic effect, leading to a greater reduction in cell viability. On the other hand, when a gene that causes resistance is knocked out or inhibited, we expect an antagonistic effect when combined with anti-HER2 therapy.

With the insights gained from this study we seek to propose updated guidelines for better patient stratification, enabling a larger number of patients to benefit from anti-HER2 therapy, as well as proposing new rational combination therapies in order to enhance the effectiveness of anti-HER2 therapies.

III. MATERIAL AND METHODS

1. Cell Culture

NCI-N87, NCI-N87 Lapatinib Resistant, NUGC4, MKN45, MKN74 and HGC-27 cell lines were cultured in RPMI-Glutamax medium (Gibco, Invitrogen) and HEK293T cell line were cultured in DMEM medium. All the media were supplemented with 10% Fetal bovine serum (Gibco, Invitrogen), 1% of Pen Strap (Gibco, Invitrogen). All cell lines were cultured at 37°C and with 5% CO₂. NCI-N87 Lapatinib Resistant cell line was accomplished by keep in culture the NCI-N87 cell line with increasing concentration of lapatinib for two month.

Table 3 shows an overview of the molecular characteristics of the cell lines used, according to the Cellossaurus website [103].

Table 3. Molecular profile of each cell line

Cell Line	HER2-status	Disease	Site	Sequence Variations
NCI-N87	Positive	Gastric tubular adenocarcinoma	Metastatic Liver	- Gene deletion: SMAD4; Zygosity=Homozygous (ATCC). - Mutation: ERBB3; p.Thr757Lys (c.2270C>A); Zygosity=Unspecified - Mutation: TP53; Simple; p.Arg248Gln (c.743G>A); Zygosity=Homozygous
NUGC4	Positive	Gastric signet ring cell adenocarcinoma	Metastatic Paragastric lymph node	Mutation: TP53, Zygosity and mutation type unknown
MKN45	Negative	Gastric adenocarcinoma	Metastatic Liver	Missense mutation: TP53; p.Arg110Cys (c.328C>T); Zygosity=Heterozygous
MKN74	Negative	Gastric tubular adenocarcinoma	Metastatic Liver	Mutation: TP53; p.Ile251Leu (c.751A>C); Zygosity=Unspecified (PubMed=1370612; PubMed=15900046)
HGC-27	Negative	Gastric carcinoma	Metastatic lymph node	- Frameshift mutation: ATR; p.Gly2316fs*9 (c.6947delG); Zygosity=Heterozygous - Missense mutation: PIK3CA; Simple; p.Glu542Lys (c.1624G>A), Zygosity=Heterozygous - Frameshift mutation: TP53; p.Pro153Alafs*28 (c.455dupC) (c.455_456insC) (p.P152fs) (c.459_460insC); Zygosity=Heterozygous

2. KO Generation

2.1. Cloning the gRNA into the plasmid

10µg of Lenti-GuidePuro (Addgene, #52963) plasmid was digested using BsmBI.v2 (New England BioLabs), by incubating at 55°C for 1hour. After the digestion, the sample was run in a DNA gel to ensure the digestion was successful. It was run at 120V until the dye line is approximately 75-80% of the way down the gel in a 0.75% agarose gel that was done with 1X TAE buffer. The gel was checked under blue light and the larger band that corresponded to the digested backbone was cut and recovered using ISOLATE II PCR and Gel Kit (Bioline). Subsequently, 50ng of recovered digested backbone was mixed with HiFi DNA Assembly Master Mix (NEBuilder®) and 2ng of insert at 50nM and incubated for 30 minutes at 50°C.

2.2 Bacteria transformation to amplify the gRNA expression plasmid/library and plasmid isolation

The Gibson assembly product was diluted 3-fold with mQ. After that, 5µL of the diluted Gibson assembly product was added to 35µL of chilled mQ and 10µL of electrocompetent Endura cells. Subsequently, the mix was transferred into a pre-chilled electroporation cuvette, was electroporated at 1,8kV and 1000µL of SOC medium was immediately added, after which it was transferred to a new tube and incubated at 37°C for at least 1hour with shaking.

After the incubation, bacteria was plated in LB-Agar-Carbenicillin plates and grew overnight at 37°C. In the next step, a colony was picked and incubated overnight in LB-carbenicillin medium at 37°C with shaking. To confirm if the cloning was successful a mini-prep was performed and the plasmids were sequenced.

In the following step, after confirmation, maxi-prep was performed using Maxi-prep kit (QIAGEN) to extract DNA and later lentivirus production.

2.3 Lentivirus production

HEK293T cells were plated in 6-well plates (one well was plated per sgRNA), with a cell density of 1×10^6 per well. 24 hours later the transfection was performed: two tubes per sgRNA were prepared, one with 2µg plasmid DNA, 2µg lentivirus packaging mix, 6µL of P3000 reagent (Invitrogen) and 250µL of OPTIMEM (Gibco, Invitrogen). The second tube with 7µL Lipofectamin 3000 (Invitrogen) and 243µL of OPTIMEM. This two mixtures were

then combined and added drop wise to the cells with 500µL of OPTIMEM. 6h later the medium was changed and 48 hours later the lentivirus particles were harvested.

2.4 Cell infection with the lentivirus

NCI-N87 cells already expressing Cas9 were plated in 6-well plates (one well per sgRNA with a cell density of 6×10^6); the same was done for MKN74 cells also already expressing Cas9 (with a cell density of 3×10^6). 2µL of Polybrene at 10mg/mL and 200µL of lentivirus were added to each well.

2.5 Infected cells selection

2.5.1 Puromycin Titration

In order to achieve a population of cells successfully infected it was performed a Puromycin titration to identify the minimum concentration that kills all cells without the vector.

NCI-N87 and MKN74 cells were plated in a cell density of 8×10^5 and 4×10^5 , respectively, per well. Increasing concentrations of Puromycin (from 1,25µg/mL to 3,3µg/mL) were added 24h after seeding. 48h after treatment cells were fixed with formaldehyde 5% and stained with Crystal Violet 0,5% and images were collected with ChemiDoc XRS⁺ (BioRad).

2.5.2 Selection

48h after the infection the medium was removed and fresh medium with 2µg/mL of Puromycin was added. This step was repeated at 96h and 144h after infection. Subsequently, the cells were maintained in culture in the conditions mentioned in the section 1 of MATERIAL AND METHODS.

2.6 Monoclones

To ensure a high percentage of KO cells, monoclones were generated. To achieve a monoclonal population, cells were plated in a cell density of 5 cells per mL in a plate of 96 wells, in order to increase the probability of only one cell per well. The clones were maintained in culture in 96-well plate until a 80-90% confluence. After this, 5 clones per gene were selected and seeded in a 48-well plate until the same confluence was reached;

they were then passaged to a 6-well plate and finally to 10 cm dishes, where several aliquots were frozen.

2.7 KO validation

After selection with puromycin the cells lines were plated in 6-well plates with a cell density of 6×10^5 per well for NCI-N87 and 2×10^5 per well for MKN74 and the cells grew for four days. After that, the cells were harvested and the gnDNA was extracted according to the manufacturer's instructions, using the NZY Tissue gDNA Isolation Kit (NZYtech).

Subsequently, a PCR reaction per each gene was performed, using per PCR reaction: 12,5µL of Multiplex Master Mix (QIAGEN); 2,5 µL of Q-solution (QIAGEN); 2,5 µL of Primer Mix at 2µM; 1µg of DNA template and H₂O up to 20 µL. This was followed by a purification of the PCR product using ExoSAP-IT *Express* Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions and finally sanger sequencing was performed and editing efficiency was analysed using TIDE [104].

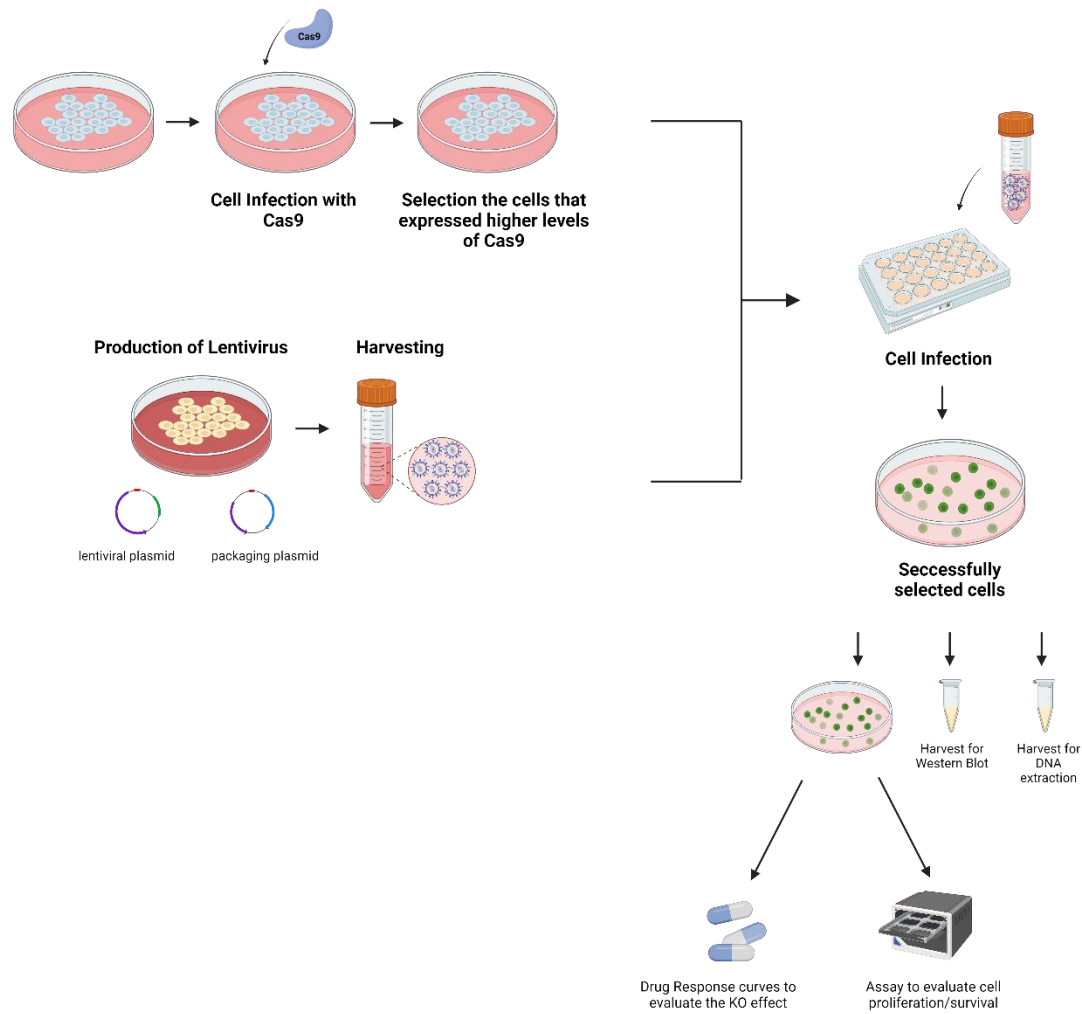


Figure 5. Diagram illustrating the workflow of the genetic validation of the selected hits. Firstly, cells are infected with Cas9 followed by selection of the ones expressing higher levels of Cas9 and lentivirus for the expression of the selected gRNAs are produced and harvested. Secondly, cells already expressing Cas9 are infected with lentivirus followed by selection with antibiotic. After successful selection, cells are maintained in culture to proceed with assays for KO validation and, in parallel, cells are harvested for Western Blot and DNA extraction for KO confirmation

3. Drug response and synergy assays

3.1 Drugs and Inhibitors

For this study, three different EGFR/HER2 inhibitors were used, each of them with different characteristics that are described in **Table 4**, and hit inhibitors available, described in **Table 5**. All drugs were dissolved in DMSO (Sigma-Aldrich) to a stock concentration of 10mM, stored at -20°C and protected from light exposure.

Table 4. Characteristics of HER2/EGFR inhibitors

Drug	Target(s)	Characteristics	Use in treatment
Lapatinib	EGFR and HER2	• Reversible dual TKI • Unlike trastuzumab is able to bind and inhibit the truncated form of HER2 [105]	• Approved for HER2-positive mBC [76, 106, 107].
Dacomitinib	EGFR and HER2	• Irreversible TKI • Irreversibly binding at a site within the ATP-binding pocket [108]	• Approved as first line of treatment for patients with mNSCLC with EGFR exon 19 deletion or exon 21 L858R substitution mutations [109]. • First-line treatment of patients with locally advanced or mNSCLC with EGFR-activating mutations [109].
Tucatinib	HER2	• Specific and reversible inhibitor of HER2 [110, 111]	• Approved for advanced unresectable or mBC HER2-positive in combination with trastuzumab and capecitabine [112]. • Approved for RAS-Wt, HER2-positive, unresectable or metastatic colorectal cancer in combination with trastuzumab [113].

Table 5. Summary of hit inhibitors

Drug	Target	Characteristics
IGS-1.76	RIC8A	• Inhibits the complex NCS-1/RIC8A
BpV(HOpic)	PTEN	• Potent and selective PTEN inhibitor

For all of this inhibitors drug response curves (DRC) were performed for each cell line used. Cells were plated in 96-well plates a cell density of 6×10^3 for NCI-N87 and $1,5 \times 10^3$ for MKN74 and then treated with increasing concentrations of each one of the inhibitors. The drugs were added 48h and 96h after seeding.

3.2 Synergy Assay

Cells were plated in 96-well plates with a cell density of 6×10^3 per well for NCI-N87 and $1,5 \times 10^3$ per well for MKN74. Two days after the cells were treated with increasing concentrations of HER2 inhibitor, and hit inhibitor. The media was refreshed (with drug treatment) two days after. And 72h later resazurin (1x) was added to the cells and incubated at 37°C for 1-2h, followed by measuring the fluorescence to assess cell viability. Cell viability data was uploaded to SynergyFinder 3.0 [114] and the results were analysed.

3.3 Drug/ Functional Assay

3.3.1 In 2D

The effect of the KO in cell proliferation and survival was evaluated when treated with HER2 inhibitors.

Cells were plated in 96-well plates with a cell density of 6×10^3 per well for NCI-N87 cell lines (the parental one and KO ones), and were treated with increasing concentrations of Lapatinib (a range between 1nM to 225nM), the MKN74 cells were plated with a cell density of $3,5 \times 10^3$ and were also treated with increasing concentration of Lapatinib (a range between 100nM to 5 μ M) at 48h, 96h and 144h after seeding. After the treatment resazurin (1x) was added to the cells and incubated at 37°C for 1-2h, followed by measuring the fluorescence to assess cell viability, in addition, the cells were fixed with formaldehyde 5% and stained with Crystal Violet 0,5% and images were collected with ChemiDoc XRS⁺ (BioRad).

After results analysis the assay was repeated this time in 6-well plates. Cells were plated with a cell density of 1×10^5 per well for NCI-N87 and a cell density of $3,37 \times 10^4$ for

MKN74, and were treated with a shorter range of increasing concentrations of lapatinib (a range between 20nM to 50nM for NCI-N87 and between 70nM to 100nM for MKN74) at 48h, 96h and 144h after seeding, resazurin (1x) was added to the cells and incubated at 37°C for 1-2h, followed by measuring the fluorescence to assess cell viability with following fix and staining with crystal violet. Following the results were updated in GraphPad software and was generated a *[Inhibitor] vs. normalized response -- Variable slope*, where our values are normalized to run between 100% down to 0% and the goal is to determine the IC50, which means the concentration of inhibitor that provokes a response of 50%.

3.3.2 In 3D

Plates of 10cm were filled with 20% agarose gel; after the agarose was gelled warm cell culture medium was added until cell plating, with a cell density of 1×10^6 cells for NCI-N87 cell lines (parental and KO cell lines).

25nM of Lapatinib was added at 48h, 96h and 144h after seeding. 48h after the last cells were collected and counted with Cell Counter (BioRad) and were compared with the control that was not treated with lapatinib and established a growth curve. Cells after collection were plated in a 6-well plates with resazurin (1x) and incubated 37°C for 1-2h, followed by measuring the fluorescence to assess cell viability.

