Tumour heterogeneity of homologous recombination phenotype in metastatic gastric cancer of mixed histology

TUMOUR HETEROGENEITY OF HOMOLOGOUS RECOMBINATION PHENOTYPE IN METASTATIC GASTRIC CANCER OF MIXED HISTOLOGY

MSc dissertation in Medicine and Molecular Oncology submitted to the FACULTY OF MEDICINE, UNIVERSITY OF PORTO

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‘’I’d rather attempt to do something great and fail than to attempt nothing and succeed.”

Robert H. Schuller

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ABBREVIATIONS LIST

A

ATM	ataxia-telangiectasia mutated
ATR	ataxia telangiectasia and Rad-3 related

B

BER	base excision repair
BRCA1	breast cancer 1
BRCA2	breast cancer 2

C

CagA	cag pathogenicity island encoded cytotoxin associated gene A
ChK2	serine threonine checkpoint kinase 2

D

DAB	diaminobenzidine tetrahydrochloride
DDR	DNA damage response
D-loop	displacement loop
DNA	deoxyribonucleic acid
DR	direct repair
DSB	double-strand breaks

F

FFPE	formalin-fixed paraffin-embedded

G

GC	gastric cancer
GERD	gastroesophageal reflux disease

H

H. pylori	*Helicobacter pylori*
HE	hematoxylin & eosin
HR	homologous recombination
HRD	homologous recombination deficient
HRP	homologous recombination proficient

I

IARC	international agency for research on cancer
IHC	immunohistochemistry
ISH	in situ hybridization
ITH	intratumour heterogeneity

M

MMR	mismatch repair
MRE11	meiotic recombination 11
MRN	MRE11-RAD50-NBS1

N

NER	nucleotide excision repair
NHEJ	non-homologous end-joining

P

PARP	poly (ADP-ribose) polymerase

R

RAD51	DNA repair protein 1
RAD52	DNA repair protein 2

S

SSA	single-strand annealing
SSB	single-strand breaks
ssDNA	3’ single-strand DNA

V

VacA	vacuolating cytotoxin A
VMS	ventana medical system

W

WHO	world health organization

ABSTRACT

Gastric cancer (GC) remains one of the most prevalent cancers worldwide and one of the most common causes of death in cancer patients. GC is well characterized for its fast progression, distant metastasis, heterogeneity and chemoresistance. The limitations in current therapy contribute to the research of new approaches. Thus, the recognition of different biologic behaviours and its correlation within various clinico-pathological features is relevant, in GC patients, enabling the understanding of therapeutic targeting and efficacy.

Homologous recombination (HR) is a critical pathway involved in DNA double-strand breaks repair. Defects in the HR pathway and homologous recombination deficiency (HRD, decreased DNA repair) specifically compromising the genomic stability, predisposing to cancer formation, which can be exploited therapeutically.

The main objective of this project was to investigate, by immunohistochemistry (IHC), a HR biomarker panel, in a cohort of gastric cancers classified as mixed tumours (characterised by the coexistence, in the same tumour, of distinct tubular/papillary and poorly cohesive components), and to compare the HR expression patterns in each of the components.

We performed immunohistochemistry (IHC) for the key regulators of the HR repair pathway: ATM, ATR, MRE11, ChK2, RAD51, RAD52, BRCA1 and BRCA2. HR was classified as: 1) HR-proficient (HRP), with positive expression of all HR biomarkers or; 2) HR-deficient (HRD), with negative expression of at least one HR biomarkers.

We observed that both the pooled samples and the separate components were mostly positive for the HR biomarkers. When we compared the expression of each biomarker in each histological component, the main observation was that more cases were positive in the tubular/papillary than in the poorly cohesive component, showing that most of the poorly cohesive components presented less positivity for all HR biomarkers assessed. Based on the results we obtained, we suggest that ATM, ATR, BRCA1 and BRCA2 may play an important role in HR pathway for gastric carcinoma. Our results also indicate intratumour heterogeneity of HR phenotype when comparing the distinct

histological components in mixed gastric carcinomas, providing support to address the challenge of tumour heterogeneity in gastric carcinoma.

Keywords: Gastric cancer | Homologous recombination | Homologous recombination deficiency | Heterogeneity

RESUMO

O cancro gástrico (CG) é uma das neoplasias malignas mais diagnosticadas em todo o mundo e uma das principais causas de morte por cancro. O CG é caracterizado, pela sua rápida progressão, metastização, heterogeneidade fenotípica e resistência à quimioterapia. As limitações da terapêutica atual têm contribuído para a pesquisa de novas abordagens. Assim, o reconhecimento de diferentes comportamentos biológicos, e a sua correlação com as várias características clínicas e patológicas dos tumores, é muito relevante, permitindo o progresso e eficácia da terapia dirigida. A recombinação homóloga (RH) é uma via fundamental e está envolvida na reparação de danos na dupla cadeia do ADN. Deficiências na via da RH podem comprometer a estabilidade genética, criando maior predisposição para o desenvolvimento de cancro, e consequentemente abrindo uma oportunidade para intervenção terapêutica.

O principal objetivo deste projeto foi investigar um painel de biomarcadores da RH, através de imuno-histoquímica (IHQ), em cancros gástricos classificados como tumores mistos (caracterizados pela coexistência, no mesmo tumor de componentes distintos, tubular/papilar e de células pouco coesas) e comparar os padrões de expressão da RH nesses componentes.

A IHQ foi realizada em proteínas-chave, reguladoras da via da RH: ATM, ATR, MRE11, ChK2, RAD51, RAD52, BRCA1 e BRCA2. A RH foi classificada como: 1) RH-proficiente (RHP), com expressão positiva para todos os marcadores da RH ou; 2) RH-deficiente (RHD), com ausência de expressão de pelo menos um dos marcadores da RH.

Verificámos a expressão dos biomarcadores da RH nos tumores no seu todo, assim como nos seus diferentes componentes. Quando comparámos a expressão de cada biomarcador nos diferentes padrões histológicos, observámos que a expressão de biomarcadores de RH é mais frequente no componente tubular/papilar do que no componente de células pouco coesas. Com base nos resultados obtidos sugerimos que a expressão de ATM, ATR, BRCA1 e BRCA2 pode desempenhar um papel importante na via da RH no carcinoma gástrico. Os nossos resultados também sugerem um fenótipo de heterogeneidade intratumoral no contexto da RH, quando comparamos os

componentes histológicos distintos nos carcinomas mistos do estômago, reforçando a existência de heterogeneidade tumoral nos carcinomas gástricos.

Palavras-chave: Cancro gástrico | Recombinação homóloga | Deficiência na recombinação homóloga | Heterogeneidade

I. INTRODUCTION

1.1. GASTRIC CANCER

1.1.1. Epidemiology

Gastric Cancer remains a leading cause of death worldwide. Despite the major technological developments in the field of diagnosis and treatment, the effect on incidence and mortality has been modest.

According to the latest update from GLOBOCAN statistics (2018), from a cluster of 36 specific cancers, gastric cancer (GC) is the 5th most common cancer diagnosis and 3rd cause of cancer death worldwide [1, 2]

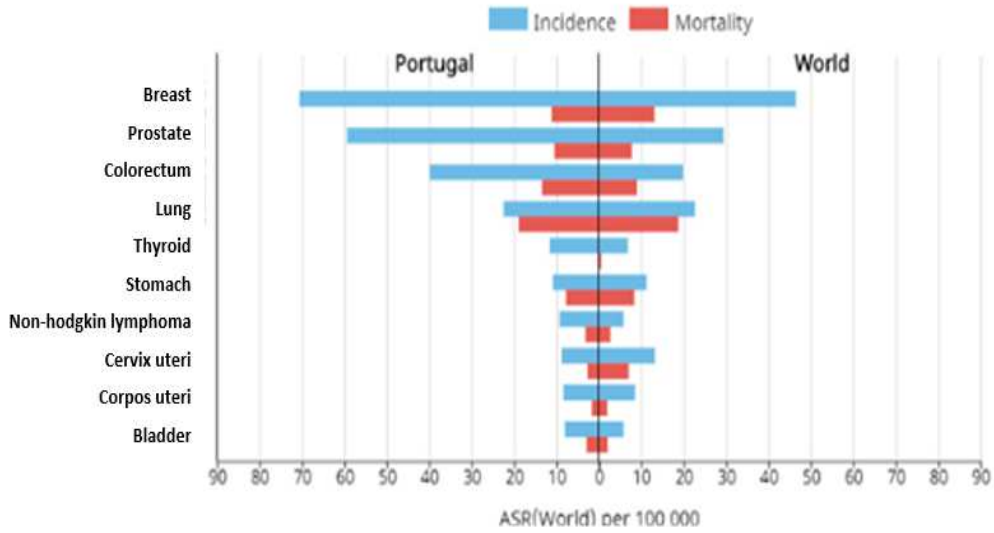

Figure 1. Cancer incidence and mortality statistics, in 2018, at Portugal from Global Cancer Observatory, IARC 1

