COLORECTAL CANCER IN-BETWEEN CLINICAL APPLICATION AND TRANSLATIONAL RESEARCH: WHERE DO WE STAND AND WHAT CAN BE IMPROVED?

CANCRO COLORETAL ENTRE A APLICAÇÃO CLÍNICA E A INVESTIGAÇÃO TRANSLACIONAL – ONDE ESTAMOS E O QUE PODE SER MELHORADO?

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ABSTRACT

Colorectal cancer remains the second deadliest type of cancer with many causes resulting in a severe outcome. It is well recognized the higher level of cellular heterogeneity of colorectal cancer respect to any other type of cancer, which plays a significant role in its diagnosis, prognosis and treatment. Colorectal cancer is a curable disease when detected in early phases, up to 90% when detected in stage I, but the absence of symptoms makes the diagnosis a problematic process. Thus, the understanding of the tumour dynamics, cancer genetics and the expression of specific tumour biomarkers is crucial for the cancer early detection. Furthermore, parallel studies demonstrated the determinant role of post-translational modification in cancer formation and progression. This review aims to resume and combine all the different aspects involved in colorectal cancer malignancy, important for clinicians and researchers to understand where we currently stand, and which improvements are required.

Keywords: *Colorectal cancer, diagnosis, heterogeneity, biomarkers, glycosylation.*

RESUMO

O Cancro colorectal é o segundo tipo de cancro mais mortal. A heterogeneidade celular deste tumor tem impacto no diagnóstico, no tratamento e prognóstico. Este tumor maligno é curável quando diagnosticado em fase inicial, e a sobrevivencia é longa (90% aos 5 anos) quando diagnosticado no estágio I, mas a ausência de sintomas torna por vezes o diagnóstico difícil. Assim, o conhecimento da biologia tumoral, da genética e a expressão de biomarcadores tumorais específicos deste tumor malign são fundamentais para protocolos de detecção mais precoces. Estudos paralelos demonstraram o papel determinante das modificações pós-transcripcionais na carcinogénese e progressão do canco colorectal. A presente revisão tem como objetivo resumir e associar os diferentes aspectos biológicos envolvidos nos distintos perfis desta neoplasia maligna, importantes na decisão médica. Pretendemos dar uma visão o que os investigadores já sabem e o que é necessário ainda fazer e conhecer.

Palavras-chave: *Cancro colorectal, diagnóstico, heterogeneidade, biomarcadores, glicosilação.*

1. INTRODUCTION

Cancer is one of the major death-related problems worldwide. In 2015, in Europe, out of the 5,217,376 deaths registered, 1,062,112 were caused by cancer, reaching roughly 20% of the population.

Colorectal cancer (**CRC**) dwells from the second to the third position as the most diagnosed malignancy and leading cancer related death, in both sexes. In 2012, just in Europe, 447,000 new cases have been registered with 215,000 deaths [1]. It is a major public concern in the Western world and its incidence is also highly increasing in developing countries (Fig. 1). Genetic mutations, comprehensive of previous or family history of polyps-formation, and environmentally-induced epigenetic factors derived from food habits, physical activity or smoking are the main responsible of CRC incidence and increase [2–6].

In the advanced stages, current therapies are not curative, with the necessity of continuous treatments, which is becoming an important burden in the society[7].

At the metastatic stages, chemo-radiotherapy is the primary therapy, which improves the treatment for the local advanced stage cancer, but is not curative [1,8].

Thus, the greatest challenge of CRC, is the necessity to early stage detection methods. The identification of adenomas (benign tumour) before carcinomas formation (malignant tumour) can help treatment, thus reducing cancer incidence and increasing overall survival (OS)[7].

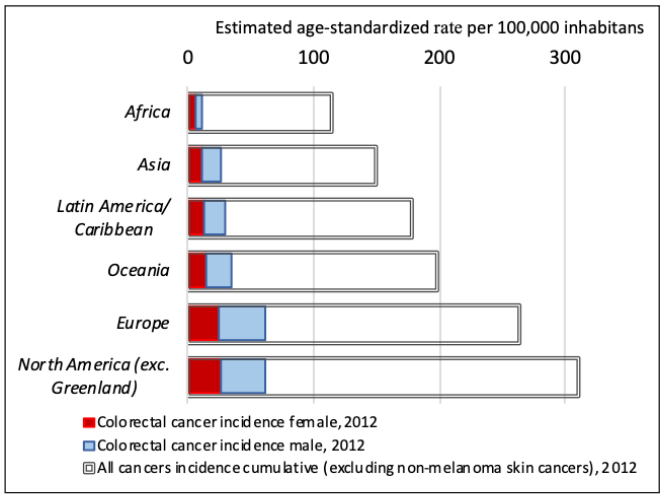

FIGURE 1 – **Comparison of all cancer incidences (transparent), excluding non-melanoma skin cancer with colorectal cancer incidence in males (blue) and females (red) on different continents. Western countries resulted in both cancer incidence and CRC formation, as a consequence of a consumerism lifestyle. The data are retrieved from GLOBOCAN 2012 project.**

2. THE ROLE OF CRC HETEROGENEITY IN THE CLINICAL DECISION

Investigating cancer heterogeneity has been one of the central focus in the last two decades. Cellular heterogeneity is often responsible for misleading diagnosis and prognosis associated with inefficient classification and reduced treatment efficacy[9].