4. Western Blot

To investigate the effect of the drugs in this study on the activation/inhibition of downstream proteins of MAPK and PI3K/AKT signalling pathways, cells were plated in 6-well plates according to optimal densities and incubated for 48h hours. Then, the drugs were added, followed by an overnight incubation period. In the next day, cells were washed with cold PBS, then lysed with RIPA buffer (25mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with Protease & Phosphatase inhibitor cocktail (Halt, Invitrogen). Samples were then centrifuged for 10 minutes at 15,000 x g at 4°C and supernatant was collected. BCA assay were performed to normalize the samples according to the manufacturer's instructions (ThermoFisher Scientific). For analysis of total protein samples, the amount of loading dye, RIPA+ and protein was calculated and prepared for a total of 80 µl per normalized sample. 15 µl per sample were loaded in a gel and separated by SDS-page Bolt 4-12% Bis-Tris Plus Gels (ThermoFisher Scientific) in MOPS buffer and run at 175V. After electrophoresis, gels were transferred to polyvinylidene fluoride membrane (PVDF) (Immobilon®-P PVDF Membrane, IPVH00010)

for 1 hour and 45 minutes at 350mA. Subsequently membranes were incubated in blocking solution (5% bovine serum albumin (BSA) in PBS with 0.1% Tween-20 (PBS-T)) for 30 minutes, and thereafter incubated with primary antibodies (**Table 6**) at 4°C overnight (ON). Membranes were then washed with PBS-T, and incubated with respective secondary antibody (**Table 7**) for 1 hour at room temperature (RT). After final wash, membranes were incubated with Clarity Western ECL Substrate (BioRad) for signal detection that were detected with ChemiDoc (BioRad).

The following part was the protein quantification for that it was used Quantity One Software (Bio-Rad), after having the values from each protein these were divided by the value of the housekeeping protein (α -tubulin) to normalize the results. After this, the phosphorylated form was divided by the total form of the protein, in PARP the cleaved form (lower band) was divided by the total form, that value of phosphorylation taken in consideration in the analyses.

Table 6. Primary antibody specifications for Western Blot.

Target Molecule	Serum	MW (kDa)	Dilutions	Supplier	Incubation conditions
Primary Antibodies					
p-HER2	Rabbit	185	1:1000	Cell Signaling Technology	4°C ON
HER2		185		Cell Signaling Technology	
p-RSK		90		Cell Signaling Technology	
p-S6		32		Cell Signaling Technology	
PARP		116/89		Santa Cruz	
RSK		90		Cell Signaling Technology	
S6		32		Cell Signaling Technology	
α -Tubulin	Mouse	50		Sigma-Aldrich	

Table 7. Secondary antibody specifications for Western Blot.

Target Molecule	Dilutions	Supplier	Incubation conditions
Secondary Antibody			
Anti-Mouse-HRP	1:10000	BioRad	1h RT
Anti-Rabbit-HRP		BioRad	

5. Statistical Analysis

Data was statistically analysed using GraphPad Prism version 10.2.3 software. Normality test was performed using Shapiro-Wilk test, to see the distributions of each condition, one-way ANOVA, with Tukey's for multiple comparison analysis was used, with a confidence interval of 95%. P-value inferior of 0,05 was required for statistical significance.

IV. PRELIMINARY DATA

When I joined the group this project was already ongoing and the CRISPR-Cas9 screen in NCI-N87 cells using the genome-wide Brunello sgRNA library and its respective bioinformatics analysis had already been performed. The results gathered until the beginning of my internship included the identification of 4 genes that conferred resistance to lapatinib and more than 20 genes that conferred sensitivity (**Figure 6**).

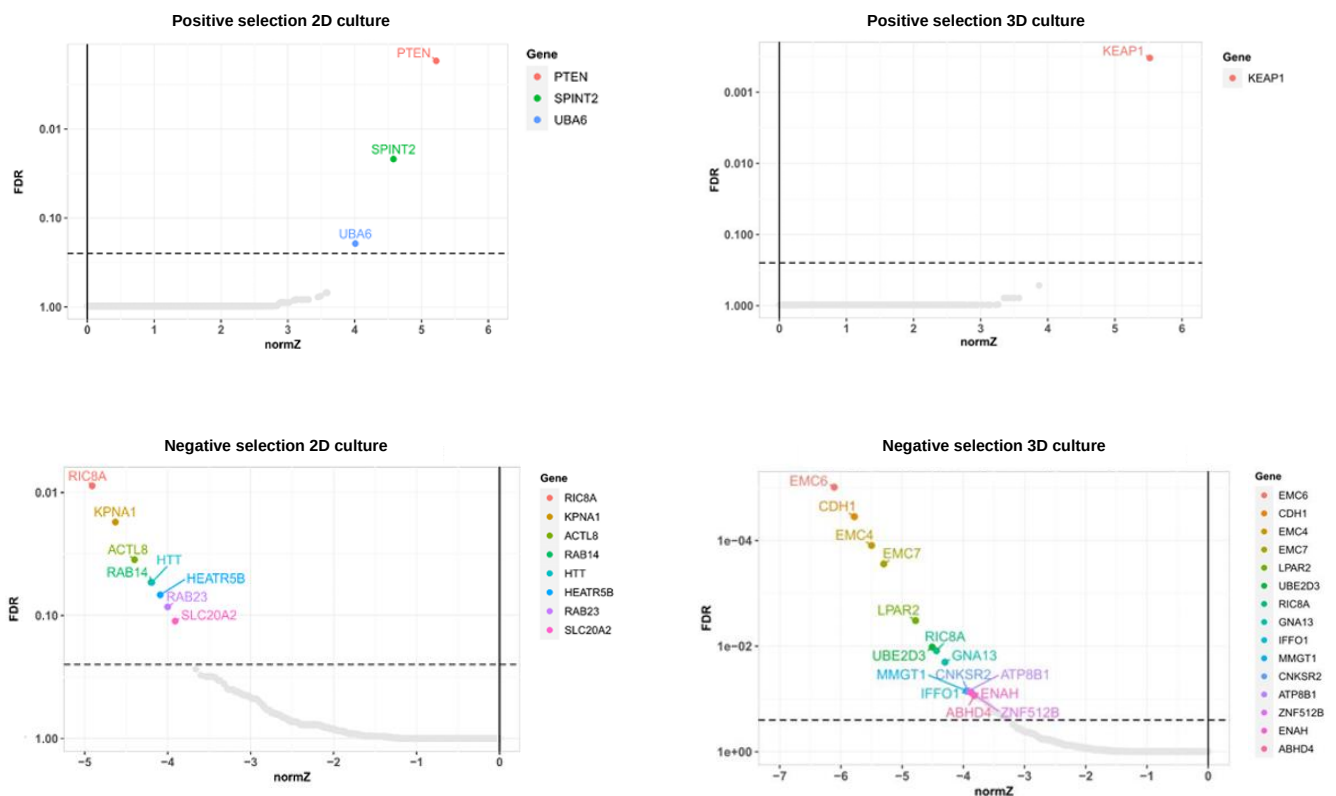


Figure 6. Significance plots for negative and positive selection, used to identify hits from the Lapatinib screen in the 2D and 3D cultures (x-axis = normalised read counts, y-axis = false discovery rate).

It was also already known that in the clinic trastuzumab is the only anti-HER2 therapy approved as a first-line of treatment for HER2-positive GC patients [41], however, *in vitro* trastuzumab has been shown not to affect cell viability [115, 116]. To validate this, a Drug Response Curve Assay using concentrations between 1µg/mL and 100µg/mL and a Western Blot for Trastuzumab treatment was performed, (**Figure 7**), confirming the data in the literature. It is because of these results that Lapatinib (and other HER2 TKIs) have been used in this study as a HER2 inhibitor instead of Trastuzumab.

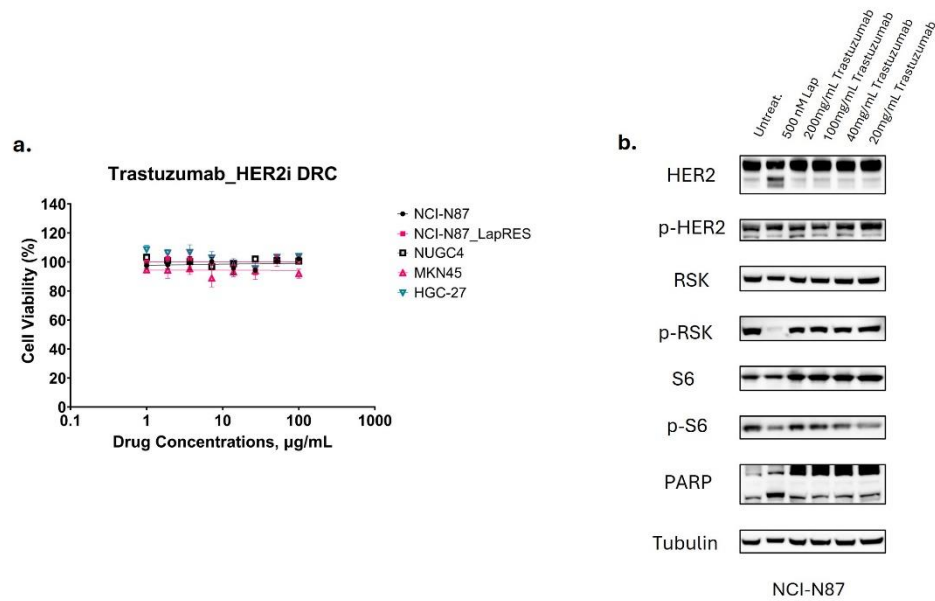


Figure 7. Response to Trastuzumab a) Drug Response Curve of Trastuzumab for NCI-N87, NCI-N87 Lapatinib Resistant, NUGC4, MKN45 and HCG-27 cell lines. Cells were treated with increasing concentrations of Trastuzumab, between 1mg/mL and 100mg/mL. b) Western Blot of effect of increasing concentrations of trastuzumab in NCI-N87 cell line, HER2 (185 kDa), p-HER2 (185 kDa), RSK (90 kDa), p-RSK (90kDa), S6 (32 kDa), p-S6 (32 kDa), PARP (116-89 kDa), Tubulin (52kDa).

V. RESULTS

1. Database analysis

In order to prioritize screen hits for validation, we analysed all the previously mentioned screen hits using *cBioPortal* [117]. First, we evaluated the percentage of alterations of each gene in TCGA Gastric Cancer Cohort compared to the percentage of alterations in the TCGA Pan Cancer Cohort (**Table 8**) then it was performed a ration between GC and Pan Cancer. The 3 hits that have higher ratio, were the ones with higher percentage of alterations in GC when compared to TGCA Pan Cancer and they were ATP8B1, RAB14 and MMGT1, all of them were genes that conferred sensitivity in the screen.

Table 8. Table of percentage of alterations of the hit genes in TCGA panel and in Gastric Cancer

Top Hits	GENE	% Alteration_PanCancer	Number of samples (10967)	% Alteration_GC	Number of samples (440)	Ratio GC/PanCancer	Data of Analysis
1	ATP8B1	2,90%	315	8%	33	2,76	03/12/2024
2	RAB14	0,80%	92	2,00%	9	2,50	03/12/2024
3	MMGT1	1,10%	124	2,70%	12	2,45	03/12/2024
4	CSNK2B	1,40%	151	3,00%	13	2,14	03/12/2024
5	RNASE9	1,40%	158	3,00%	15	2,14	03/12/2024
6	SPTBN1	3,00%	380	6,00%	27	2,00	03/12/2024
7	LYZL1	1,00%	110	2,00%	9	2,00	03/12/2024
8	DAPK3	1,50%	166	3,00%	14	2,00	03/12/2024
9	RNASE6	1,20%	131	2,30%	10	1,92	03/12/2024
10	ABHD4	1,60%	170	3,00%	13	1,88	03/12/2024
11	ACTL8	1,60%	173	3,00%	13	1,88	03/12/2024
12	KDSR	1,70%	184	3,00%	15	1,76	03/12/2024
13	EMC1	1,70%	191	3,00%	14	1,76	03/12/2024
14	PTEN	7,00%	329	12,00%	51	1,71	03/12/2024
15	CNKS2R	3%	354	5%	24	1,67	03/12/2024
16	CD58	1,80%	199	3,00%	13	1,67	03/12/2024
17	EMC6	0,70%	82	1,10%	5	1,57	03/12/2024
18	ZNF512B	4%	392	6%	27	1,50	03/12/2024
19	ARHGEF19	2,70%	77	4,00%	16	1,48	03/12/2024
20	RAB23	1,40%	151	2,00%	9	1,43	03/12/2024
21	UBA6	2,10%	231	3,00%	13	1,43	03/12/2024
22	CDH1	8,00%	391	11,00%	48	1,38	03/12/2024
23	EMC3	1,50%	162	2,00%	9	1,33	03/12/2024
24	ENAH	3%	332	4%	19	1,33	03/12/2024
25	TOP3B	2,40%	69	3,00%	14	1,25	03/12/2024
26	GNA13	2,20%	239	2,70%	12	1,23	03/12/2024
27	HEATR5B	5,00%	131	6,00%	25	1,20	03/12/2024
28	RIC8A	1,70%	183	2,00%	9	1,18	03/12/2024
29	HTT	7,00%	194	8,00%	34	1,14	03/12/2024
30	RGS4	4%	403	4%	16	1,00	03/12/2024
31	ISLR	2,00%	56	2,00%	9	1,00	03/12/2024
32	SPINT2	3,00%	89	3,00%	13	1,00	03/12/2024
33	IFFO1	3,00%	86	3,00%	14	1,00	03/12/2024
34	EMC7	1,30%	138	1,1%	5	0,85	03/12/2024
35	RPS19BP1	0,60%	65	0,50%	2	0,83	03/12/2024
36	TDG	1,10%	116	0,90%	4	0,82	03/12/2024
37	SLC20A2	5,00%	521	4,00%	18	0,80	03/12/2024
38	LPAR2	1,40%	148	1,10%	5	0,79	03/12/2024
39	KEAP1	4%	441	3%	14	0,75	03/12/2024
40	EMC4	1%	109	0,70%	3	0,70	03/12/2024
41	F11R	4,00%	447	2,70%	12	0,68	03/12/2024
42	S100A12	4%	392	2,00%	9	0,50	03/12/2024
43	UBE2D3	0,40%	10	0,20%	1	0,50	03/12/2024
44	KPNA1	2,10%	61	0,50%	2	0,24	03/12/2024

For the following analysis, also using *cBioPortal*, we tried to identify which genes were more altered in HER2-positive GC, when compared to all cases of GC. For that it was compared the percentage of alteration of TCGA Gastric Cancer Cohort with the TCGA Gastric Cancer HER2 amplified Cohort (**Table 9**). Similarly, to the previous result it was performed a ration between GC with HER2 amplified and GC. RAB23 seem to be the hit with more alterations in HER2-positive GC, followed by RSP19BP1 and KPNA1. Next, we analysed the overall survival (SUPPLEMENTARY RESULTS, **Table 14**), and in this case, a high number of hits do not have information regarding the survival in HER2-positive GC, due to the small amount of samples, so it was impossible to take meaningful conclusions about that analysis.