In Portugal, GC is a public health concern, with an incidence of nearly 3000 new cases and mortality of approximately 2500 patients, per year, in both sexes and all ages **(Figure 1)**.[1, 3]

GC incidence rates vary wildly between men and women, being 2.2 times more frequent in males than females. Regarding mortality rates, GC is one of the most important oncological causes of death for males.[1, 2]

Across different countries and cultures, the incidence of GC is also highly variable. Incidence rates are highest in Eastern and Central Asia and Latin America when

compared to Northern America, Northern Europe, and North and East Africa where the incidence is lower.[1, 2] These variations, in incidence rates, are most probably explained by environmental factors. [4, 5]

Concerning survival rates, these tend to be poorer in developing countries, presumably because of a late stage at diagnosis and limited access to appropriate treatment. Following the incidence and mortality rates, survival tends to be higher amongst females than males.[2, 6]

The disparity in gastric cancer survival, across different settings, is largely related to the relative heaviness of cancers with different characteristics over the subsite, histological type and stage. The countries with higher incidence rates of GC, in general, have better survival rates than countries with lower incidence.[7]

However, worldwide, the incidence and mortality, over the past 15 years have decreased, due to the fact of the decrease in the prevalence of *Helicobacter pylori (H. pylori),* reduced intake of salt and a healthy diet, including higher intake of vegetables and fruit. [3, 8]

1.1.2. Risk Factors

GC is a heterogeneous disease that can be induced through the interaction of both genetic and environmental factors.[9]

Regarding environmental factors, *H. pylori* infection, smoking, lifestyle, gastroesophageal reflux disease (GERD) and genetic susceptibility are described as being the main risk factors for gastric cancer development.[8]

H. pylori infection has been recognized as a major risk factor for gastric cancer due to persistent infection of the gastric mucosa and carcinogenesis-promoting effect of *H. pylori* itself. According to IARC (International Agency for Research on Cancer)/WHO (World Health Organization), *H. pylori* was classified, in 1994, as a group 1 carcinogen. [10] The mechanism is well established: the inflammatory process promoted by *H. pylori* can progress to atrophic gastritis, intestinal metaplasia, dysplasia and, lastly, gastric

carcinoma. Inflammation is a well-known hallmark of cancer and, most importantly, promotes cancer development.[11, 12] Although approximately half of the world's population is infected with *H. pylori*, only a small percentage of infected individuals will develop cancer.[13] This suggests that additional factors contribute to the carcinogenic process, including host genetics, lifestyle habits, environmental factors and *H. pylori* virulence factors, categorized by CagA (cag pathogenicity island encoded cytotoxin associated gene A) and the VacA (vacuolating cytotoxin A). [14]

H. pylori eradication in subjects with superficial gastritis, intestinal metaplasia or chronic atrophic gastritis possibly can suppress disease development to dysplasia or gastric cancer.[15]

Regarding different tumour types, smoking, diet and physical activity are also identified risk factors associated with an increased risk of developing cancer.[16-18] Several studies show that smoking not only increases the risk of developing GC but is linked also to higher rates of recurrence and mortality.[16, 19, 20]

Regarding diet, the intake of large amounts of salt, processed foods and low consumption of fruit and vegetables are associated with an increased risk of stomach cancer, especially when combined with *H. pylori* infection.[21] Also, the gastroesophageal reflux disease, and the persistence of this condition may also lead to precancerous lesions and, lastly, to cancer.[22]

Apart from the above-mentioned risk factors, many others may trigger GC development. Genetic susceptibility (expressed in single nucleotide polymorphisms), epigenetic alterations, somatic gene mutations, chromosomal instability or microsatellite instability and defects in DNA repair pathways, all may trigger tumorigenesis, some by predisposing cells to additional genetic alterations.[23, 24] Thereby, over recent years, many studies have been performed, to understand DNA damage repair mechanisms and its defects. In GC, deficiencies were identified in homologous recombination, a DNA repair pathway.[25] Therefore, the categorization of carcinogenic events is complex and results from multiple overlap pathways.[26]

1.1.3. Etiology

The majority of stomach cancers are adenocarcinomas (90-95%) and based on their anatomical site can be divided into proximal and distal cancer. Proximal stomach cancers arise in the region of the oesophago-gastric junction. They usually result from chronic gastritis and inflammation derived from a variety of environmental factors. Distal stomach cancers are more prevalent and frequently arise in the lower portion of the stomach.[8] The pathogenesis of distal cancers is highly associated with *H. pylori* infection.[27]

Etiologic factors related to proximal ("cardia") gastric cancers comprise GERD and obesity, while *H. pylori* infection and dietary factors play a very important role in distal (non-cardia) gastric cancers.[8, 27, 28]

1.1.4. GC classification

Clinically, GC can be classified as early or advanced. Early GC describes an invasive carcinoma that is restricted to the mucosa or mucosa and submucosa, with or without lymph-node metastasis and regardless of tumour size. On the other hand, advanced GC refers to an infiltrative carcinoma into the *muscularis propria* and beyond. Regarding prognostic classification, early GC has a better prognosis as evaluated by the 5-year survival rate. By contrast, advanced GC has a poor prognosis with an impact on the shorter survival of the patients.[29, 30]

Along the past years, several histopathological classifications have been proposed to thorough classify all morphologies found in the GC, trying to address the heterogeneity of GC at the cellular, architectural and molecular level.[31]

From the multiple classifications proposed, the most commonly used are Lauren's and WHO classifications **(Table 1)**. [32, 33]

Table 1. WHO classification of gastric carcinoma

WHO (2019)
Papillary Tubular, well-differentiated Tubular, moderately-differentiated
Tubular, poorly-differentiated (solid)
Poorly cohesive, SRC phenotype Poorly cohesive, other cell types
Mucinous
Mixed
Histological variants: Adenosquamous carcinoma Squamous cell carcinoma Undifferentiated carcinoma Carcinoma with lymphoid stroma Hepatoid carcinoma Adenocarcinoma with enteroblastic differentiation Adenocarcinoma of fundic gland type Micropapillary adenocarcinoma

In Lauren's classification, two main types of GC are identified: intestinal and diffuse. [32] Intestinal GC is characterized by the formation of glandular structures with different degrees of differentiation. Diffuse type is characterized by scattered poorly cohesive cells or small clusters of cells with slight or no gland formation.[32] The WHO classification subdivides gastric adenocarcinomas into five main subtypes according to the morphological pattern: tubular, papillary, mucinous carcinoma, poorly cohesive cell carcinoma (including signet ring cell carcinoma), and mixed carcinoma. In addition to these, there are other rare histological variants, which represent about 5% of all GCs, including but not limited to adenosquamous carcinoma, hepatoid carcinoma, and gastric carcinoma with lymphoid stromal **(Figure 2).**[29, 33, 34]

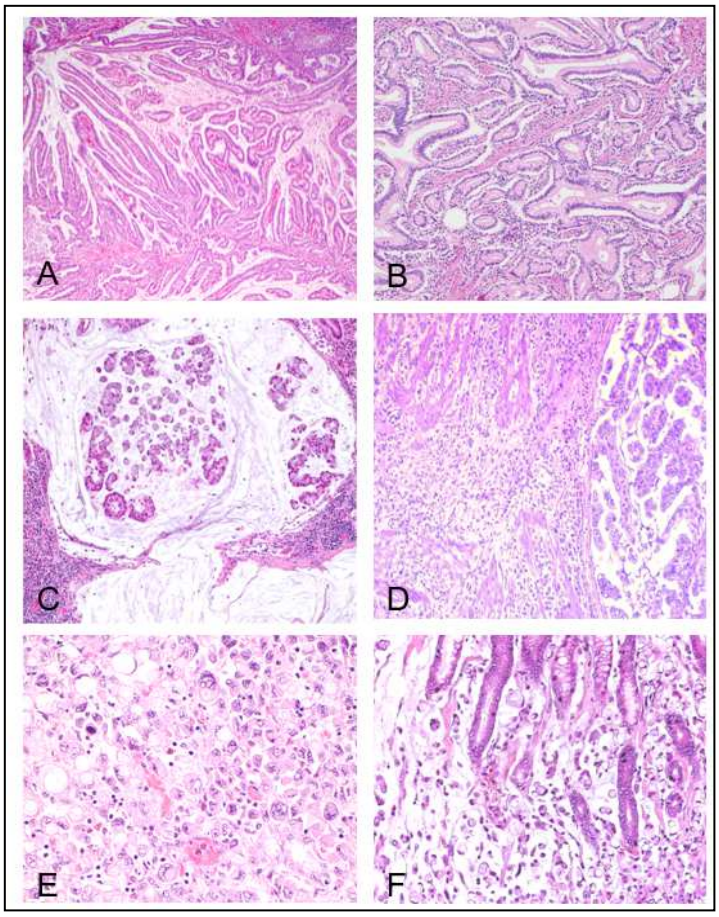

Figure 2. Morphologic heterogeneity in gastric cancer. Different histological patterns represented according to the WHO classification: (A) papillary; (B) tubular; (C) mucinous; (D) mixed; (E) poorly cohesive SOE; (F) poorly cohesive with signet ring cells.