CRC displays a high level of heterogeneity and thus of high molecular complexity.

From the pathological point of view, CRC histological analysis is essential for a correct diagnosis and prognosis. As shown in table 1, carcinomas account for 99.5% of all CRC, of which 92% are adenocarcinomas, originated from the normal epithelium of colorectal mucosa [6,7]. In the remaining 8%, a highly heterogeneous population, with sarcomas originating from the connective tissue, and carcinomas could be identified (Table 1).

The histological analysis is also essential for the determination of the stage and the grade of the tumour. The stages determine the level of extension of the tumour resulted from the uncontrolled growth, while the grade measures the degree of differentiation of the tumour cells and is a stage-independent measurement[10,11].

The main staging system is the TNM that aims to classify tumour progression in different groups The TNM staging is useful to determine the stage of the cancer, by a combination of three parameters: the *T* describing the size and wall penetration of the tumour, the *N* representing the lymph nodes involved and *M* eventual metastasis present[5]. The TNM scores

allows to classify each patient in different stages to which corresponds a particular survival expectancy:

i) *in situ or stage 0*, defines the presence of abnormal tissue,
ii) *localized or stage I* where the cancer is present only in the organ,
iii) *regional or stages –II /-III* with the tumour spreading to regional lymph nodes
iv) *distant or stage IV* with a spread into distant parts of the body (metastasis).

On the other hand, although the identification of the correct cancer stage and grade is fundamental to assign the correct therapy, the full understanding of the origin of CRC heterogeneity remains crucial for choosing the most efficient and effective treatment. Many theories have been postulated to explain the origin and function of the molecular heterogeneity in the tumour microenvironment [12].

In the first theory, the cancer stem model, it is proposed that a minor group of cells, called stem-cells, is responsible of tumour initiation and progression since they often carry specific proteins capable of inducing metastasis and tumour progression. In the second model, of clonal evolution, a more classical Darwinian model is pursued, where the mutations are acquired upon previous mutations and the fittest clone survive upon the other clones. Finally, in the third model, of the big bang, the mutations responsible for cancer development and progression happen mainly in early CRC formation[13].

These models have been theorised for a better comprehension of tumour progression, and instead of been contradictory, they can be considered complementary solutions to the problem.

In general, heterogeneity can be subdivided in inter-tumours variety, among different primary tumours of the same type in the same patient, or intra-tumour variety within the same tumours. It can also be sub-grouped into spatial and temporal heterogeneity, defining respectively, the diversity derived from different areas of the same neoplasm and the variability occurred throughout the time[14].

Table 1 – **Percent Distribution by Histology among Histologically Confirmed Cases, both sexes, USA, from 2011-2015.Different types (highlighted in darker-grey) and respective subtypes (light grey) of CRC carcinoma. Data collected by SEER 18 areas, adapted from SEER Cancer Statistics Review 1975-2015.**

Adenocarcinoma	92.10%
Adenocarcinoma, NOS	63.20%
Adenocarcinoma in adenomatous polyps	8.80%
Mucinous adenocarcinoma	6.30%
Adenocarcinoma in villous adenoma	1.90%
Signet ring adenocarcinoma	1.10%
Mucin-producing adenocarcinoma	0.70%
Other adenocarcinomas	10.20%
Other specific carcinomas	5.90%
Neuroendocrine carcinoid	4.40%
Others	1.50%
Epidermoid carcinoma	0.70%
Squamous cell carcinoma	0.70%
Unspecified, Carcinoma, NOS	0.80%

CRC among all cancers is considered one of the most heterogeneous, with differences in molecular expression, cellular subtypes and cancer location. Diversities among left, right colon and rectum have been investigated underlying genetic and immunological differences in colorectal cancer[15]. As a consequence, the creation of a new classification, including all the variables that contribute to diversity, has become fundamental for impact on colorectal cancer screening and therapy.[13].

3. FACTORS INDUCING HETEROGENEITY AND NEW CLASSIFICATION

The origin of the complex heterogenetic tumour environment can be related both to genetic and

to epigenetic factors and differential tumour environment.