Table 9. Table with the percentage of each alteration in Gastric Cancer and Gastric Cancer with amplification of HER2

Top Hits	GENE	% Alteration_GC	Number of samples (440)	% Alteration_HER2_amp	Number of samples (58)	Ratio HER2_amp/GC	Data of Analysis
1	RAB23	2,00%	9	7,0%	4	3,50	03/12/2024
2	RPS19BP1	0,50%	2	1,7%	1	3,40	03/12/2024
3	KPNA1	0,50%	2	1,7%	1	3,40	03/12/2024
4	CSNK2B	3,00%	13	9,0%	5	3,00	03/12/2024
5	EMC7	1,1%	5	3%	2	2,73	03/12/2024
6	RIC8A	2,00%	9	5,0%	3	2,50	03/12/2024
7	S100A12	2,00%	9	5,0%	3	2,50	03/12/2024
8	EMC4	0,70%	3	1,7%	1	2,43	03/12/2024
9	KDSR	3,00%	15	7,0%	4	2,33	03/12/2024
10	ACTL8	3,00%	13	7,0%	4	2,33	03/12/2024
11	RNASE6	2,30%	10	5,0%	3	2,17	03/12/2024
12	TDG	0,90%	4	1,7%	1	1,89	03/12/2024
13	GNA13	2,70%	12	5,0%	3	1,85	03/12/2024
14	ENAH	4%	19	7,0%	4	1,75	03/12/2024
15	SLC20A2	4,00%	18	7,0%	4	1,75	03/12/2024
16	EMC1	3,00%	14	5,0%	3	1,67	03/12/2024
17	ABHD4	3,00%	13	5,0%	3	1,67	03/12/2024
18	RNASE9	3,00%	15	5,0%	3	1,67	03/12/2024
19	LPAR2	1,10%	5	1,70%	1	1,55	03/12/2024
20	EMC6	1,10%	5	1,7%	1	1,55	03/12/2024
21	CNKSRR2	5%	24	7,0%	4	1,40	03/12/2024
22	RGS4	4%	16	5%	3	1,25	03/12/2024
23	ARHGEF19	4,00%	16	5,0%	3	1,25	03/12/2024
24	ZNF512B	6%	27	7,0%	4	1,17	03/12/2024
25	ATP8B1	8%	33	9,0%	5	1,13	03/12/2024
26	MMGT1	2,70%	12	3%	2	1,11	03/12/2024
27	F11R	2,70%	12	3,0%	2	1,11	03/12/2024
28	KEAP1	3%	14	3,0%	2	1,00	03/12/2024
29	UBA6	3,00%	13	3,0%	2	1,00	03/12/2024
30	IFFO1	3,00%	14	3,0%	2	1,00	03/12/2024
31	EMC3	2,00%	9	1,7%	1	0,85	03/12/2024
32	RAB14	2,00%	9	1,7%	1	0,85	03/12/2024
33	DAPK3	3,00%	14	2,0%	1	0,67	03/12/2024
34	CD58	3,00%	13	1,7%	1	0,57	03/12/2024
35	SPINT2	3,00%	13	1,7%	1	0,57	03/12/2024
36	TOP3B	3,00%	14	1,7%	1	0,57	03/12/2024
37	SPTBN1	6,00%	27	3,0%	2	0,50	03/12/2024
38	CDH1	11,00%	48	5,0%	3	0,45	03/12/2024
39	PTEN	12,00%	51	5,0%	3	0,42	03/12/2024
40	HTT	8,00%	34	3,0%	2	0,38	03/12/2024
41	HEATR5B	6,00%	25	1,7%	1	0,28	03/12/2024
42	LYZL1	2,00%	9	0,0%	0	0,00	03/12/2024
43	ISLR	2,00%	9	0,0%	0	0,00	03/12/2024
44	UBE2D3	0,20%	1	0,0%	0	0,00	03/12/2024

After combining the results of these analyses, the significance ranking of the screen hits and literature research on the screen hits, we decided to validate the following hits: RIC8A and RAB14. RIC8A was the only hit simultaneously in the 2D and 3D screens, and regarding the parameters analysed didn't stand out in percentage of alterations but showed a significant reduction in OS. This hit was firstly described as an guanine nucleotide exchange factor (GEF) and a more recent study supported the hypothesis that this protein is also important for maintenance of appropriate levels of a subset of G protein subunits [118]. RAB14 stood out in the comparison of percentage of alterations in TCGA Pan Cancer and GC. It is part of the RAS oncogenic family which regulate membrane vesicle trafficking, signal transduction and recycling of various membrane receptors [119]. In particular, the Rab family have been found to play various roles during the development and progression of human cancers [120, 121].

2. Cell line characterization

In this work we used the NCI-N87 cell line to mimic HER2-positive GC that is responsive to anti-HER2 therapy and MKN74 to mimic HER2-negative GC (resistant to HER2i). Additionally, we also generated NCI-N87 cells resistant to Lapatinib (NCI-N87_LapRES, see methods) to mimic HER2-positive GC that develops resistance to anti-HER2 therapy. The morphology of these cell lines is quite different; NCI-N87 are much smaller than MKN74 cells, however, both of them form colonies with well-defined edges. The NCI-N87 cells made resistant to Lapatinib have the same morphology as the parental cells (**Figure 8**).

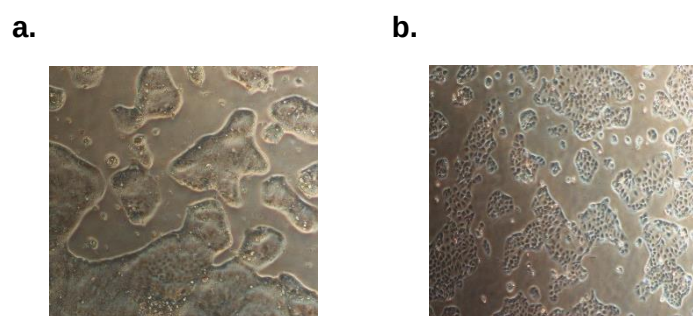


Figure 8. Morphology of cell line models. a) NCI-N87 cell line. b) MKN74 cell line. Both images were taken in an optical microscope at a 40x magnification.

To validate our hypothesis that there might be genetic alterations that affect the efficacy of anti HER2 therapy, we first characterized the cell lines according to their sensitivity to Lapatinib (an HER2 and EGFR inhibitor), Dacomitinib (also a dual inhibitor) and Tucatinib (a HER2-specific inhibitor) (**Figure 9**). This assay was also done for all the GC cell lines available in the lab (SUPPLEMENTARY RESULTS, **Figure 19**). Our results are in concordance with what is already known in literature; NCI-N87 cells are the most sensitive to HER2 inhibitors because they have the highest expression of HER2 (SUPPLEMENTARY RESULTS, **Figure 20**) and MKN74 the ones that responds the less [122, 123]. Of note, even though NCI-N87_LapRES were generated by long term exposure to lapatinib, these cells also exhibited resistance to the other HER2 inhibitors Dacomitinib and Tucatinib.

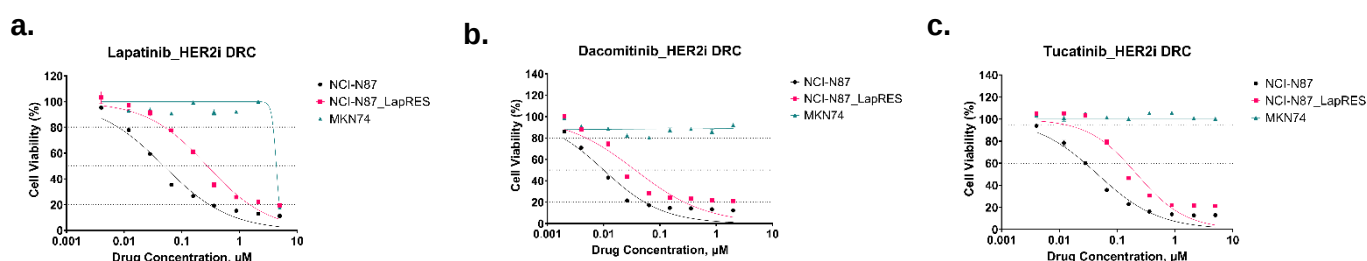


Figure 9. Dose Response Curves graphs for NCI-N87, NCI-N87 Lapatinib Resistant and MKN74 cell lines. a) Dose response curve for Lapatinib, with concentrations between 0,004 μ M and 5 μ M. b) Dose response curve for Dacomitinib, with concentrations between 0,002 μ M and 2 μ M. c) Dose response Curve for Tucatinib, with concentrations between 0,004 μ M and 5 μ M.

3. Pharmacological validation

Aiming to speed up the translation of our findings to the clinic we researched if any of our hits had pharmacological inhibitors available. RIC8A (Hit1) had an inhibitor available (IGS-1.76) but RAB14 didn't. Therefore, we selected another hit for this task - PTEN (Hit2), which conferred resistance in the screen and it is a very well-known gene in oncology that also has a pharmacological inhibitor available, BpV(HOpic).

To make sure that the cell death was not due to toxicity of the inhibitors, the first step was to characterize the sensitivity of the cells to RIC8A and PTEN inhibitors, similarly to what was done for the HER2 inhibitors (**Figure 10**). In this case, none of the cell lines presented a high sensitivity to any of the inhibitors. This was also performed for the other cell lines (SUPPLEMENTARY RESULTS, **Figure 21**).

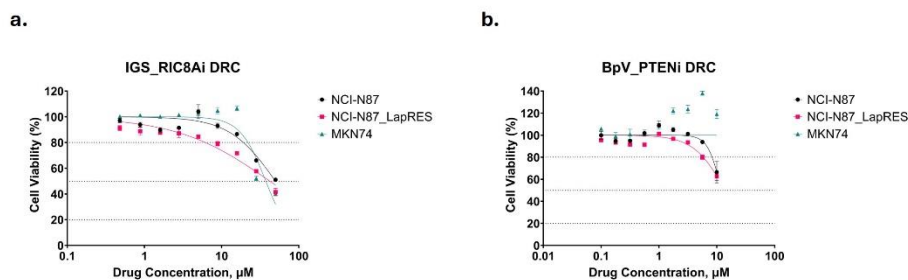


Figure 10. RIC8A and PTEN inhibitors Dose Response Curves for NCI-N87, NCI-N87 Lapatinib Resistant and MKN74 cell lines. a) Dose response curve for RIC8A inhibitor, IGS-1.76, with concentrations between 0,47μM and 50μM. b) Dose response curve for PTEN inhibitor, BpV(HOPic), with concentrations between 0,1μM and 10μM.

To assess if the combination of HER2i/ EGFRi and RIC8Ai or PTENi was synergistic, the cell viability matrixes were uploaded in SynergyFinger and analysed in GraphPad using the One-way ANOVA (SUPPLEMENTARY RESULTS, **Figure 22**). **Figure 11a** indicates that the combination of HER2i and RIC8Ai is not synergistic in NCI-N87 cells (scores are below 10). Regarding the combination of HER2i and PTENi (**Figure 11b**), we obtained synergy scores below -10 which indicates antagonism, therefore supporting the screen finding that PTEN loss induces resistance to HER2 inhibition. In **Figure 11c** it is possible to see an impact of Lapatinib and RIC8Ai in cell growth when compared to untreated, but when treated in combination the cells grow even less. In **Figure 11d** it was expected to see a rescue in cell growth when cells are treated with Lapatinib and PTENi but that is not very evident. Western blot was performed to evaluate the effect of the combinations in the PI3K and MAPK pathways, by evaluating the total and phosphorylated levels of HER2, RSK and S6, to assess the effect of the single agents and combinations in the downstream signalling pathway; besides these, we also blotted for PARP to evaluate if there is more apoptosis, assessed by the levels of its cleaved form (lower band) (**Figure 11f**). We didn't observed very evident changes in the WB the only result important to me mentioned is a very evident increment in cleaved PARP in the combination of Lapatinib with PTENi, as it is possible to see in the WB quantification in **Figure 11g**,

MKN74 cells were also treated with combinations of HER2i/ EGFRi and RIC8Ai (SUPPLEMENTARY RESULTS, **Figure 23a**); in this case we did not use PTENi since the cells do not have a significant cell growth decrease with HER2i/ EGFRi and the rescue effect was not observed in the optimization process (SUPPLEMENTARY RESULTS, **Figure 23b**). Similarly to what was done in NCI-N87 cell line, the cell viability matrixes were uploaded in SynergyFinger and analysed in GraphPad using One-way ANOVA. In **Figure 12a** it is possible to observe the graph with different drug combinations and there is a statistically significant difference between the combination of RIC8Ai with Lapatinib and

Tucatinib (p-value of 0,0219), having a synergistic effect in combination with Lapatinib (score higher 10), which we can also observe in the cell growth assay (SUPPLEMENTARY RESULTS, **Figure 23c**). Regarding the effect in the biochemistry, we blotted for the same proteins as for NCI-N87 to assess the activity of the MAPK and PI3K pathways, but we could not observe any significant differences in any of the conditions, as it is possible to see in the **Figure 12b-c**.

The treatment with combinations of HER2i/ EGFRi and RIC8Ai or PTENi was also performed in the remaining GC cell lines (SUPPLEMENTARY RESULTS, **Figure 24**). Importantly, we observed a significant difference in the combination of Lapatinib with RIC8Ai when compared to Tucatinib with RIC8Ai in NUGC4 and MKN45 cell lines (SUPPLEMENTARY RESULTS, **Figure 24c, e**), being the higher synergy score when the treatment was with Lapatinib and RIC8Ai, similarly to what is presented in MKN74 cell line, which could mean that a dual inhibition is necessary to have a better response to the treatment.

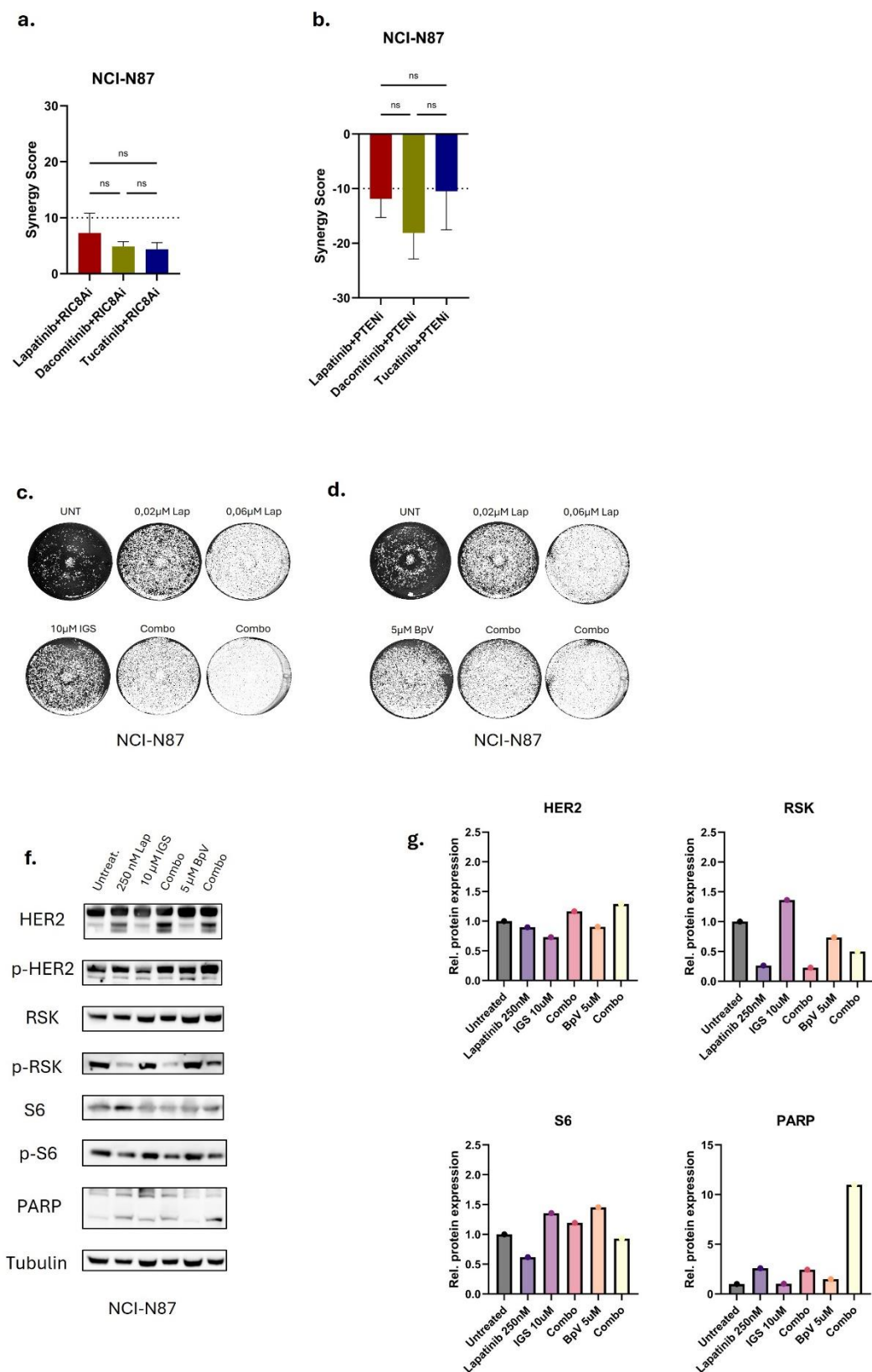


Figure 11. Pharmacological Validation in NCI-N87. Synergy graph of HER2i and EGFRi with RIC8Ai (a) or PTENi (b). Results from three independent experiments. Long Term Colony Formation with combinations of Lapatinib and IGS (c) or BpV (d), cells were seeded and treated with the combinations every 48h until the end of 3 cycles of treatment, followed by fix and staining with formaldehyde 5% and Crystal Violet 0.5%, respectively. Blots of proteins from the PI3K and MAPK pathways (f) and quantification of blotted proteins (g), results from one experiment.