1.2. HOMOLOGOUS RECOMBINATION

1.2.1. Types of DNA damage and repair mechanisms

The genome is constantly vulnerable to damage, and if in one hand, its preservation is very important for the protection of its integrity, on the other hand, its maintenance and evolution depends on some mutations, while also, some of those mutations may become responsible for developing diseases and even cancer. [35]

Therefore, the capacity of the cells to repair the DNA damage is crucial. Consequently, targeting defective DNA repair pathways has turned into an area of great interest in multiple solid tumour types, including GC.[36]

DNA damage rises from different mechanisms – endogenous and exogenous – and comprises multiple aspects. Endogenous features include oxidation, hydrolysis or alkylation of bases and errors in DNA replication, and exogenous factors embrace ultraviolet light, ionizing radiation and chemicals.[36, 37]

To counteract DNA damage and the consequences that would come along with it, initially, the cell must recognize the damage and then, activate the cell cycle checkpoints and pause the cell cycle, in order to repair the damage **(Figure 3)**. [38 39]

Cells have a variety of mechanisms, used according to the nature of the damage to be repaired and the implication in the cell cycle. Those mechanisms are described as DNA damage response (DDR) which recognizes a DNA lesion, signals it and initiates sophisticated pathways resulting in DNA repair. [40]

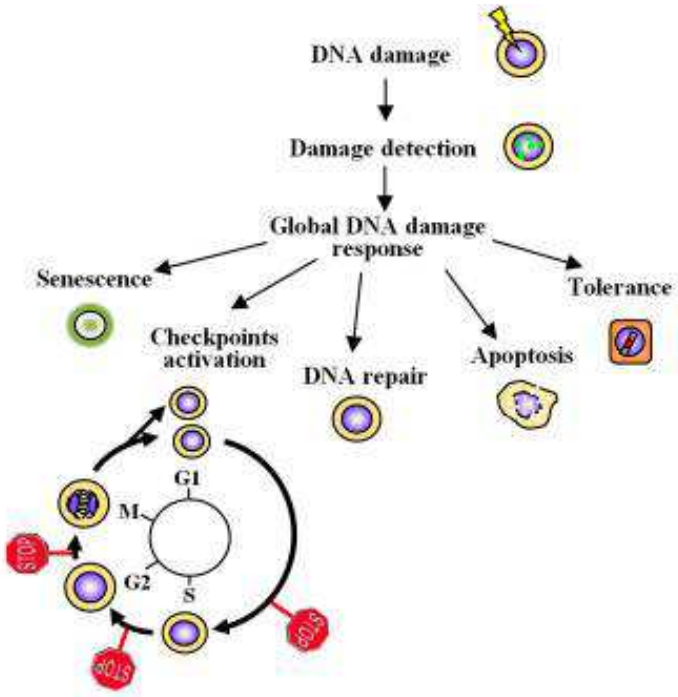

Figure 3. DDR promotes the appropriate cellular reaction, according with the DNA lesion detected. (Adapted from: Zannini, L., Delia, D. & Buscemi, G. CHK2 kinase in the DNA damage response and beyond, in J Mol Cell Biol, Vol. 6 442-457 (2014)).

When one DNA strand is affected, known as a single-strand break (SSB), but the complementary strand is intact and available as a template, the repair pathways employed are Mismatch Repair (MMR), Base Excision Repair (BER), Nucleotide Excision Repair (NER) and Direct Repair (DR). In case of both DNA strands are severed, identified as Double Strand Breaks (DBS), the most deleterious DNA lesion, repair mechanisms used embrace Homologous Recombination (HR), Non-homologous End Joining (NHEJ) and Single Strand Annealing (SSA) **(Table 2)**.[41]

Table 2. Types of DNA damage and repair mechanisms.

Type of DNA Damage	*Repair Mechanism*	*Description*
Single strand	Mismatch Repair (MMR)	Detect and repair errors in copied DNA sequences.
	Base Excision Repair (BER)	Repair simple DNA base lesions which do not distort DNA's helix structure, usually caused by endogenous factors.
	Nucleotide Excision Repair (NER)	Repair bulky DNA lesions which distort DNA's helix structure, usually caused by exogenous causes.
	Direct Repair (DR)	Restores, directly, the native nucleotide residue by removing the non-native chemical modification.
Double-strand Break	Homologous Recombination (HR)	Repair DSB and lesions which stall DNA replication forks.
	Non-homologous end joining (NHEJ)	Repair DSB through ligation of two DNA DSB's without using an intact strand as a template.
	Single strand annealing (SSA)	Repair breaks between two repeat sequences.

Adapted from: Young, K., Starling, N. & Cunningham, D. Targeting deficient DNA damage repair in gastric cancer. Expert Opin Pharmacother **17**, 1757-1766 (2016).

Defects in the DDR machinery are associated with severe human diseases that are categorized by a predisposition to cancer resulting from increased sensitivity to specific DNA damaging agents. [42]

In this study, we focused our attention on homologous recombination and its defects.

1.2.2. Homologous Recombination network

Homologous recombination is a critical pathway that contributes to the maintenance of genomic integrity providing a faithful repair.[43] HR acts not only in mitotic cells but also during meiosis, when DNA DBS are repaired.[44] HR is then involved in the crossover formation during meiosis, an important step for alignment of the homologous chromosomes and their subsequent segregation.[45] Also, this repair pathway is responsible for generating genetic diversity.[46] Furthermore, HR also plays roles in the recovery of stalled and broken replication forks and, even more important, in the restoration of genomic integrity upon the formation of DNA DSBs and interstrand crosslinks. In contrast to other repair pathways, HR provides a precise repair mechanism due to the use of the information stored in the undamaged sister chromatid, and for this reason, this repair pathway is identified to be accurate and error-free.[35, 46] The requirement of a homologous sequence present on the sister chromatid restricts HR to S and G2 phases of the cell cycle.[47]

The initial data on HR mechanism was obtained through studies in yeast, *Saccharomyces cerevisiae.*[48] Although the HR process in high eukaryotes differs in few factors when compared to yeast HR, the principle of repair is still the same.[49]

Mechanically, HR can be divided into several steps: *(i)* nucleolytic resection of the DNA DSB ends; *(ii)* formation of a nucleoprotein filament; *(iii)* homology search and strand invasion; and *(iv)* DNA repair synthesis.[50]

Thereby, to make this sophisticated process effective, a complex and hierarchical network of proteins is implicated. Those proteins are mostly responsible for *(i)* detection of the presence of DNA lesions (sensors); *(ii)* signalling and magnification of the DNA damage signal (mediators); and *(iii)* repair of DNA damage, known as effectors.[51, 52]

After DNA damage occurs, the complex MRN (MRE11-RAD50-NBS1) which acts as a sensor and effector protein, plays a crucial role for detecting DNA damage and triggering the DNA damage response, activating and recruiting the ataxia-

telangiectasia mutated (ATM) kinase to sites of DSBs. Likewise, MRE11 has endonuclease activity and, in conjunction with BRCA1-dependent process, initiates DNA-end resection, a procedure to generate 3' single-strand DNA (ssDNA) overhangs, which is a critical step in the commitment to repair DBSs and necessary for strand invasion during HR.[53]

Mutually, ATM and ataxia telangiectasia and Rad-3 related (ATR), which are two major key players in HR and regulators of the DNA damage response, are activated in the presence of oxidative stress and formation of DSB. These two protein kinases belong to the phosphatidylinositol 3-kinase family of serine/threonine protein kinases and both upregulate cell cycle checkpoints, inducing cell cycle arrest (in G1 and G2) and DNA repair. ATM activation has an essential position in DDR, acting as a transducer, which propagates, upon DNA damage, a message alert and initiates extensive cell responses through phosphorylation of downstream effector proteins, including p53 and the checkpoint kinase Chk2. ATM, also revealed to be involved in chromatin relaxation and nucleosome remodelling, regulating the transcription of genes that are needed to modulate cell responses following DNA damage.[54-57]