Among the various genetic modifications, chromosomal instability (CIN), microsatellite instability (MSI), CpG island methylator phenotype (CIMP) and somatic copy number alterations (SCNAs) are considered the major cause for alterations in signalling pathways involved in CRC. In particular, the Adenomatous polyposis coli (APC) protein activator of the WNT proto-oncogene pathway, the RAS-MAPK cascade (with NRAS, BRAF and PIK3), the MYC transcription factor, the transforming growth factor (TGF)-β and the p53 pathways, are responsible for cell proliferation and uncontrolled growth [6,16,17]. For instance, the cause for around 90% of sporadic colon cancers is the aberrant activation of the Wnt/β-catenin signalling pathway induced by APC mutations. On the other hand, mutations in the epidermal growth factor receptor (EGFR) is responsible for upregulating the oncogenic PTEN/PI3K/Akt and RAS/RAF/MEK/ERK signalling pathways. However, the upregulated genes may differ between tumour types, underlying the need for mutation profiling for all CRC tumours[7]. Besides alterations in signalling pathways, other aspects of host-tumour interaction are crucial for the tumour microenvironment progression such as extracellular matrix, supporting stromal cells, metabolism and immune cells interactions[6,18]. As a consequence, the predictive value of the TNM staging is limited for outcome estimation and more precise strategies are needed.

Guinney *et al.* (2015) after evaluating six subtyping algorithms, that combined all the factors contributing to cancer heterogeneity, suggested a new sub-classification for CRC cancer types, denominated consensus molecular subtypes (CSM), defined by 4 subgroups:

a) CSM1 (14%), *MSI immune*, characterized by an overexpression of genes involved with immune infiltrate combined with a strong activation of immune evasion pathways associated with higher MSI.
b) CSM2 (37%), *the canonical*, shows an elevated SCNA with an upregulation of WNT and MYC proto oncogenes activation.
c) CSM3 (13%), *the metabolic*, with mutations in KRAS, presents a heterogeneous MSI associated with high metabolic activity.
d) CSM4 (23%), *the mesenchymal*, with the worst outcome, present activation of TGF-β, angiogenesis and epithelial-to-mesenchymal transition (EMT) necessary for migration and infiltration[19].

The CSM classification is an essential tool that combines the different variables for a better understanding of the complex tumour dynamics. Nevertheless, a 5th subgroup containing the 13% of unclassified cases of CRC was later identified, suggesting the necessity of further refinement of the CRC-CSMs, including new variables such as post-transcriptional and epigenetic factors[19].

4. CRC DETECTION

The current diagnostic and prognostic process of CRC involve very complicated procedures that involve patients' symptoms, total colonoscopy, and biomarkers [20]. Image techniques such as TC-scanning and PET-CT scans, echo-endoscopy are examples of the standard evaluation panoply.

Staging, while mandatory to fit treatment, remains, therefore, a burden for the health system and patient quality of life.

One major problem is the identification of early symptoms since lower pain can be a result of many intestinal diseases, most of the time not being cancer-related. As presented in the review by Vega *et al.*[20], many metadata analysis have been performed to find implications between symptoms and CRC. Among the different signs and symptoms, correlation is very poor. Yet, some signs and symptoms such as weight loss, iron deficiency anaemia and whole bleeding

show high specificity but low sensitivity (85%-92%). In other cases, age (>50), family history and faecal occult blood test appeared more sensitive (75%-91%) than any other symptoms. However, the presence of one or other symptoms could not be directly correlated to CRC[20–23]. Moreover, left-colon (LC) and the right colon (RC) have disclosed different behaviour in the symptoms, microbiome, clinical-, chromosomal- and molecular characteristics, with RC having a worse prognosis and suggesting different tumour entities[15,24]. In case of symptoms suspect of CRC, many screening programs have been developed, especially in the western countries, to help early diagnosis[20].

The main instrument used for CRC diagnosis is the colonoscopy. The endoscope not only detect polyp's formation in the intestine, but also cut the abnormal mass in situ for tissue biopsy and diagnosis. In particular, sigmoidoscopy is a colonoscopy that is performed only in the lower part of the colon, including the rectum and is less complicated than a full colonoscopy, with no need for sedation. Besides being an invasive technique, the availability of the instruments for colonoscopy is limited, and it has associated complications such as bleeding and perforation with the associated bacterial infections, including antibiotic-resistant bacteria such as *Escherichia coli* and *Klebsiella*[25]. Additionally, CRC endoscopy has shown to have limited value as a diagnostic tool in symptomatic patients, having high sensitivity but lacking specificity in recognizing cancer cells[20]. Therefore, significant efforts were put in the investigation and funding of biomarkers accessible by non-invasive techniques, as blood and stools. These biomarkers include genetic, proteomic and cellular fragments that can be released by the cancer cells into the circulation of the patient. They can be detected through tests like the faecal immunohistochemistry test (FIT) and blood or faecal haemoglobin (FOBT)[7]. Current detection of liquid biomarkers, found in non-solid biological tissue, also include micro-RNA (miRNA), circulating tumour DNA (ctDNA) and protein such as the carcinoembryonic antigen (CEA)[26]. Liquid biomarkers used for diagnostic and prognostic purposes are indeed contributing to the development of personalized treatment and targeted therapy.