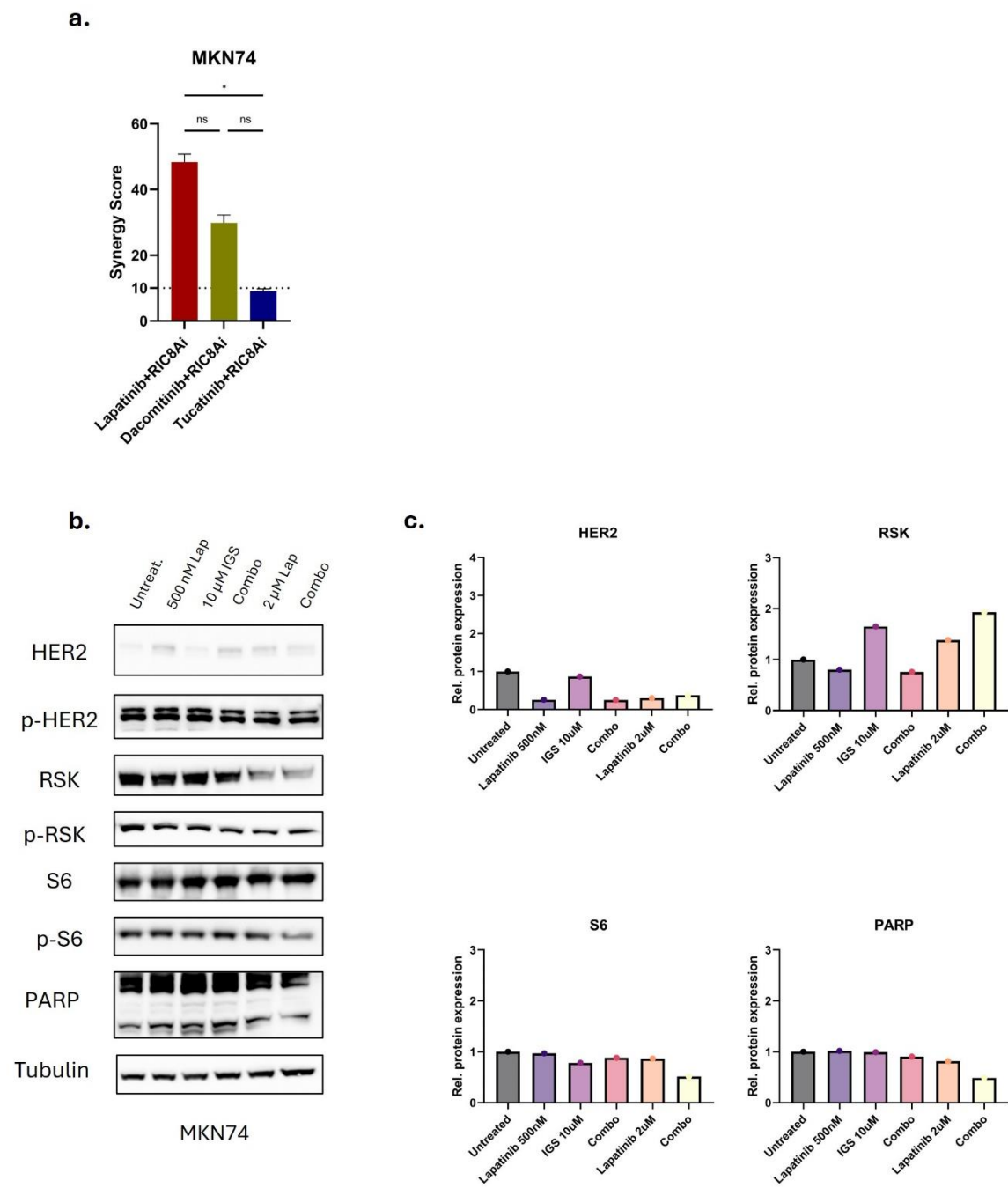


Figure 12. Pharmacological Validation in MKN74 cell line. Synergy graph of HER2i and EGFRi with RIC8Ai (a). Results from three independent experiments. Blots of proteins from the PI3K and MAPK pathways (b) and quantification of blotted proteins(c), results from one experiment.

4. Genetic validation

To validate the screen hits genetically, we used CRISPR/Cas9 to generate RIC8A and RAB14 KO cells. As a control we used LHX5 because this gene is not expressed in our cell lines since it encodes for a protein involved in the differentiation and development of the forebrain.

4.1 Cas9 expression

We started by expressing Cas9 in our cell line panel. After infection with the lentivirus produced from the LentiCas9-Blast (Addgene #52962), Cas9 expression was assessed using Western-Blot, which confirmed that all the cell lines had Cas9 expression (**Figure 13**).

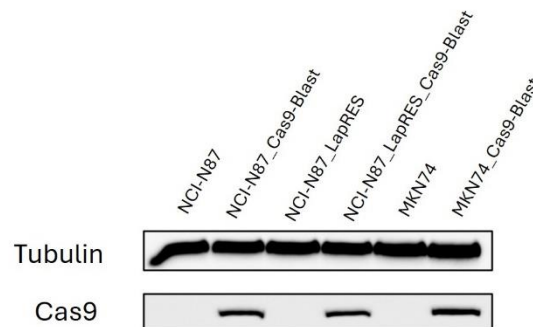


Figure 13. Confirmation of Cas9 Expression in NCI-N87, NCI-N87 Lapatinib Resistant and MKN74 cell lines. Tubulin (52kDa) was used as a housekeeping gene for Cas9 (70kDa).

4.2 Puromycin titration

Another important step before performing the KO was to optimize the concentration of Puromycin that would be used to select the cells carrying our construct-of-interest. We performed a titration with increasing concentrations in both cell lines. The goal was to find the minimum concentration of Puromycin that killed all cells. In NCI-N87 cell line, as observed in the **Figure 14a**, at the concentration of 1,8µg/mL there was still some cells but there was none in the 2µg/mL concentration, so that was the one selected. In MKN74 cell line, as shown in the **Figure 14b**, at the concentrations of 2µg/mL and 2,2µg/mL there were still some cells so we selected the concentration of 2,5µg/mL.

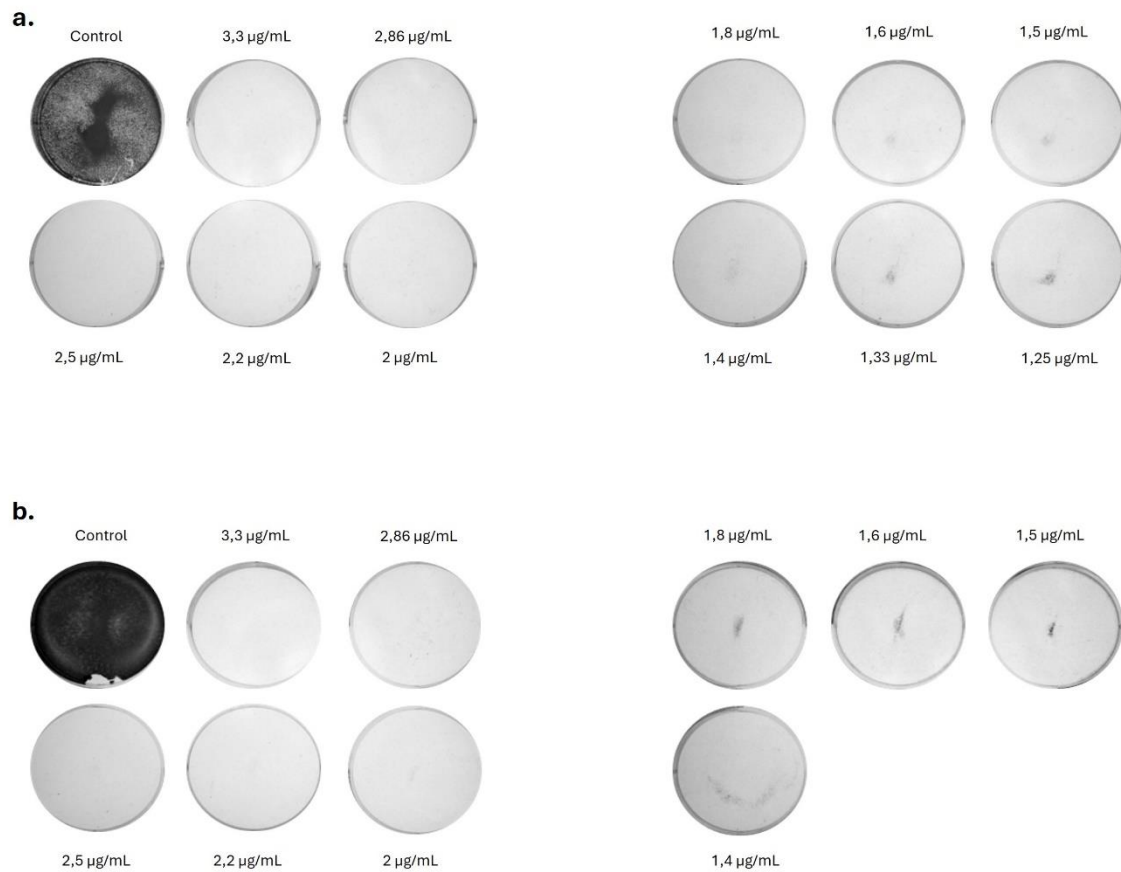


Figure 14. Puromycin titration for NCI-N87 (a) and MKN74 (b) cell lines. Cells were seeded and 24h later treated with increasing concentrations of puromycin, 48h later they were fixed and stained with Formaldehyde 5% and Crystal Violet 0,5%, respectively.

4.3 KO confirmation

After KO generation we assessed the presence of the KO and using TIDE, a software that precisely determine the spectrum and frequency of targeted mutations generated in a pool of cells by genome editing [104] , we determined the percentage of editing that was achieved in the polyclonal and monoclonal cell lines (**Table 10**). In the polyclonal cell lines, with the exception of the NCI-N87 cell line with LHX5 KO we accomplished an editing efficiency of at least around 50%.

We selected five monoclonal per each polyclonal cell line; after sequencing and determining the percentage of editing we used the ones with higher percentages for the following experiments. Interestingly, the percentage of editing in MKN74 cell line was much lower than in NCI-N87.

Another aspect that was taken into consideration was the morphology of the polyclonal cell lines. It was possible to observe an alteration in colony hedges in NCI-N87 in the presence of KO in RIC8A (**Figure 15a**).

Table 10. Table with the percentage of edition in the polyclonal and monoclonal cell lines. In grey there are the polyclonal cell lines and at white the monoclonones derived from that polyclonal cell lines.

Cell Line	Edition %
NCI_poli_RIC8A	54%
NCI_RIC8A_clone1	2,50%
NCI_RIC8A_clone2	2,90%
NCI_RIC8A_clone3	84,90%
NCI_RIC8A_clone4	29,70%
NCI_RIC8A_clone5	92,30%
NCI_poli_RAB14	50,40%
NCI_RAB14_clone1	76,30%
NCI_RAB14_clone2	72,10%
NCI_RAB14_clone3	25,20%
NCI_RAB14_clone4	18,10%
NCI_RAB14_clone5	26,20%
NCI_poli_LHX5	29,80%
NCI_LHX5_clone1	32,20%
NCI_LHX5_clone2	14,50%
NCI_LHX5_clone3	28,30%
NCI_LHX5_clone4	32,70%
NCI_LHX5_clone5	23,30%
MKN74_poli_RIC8A	41,20%
MKN74_RIC8A_clone1	25%
MKN74_RIC8A_clone2	40,50%
MKN74_RIC8A_clone3	67,80%
MKN74_RIC8A_clone4	32,70%
MKN74_RIC8A_clone5	8,60%
MKN74_poli_RAB14	49,10%
MKN74_RAB14_clone1	22,80%
MKN74_RAB14_clone2	20,80%
MKN74_RAB14_clone3	44,90%
MKN74_RAB14_clone4	44,40%
MKN74_RAB14_clone5	33%
MKN74_poli_LHX5	55,10%
MKN74_LHX5_clone1	-
MKN74_LHX5_clone2	-
MKN74_LHX5_clone3	7,60%
MKN74_LHX5_clone4	22,10%
MKN74_LHX5_clone5	-

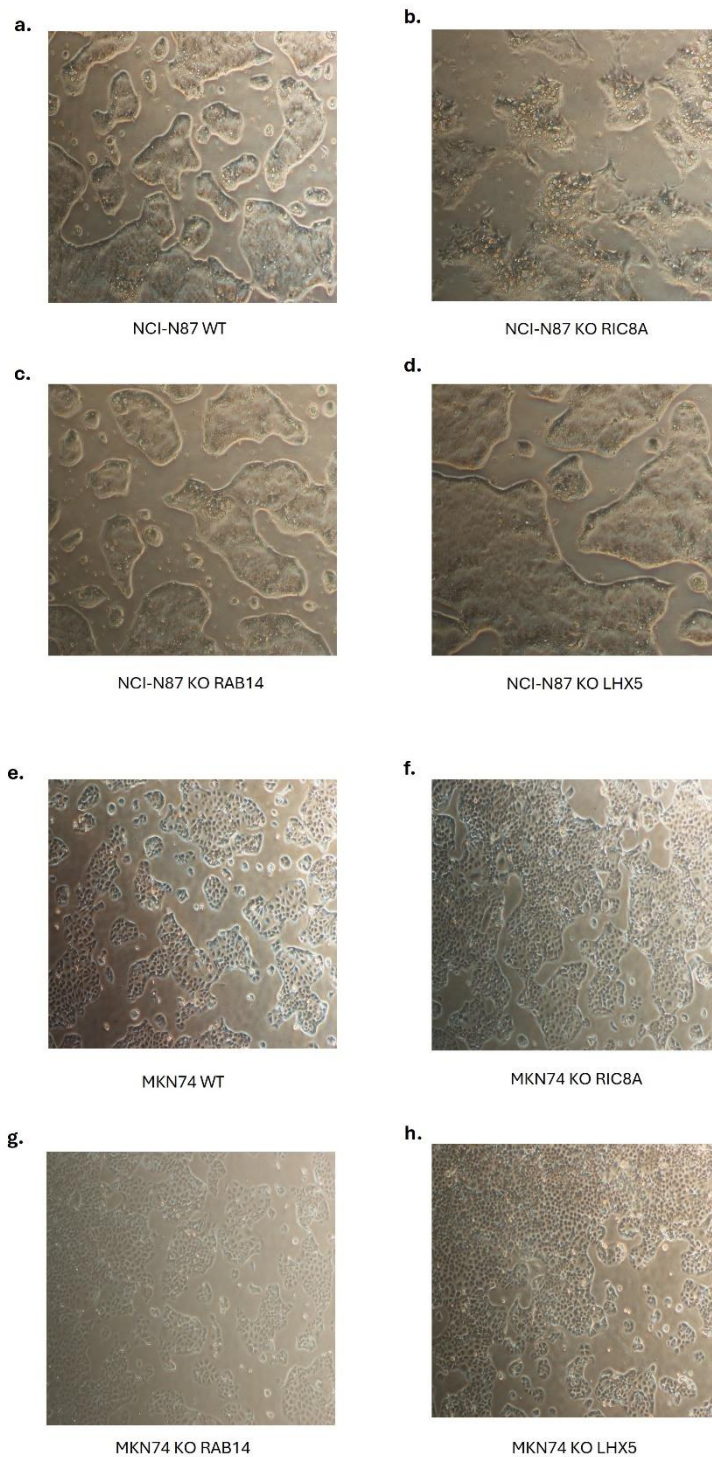


Figure 15 Morphology of polyclonal cell lines. a) NCI-N87 WT, b) NCI-N87 KO RIC8A, c) NCI-N87 KO RAB14, d) NCI-N87 WT LHX5, e) MKN74 WT, f) MKN74 KO RIC8A, g) MKN74 WT RAB14, h) MKN74 WT LHX5. All images were taken in an optical microscope at a 40x magnification.

4.4 Functional assay

After KO confirmation we evaluated its effect on cell proliferation and survival when treated with HER2i/ EGFRi.

In a first step, cell proliferation and survival was evaluated with increased concentrations of Lapatinib. Cells were plated in a 96-well plate and treated for ~7 days (refreshing media and drugs every 48h), after which the cells were fixed and stained and it was possible to verify that all the cell lines (WT and KO ones) proliferated in similar ways (**Figure 16a**). To have a more quantitative assay, we dissolved the crystal violet staining in 30% acetic acid and measured its absorbance at 590nm and the results were in concordance to what was observed in the colony formation (**Figure 16b**). With this assay it was also possible to verify that the range of lapatinib concentrations was too high, and it was better to use a range between 10nM and 50nM where we should be able to see more differences.

After this assay, cells were plated in 6-well plates and treated with increasing concentrations of lapatinib for ~14 days (refreshing media and drugs every 48h). It was possible to verify that when compared to WT cell line, the cell line with KO RAB14 was the only one that showed a visible reduction in cell viability (**Figure 16c**) (IC₅₀ in WT was 42,22nM and with KO RAB14 was 35,62nM, **Table 11**). However there is no evident difference in the crystal violet staining in presence of the KO (SUPPLEMENTARY RESULTS, **Figure 27a**).