ATR is activated, not only in response to DSB but also to multiple DNA lesions that induce the formation of single-strand breaks.[54] Activation of ATR initiates a signalling cascade that coordinates cell cycle progression, preventing cells from entering into mitosis with unreplicated or damaged DNA.[58] Equally, ATR is necessary for the stabilization of stalled replication forks and for triggering the replication stress response.[59]

The serine/threonine-protein kinase Chk2 activation is initiated by ATM phosphorylation. Chk2 is involved in the process of targeting proteins involved in p53 signalling and apoptosis.[60] Concerning HR, ChK2 is the primary effector kinase to be activated and to spread the DNA damage signal to downstream effectors of the DDR.[39] Among these, Chk2 phosphorylates BRCA2, which is critical for RAD51 localization to DSB. [61] The kinase also facilitates HR through BRCA1 phosphorylation.[62]

The next steps into HR, corresponds to the dominant reactions of this pathway, namely, homology search and DNA strand invasion mediated by RAD51, RAD52 and BRCA2.[63-65]

RAD51 is vital to DSB repair, by HR, and central for cell survival.[66] In combination with other HR mediator proteins, such as BRCA2 and RAD51 paralogs [67], RAD51 is recruited to the DSB sites, though BRCA2, and polymerizes onto the resection-generated ssDNA ends, forming a nucleoprotein filament that promotes strand invasion and exchange between homologous DNA sequences.[68]

RAD52 interacts physically with RAD51 promoting the formation of RAD51 nucleoprotein filament and modulating its DNA strand-exchange activity, as well as, promote DNA annealing.[69, 70]

Strand invasion is then completed with the formation of a displacement loop (D-loop).[71] This repair pathway converts the D-loop into a fork-like replication structure,[72] and DNA synthesis is then carried out until the chromosome end is reached, leading to a restoration of genomic integrity **(Figure 4)**.[72-74]

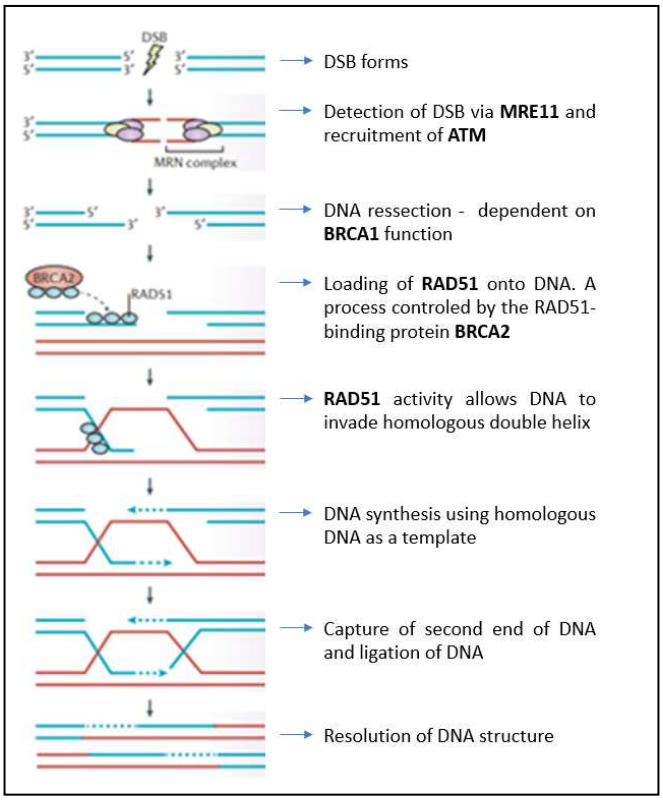

Figure 4. Schematic representation of DNA double-strand break repair by homlogous recombination.
Adapted from: Lord, C.J. & Ashworth, A. BRCAness revisited. Nat Rev Cancer 16, 110-120 (2016).

1.2.3. Balancing between HRD and therapeutic targeting

Defects in DNA repair pathways, including HR repair, are recognized as a hallmark of cancer.[75] Defective DSB repair was shown to result in mutations or chromosomal aberrations underlying carcinogenesis. More precisely, defective genome maintenance is a leading point to carcinogenesis, as it tolerates the accumulation of genetic errors.[76]

However, if on the one hand HR deficiency drives tumorigenesis on the other can be exploited therapeutically.[77] The absence or altered HR proteins are frequently associated with improved therapeutic effect. Homologous recombination deficient (HRD) tumours are generally more sensitive to DNA damage that requires HR for repair, including platinum-induced DNA replication lesions.[78, 79]

Based on the principle of synthetic lethality, which happens when a mutation in either of two genes individually has no effect but combining the mutations leads to death, new molecularly targeted therapeutic strategies have been developed.[80] This therapeutic concept, based on synthetic lethality, was initially performed in yeast and proposed by Hartwell et al. in 1997.[81] Focus on this premise, Poly (ADP-ribose) polymerase (PARP) inhibitors, were recognized as a class of drugs that target tumours with DNA repair defects and have received significant interest.[77, 82]

PARPs are a family of enzymes involved not only in multiple cellular processes but also in DNA repair. PARP inhibitors were designed to interfere with the DNA repair function.[83] PARP inhibition results in persistent DNA SSB and consequent stalling of DNA replication forks, causing DSB formation.[83] Therefore, exploiting the synthetic lethality concept, in normal cells such DSB would be repaired by HR, but patients harbouring mutations in HR components, tumour cells are deficient in HR, making this unfeasible.[84, 85] Instead, the tumour cells need to resort to the nonconservative repair mechanisms resulting in genomic instability and eventual cell death through apoptosis.[25]

In the past years, innumerable studies, including Study 39 (NCT01063517), GOLD study (NCT01924533) or PATRIOT study (NCT02223923), have been conducted regarding this promising therapeutic mechanism.[25, 86, 87] Nevertheless, some studies might obtain

daunting results, such as non-effective therapeutic response or resistance to therapy, and one of the related causes may be due to heterogeneity.[88, 89]

1.3. HETEROGENEITY

1.3.1. Heterogeneity in cancer

Within tumours, diversity can arise across numerous biological scales, from epigenetic and genomic differences between single cells to macroscopic diversity and morphological differences between tumour regions.[90] The biological scales at which heterogeneity can occur to a large extent reflect the history of research into intra-tumour and inter-tumour heterogeneity itself, from macroscopic to single nucleotide, from past to present.[91] Today, in recognition of its clinical importance, the pathologist's grading system of gastric cancer incorporates an element of heterogeneity.[91]

1.3.2. Heterogeneity in GC

GC represents a tumour model were heterogeneity is undoubtedly characterised, from various standpoints – aetiologic, environmental, genetic, morphologic and molecular.[92-94] Heterogeneity in GC is highly associated with geographic variations, macroscopic and microscopic features, as well as molecular variations. [29] This evidence can arise both between tumours (intertumour heterogeneity) and within tumours (intratumour heterogeneity, ITH), and it is caused by genetic and non-genetic factors.[95] Intertumour heterogeneity leads to the classification of tumour subtypes, distinguishing whether their molecular profiles correlate with their morphologies and expression of specific markers.[96] Moreover, tissue variation also occurs within individual tumours, intratumour heterogeneity, meaning the existence of distinct subpopulations of cancer cells within tumours, within various metastatic sites, and between metastatic sites and primary foci.[91, 96]

Regarding the histopathologic and molecular classifications, proposed over the years, there is clearly a high variability in GC.[97]

Concerning the morphologic viewpoint, a precise example of this morphological disparity is the mixed type carcinoma, identified in GC.[98] Mixed gastric carcinoma represents up to 25% of all GC, [99] and usually is composed by two different histological components within the same tumour – tubular/papillary and poorly cohesive components. The two distinct patterns may be intermingled, adjacent or completely separated.[100] Therefore, the percentage and distribution of both components may vary significantly among samples.[101] The recognition of this variation, namely ITH, add complexity to histological classifications and concerns about the accuracy of predictive biomarkers.[91]

Likewise, mixed carcinoma is associated with aggressive clinical features as well as poor outcomes.[102] Further, mixed carcinomas may display a dual metastatic pattern – hematogenous metastases and peritoneal dissemination with lymph node metastases – suggesting a cumulative effect of the adverse behaviours of both components.[103]

1.4. PROBLEM STATEMENT AND OBJECTIVES

Gastric cancer, apart from morphologic evidence of heterogeneity, shows hallmarks of genome instability that support the development of ITH.[92]

ITH allows tumour cells to self-organize, invade, metastasize, and develop drug resistance. Simultaneously, GC heterogeneity might explain the poor clinical outcomes of patients with advanced disease and could represent a putative contributor to treatment failure.[91, 95] Thus, understanding the extremely heterogeneous nature of gastric cancer may provide crucial insights to our understanding of cancer development, progression and therapeutic resistance.[104]

This study focused on gastric cancers classified as mixed tumours, and the main objective was to characterize the HR protein expression pattern, assessed by immunohistochemistry, and compare it in both components of the tumour, thus providing relevant information regarding the HR expression in distinct patterns of gastric cancer.