5. IMPROVING CRC SCREENING

One of the most significant challenges is to find a good screening program, accessible and less invasive, with different diagnostic tools that would allow physicians to detect the disease at early stages. Advanced stages are not curable and as shown in figure 2A, with data obtained by the *Surveillance, Epidemiology, and End Results* (SEER) US cancer program: early diagnosis of CRC increases the chances of survival, from 90% when detected in stage I or local, with a drop to 14% when detected in metastasis, or distant phase [7,27]. However, data retrieved by the International Agency for Research on Cancer and the project CONCORD-2, show that up to 2009 the screening programs were not able to improve the 5-years survival rate, suggesting that instruments and biomarkers for early detections still lacked sensitivity or specificity (Fig. 2B) [28,29]. Moreover, as researched in the statistical study of 2014, the invasiveness feature of the CRC screening, it is not well accepted by the population, who avoid volunteering for the test. The study, entitled "self-reported last colorectal cancer screening test" showed that more of than 50% of the population enquired, in-between 25 and 64 years, did not perform any colorectal cancer screening test up to this date[30](Fig.2C). A meta-analysis conducted on clinical trials acquired from *ClinicalTrials.gov* showed that from 1971 to 2018 most of the trials presented, focused on the different treatments for CRC but few efforts were made on the discovery of new tools for diagnosis, prognosis, prevention and screening (Fig. 2D). Although finding the cure is and remains a fundamental objective, we must not forget the impact that prevention and early diagnosis would have in colorectal cancer treatment and statistics.