It was also important to verify if having a higher percentage of editing influenced the results, so we repeated the same assay in 96-well plates for the monoclonal cell lines with the same range of lapatinib concentrations and treated the cells for ~7day (refreshing media and drugs every 48h). After fluorescence measurement and analysis it was possible to verify that both of RIC8A clones show a reduction in cell viability (**Figure 16d**). However, when compared to the polyclonal cell line which has an IC₅₀ of 41,19nM the clone C3 showed a much more evident reduction in IC₅₀ to 18nM (**Table 11**; SUPPLEMENTARY RESULTS, **Figure 27b**). In the case of RAB14, the clone C1 is the one with more evident reduction in IC₅₀ to 8,3nM (**Table 11**; SUPPLEMENTARY RESULTS, **Figure 27c**). Besides these clones, the monoclonal of LHX5 also showed reduction in cell viability, however it was not significant (**Figure 16d**). Regarding the cell proliferation, no significant change was identified, similar to what happened in the polyclonal cell lines (SUPPLEMENTARY RESULTS, **Figure 27d**).

Subsequently, we evaluated the KO effect in cell proliferation and survival when treated, in 3D spheroid cultures. However, the results cannot be reliable since, for example,

we obtained lower cell counts after 7 days in culture then the number of cells that were originally plated (and in the microscope it was visible that the spheroids had grown in size). These results could be due to the difficulty in counting these cells since they grow in clumps which difficults putting them in single cell to use the Cell Counter; besides that, since they are in clumps it could be more difficult for the resazurin to assess the core, thereby affecting the results (SUPPLEMENTARY RESULTS, **Figure 28**).

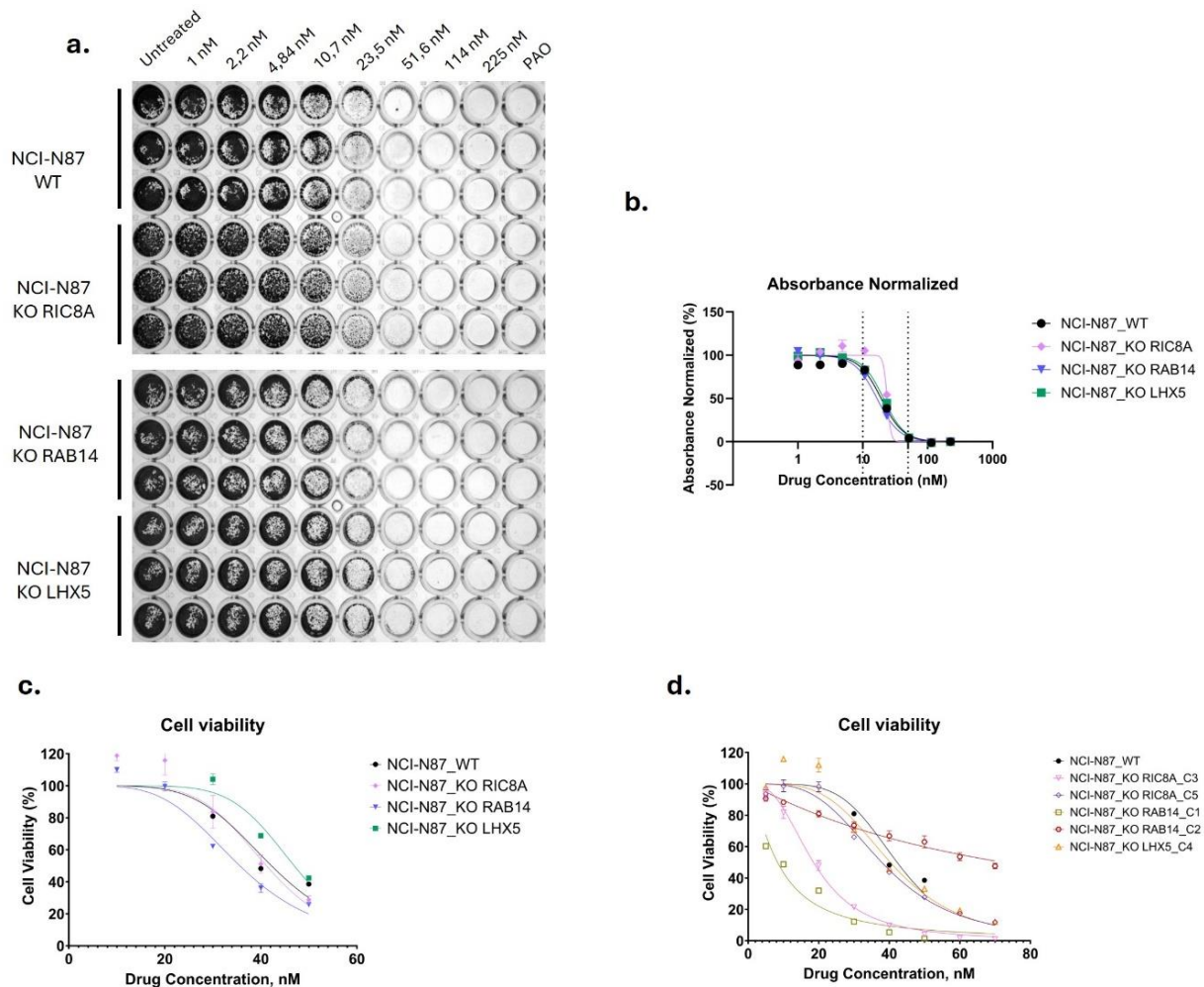


Figure 16 Genetic validation in NCI-N87 cell line. Colony Formation with increasing concentrations of lapatinib between 1nM-225nM, cells were seeded and treated with the combinations every 48h until the end of 3 cycles of treatment, followed by fix and staining with formaldehyde 5% and Crystal Violet 0,5%, respectively (a). Graph of Crystal Violet absorbance, measured at 590nm, results from three replicates (b). Cell Viability graph of polyclonal cell lines (c) and monoclonal cell lines (d) after treatment with lapatinib and incubated at 37°C for 1h-2h with resazurin (1x). Both of the graphs are results from three independent experiments.

Table 11. IC50 values of Lapatinib in NCI-N87 cell lines. Polyclonal ones and monoclones

Cell Line	IC50 Lapatinib (nM)
NCI-N87 WT	42,22
NCI-N87 KO RIC8A	41,19
NCI-N87 KO RIC8A C3	18,51
NCI-N87 KO RIC8A C5	37,87
NCI-N87 KO RAB14	35,62
NCI-N87 KO RAB14 C1	8,31
NCI-N87 KO RAB14 C2	72,23
NCI-N87 KO LHX5	46,95
NCI-N87 KO LHX5 C4	40,28

Regarding MKN74 cell line, we followed the same setup as for the NCI-N87 cell line; the cells were plated in a 96-well plate and treated with increasing concentrations of lapatinib for ~7 days (refreshing media and drugs every 48h). Surprisingly, the behaviour of the monoclones was different from what was previously demonstrated in **Figure 9a**, since with 100nM of Lapatinib 80% of the cells were already dead (**Figure 17a**).

After this assay, the polyclonal cells were plated in 6-well plates and treated with increasing concentrations of lapatinib for ~14day (refreshing media and drugs every 48h), followed by analysis. In the polyclonal cells there was a significant reduction in IC50 from 5,4µM in the WT to 3,7µM in the KO in RAB14 (**Figure 17a, Table 12**), was also possible to observe a reduction in cell viability with KO in this gene especially in the higher concentrations of the drug (**Figure 17b**).

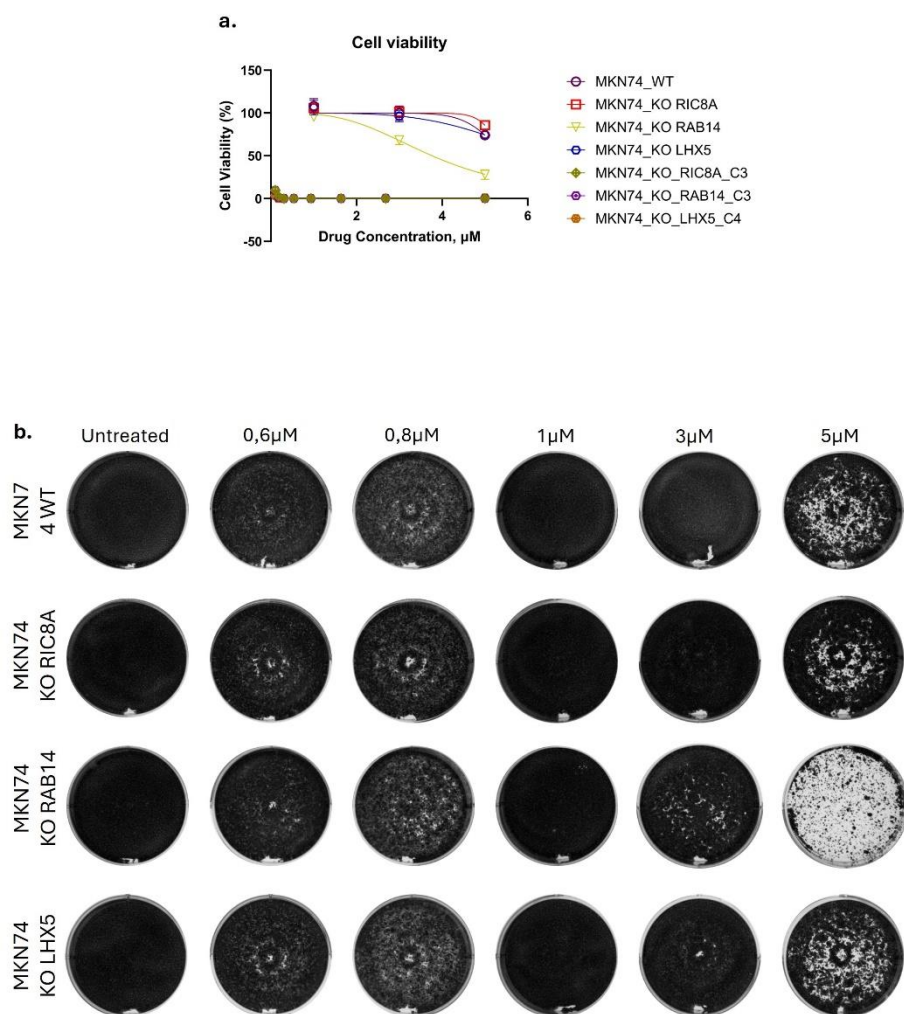


Figure 17. Genetic Validation in MKN74 cell line. Cell Viability graph of polyclonal cell lines and monoclonal cell lines (a) after treatment with lapatinib. Results from three independent experiments. Long Term colony formation with increasing concentrations of Lapatinib (b), cells were seeded and treated with the combinations every 48h for ~14 days, followed by fix and staining with formaldehyde 5% and Crystal Violet 0,5%, respectively

Table 12. IC50 values of Lapatinib in MKN74 cell lines. Polyclonal ones and monoclones

Cell Line	IC50 Lapatinib (μM)
MKN74 WT	5,46
MKN74 KO RIC8A	5,44
MKN74 KO RIC8A C3	0,042
MKN74 KO RAB14	3,77
MKN74 KO RAB14 C3	0,053
MKN74 KO LHX5	6,39
MKN74 KO LHX5 C4	0,046

VI. DISCUSSION

Gastric Cancer is a serious public health problem, being the third most deadly cancer in Portugal [124]. The high mortality rate is mostly due to the absence of significant symptoms in early stages and the lack of screening programs. Because of this, it is generally only diagnosed at an advanced stage of the disease, where effective treatment options are scarce [33].

Approximately 1 in 5 gastric cancer tumours have overexpression of HER2 [5, 6], which correlates with poorer outcomes and more aggressive disease [52, 59]. Of the patients that can benefit from anti-HER2 therapy a huge number of them exhibit primary resistance, and even the ones that initially respond to the treatment eventually develop a secondary resistance [81, 82].

Aiming to identify novel genes involved in anti-HER2 therapy resistance and sensitivity in HER2-positive GC, a genome-wide CRISPR screen had recently been performed in the lab. The main goal of this project was to validate some of the hits obtained in the screen pharmacologically and genetically.

In the pharmacological validation, we aimed to explore the roles of PTEN and RIC8A in resistance and sensitivity, respectively, to anti-HER2 therapy. PTEN functions as a traditional tumour suppressor, primarily regulating the PI3K/AKT pathway to maintain cellular homeostasis. Its activity is frequently disrupted in a significant number of human cancers [125] and there is evidence that the silencing of PTEN promotes metastatic behaviour and therapy resistance in highly aggressive cancers [126, 127]. Our screening results support this, showing that PTEN loss may lead to resistance to anti-HER2 therapy in HER2-positive gastric cancer. In synergy assays conducted on NCI-N87 cells, we observed a strong indication of resistance, with synergy scores consistently below -10 in all drug combinations, suggesting an antagonistic interaction between PTEN loss and anti-HER2 therapy. However, this antagonistic effect was not observed in the other HER2 positive cell lines (NCI-N87 Lapatinib resistant and NUGC4). This suggests that a high threshold of HER2 expression could be necessary for PTEN loss to fully drive resistance to HER2-targeted therapies. Additional studies are necessary to assess the effect of PTEN in the response to therapy using KO since the available inhibitor might not be very specific.

In contrast to PTEN, RIC8A inhibition, as observed in our initial screen, increased sensitivity to anti-HER2 therapies. This goes along with a study that performed a CRISPR screen on an NSCLC cell line with an EGFR mutation [128], the authors identified RIC8A as the most significant synergistic hit from the screen, they verify that RIC8A has dual functions, acting both as a molecular chaperone and a guanine nucleotide exchange factor. Additionally, they concluded that RIC8A positively regulates YAP signalling, influencing

EGFR TKI sensitivity in EGFR-mutant NSCLC cells. As a result, targeting RIC8A could be a promising strategy to prevent EGFR TKI resistance in lung cancer. In our study, the majority of combinations yielded synergy scores above 10, indicating a strong synergistic effect when RIC8A is inhibited. The highest synergy was observed in combinations with Lapatinib and Dacomitinib, suggesting that dual inhibition of ERBB receptors may be necessary to fully exploit the therapeutic potential of RIC8A inhibition. This could imply that targeting multiple ERBB family members simultaneously provides a more robust anti-tumour response when RIC8A is suppressed. Interestingly, the most significant synergy was seen in HER2-negative cell lines, particularly MKN74. This is noteworthy because MKN74 is not typically reliant on HER2 expression, yet it displayed a strong synergistic response, especially evident in colony formation assays, where cell growth was significantly reduced. This suggests that RIC8A inhibition might sensitize even HER2-negative or low-HER2-expressing cancers to anti-HER2 therapies by affecting broader signalling pathways related to ERBB receptor activity. These results are very pertinent since around 80% of the patients with GC express low levels of HER2 and these results suggest a potential approach for overcoming challenges in therapy for these patients, once they can start benefit from anti-HER2 therapy when RIC8A is also inhibited. To follow up on these encouraging results we performed the KO, since the results from the pharmacological inhibitors could be due to off-target effects from the drug and not necessarily from the inhibition itself.

We successfully edited the cells, having achieved a satisfying percentage of gene editing. Following RIC8A knockout, we observed a surprising change in cell morphology, with the most pronounced phenotype seen in the NCI-N87 cell line. This aligns with existing literature, which indicates that RIC8A plays a critical role in processes such as gastrulation and neurogenesis in mice [129]. Additionally, studies have shown that RIC8A is essential for proper actin cytoskeleton organization [118, 130] consistent with the morphological alterations we observed in our cells. In terms of cell viability, the expected reduction was not evident in the polyclonal NCI-N87 knockout cells. However, in one of the monoclonal, a decrease in viability was noted. This result should be interpreted cautiously, as the monoclonal with the higher editing percentage unexpectedly showed greater viability. Thus, we cannot definitively attribute the viability reduction to the degree of gene editing. RIC8A was also identified as a hit in the 3D screen, leading us to assess its knockout effects in 3D spheroid culture. However, we faced challenges with cell counting and viability measurement. Since the cells formed clumps, it was difficult to obtain single cells which is imperative for an accurate counting. Moreover, resazurin, used to measure viability, was not metabolized as expected, likely because the assay (CellTiter-Blue) is optimized for 2D cultures, not 3D spheroids. Moving forward, we plan to repeat the 3D assay using CellTiter-

Glo, which is better suited for spheroid cultures [131] and refine our cell counting technique to address these challenges.

Knocking out RAB14 led to a significant reduction in cell viability in both NCI-N87 and MKN74 cell lines. In MKN74, this reduction in cell proliferation was also evident in colony formation assays, suggesting that the absence of RAB14 enhances the response to anti-HER2 therapy. These findings align with a study [132] showing that RAB14 is often overexpressed in gastric cancer, and its depletion inhibits AKT phosphorylation, thereby suppressing cell proliferation. Additionally, research on bladder cancer [133] demonstrated that RAB14 knockdown reduces the expression of classical autophagy markers, indicating its role in regulating cancer cell autophagy. The study also found that RAB14 suppression inhibits the AKT pathway, inducing autophagy and hindering malignant progression. This dual inhibition of the AKT/PI3K pathway when RAB14 is knocked out may explain the increased sensitivity to anti-HER2 therapy, even in cell lines that do not overexpress the ERBB receptor **Figure 18**.