II. MATERIALS AND METHODS

2.1. SAMPLES

2.1.1. Clinical samples and patient information

This retrospective cohort study analysed a series of 35 GCs, retrieved from the files of the Anatomic Pathology Service from *Centro Hospitalar Universitário São João, Instituto Português de Oncologia de Lisboa, Instituto Português de Oncologia do Porto* and *Hospital da Senhora da Oliveira*. All eligible subjects were identified by systematically searching clinical records and cross-referencing pathology databases.

The cases included in the study fulfil the following criteria: age of 18 years or above, confirmation of metastatic GC, platinum-based chemotherapy as 1st line of palliative treatment from January 2010 to December 2015. Detailed demographic and clinico-pathological information was not available for this study, which is a sub study, specifically a *post hoc* analysis of an ongoing study and is focused only on the heterogeneity of homologous recombination phenotype in metastatic gastric cancer of mixed histology.

The study was performed under ethical principles, and regulatory requirements subject data protection.

2.2. IMMUNOHISTOCHEMISTRY

2.2.1. FFPE Samples

After receiving the samples (formalin-fixed and paraffin-embedded), from the various centres involved in this study, all blocks were anonymized, and a corresponding serial number was assigned to each.

FFPE samples were sectioned at 3µm using MICRON HM 325 microtome and the sections obtained, to perform IHC, were placed onto SuperFrost Plus (Fisher Scientific, Pittsburgh, PA) microscope slides. Sections were kept in an incubator for 1 hour at 60 °C to allow the tissue attachment on the slide. One section was stained with H&E to confirm the type of the tumour.

2.2.2. Ventana Autostainer

In this project, protein immunodetection was performed using a Ventana Autostainer (Model Benchmark Ultra) with the OptiView Dab Detection kit - Ventana Medical System, Roche, Tucson, USA - (VMS, USA). Ventana Autostainer is automated staining equipment that allows users to make several protocols of several techniques simultaneously, such as IHC and *in situ* hybridization (ISH) techniques. The equipment is computer controlled and automates the process from deparaffinization to counterstaining. Benchmark Ultra is valid for the various techniques and has demonstrated reproducible results and effective quality control in the diagnosis of neoplastic diseases. In addition, the implementation and use of this technology allow greater labour flexibility, monitors user time, improves workflow and decreases human interference or error.

2.2.3. OptiView DAB KIT/Reagents

The OptiView DAB IHC Detection Kit, which is a cocktail of secondary antibodies designed to recognize the specific primary antibody. The secondary antibodies are recognized by an enzyme bound to a tertiary antibody that is visualized with hydrogen peroxide substrate and 3, 3' – diaminobenzidine tetrahydrochloride (DAB) chromogen, which produces a brown precipitate easily detected by light microscopy. Each step involves a defined period of incubation at a certain temperature. After each incubation step, the VENTANA Benchmark Series instrument washes the sections to remove unbound serums and applies a liquid coverslip, which minimizes the evaporation of the aqueous reagents from the slide.

Commercial pre-formulated buffers CC1 and CC2 (VMS, USA) were used in antigen retrieval. OptiView Cooper (VMS, USA), OptiView H_2O_2 (VMS, USA), OptiView HRP Multimer (VMS, USA) together with OptiView DAB substrate (VMS, USA) were used for the detection of primary antibodies and the blocking of endogenous peroxidase was done using OptiView Peroxidase Inhibitor (VMS, USA). Counterstaining was performed

using Haematoxylin (VMS, USA) and Bluing Reagent (VMS, USA). Deparaffinization solution was EZPrep (VMS, USA), and the buffer was Reaction Buffer (VMS, USA). When necessary, depending on antibody and protocol used, an amplifier kit and solution to reduce the background staining was used. The OptiView Amplification kit (VMS, USA) was used to increase the signal, when necessary, after the bounding of secondary antibodies. The Discovery Ab Diluent (VMS, USA), which helps to prevent the background signal, was used before the primary antibody bounding and only when necessary. Entellan (Merck, Darmstadt, Germany) was applied for mounting.

2.2.4. Antibody optimization

All IHC assays were performed using specific monoclonal or polyclonal antibodies. The primary antibodies are described in **Table 3**. The diluent used for the preparation of primary antibody dilutions was Antibody Diluent Reagent Solution (Life Technologies).

Table 3. Antibody details according to manufacturers.

Antibody	Immunoreactivity	Clonality/Clone	Positive Control	Brand
ATM	Nuclear	Rabbit monoclonal/ ab32420	Testis	Abcam, Cambridge, UK
ATR	Nuclear	Rabbit monoclonal/ ab178407	Testis	Abcam, Cambridge, UK
MRE11	Nuclear	Rabbit polyclonal/#4895	Thyroid	Cell Signalling, Beverly, USA
ChK2	Nuclear	Mouse monoclonal/#3440	Colon	Cell Signalling, Beverly, USA
RAD51	Nuclear	Rabbit monoclonal/ ab133534	Testis	Abcam, Cambridge, UK
RAD52	Nuclear	Rabbit polyclonal/ ab117097	Prostate	Abcam, Cambridge, UK
BRCA1	Nuclear	Mouse monoclonal/ MS110	Breast	Calbiochem/Merck, Darmstadt, Germany
BRCA2	Nuclear	Rabbit polyclonal/ CA1033	Breast	Calbiochem/Merck, Darmstadt, Germany

2.2.5. IHC protocol

Briefly, the standard protocol used for immunodetection started with the deparaffinization of the sections using EZPrep (VMS, USA) @ 72 ºC. Then, the sections were washed with Reaction Buffer (VMS, USA). The antigen retrieval necessary for the bound of the primary antibody was achieved using Cell Conditioning solution 1 or 2 (Roche), depending on the antibody, at a temperature ranging from 95 to 100 ºC. Before adding the primary antibody, the endogenous peroxidase from the tissues was inhibited, due to its possible interference with the bounding reaction, and a background inhibitor, Diluent Option (VMS, USA), was also used to decrease unspecific background signal. Then, the primary antibodies were incubated using the optimized dilutions and time. In this study, diluted primary antibodies were added manually onto each slide during staining run, applying a volume of 100 µl per slide for each staining. The detection of the primary antibody was accomplished using the OptiView Horseradish Peroxidase Universal Multimer (VMS, USA) and the signal revelation was obtained with OptiView cocktail (VMS, USA) – DAB, H_2O_2 and Cooper. To visualize the cells in the tissue, Haematoxylin (VMS, USA) and a counterstained, Bluing Reagent (VMS, USA) was used (this step result in the medium blue colouration of cell nuclei). After counterstaining slides were collected and washed in tap water with EZPrep, to remove oil coverslip. Finally, slides were dehydrated using a series of ethanol and xylene and mounted for light microscopy.

Before achieving the perfect conditions to each selected antibody of this study, several tests were performed **(Table 4)**.

Table 4. Antibodies optimization.

Antibody	Dilution	Antigen Retrieval	Incubation Time
ATM	[1:100-1:600]	[8'-32'] with CC1	[16'-40']
ATR	[1:50-1:250]	[16'-48'] with CC1	[16'-48']
MRE11	[1:500-1:600]	[8'-32'] with CC1	[8'-32']
ChK2	[1:200-1:300]	[8´-32'] with CC1	[8'-36']
RAD51	[1:100-1:400]	[16'-40'] with CC1	[24'-52']
RAD52	[1:100-1:350]	[16'-40'] with CC1	[24'-48']
BRCA1	[1:100-1:300]	[16'-64'] with CC2	[16'-60']
BRCA2	[1:25-1:100]	[16'64'] with CC1	[16'-52']

2.2.6 Scoring

The IHC results were evaluated by two independent observers. Cancer cells showing nuclear reactivity, regardless of the presence of cytoplasmic staining, were considered for scoring. Interpretation of the IHC results was made according to the criteria described by Kim *et al*., [105], as follows: intensity was graded as 0 (totally negative), ± (equivocal staining, signal observed only at high-power microscopy with ×40 eyepiece), 1+ (weakly positive), 2+ (moderately positive) and 3+ (strongly positive). The extension of staining was also assessed, whereby cases fall into one of the following categories: <10% of stained cells; 10-25% of stained cells; 25-50% of stained cells; 50-75% of stained cells and >75% of stained cells. The criteria for negative cases was set as less than 10% of cells stained as weakly positive (+/3) or more than 90% of cells showing totally negative (0) or equivocal staining (±). For example, if more than 90% of tumour cells showed equivocal (±) or negative (0) staining and less than 10% show any positive (+, ++ or +++/3) staining, a case was defined as negative **(Table 5)**. The HRD status was determined by the absence of at least one of the proteins studied, according to the criteria described by Zhang et al. [106]

Table 5. Schematic representation of the scoring assessment

	Extent					
Intensity	**Absent**	**1-10%**	**11-25%**	**26-50%**	**51-75%**	**>75%**
0	Negative	Negative	Negative	Negative	Negative	Negative
Equivocal	Negative	Negative	Negative	Negative	Negative	Negative
+	Negative	Negative	Positive	Positive	Positive	Positive
++	Negative	Negative	Positive	Positive	Positive	Positive
+++	Negative	Negative	Positive	Positive	Positive	Positive

2.3. STATISTICAL ANALYSIS

SPSS (26.0, IBM Corp, Armonk, NY) was used for statistical analysis. Summary statistics were implemented to describe the included population. The results from the cohort were analysed with nonparametric tests, as appropriate for each variable. The different variables were analysed according to the Proportion of Agreement, Cohen's Kappa Coefficient and McNemar's test. In all statistical analysis, $P \leq 0.05$ was considered significant.