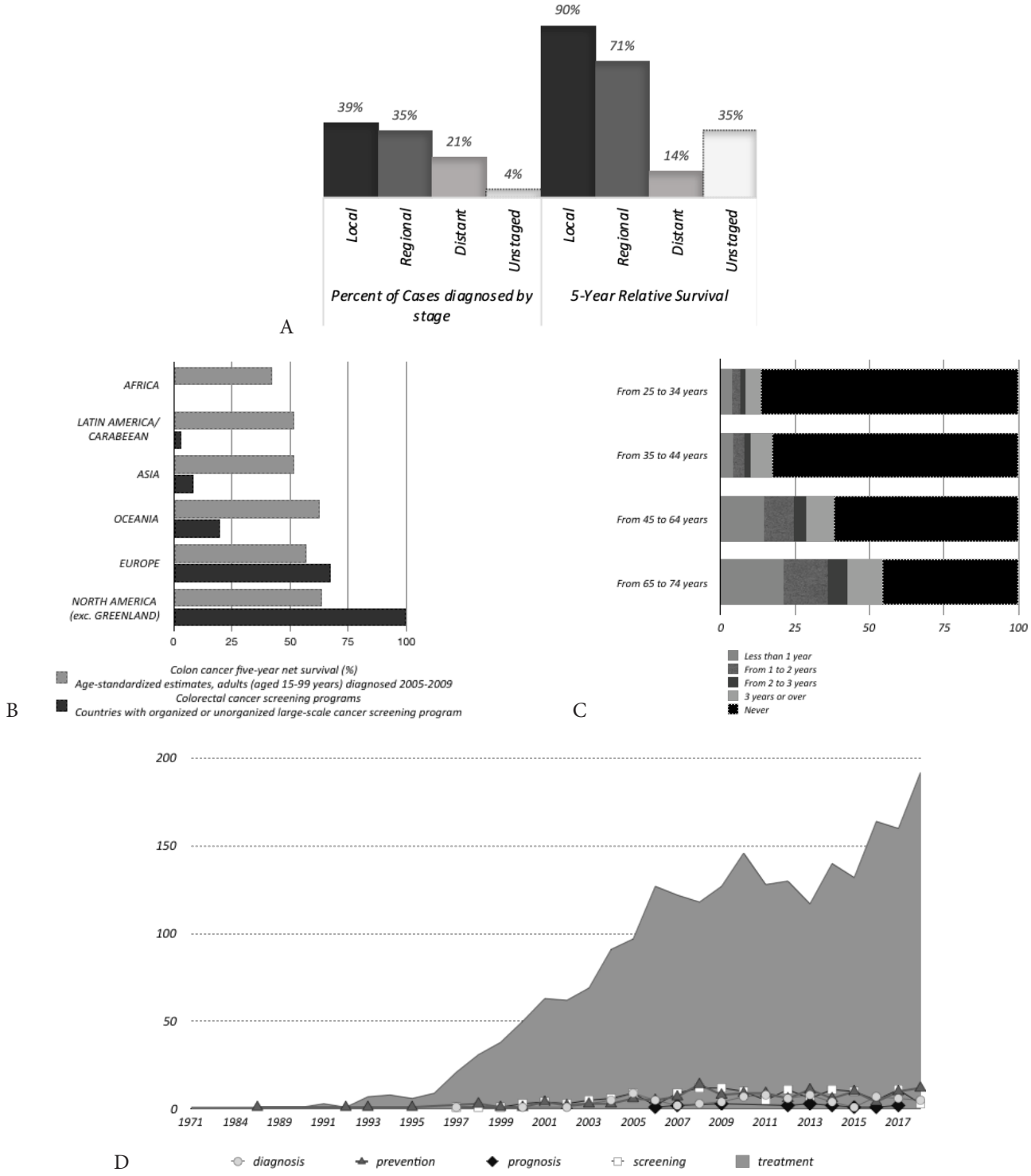

Figure 2 – **Statistical analysis of CRC in clinics. A) Cancer of the Colon and Rectum 5-Year SEER Conditional Relative Survival and 95% Confidence Intervals Probability of surviving the next five years given the cohort has already survived 0, 1, or 3 years 1998-2014 by stage at diagnosis. B) Correlation between colorectal cancer program screening and colon cancer five-year net survival worldwide 2005-2009 C) Self-reported last colorectal cancer screening test by sex, age and educational attainment level.2014. Data by Eurostat. D) Trends of CRC clinical trials research types: From 1971 to 2018, most of the efforts throughout the four clinical trial phases were put on treatment investigation. Study for new methods of prevention, diagnosis, screening and prognosis has slightly increased in the last 15 years. Data retrieved from ClinicalTrials.gov on the 28th October of 2018: presented 2331 of the 4911 cases.**

6. BIOMARKERS IN USE

Nowadays, the discovery of new biomarkers and the validation of the existing ones is crucial to facilitate the diagnostic process that leads to the understanding of a correct prognosis and treatment. Biomarkers or tumour markers can be genetic (DNA, RNA and miRNA), proteins (such as antibodies or glycoproteins) or may be the results of epigenetic factor (i.e. glycosylation)[31]. In general, they are referred to as tumour associated antigens (TAAs) and are expressed by the cancer cells in quantity proportionated to the number or mass of the neoplasm formation. The TAA concentration in the body fluids can help in early diagnosis stages, but also to monitor the treatment and follow-ups[32].

Among the different biomarkers, DNA, RNA and proteins have been mainly tested. Common DNA markers used for detection are KRAS, TP53, APC and MSI. Faecal and serum testing, such as the BEAMing technology, have shown high specificity in CRC, in detecting circulating tumour DNA (ctDNA), up to 98%[32,33]. RNA and micro RNA (miRNA, non-coding oligonucleotides) have been investigated as stool and blood biomarkers. MMP7, PTGS2, TP53 and MYBL2 (cancer-specific genes) isolated from colonocytes lack sensitivity for CRC detection, because RNA is particularly subjected to degradation in the stool[34].

Protein biomarkers are more stable and seem to be a more promising approach. However, at the moment, only few protein biomarkers are in use. Carcinoembryonic antigen (CEA), a glycoprotein overexpressed in many tumours, cannot distinguish malignant from benign neoplasm but is in use as a prognostic tool: its centration after R0 resection surgery, the complete removal of the tumour mass, is useful for understanding the success of the treatment, since its level decline after six weeks. In case of continuous high concentrations post-surgery, suggests metastasis and infiltration [32,35]. CA19.9 is a carbohydrate antigen, often associated with mucins, high molecular weight glycoproteins, and is used as a prognostic factor: high levels determine a poor prognosis. However, it is less sensitive than CEA. Further studies, that combined an algorithm with CA19.9, CEA and other two carbohydrates antigens, CA72-4 and CA242, showed and improved diagnostic power[36].

Further promising markers are the circulating tumour cells (CTCs). Tumour cells detach from the primary tumour into the bloodstream in the early phases of progression. It could become a significant prognostic factor detecting the gravity of tumour stage. Until now, CTCs are isolated through positive selection for an epithelial cell surface marker—, epithelial cell adhesion marker (EpCAM), overexpressed in cancer cells. However, when the cells undergo EMT, the EpCAM expression drops and the sensitivity of the detection, decreases. [26,37,38].