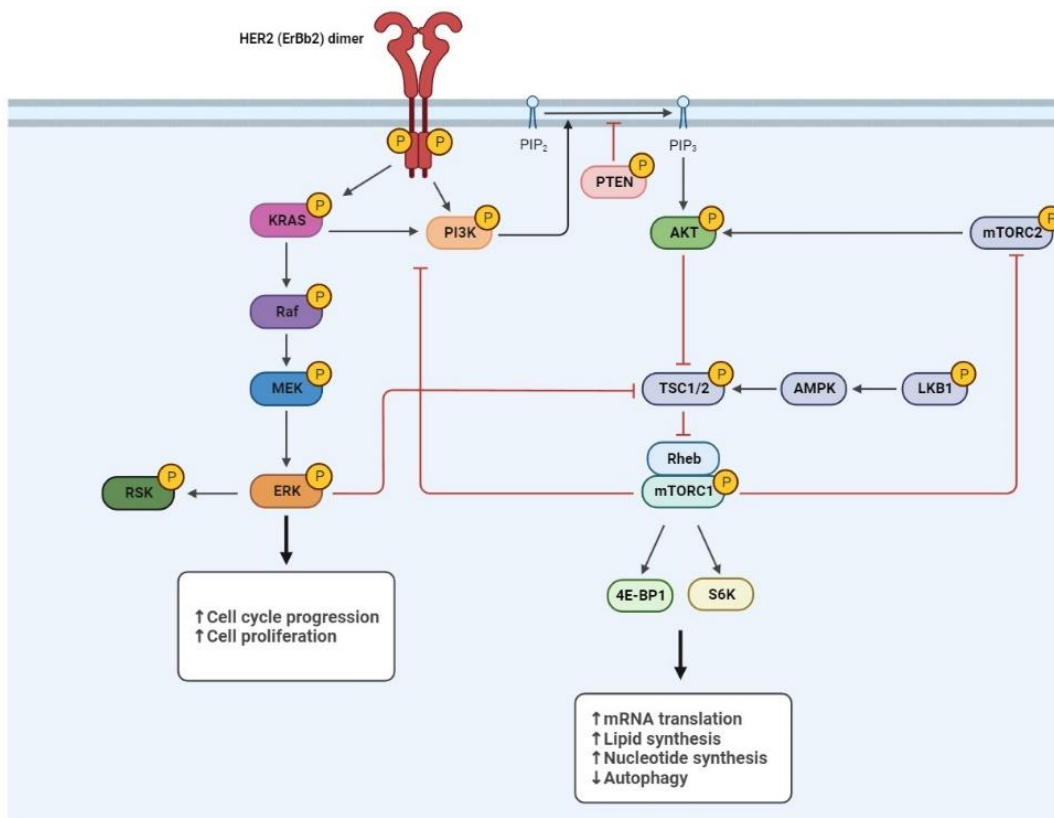


Figure 18. Overview of AKT/PI3K signalling pathway. Created with BioRender.com

We encountered several challenges with the growth and response of the MKN74 cell line to Lapatinib. While both the literature and our initial observations suggested that this cell line is resistant to Lapatinib, there were instances during the experiments where the cells exhibited unexpected sensitivity, dying at very low drug concentrations. As a result, it was difficult to assess the drug response in the monoclonal populations.

Given this variability, further studies are required. Particularly, we need to perform the genetic validation in more cell lines and, as planned for the NCI-N87 cell line, conduct 3D spheroid culture assays. Additionally, our future plans include conducting in vivo validation to confirm whether the results align with those observed in vitro, bringing the effects of Trastuzumab closer to clinical outcomes. Follow-up studies will also focus on uncovering the mechanisms of action behind the validated hits.

VII. CONCLUSIONS

Even though more work and additional studies are required, with this project we took a step forward in the understanding of the biology behind the lack of efficacy of anti-HER2 therapies in HER2-positive GC patients.

We identified two novel genes involved in the modulation of response to HER2 inhibition. We show that RAB14 loss sensitizes both HER2-positive and HER2-negative NCI-N87 and MKN74 cells, respectively, to HER2 inhibitor lapatinib. We have also shown encouraging data supporting that RIC8A may have a similar effect to RAB14. Additionally, our data supports previous reports showing that PTEN loss induces resistance to HER2 TKIs. Together, these three promising genes (PTEN, RAB14 and RIC8A) have potential to be used as biomarkers of predictive response to anti-HER2 therapy.

VIII. APPENDIXES

Appendix 1

Table 13. Summary of Clinical Trials. For patients with GC, including phase of the trial, drug that was tested and the target, the conditions (arms) used to test the drugs and the main conclusions.

Clinical Trial	Phase	Last update	Drug	Target	Arms	Conclusions
ToGA (NCT01041404) [4]	III	05-11-2014	Trastuzumab	HER2	Chemotherapy (ChT)	Improve in median Overall Survival (mOS) from 11,1 months in chemotherapy alone to 13,8 months in chemotherapy plus trastuzumab. FDA approved trastuzumab in combination with chemotherapy as a first-line of treatment for HER2-positive GC.
					Chemotherapy + Trastuzumab	
GATSBY (NCT01641939) [134]	II/III	12-05-2017	Trastuzumab emtansine	HER2	Trastuzumab emtansine + Taxane	Trial failed to show a survival benefit (mOS 7,9 months versus 8,6 months taxane alone).
					Taxane alone	
JOSHUA (NCT01461057) [135]	II	09-08-2018	Pertuzumab	HER2	Pertuzumab + trastuzumab + chemotherapy (with two doses of pertuzumab)	There was durable responses when combining higher doses of pertuzumab
JACOB (NCT01774786) [71]	III	30-12-2020	Pertuzumab	HER2	Pertuzumab + trastuzumab + chemotherapy	Trial failed to show improved OS in patients when added pertuzumab to trastuzumab plus chemotherapy
					Placebo + trastuzumab + chemotherapy	
LOGIC (NCT00680901) [73]	III	03-04-2024	Lapatinib	HER2	Lapatinib + Chemotherapy	Trial ongoing but no significant improvement in OS (12,2 months versus 10,5 months)
					Placebo + Chemotherapy	
TyTAN (NCT00486954) [74]	III	20-06-2013	Lapatinib	HER2	Lapatinib + Paclitaxel	There was no significant improvement in OS when added lapatinib to paclitaxel (OS 11,0 months versus 8,9 months).
					Paclitaxel alone	
DESTINY-Gastric01	II	18-03-2022	Trastuzumab deruxtecan	HER2	Trastuzumab deruxtecan	Results showed and improvement in OS with

(NCT03329690) [136]					Chemotherapy	Trastuzumab deruxtecan when compared with chemotherapy (mOS 12,5 months versus 8,4 months)
DESTINY-Gastric02 (NCT04014075) [137]	II	09-04-2024	Trastuzumab deruxtecan	HER2	Trastuzumab deruxtecan	Drug approved as second-line therapy in HER2-positive advanced GC with prior treatment trastuzumab-based
CP-MGAH22-05 (NCT02689284) [138]	I/II	04-08-2022	Margetuximab	HER2	Margetuximab + pembrolizumab (with two different doses of margetuximab)	The combination therapy showed adequate safety and tolerability and promising synergistic anti-tumour effect
EXPAND (2007-004219-75) [139]	III	17-02-2013	Cetuximab	EGFR	Cetuximab + chemotherapy	Failed to show a significant survival benefit of cetuximab when combined with chemotherapy, median progression-free survival (PFS) 4,4 months versus 5,6 months
					Chemotherapy alone	
REAL3 (NCT00824785) [140]	III	11-12-2012	Panitumumab	EGFR	Panitumumab + chemotherapy	Failed to show a significant survival benefit of panitumumab when combined with chemotherapy (mOS 8,8 months for P+ChT versus 11,3 months).
					Chemotherapy alone	
AVAGAST (NCT00548548) [141]	III	14-06-2017	Bevacizumab	VEGF	Bevacizumab + Chemotherapy	Trial failed to reach the primary endpoint of OS. The combination of bev+ChT was associated with significant increases PFS and overall response rate.
					Placebo + Chemotherapy	
AVATAR (NCT00887822) [141]	III	01-03-2027	Bevacizumab	VEGF	Bevacizumab + Chemotherapy	In AVATAR the addition of bevacizumab to ChT did not improve outcomes in Chinese patients, with no significant alterations in mOS and safety findings previously included in AVAGAST.
					Placebo + Chemotherapy	

REGARD (NCT00917384) [142]	III	25-09-2019	Ramucirumab	VEGF	Ramucirumab + Best Supportive Care (BSC)	Compared with placebo ramucirumab significantly improve mOS (mOS 5,2 months Ram+BSC versus 3,8 months placebo).
					Placebo + BSC	
RAINBOW (NCT01170663) [143]	III	18-09-2019	Ramucirumab	VEGF	Ramucirumab + paclitaxel	Combination of ramucirumab with paclitaxel significantly increase mOS when compared with the combination with placebo (mOS 9,6 months versus 7,4 months).
					Placebo + paclitaxel	
RAINFALL (NCT02314117) [144]	III	26-08-2021	Ramucirumab	VEGF	Ramucirumab + Cisplatin + Capecitabine	Trial failed in improve significantly the mOS with the addition of ramucirumab to ChT (11,2 months versus 10,7 months).
					Placebo + Cisplatin + Capecitabine	
INTEGRATE IIa (NCT02773524) [144]	III	13-01-2022	Regorafenib	VEGF	Regorafenib + BSC	Treatment with regorafenib improves the mOS when compared with placebo (mOS 4,5months versus 4,0 months).
					Placebo + BSC	
REGONIVO (NCT03406871) [145]	Ib	26-04-2021	Regorafenib	VEGF	Nivolumab + Regorafenib	Combination of regorafenib with nivolumab has a safety profile and encouraging anti-tumour activity
INTEGRATE IIb (NCT04879368) [144]	III	29-02-2024	Regorafenib	VEGF	Regorafenib + Nivolumab	Still ongoing with no published results yet
					Chemotherapy	
GRANITE-1 (NCT00879333) [146]	III	03-11-2015	Everolimus	mTOR	Everolimus + BSC	Everolimus did not improve significantly the mOS when compared with BSC (5,4 months everolimus versus 4,3 months BSC).
					Placebo + BSC	
RILOMET-1 (NCT01697072) [147]	III	09-02-2016	Rilotumumab	HGF	Rilotumumab + Chemotherapy	Adding rilotumumab to chemotherapy it was not benefit for MET positive GC, being the mOS worse when rilotumumab used (mOS 8,8 months versus 10,7 months).
					Placebo + Chemotherapy	
MET-Gastric	III	22-11-2016	Onartuzumab	MET	Onartuzumab + mFOLFOX6	The combination of onartuzumab to first-line

(NCT01662869) [148]					Placebo + mFOLFOX6	mFOLFOX6 had no significantly improvement in clinical benefits (mOS 11 months onartuzumab versus 11,3 months placebo).
GOLD (NCT01924533) [149]	III	22-03-2024	Olaparib	PARP	Olaparib + paclitaxel Placebo + paclitaxel	Trial did not met is first primary endpoint showing no significant improvement in OS with the combination of Olaparib with paclitaxel (mOS 5,26 months versus 4,14 months placebo).
ATTACTION-2 (NCT02267343) [150]	III	17-11-2021	Nivolumab	PD-1	Nivolumab Placebo	Trial showed promising results that nivolumab may be a treatment option for heavily pretreated patients.
CheckMate-032 (NCT01928394) [151]	I/II	10-01-2024	Nivolumab Ipilimumab	PD-1 CTLA-4	Nivolumab Nivolumab + Ipilimumab Nivolumab +Ipilimumab + Cobimetinib	Results showed that Nivolumab + Ipilimumab provide clinically relevant and durable anti-tumour activity with safety profile.
CheckMate-649 (NCT02872116) [152]	III	04-12-2023	Nivolumab	PD-1	Nivolumab + Ipilimumab XELOX (Oxaliplatin + Capecitabine) FOLFOX (Oxaliplatin + Leucovorin + Fluorouracil) Nivolumab + XELOX Nivolumab + FOLFOX	Nivolumab in combination with chemotherapy showed better results with a safety profile when compared with chemotherapy alone (mOS 13,8 months versus 11,6 months chemotherapy alone). Nivolumab plus chemotherapy represents a new standard first-line treatment for previously untreated patients with advanced gastric, gastro-oesophageal junction, or oesophageal adenocarcinoma
KEYNOTE-059 (NCT02335411) [153]	II	08-08-2022	Pembrolizumab	PD-1	Pembrolizumab monotherapy, previously treated Pembrolizumab + chemotherapy, treatment naïve	In monotherapy, pembrolizumab demonstrate promising anti-tumour activity and manageable safety profile.

					Pembrolizumab monotherapy, treatment naive, PD-L1 positive	
KEYNOTE-061 (NCT02370498) [154]	III	06-06-2022	Pembrolizumab	PD-1	Pembrolizumab	Pembrolizumab did not significantly improve OS when compared to paclitaxel (mOS 9,1 months versus 8,3 months)
					Paclitaxel	
KEYNOTE-181 (NCT02564263) [155]	III	13-03-2023	Pembrolizumab	PD-1	Pembrolizumab	When compared to chemotherapy, pembrolizumab improved mOS (9,3 months versus 6,7 months).
					Chemotherapy	
KEYNOTE-062 (NCT02494583) [156]	III	13-04-2023	Pembrolizumab	PD-1	Pembrolizumab Monotherapy	Pembrolizumab in monotherapy was not inferior to chemotherapy, however the combination of pembrolizumab plus chemotherapy did not reveal any survival benefit.
					Pembrolizumab + Chemotherapy	
					Placebo + Chemotherapy	
KEYNOTE-859 (NCT03675737) [157]	III	17-01-2024	Pembrolizumab	PD-1	Pembrolizumab + Chemotherapy	Combination of pembrolizumab with chemotherapy revealed a significantly and clinically relevant improvement in OS (mOS 12,9 months versus 11,5 months). FDA approved the combination of pembrolizumab with chemotherapy as first-line treatment of adults with locally advanced unresectable or metastatic HER2-negative gastric or gastroesophageal junction (GEJ) adenocarcinoma.
					Placebo + Chemotherapy	
KEYNOTE-811 (NCT03615326) [158]	III	05-01-2024	Pembrolizumab	PD-1	Pembrolizumab + Trastuzumab + Chemotherapy	Combination with pembrolizumab significantly increased PFS, specifically in patients with tumours with a PD-L1 combined positive score of 1 or more.
					Placebo + Trastuzumab + Chemotherapy	

JAVELIN Gastric 300 (NCT02625623) [159]	III	24-11-2020	Avelumab	PD-L1	Avelumab + BSC	Trial did not met the first endpoint of OS, however, avelumab showed a more manageable safety profile
					Physicians choice of chemotherapy + BSC	
JAVELIN Gastric 100 (NCT02625610) [160]	III	06-09-2022	Avelumab	PD-L1	Avelumab	Avelumab did not improved the OS when compared to chemotherapy (mOS 10,4 months versus 10,9 months chemotherapy)