III. RESULTS

3.1 RESULTS

3.1.1 Samples of mixed gastric carcinoma

In this work, we used 35 samples of mixed gastric carcinoma, retrieved from several Portuguese institutions. To assess the correct patient selection in H&E stained section for each case was performed, to confirm the histological diagnosis by the presence of two distinct patterns (tubular/papillary and poorly cohesive), within the same sample. The histological classification of the tumours was expressed according to WHO classification. [33] All 35 samples were confirmed to be of the mixed histological type, as shown in **Figure 5**.

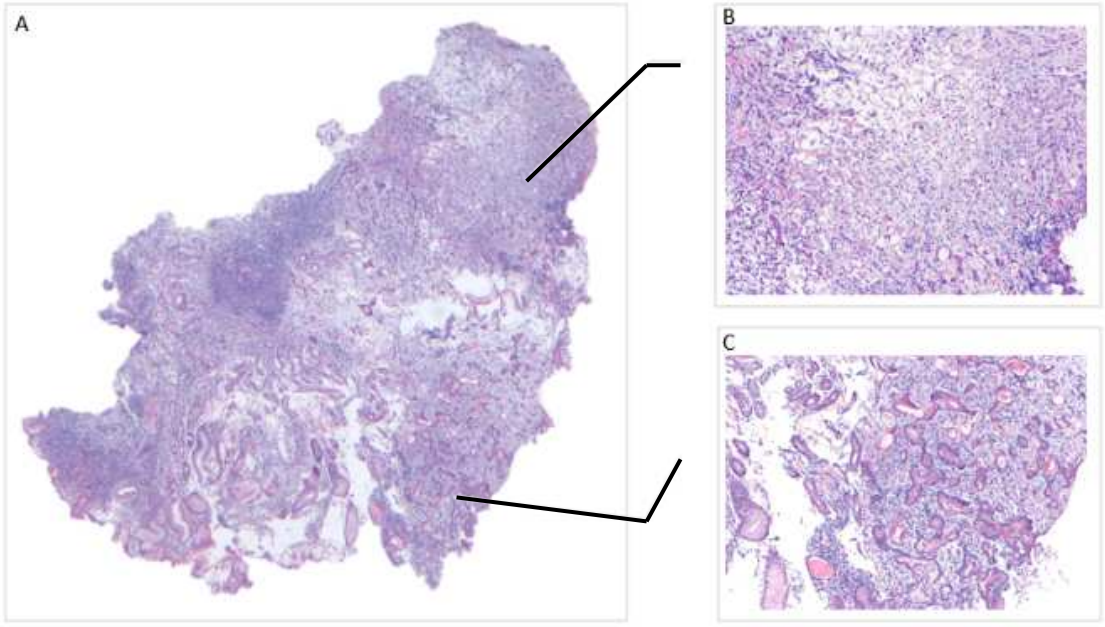

Figure 5. Mixed gastric carcinoma: **(A)** biopsy of mixed histology, composed by two patterns (tubular and poorly cohesive) (H&E, 20X); **(B)** detail of the poorly cohesive component (100X, H&E); and **(C)** detail of the tubular component (40X, H&E).

3.2. IHC

3.2.1 Homologous recombination status and biomarkers expression

We performed IHC for the key regulators of the homologous recombination repair pathway: ATM, ATR, MRE11, ChK2, RAD51, RAD52, BRCA1 and BRCA2. For all antibodies, a preliminary optimization step was necessary to define Ventana Autostainer (Roche) protocols. For this, several of the adjustable steps in the IHC

reaction, namely, antigen retrieval, enzymatic digestion and incubation time were tested using different tissues and including, for each slide, a positive and negative control. This optimisation step was crucial for optimal and trustable results. The optimal conditions are detailed in **Table 6**.

Table 6. Details of antibody conditions used in immunodetection.

Antibody	Dilution	Antigen Retrieval	Incubation Time
ATM	1:500	16 min	32 min
ATR	1:200	32 min	38 min
MRE11	1:600	24 min	32 min
ChK2	1:300	16 min	32 min
RAD51	1:200	24 min	44 min
RAD52	1:300	24 min	24 min
BRCA1	1:300	40 min	40 min
BRCA2	1:100	48 min	40 min

IHC results were analysed for pooled samples (both tubular/papillary and poorly cohesive components as one), as well as for the separate counterparts. Of notice, we observed multiple scenarios for the expression of each biomarker (**Figure 6**). For example: 1) for ATR, some cases presented negative expression in both components (**Figure 6A** and **6B**); 2) for MRE11, some cases were positive for both components (**Figure 6C**); 3) for Chk2, some cases just presented positivity in the tubular component, with the poorly cohesive presenting no expression of this biomarker (**Figure 6D**); 4) also for Chk2, few cases expressed positivity in both components but with different intensity (**Figure 6F**); 5) for ATM, some cases did not present expression in any of the components (**Figure 6E**). As expected, all cases presented positivity in lymphocytes (internal control) (**Figure 6E**).