Nevertheless, as presented in a study of Neves *et al.* (2019), CTCs can also offer another specific tumour antigen, named sialyl Tn (STn, sialylated Thomsen-nouvelle antigen) which is an aberrant truncated O-glycan. It decorates glycoproteins and has been recognized to be overexpressed in advanced bladder and colon cancer[39,40]. Using a size-based microfluidic device, they have shown that the majority of CTCs from the blood of patients with metastatic bladder and colorectal cancers (>90%) correlate with high STn expression[41].

Tumour markers other than diagnostic and prognostic tools can be used as predictive tools. While as a prognostic tool it determines the overall outcome of the disease, independent of the treatment, as a predictive marker it allows distinguishing between different therapies that will be effective[42,43]. The role of the predictive biomarkers has gained importance for personalized medicine and targeted therapy. MSI, chromosome 18q loss of heterozygosity (18qLOH), p53, KRAS, BRAF, NRAS, PIK3CA mutations, PTEN expression were all studied as indicators for adjuvant therapy that may include chemotherapy (5-fluorouracil, oxaliplatin or irinotecan), radiation therapy, hormone therapy, targeted therapy (e.g. anti-VEGF or anti-EGFR: bevacizumab or cetuximab, panitumumab) or biological therapy[44].

It has been described the role of RAS^{mut} as a negative predictive biomarker for anti-EGFR treatment, and the reduced efficiency of the therapy with RAS^{wt}/$BRAF^{mut}$ patients.

$BRAF^{mut}$, near always mutated in position V600E, is mostly located in the right-side colon and are negative prognostic for metastatic CRC (mCRC). MSI has a predictive value in immune checkpoint therapies[1]. MSI or microsatellite stability (MSS) and the presence or absence of 18q chromosome deletion are requirements for therapies based on 5-FU[32].

However, most of the studies showed contradictory results, excluding few established cases, such as the role of KRAS gene in anti-EGFR therapy. Further work on function definition and adequate validation remains to be done (Table 2)[44].

7. THE ROLE OF THE CONSENSUS MOLECULAR SUBTYPES (CSM) CLASSIFICATION IN PROGNOSIS AND TREATMENT

At present, the only curative treatment is surgery with an R0 resection that consists of the complete removal of the tumour mass, to be distinguished from the R1 (microscopic residual tumour) or R2 (macroscopic residual tumour) resections[45]. Nevertheless, the CSM classification is becoming the most crucial tool for researchers and clinician to combine and correlate the role of the different tumour marker for a correct CRC prognosis and treatment. The classification takes into account the different outcomes of the therapies and the proper therapies are associated with the biomarker's expression: ESMO guidelines have already adopted and adapted the therapies indication following the genetic, epigenetic and proteomic profile, associated to the different CSM classification[1,19].

Trinh *et al.*[46] validated the prognostic value of CRC subtyping using five additional markers (CDX2, FRMD6, HTR2B, ZEB1 and KER).

Using immunohistochemistry (IHC) technique on four independent CRC patients' cohorts, they have created a tool for CRC classification based on semi-quantitative pathology scoring. The MSI status identifies the CSM1 subtype, the CDX2 a marker highly expressed in epithelial-like tumour selects the CSM2/3 subtype. While HTR2B and FRMD6 expressed in mesenchymal like cells selects the CSM4 subtype.

The markers ZEB1 and cytokeratin (AE1/AE3) confirms the EMT transition of each subtype. The IHC analysis permit to confirm the stratification of CRC groups and verify the therapeutic benefit for each subtype[47]. It was proven an increase of OS of epithelial like tumour $KRAS^{wt}$/$BRAF^{wt}$ patients in treatment of anti-EGFR (cetuximab) to a standard regimen of capecitabine, oxaliplatin and anti-VEGF (bevacizumab), in advanced CRC patients. Same therapy on CSM4 mesenchymal-like, with $KRAS^{mt}$/$BRAF^{mt}$ was not beneficial[47]. On the downside this robust IHC classifier can be used more as a tool for the stratification than a prognostic or predictive marker with a missing marker that allows distinguishing CSM2 from CSM3 epithelial-like tumour.

8. GLYCOSYLATION AS THE MISSING LINK

Genomic studies and their correlation with tumour development and treatment success has been the primary focus of the last decade. The significance of these studies in CRC research is undeniable in diagnosis and prognosis. However, as previously discussed, the heterogeneity of CRC is still responsible for misleading diagnosis. For this reason, more studies on tumour microenvironment have been equally in-depth, for the understanding of cancer dynamics. The importance of epigenetic factors influencing post-translational modification cells mechanisms has emerged. Among these, aberrant glycosylation is highly correlated with CRC phenotype and the translation from bench to the clinic may results in new improvement not only in treatment but also in diagnosis and in discovering new biomarkers[31,48].