IX. SUPPLEMENTARY RESULTS

Table 14. Table of Median Overall Survival of the screen hits

		Median Months Overall Survival (95% CI)				
		Gastric Cancer		HER2+ Gastric Cancer		
#	GENE	Amplification	Deletion	Amplification	Deletion	Date Analysis
1	EMC6	NA	25,53 (7,43-NA)	-	1,41	24/05/2024
2	CDH1	NA	12,36 (11,20-NA)	NA	NA	24/05/2024
3	EMC4	17,85 (17,84-NA)	18,21 (18,20-NA)	NA	18,21 (18,20-NA)	24/05/2024
4	EMC7	17,85 (17,84-NA)	14,01 (14,00-NA)	NA	18,21 (18,20-NA)	24/05/2024
5	LPAR2	18,35 (18,19-NA)	NA	NA	N/A	24/05/2024
6	UBE2D3	-	NA	-	-	24/05/2024
7	RIC8A	18,49 (18,22-NA)	18,17 (4,54-NA)	18,22 (18,20-NA)	4,53 (1,41-NA)	24/05/2024
8	GNA13	NA	31,24 (31,24-NA)	NA	-	24/05/2024
9	IFFO1	NA	31,24 (5,72-NA)	NA	-	24/05/2024
10	MMGT1	25,08 (2,30-NA)	NA	NA	N/A	24/05/2024
11	CNKSR2	17,51 (13,47-NA)	18,17 (10,26-46,22)	13,47 (13,47-NA)	10,45 (10,45-NA)	24/05/2024
12	ATP8B1	12,83 (12,81-NA)	22,82 (21,35-NA)	N/A	NA	24/05/2024
13	ZNF512B	27,37 (21,44-NA)	NA	21,45	-	24/05/2024
14	ENAH	17,87 (11,04-NA)	NA	3,49	-	24/05/2024
15	ABHD4	37,88 (18,15 - NA)	20,02 (20,01-NA)	NA	20,01 (20,01 - NA)	24/05/2024
16	DAPK3	NA	12,36 (11,43-NA)	-	1,38	24/05/2024
17	TOP3B	NA	20,01 (20,01-NA)	-	20,02	24/05/2024
18	F11R	NA	NA	11,78	-	24/05/2024
19	KDSR	44,71	22,83 (21,37-NA)	NA	NA	24/05/2024
20	ISLR	NA	2,89 (2,89-NA)	7,6	-	24/05/2024
21	RGS4	31,28 (31,24-NA)	9,86 (9,86-NA)	10,46 (2,66-NA)	N/A	24/05/2024
22	ARHGEF19	NA	NA	13,49	-	24/05/2024
23	RNASE9	37,88 (18,15-NA)	20,01 (20,01-NA)	NA	20,01 (20,01-NA)	24/05/2024
24	LYZL1	NA	5,72 (5,72-NA)	10,46	-	24/05/2024
25	TDG	18,19	-	NA	-	24/05/2024
26	S100A12	NA	83,24 (83,18-NA)	NA	-	24/05/2024
27	CD58	18,33 (18,28-NA)	14,01 (13,99-NA)	22,04	NA	24/05/2024
28	EMC3	18,75 (18,73-NA)	13,99 (7,04-NA)	NA	7,04 (7,04-NA)	24/05/2024
29	RPS19BP1	NA	NA	NA	-	24/05/2024
30	RNASE6	37,88 (18,13-NA)	20,01 (20,01-NA)	NA	20,01 (20,01-NA)	24/05/2024
31	EMC1	NA	NA	NA	-	24/05/2024
32	CSNK2B	52,57 (26,05-NA)	NA	26,05	-	24/05/2024
33	SPTBN1	NA	NA	NA	-	24/05/2024
34	KEAP1	15,79 (15,77-NA)	NA	15,79 (15,77-NA)	NA	24/05/2024
35	KPNA1	18,22 (18,20-NA)	14,01 (14,00-NA)	18,22	-	24/05/2024
36	ACTL8	NA	NA	NA	4,54 (4,53-NA)	24/05/2024
37	RAB14	9,95 (6,61-NA)	0,56 (0,56-NA)	9,05 (9,04-NA)	NA	24/05/2024
38	HTT	NA	25,53 (13,93-NA)	NA	-	24/05/2024
39	HEATR5B	NA	7,53 (7,53-NA)	8,28	-	24/05/2024
40	RAB23	26,05 (21,42-NA)	9,04 (9,03 - NA)	21,44 (7,99-NA)	9,04 (9,03-NA)	24/05/2024
41	SLC20A2	28,13 (21,44-NA)	NA	28,09 (21,42-NA)	NA	24/05/2024
42	UBA6	18,13 (9,31-NA)	NA	-	NA	24/05/2024
43	SPINT2	25,07 (23,98-28,13)	14,01 (13,99-NA)	28,09	-	24/05/2024
44	PTEN	NA	18,36 (14,00-35,90)	-	10,46	24/05/2024

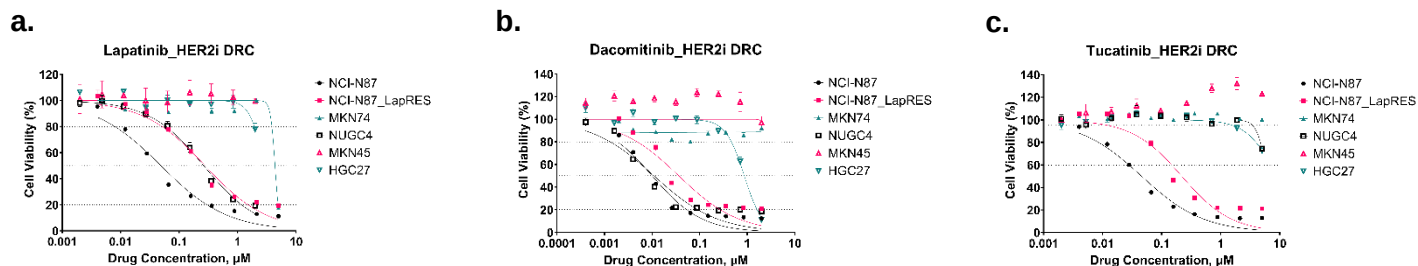


Figure 19. Dose Response Curves graphs for NCI-N87, NCI-N87 Lapatinib Resistant, NUGC4, MKN45, MKN74, HGC-27. a) Dose response curve for Lapatinib, with concentrations between 0,004 μ M and 5 μ M. b) Dose response curve for Dacomitinib, with concentrations between 0,002 μ M and 2 μ M. c) Dose response Curve for Tucatinib, with concentrations between 0,004 μ M and 5 μ M.

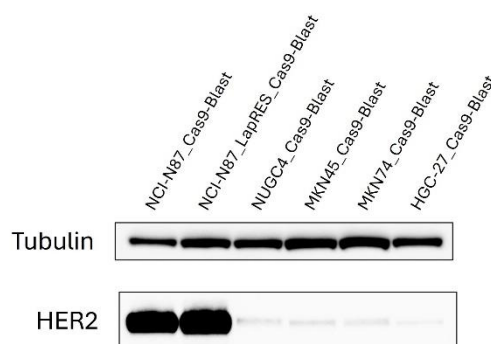


Figure 20. HER2 expression in the cell lines used in study. HER2 (185kDa) expression assed with Western Blot and Tubulin (52kDa) was used as housekeeping gene.

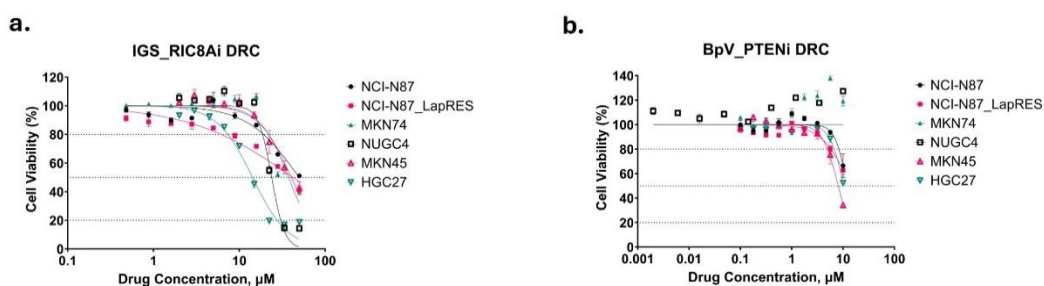


Figure 21. Dose Response Curve for Hit inhibitors for NCI-N87, NCI-N87 Lapatinib Resistant and MKN74 cell lines. a) Dose response curve for RIC8A inhibitor, IGS-1.76, with concentrations between 0,47 μ M and 50 μ M. b) Dose response curve for PTEN inhibitor, BpV(HOpic), with concentrations between 0,1 μ M and 10 μ M.

a.		Lapatinib (μM)					
IGS (μM)			0,004	0,01	0,017	0,024	
		100	91,03	79,61	69,49	61,77	
	5	93,3	86,91	73,38	61,98	55,37	
	10	89,94	81,7	68,92	59,32	52,69	
	15	86	75,84	63,37	56,24	49,7	
		Dacomitinib (μM)					
IGS (μM)			0,001	0,0023	0,0036	0,005	
		100	92,53	85,56	78,35	71,89	
	5	97,17	93,45	80,18	70,41	62,05	
	10	94,15	91,89	78,34	67,75	59,37	
	15	82,37	80,73	71,5	60,95	51,58	
		Tucatinib (μM)					
IGS (μM)			0,004	0,0093	0,014	0,02	
		100	96,3	89,21	83,77	79,23	
	5	95,77	88,14	84,13	79,53	72,64	
	10	94,59	87	79,22	79,17	71,6	
	15	85,08	79,23	76	69,97	64,62	
b.		Lapatinib (μM)					
BpV (μM)			0,008	0,016	0,024	0,032	
		100	80,08	78,35	64,37	61,86	
	2	111,93	94,67	88,51	80,49	69,07	
	6	109,82	97,04	91,89	83,45	74,31	
	10	95,11	97,24	77,87	74,02	60,74	
BpV (μM)			0,002	0,004	0,006	0,008	
		100	87,57	76,46	64,75	55,22	
	2	113,25	102,36	91,19	87,18	75,72	
	6	114,07	106,68	99,84	92,82	84,26	
	10	99,64	92,74	89,34	84,63	74,68	
BpV (μM)			0,004	0,0093	0,014	0,02	
		100,00	91,40	87,78	80,12	75,18	
	2	116,14	106,91	106,27	98,34	89,14	
	6	113,18	107,42	102,55	98,44	90,78	
	10	100,69	103,67	102,46	97,36	87,21	

Figure 22. Pharmacological validation in NCI-N87 cell line Matrix of average percentage of cell viability after treatment with Lapatinib with RIC8Ai (a) or PTENi (b), average of three independent experiments.

a.

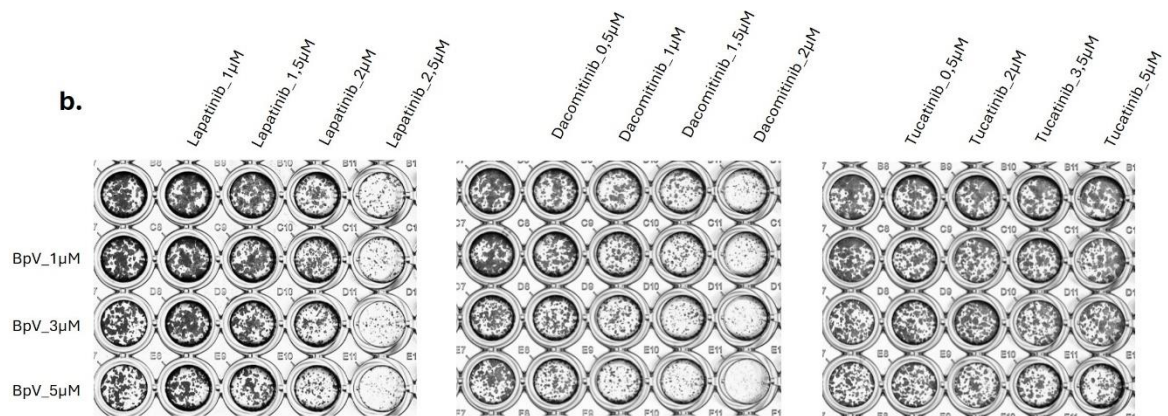
a.

		Lapatinib (μM)				
			1	1,5	2	2,5
IGS (μM)		100,00	97,14	87,88	81,28	60,78
	10	85,33	82,16	53,98	31,58	20,31
	16	91,16	44,62	25,22	17,90	15,23
	22	79,26	26,30	16,11	12,73	14,13

		Dacomitinib (μM)				
			0,5	1	1,5	2
IGS (μM)		100,00	92,74	85,15	79,79	71,11
	10	85,37	77,12	81,34	64,20	38,10
	16	93,53	77,25	60,89	34,58	21,89
	22	85,86	57,16	35,03	18,98	16,12

		Tucatinib (μM)				
			0,5	2	3,5	5
IGS (μM)		100,00	102,18	99,80	99,22	94,52
	10	91,67	92,27	96,25	99,34	97,61
	16	105,48	106,10	95,47	82,43	64,16
	22	85,57	84,81	65,66	47,76	47,80

b.



c.

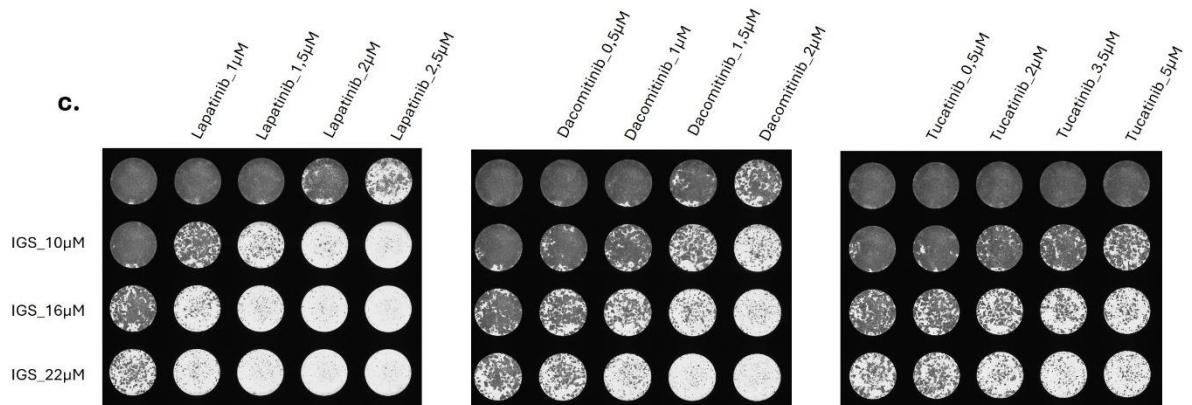


Figure 23. Pharmacological validation in MKN74 cell line. Matrix of average percentage of cell viability after treatment with Lapatinib with RIC8Ai (a), average of three independent experiments. Colony Formations of drug combination of HER2i/EFGRi and PTENi (b) or RIC8Ai (c).

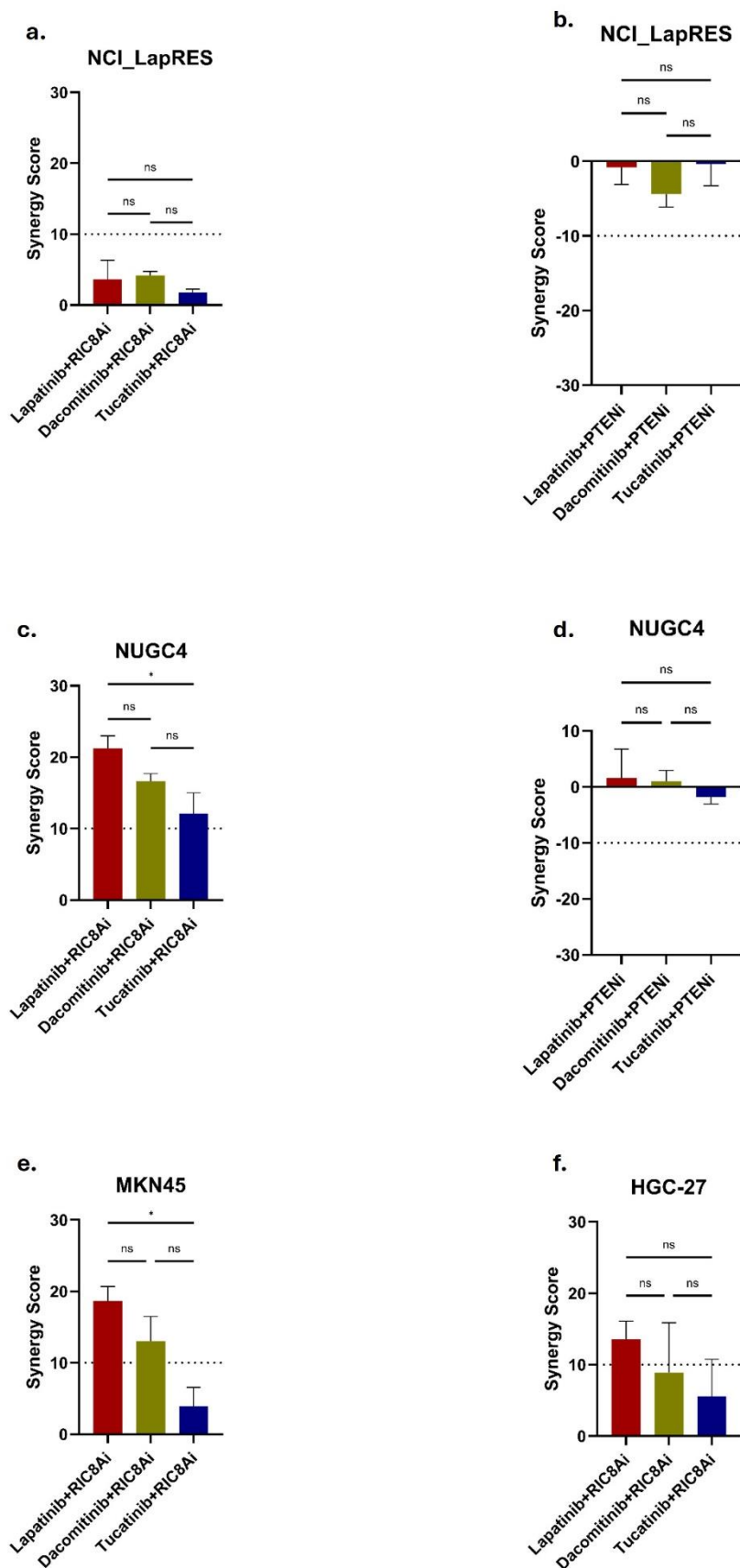


Figure 24. Pharmacological validation in NCI-N87 Lapatinib Resistant, NUGC4, MKN45, HGC-27 cell lines.. Synergy graphs of HER2i with RIC8Ai (a,c,e,f) or PTENi (b,d). Results from three independent experiments. *p-value <0,05.