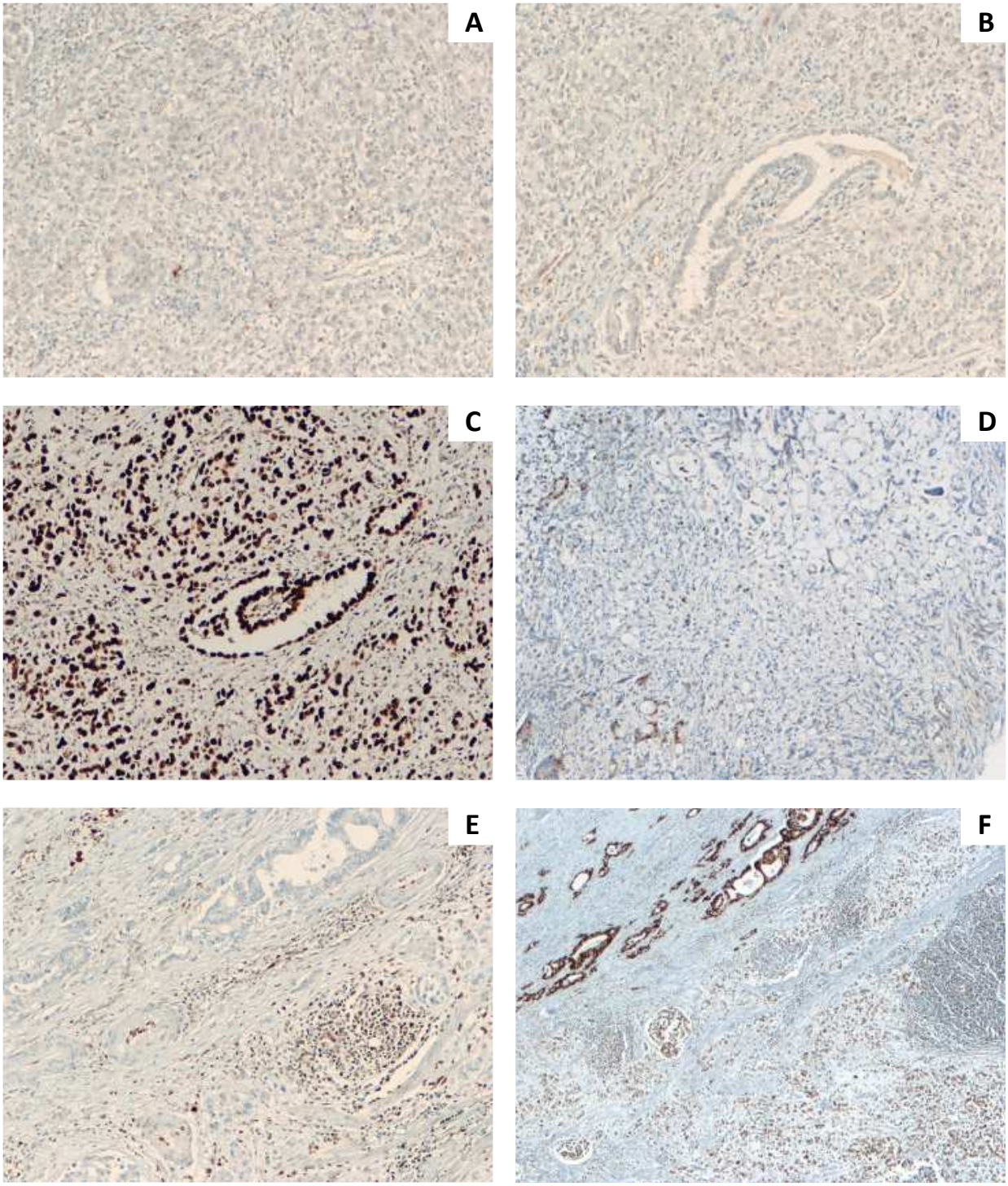

Figure 6. Expression of proteins studied by IHC staining. **A** and **B** are from the same sample and demonstrate negativity in both components for ATR biomarker. **A** corresponds to the poorly cohesive component and **B**, the tubular component. **C**, MRE11 positive expression in both components. **D**, ChK2 expression showing positivity in the tubular component and negativity in the poorly cohesive pattern. **E** ATM negativity expression in both components and lymphocytes positivity as an internal control. **F**, ChK2 expression showing positivity in both components but with different intensity. Original magnifications: A, C and E X100; B, D and F X40.

After the analysis of each IHC, a score was given to each sample and biomarker: positive or negative. We observed that both the pooled samples and the separate components were mostly positive for the HR biomarkers. For example, ATM was considered positive in 27/35 cases for the pooled sample, 28/35 cases for the tubular/papillary component and 24/35 cases for the poorly cohesive component (**Table 7**). As we compared the expression of each biomarker in each histological pattern, the main observation was that, slightly but consistently, more cases were positive for the selected biomarkers in the tubular/papillary component than in the poorly cohesive component. For example, RAD52 was considered positive in 32/35 cases for the tubular/papillary component, while the poorly cohesive component only displayed positivity in 28/35 cases (**Table 7**). The same scenario was observed for all proteins, showing that the poorly cohesive component presented less positivity for all HR biomarkers assessed.

Table 7. Comparison of all biomarker's expression in the different histological components.

		POOLED SAMPLE n(%)	TUBULAR COMPONENT n(%)	POORLY COHESIVE COMPONENT n(%)
ATM	+	27 (77,1%)	28 (80%)	24 (68,6%)
	–	8 (22,9%)	7 (20%)	11 (31,4%)
ATR	+	24 (68,6%)	23 (65,7%)	22 (62,9%)
	–	11 (31,4%)	12 (34,3%)	13 (37,1%)
MRE11	+	33 (94,3%)	34 (97,1%)	32 (91,4%)
	–	2 (5,7%)	1 (2,9%)	3 (8,6%)
ChK2	+	34 (97,1%)	34 (97,1%)	32 (91,4%)
	–	1 (2,9%)	1 (2,9%)	3 (8,6%)
RAD51	+	33 (94,3%)	33 (94,3%)	32 (91,4%)
	–	2 (5,7%)	2 (5,7%)	3 (8,6%)
RAD52	+	32 (91,4%)	32 (91,4%)	28 (80%)
	–	3 (8,6%)	3 (8,6%)	7 (20%)
BRCA1	+	31 (88,6%)	31 (88,6%)	29 (82,9%)
	–	4 (11,4%)	4 (11,4)	6 (17,1%)
BRCA2	+	26 (74,3%)	25 (71,4%)	23 (65,7%)
	–	9 (25,7%)	10 (28,6%)	12 (34,3%)

Next, the observed expression of all biomarkers was combined into a positive/negative expression for HR, for the pooled sample and for both components separately (HR-status). With this approach, all samples were either classified as: 1) HR-proficient (HRP), *i.e.* with positive expression of all HR biomarkers or; 2) HR-deficient (HRD), *i.e.* with negative expression of at least one HR biomarkers.

Regarding the pooled sample, we observed that most cases (24 cases, 68,6%) were HRD, whereas 11 cases (31,4%) showed HRP (**Table 8**). When we analysed the counterparts separately, tubular/papillary and poorly cohesive components, our results demonstrated an increase of HRD status on the poorly cohesive counterpart (80%) comparing with the pooled sample (68,6%).

Table 8. HR status in all samples and the different components.

	POOLED SAMPLE	**TUBULAR COMPONENT**	**POORLY COHESIVE COMPONENT**
HRP	11 (31,4%)	11 (31,4%)	7 (20%)
HRD	24 (68,6%)	24 (68,6%)	28 (80%)

After analysing all the cases for all the HR biomarkers, we examined in more detail the results obtained for the main patterns of IHC expression. The four possible patterns of expression were defined as: pattern *(i)* for HRD in both components; pattern *(ii)* for HRD in the tubular/papillary component and HRP in the poorly cohesive component; pattern *(iii)* for HRP in tubular component and HRD in the poorly cohesive component; and, pattern *(iv)* for HRP in both components. The results are detailed in **Table 9**.

Regarding the HR expression in both patterns (pattern *i*), the majority of cases (*n*=23, 65,7%), presents HRD in both components while only 6 cases (17,1%) presented HRP. Concerning the HR expression on each studied protein, we observed that the proteins with higher number of cases classified as HRD, in both components, were ATR (31,4%) and BRCA2 (25,7%). Focusing on patterns where the components present distinct HR-status, we observed more cases with the expression pattern (*iii*) (HRP in

tubular/papillary component and HRD in the poorly cohesive component) than with pattern (*ii*) (HRD in tubular/papillary component and HRP in the poorly cohesive component). In particular, for ATM we observed 5 cases (14,3%) presenting pattern (*iii*) versus 1 case (2,9%) as observed in **Table 9**. A similar scenario was observed for the remaining biomarkers.

Table 9. Results of main patterns of HR expression, in both components.

HR STATUS

TUBULAR COMPONENT	POORLY COHESIVE COMPONENT: HRD	POORLY COHESIVE COMPONENT: HRP
HRD	23 (65,7%)	1 (2,9%)
HRP	5 (14,3%)	6 (17,1%)

ATM

TUBULAR COMPONENT	POORLY COHESIVE COMPONENT: HRD	POORLY COHESIVE COMPONENT: HRP
HRD	6 (17,1%)	1 (2,9%)
HRP	5 (14,3%)	23 (65,7%)

ATR

TUBULAR COMPONENT	POORLY COHESIVE COMPONENT: HRD	POORLY COHESIVE COMPONENT: HRP
HRD	11 (31,4%)	1 (2,9%)
HRP	2 (5,7%)	21 (60,0%)

MRE11

TUBULAR COMPONENT	POORLY COHESIVE COMPONENT: HRD	POORLY COHESIVE COMPONENT: HRP
HRD	1 (2,9%)	0 (0,0%)
HRP	2 (5,7%)	32 (91,4%)

CHK2

TUBULAR COMPONENT	POORLY COHESIVE COMPONENT: HRD	POORLY COHESIVE COMPONENT: HRP
HRD	1 (2,9%)	0 (0,0%)
HRP	2 (5,7%)	32 (91,4%)

RAD51

TUBULAR COMPONENT	POORLY COHESIVE COMPONENT: HRD	POORLY COHESIVE COMPONENT: HRP
HRD	2 (5,7%)	0 (0,0%)
HRP	1 (2,9%)	32 (91,4%)

RAD52

TUBULAR COMPONENT	POORLY COHESIVE COMPONENT: HRD	POORLY COHESIVE COMPONENT: HRP
HRD	3 (8,6%)	0 (0,0%)
HRP	4 (11,4%)	28 (80,0%)

BRCA1

TUBULAR COMPONENT	POORLY COHESIVE COMPONENT: HRD	POORLY COHESIVE COMPONENT: HRP
HRD	4 (11,4%)	0 (0,0%)
HRP	2 (5,7%)	29 (82,9%)

BRCA2

TUBULAR COMPONENT	POORLY COHESIVE COMPONENT: HRD	POORLY COHESIVE COMPONENT: HRP
HRD	9 (25,7%)	1 (2,9%)
HRP	3 (8,6%)	22 (62,9%)

In order to understand the interrater agreement (the measurent of the extent to which data collectors assign the same score to the same variable), between the tubular/papillary and poorly cohesive patterns and its status, HRD or HRP, we calculated its proportion of agreement and validate its significance using the Cohen's Kappa coefficient and the Mcnemar test (**Table 10**).