Table 2 – **Genetic tumour markers original function, mutation and consequences of altered pathway. Preferences of colon location and predictive potential are also listed. Data adapted from different publications, mainly the reviews of Zarkavelis *et al.* and Salem *et al.* [24,85–91].**

MARKER	FUNCTION	MUTATION	CONSEQUENCE	COLORECTAL LOCATION	PROGNOSTIC AND PREDICTIVE VALUE
KRAS/NRAS	proto oncogene encodes a GTPase protein (KRAS)	KRAS: exon 2 (codons 12 and 13) or exon 3 o NRAS: exons 2, 3 and 4 of	permanent activation of the RAS (RAS/RAF/MAPK) pathway	NRAS more present in the left-side	Resistance to anti-EGFR
BRAF	encodes serine threonine kinase proteins	8% of CRC carry the distinct BRAF V600E mutation	direct downstream target of KRAS	More present in the right side associated with high MSI	resistance to BRAF inhibitors
DNA MMR (mismatch repair) genes /MSI Microsatellites instability	MMR gene ability to fix DNA errors occurring during replication	inactivated as a result of sporadic MLH1 promoter hypermethylation or germline mutations in MLH1, MSH2, MSH6 and PMS2 genes	MSI are originated from MMR deficient or proficient tumours and thus genomic instability	Higher in right-side of the colon	High MSI doesn't benefit of 5FU but have better outcomes in stage II
CpG island methylator phenotype (CIMP)	CpG island are regions with a high frequency of CpG site (i.e., cytosine residues preceding guanines)	Aberrant methylation of CpG islands	hypermethylated genes, such as SLC5A8, ITGA4, SFRP2, CDKN2A, HLTF, and MGMT	High in the right side associated with KRAS wt and BRAF mut	Worse survival outcomes and poorer response to anti-EGFR
EGFR/HER family EGFR	Receptor of epidermal growth factor (EGF) that stimulates cell growth and differentiation	Increased gene copies	Uncontrolled cell growth when constantly activated	Mostly Right sided	HER2 could be responsible for EGFR resistant pathway
TP53	TP53 protein has role in cells growth arrest DNA repair	Point mutation in codon 72	Promotes malignant process and carcinogenesis	Mostly rectal	TP53 high mutation suggest worse survival
APC/Đ-catenin	APC (adenomatous polyposis) tumour suppressor protein	promoter hypermethylation and somatic mutations	Mutations of APC activates the Wnt pathway and inactivates glycogen synthase-kinase-3Đ and Đ-catenin	Mostly rectal	APC hypermethylation for early diagnosis
miRNA	microRNA are highly stable structures with a hairpin-loop shape and small size	Expression associated with different mutations of other markers	microRNAs, play a key role in tumour suppression or growth	Depends on miRNAs. i.e. miRNA146a and 147b higher in the left side	1. miR-31: present with $BRAF^{mt}$. potential predictive biomarker 2. miR-99a and miR-125b: good prognosis with $KRAS^{wt}$ responding to EGFR therapies 3. miR-181 poor prognosis with KRAS wt responding to EGFR therapies 4. miR-622: poor responders to radiation therapy in rectal cancer
18qLOH	Chromosome loci	Loss of heterozygosis At D18S58 and D18S61 loci	Allelic imbalance	Distal colon, Left sided	greater survival rate when treated with capecitabin
PIK3CA	Encodes for human p110Đ protein is encoded by the *PIK3CA* gene	GLU542, GLU545, and HIS1047	Highly oncogenic	Decreasing mutation from right to left to rectal	Implications with anti-EGFR treatment resistance and increased benefit from aspirin therapy
PTEN	Phosphatase and tensin homolog (PTEN) is a tumour suppressor protein	Mutation and deletion of PTEN	Increase cell proliferation and reduce cell death	Rectal tumour mainly but also right and left side	Loss of expression may induce anti-EGFR resistance
MYC	protooncogene	Constitutively expressed	Cell proliferation	Left sided	Unfavourable prognostic
WNT	Group on signal pathways protooncogene	Overexpression of WNT ligands	Involved with EMT	Left sided	Early stages of carcinogenesis
***TGF*-b**	Transforming growth factor cytokine, stop cell cycle at G1	Inhibition of TGF-beta	Proliferation, angiogenesis and immunosuppression	Left sided	TGF-beta activity can induce High risk of CRC relapse upon treatment