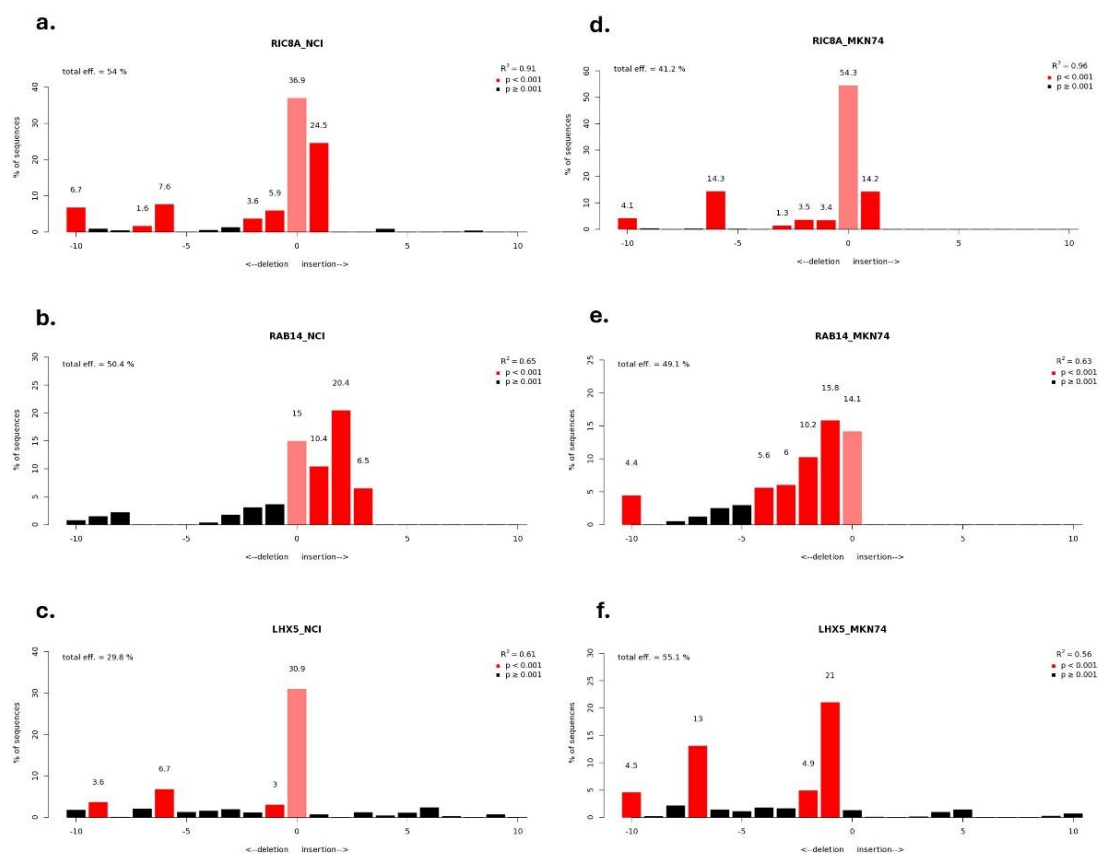


Figure 25. Graphs of editing efficiency in the polyclonal cell lines. a) NCI-N87 KO RIC8A, b) NCI-N87 KO RAB14, c) NCI-N87 KO LHX5, d) MKN74 KO RIC8A, e) MKN74 KO RAB14, f) MKN74 KO LHX5

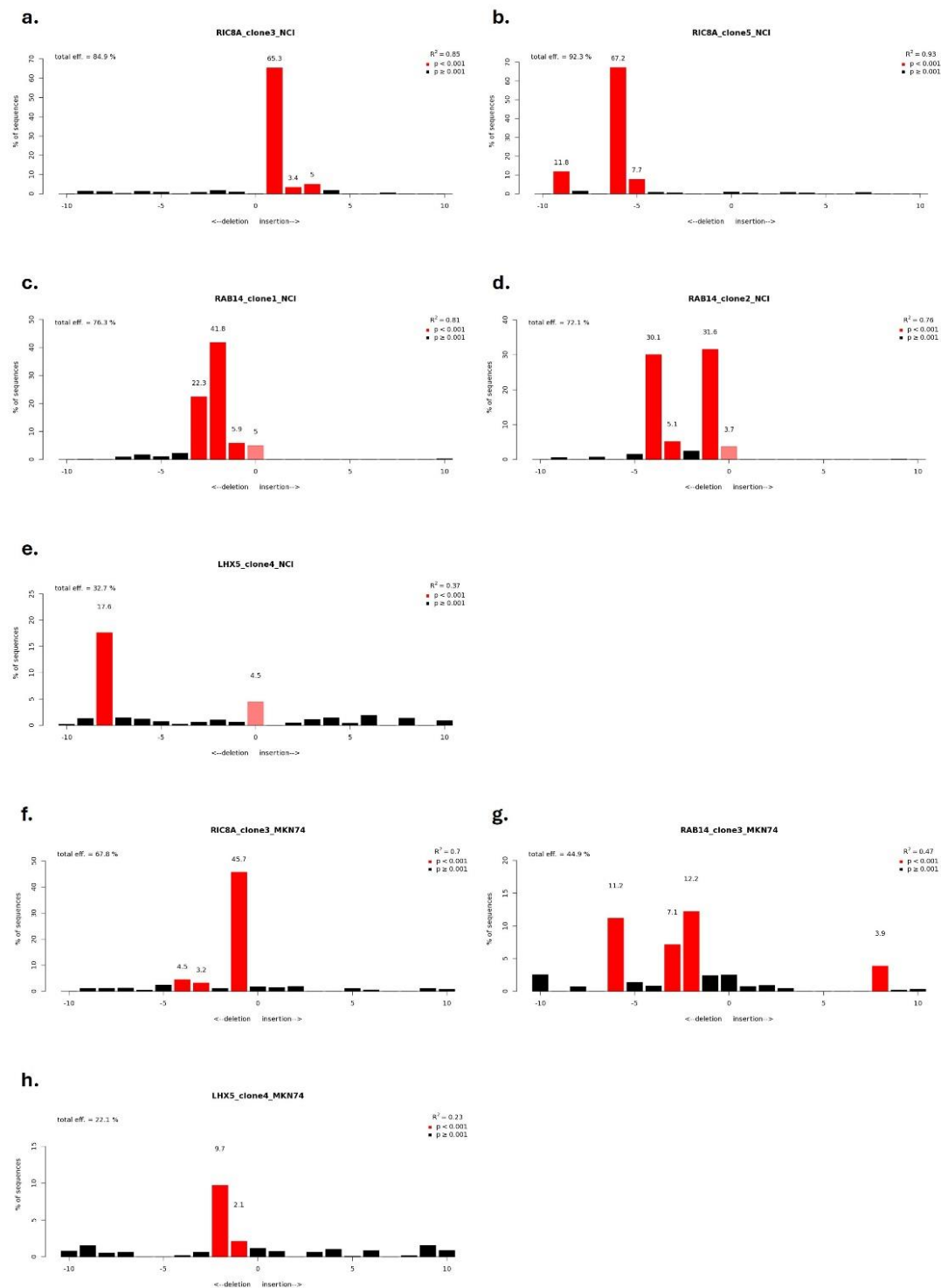


Figure 26. Graphs of edition in the polyclonal cell lines and percentage of edition. a) NCI-N87 KO RIC8A clone 3, b) NCI-N87 KO RIC8A clone 5, c) NCI-N87 KO RAB14 clone 1, d) NCI-N87 KO RAB14 clone 2, e) NC-N87 KO LHX5 clone 4, f) MKN74 KO RIC8A clone 3, g) MKN74 KO RAB14 clone 3, h) MKN74 NO LHX5 clone 4.

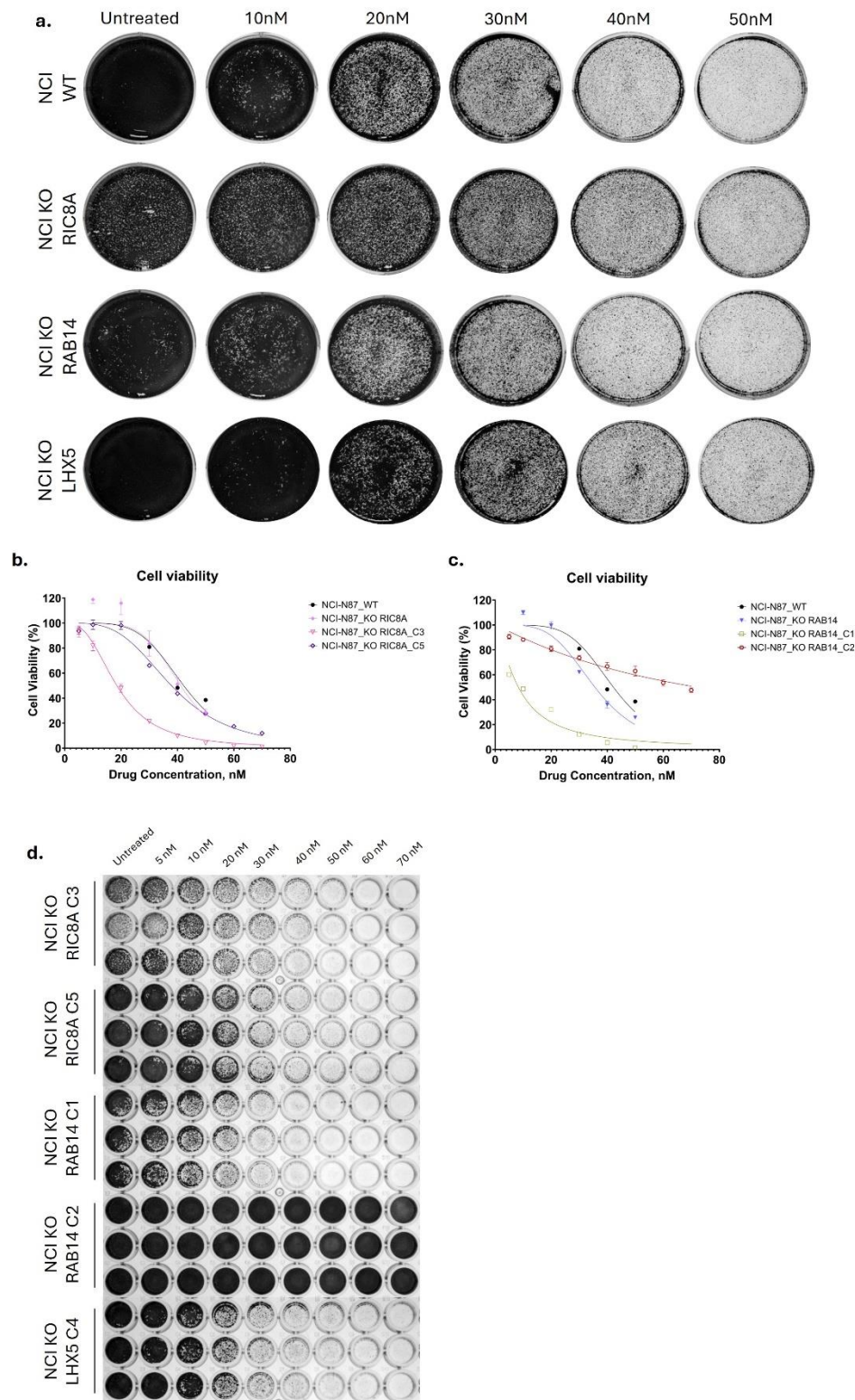


Figure 27. Genetic Validation in NCI-N87 cell line. Long Term Colony Formation with increasing concentrations of Lapatinib (a), cells were seeded and treated with the combinations every 48h until the end of ~14days of treatment, followed by fix and staining with formaldehyde 5% and Crystal Violet 0,5%, respectively. Graphs of cell viability of RIC8A KO (b) and RAB14 KO (c) with polyclonal and monoclonal cell lines. Colony Formation of the monoclonal cell with increasing concentration of lapatinib, cells were treated for ~7 days every 48 hours, followed by fix and staining with formaldehyde 5% and Crystal Violet 0,5%, respectively (d).

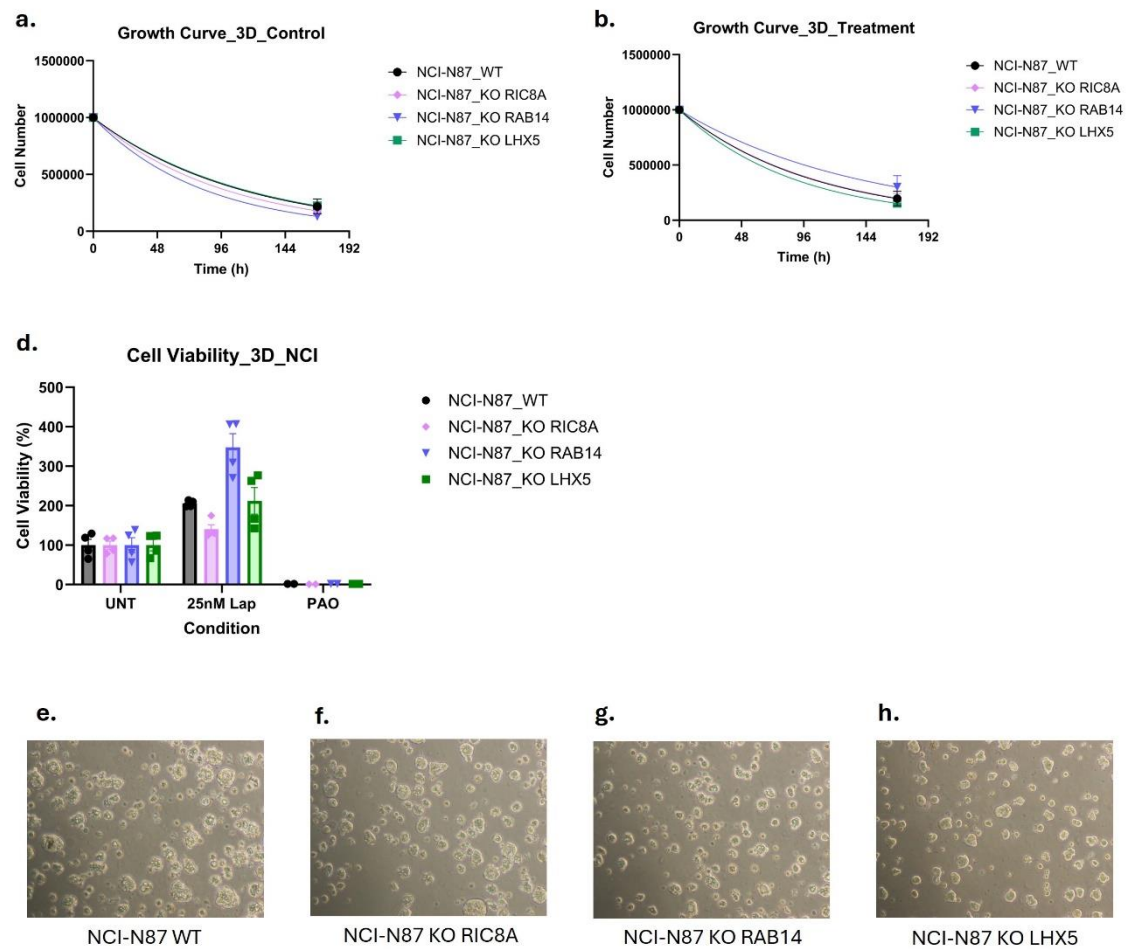


Figure 28. Genetic Validation in NCI-N87 cell line in 3D spheroid culture. Growth curve of 3D culture of control (a) and after treatment with 25nM lapatinib (b), results from two measurements. Cell viability bar graph of 3D culture without and with treatment with 25nM of lapatinib (c), results from four measurements. Cell growth morphology in 3D spheroid culture of NCI-N87 WT (e), NCI-N87 KO RIC8A (f), NCI-N87 KO RAB14 (g) and NCI-N87 KO LHX5 (h), all images were taken in an optical microscope at a 100x magnification.

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