Table 10. The proportion of agreement between HRP and HRD.

	PROPORTION OF AGREEMENT		KAPPA STATISTICS		MCNEMAR TEST
	%	95% CI	κ	95% CI	*P*-value
HR	82,9	[66,4-93,4]	0,559	[0,257-0,861]	0,219
ATM	85,7	[69,7-95,2]	0,576	[0,245-0,907]	0,999
ATR	85,7	[69,7-95,2]	0,689	[0,438-0,940]	0,999
MRE11	97,1	[85,1-99,9]	0,653	[0,026-1,000]	0,999
CHK2	94,3	[80,8-99,3]	0,478	[(-0,122)-1,000]	0,500
RAD51	97,1	[85,1-99,9]	0,785	[0,379-1,000]	0,999
RAD52	88,6	[73,3-96,8]	0,545	[0,171-0,919]	0,125
BRCA1	94,3	[80,8-99,3]	0,768	[0,464-1,000]	0,500
BRCA2	88,6	[73,3-96,8]	0,736	[0,495-0,977]	0,625

Cohen's kappa is a robust statistic test, useful for either interrater or intrarater reliability testing, which allows the correlation of coefficients. The Kappa result was interpreted as follows: values ≤ 0 as indicating no agreement and 0.01–0.20 as none to slight, 0.21–0.40 as fair, 0.41– 0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect agreement. [107] McNemar test is used to compare paired proportions, and we use it, to find a change in proportion for the paired data. [108] The result of $P \leq 0.05$ was considered significant.

The HR proportion of agreement, between HRP and HRD, is 82,9% (95% CI: 66,4-93,4), with a moderate agreement, 0,559 (95% CI 0,257-0,861) and with a *p*-value of 0,219. Regarding the proportional of the agreement for each studied protein, we obtained a good ratio of agreement, varying between 85,7% (95% CI: 69,7-95,2) and 97,1% (95% CI: 85,1-99,9). Concerning the Kappa results we achieved for ATM, ChK2 and RAD52 a moderate result and for ATR, MRE11, RAD51, BRCA1 and BRCA2 a more expressive result. The *p*-value revealed that our analysed had no statistical significance.

IV. DISCUSSION

4.1 DISCUSSION

Gastric cancer is one of the leading causes of cancer-related death worldwide, and numerous reports have suggested that HRD could play a role in gastric tumorigenesis. [106] However, a correlation between a systematic analysis of the HR biomarkers and morphologic differences within the same tumour (intratumour heterogeneity) is mostly missing. In the present study, we aimed to assess in gastric cancers classified as mixed tumours, the heterogeneity phenotype in the homologous recombination, through immunohistochemistry. With this purpose, we collected 35 cases of mixed histology and analysed eight proteins (ATM, ATR, MRE11, ChK2, RAD51, RAD51, BRCA1 and BRCA2) which are involved in the homologous recombination process.

Heeke *et al*.[109] analysed the molecular profiles of approximately 52,500 different solid tumour types and identified pathogenic mutations in 25 HR genes, including, ATM, ATR, BRCA1, BRCA2, ChK2, MRE11 AND RAD51. In this study, the overall frequency of HR genes mutation detected was 17,4%, and the gastroesophageal was one of the most commonly mutated lineages with 20,8% (*n*=619). Overall, they also found that BRCA2 (3,0%), BRCA1 (2,8%), ATM (1,3%) and ATR (1,3%) were the most commonly mutated genes.

Concerning the obtained results for each biomarker that we analysed, our findings suggest that expression of ATM, ATR, BRCA1 and BRCA2 may play an important role in GC and may serve as a useful predictive biomarker. Also, it reveals that most of our studied cases have an HRD profile. Moreover, we observed suggestive tumour heterogeneity, when comparing the two different components present in the mixed gastric cancers, in which, the poorly cohesive counterpart presents a higher HRD profile.

Zhang ZZ *et al.*[106] studied also 120 patients with gastric adenocarcinoma and analysed the IHC expression of 5 biomarkers related to HR (ATM, ATR, MRE11, BRCA1 and MDC1), demonstrating that 48,1% of the cases had loss of expression of at least one protein, the HRD group. They also suggested that ATM, ATR and MRE11 deficiencies are commonly found in GC patients. Interestingly, the authors additionally found that the loss of expression of ATM was significantly correlated with poor differentiation,

lymph node metastasis and poor 5-year survival, and that the loss of expression of BRCA1 was significantly associated with patients of diffuse Lauren's type. These findings are in keeping with our results as we observed a relevant HRD profile in the poorly cohesive counterpart, usually with a more aggressive behaviour.

Alexandrov *et al*.[110] analysed the expression of BRCA1 and BRCA2 and suggested a link between BRCA1 and BRCA2 mutations and the risk of GC development. The mutations of the BRCA2 gene, rather than the BRCA1 gene, had a higher impact on the survival of patients. In our analysis, we observed a higher loss of expression in BRCA2 when compared to BRCA1. In another study, Semba *et al*.[111] described also that mutations of BRCA1 were not so commonly found in gastric cancer, a finding that is in agreement with our results.

Concerning the heterogeneity found in our cases, we defined four main patterns of expression, where we found the following status: an HRP profile in both components; an HRP profile in the tubular/papillary component and HRD profile in the poorly cohesive component or vice-versa, and also HRD profile in both components.

Interestingly, the frequency of HRD profile was higher in the poorly cohesive component than in the pooled samples. Further, when we compared the phenotypes, we observed that HRP was more frequent in the tubular/papillary component than in the poorly cohesive component. It remains to be clarified the impact of the tumour heterogeneity of homologous recombination phenotype in the biological behaviour of mixed gastric carcinomas, characterised by more aggressive features than "pure" tubular/papillary and poorly cohesive gastric cancers, including larger tumour size, deeper invasion, lymphatic invasion, and lymph node metastases. Heterogeneity is a hallmark well recognised in GC suggesting an important role in prognosis and response to treatment. In the emerging new areas of targeted therapy, tumour heterogeneity can be an issue not only in-patient selection for clinical trials but also in the success of a personalised treatment based on the biomarker strategy.

There are some limitations in this study to note. Regarding the cases included in this cohort, we used both biopsies and surgical specimens, and it is possible that different sampling may have affected our results. For example, in the immunohistochemistry

procedure biopsies provided cleaner staining than surgical specimens; however, the representativity of the tissue is limited in biopsies, increasing the difficulty of measuring the expression of the biomarkers. On the other hand, surgical specimens provide the right amount of tissue, but the evaluation of the expression becomes more difficult, as the background can impair the precise evaluation of the biomarkers expression. The above-mentioned background is due to pre-analytic interferences, namely, in surgical specimens due to fixation deficiency, which increases the background of the IHC staining.

The limited number of samples included in this study is another issue, likely underlying the lack of statistical significance. Also, the absence of information regarding the clinicopathological features prevented us from studying any possible associations between HR-status and demographic and other pathological features of the cases. Another limitation stems from the fact that, in this series, all patients were under treatment with platinum-based chemotherapy. It remains to be elucidated how much chemotherapy affects the HR-status of the tumours. However, our results demonstrate the importance of HR heterogeneity in GC that ultimately represents a considerable unaddressed challenge for the clinicians.

V. CONCLUSION

5.1 CONCLUSION

Gastric cancer is a widely heterogeneous disease, and the identification of new predictive biomarkers and the development of personalised therapeutic targets has clinical relevance. Also, addressing biomarker heterogeneity is critical for the success of a customized medicine biomarker strategy.

Our data show that HRD is commonly observed in gastric cancer and suggest that ATM, ATR, BRCA1 and BRCA2 may serve as putative predictive biomarkers. Our study demonstrates that there is intratumour heterogeneity of the HR-status, expressed in the pattern of expression of each biomarker in the different components of mixed gastric carcinomas. This study provides a major contribution to the analysis of HR since an intensive optimization of the immunohistochemical technique was performed for a large panel of biomarkers. The information herein obtained may be useful for future studies addressing HR-status in gastric cancer or other tumour models. Larger series of cases will be necessary to confirm the major findings of this study.

For the future, a relevant challenge is the identification of the most informative signature of HRD, by the comparison of the immunohistochemical signatures of HRD with newer assays undergoing clinical validation, including 1) somatic mutations in homologous recombination genes, 2) “genomic scar” assays using array-based comparative genomic hybridization (aCGH), single nucleotide polymorphism (SNP) analysis or mutational signatures derived from next-generation sequencing, 3) transcriptional profiles of HRD, and 4) phenotypic or functional assays of protein expression and localization.[112]

The validation of the methods to measure HRD is crucial to aid decision-making for patient stratification and translational research in PARP inhibitor trials.

VI. REFERENCES

6.1 REFERENCES

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