Glycosylation is one of the most common and important post-translational modification that affects key biology processes such as cell-cell interaction, growth, adhesion etc[49]. Glycosylation occurs both on proteins and lipids: on the former, the sugar structures can be borne on asparagine (N-Glycans) or serine/threonine (O-Glycans) residues while on the latter, the dominant class is recognized mostly in the glycosphingolipids class (GLS)[50]. The genome does not directly encode glycans, but the enzymes involved in its biosynthesis. In turn, their biosynthesis depends on metabolism signal transduction and the cellular status[51]. Several studies revealed that aberrant glycosylation is a universal feature in various steps of malignant transformation and tumour progression[52]. Aberrant glycosylation leads to dysregulation of essential cellular processes. Additionally, it induces the novel biomarkers that can distinguish healthy from cancerous tissue, a vital characteristic in therapeutic approach[53]. For instance, the expression of the STn antigen is expressed specifically in tumour tissue and, in bladder cancer, it has been reported overexpressed in 75% of high-grade bladder tumours presenting elevated proliferation rates and high risk of recurrence/progression expressed STn. Thus, targeting STn could be a new valid therapy approach[54]. Likewise, aberrant glycosylation is a feature of all CRC. A general increase in N-glycan β1,6-branching, (poly-) N-acetyllactosamine extensions and (truncated) high-mannose has been proven, as well as, higher levels of core 1 glycans, (sialyl) T-antigen, (sialyl) Tn-antigen, and a generally higher density of O-glycans. Increase in sialylation and fucosylation results in a high expression of Lewis antigens and their sialylated derivates[31]. All these markers can be considered target for personalized therapy, being involved in main tumour cell functions such as tumorigenesis, metastasis, modulation of immunity, and resistance to antitumour therapy.

Two are the main mechanisms involved in glycan structure changes: incomplete synthesis and neosynthesis[50]. Examples of incomplete synthesis can be attributed to the misregulation or silencing of glycosyltransferases and glycosidases, key enzymes responsible for the glycosylation processes, which occur in the formation and accumulation of aberrant O-glycans such as SLewis ^{X} and STn [55]. Neosynthesis can originate from altered expression of enzymes involved in the glycosylation pathways, possibly related to hypoxic conditions in tumours[50]. The healthy colon mucosa presents bisecting N-acetylglucosamines (GlcNAc) on N-glycans as well as core 3 and core 4 O-glycans, globo-type glycosphingolipid (GSL) glycans, and disialylated gangliosides[31].

Many glycan modifications have been found and investigated throughout the stages of CRC. Lewis antigens and its sialylated derivatives (Le^x/sLe^x and Le^a/sLe^a) are the most prominent epitopes on both glycoproteins and glycolipids. Its overexpression is related to CRC malignant transformations and may lead to increased tumour cell adhesion and motility, thereby resulting in metastasis[56]. Some alterations, such as gangliosides GD3 and GM2 as well as the globo-type GSL Gb3 are specifically related to angiogenesis[57]. Many protein carriers, such as the mucin MUC1, CD44v6, and CEA have been identified carrying T- ST- (sialylated/Thomsen-Friedenreich (T)-antigen) and STn tumour specific antigens[31]. Mucins, for instance, are major secretory products of the colon and are hyper glycosylated in CRC, due to the overexpression of the β1,3-galactosyltransferase. This aberrant glycosylation is associated with poor survival, cancer progression, and metastasis[58].

Nowadays, the glycoproteins carcinoembryonic antigen (CEA) and carbohydrate antigen sLe^a (CA19.9), as already mentioned, are the most widely applied serum biomarkers in clinics. Increased serum level of CEA or sLe^a indicates the presence of CRC[59]. Other biomarkers such as highly fucosylated haptoglobin (Fuc-Hpt) are elevated in certain CRC patients, in relation to the proximity of the tumour to the liver and distance metastasis. The study of the combination of Fuc-Hpt and CEA, has shown to be a promising novel prognostic marker in CRC[60].

A different approach adopted by Rho *et al.* (2014) used a microarray paired with specific antibodies

against sLe^x/sLe^a. This enable discovering new biomarkers that can distinguish between stages III, IV and healthy control in CRC [61]. Croce *et al.* (2005) have also investigated the α1-acid glycoprotein (AGP) which has been identified as a putative carrier of sLe^x antigen in colorectal carcinoma[62].

The role of aberrant glycosylation has shown to have an effective correlation with CRC formation and has disclosed a great potential for use as non-invasive biomarker. However, a characterisation between aberrant glycosylation and cancer stages is still needed to depict the right candidates for early diagnosis.

9. THE ROLE OF GLYCOSYLATION IN THERAPY

The tumour-associated carbohydrate antigens (TACAs) show absence or low expression in normal healthy tissues. These features facilitates TACAs to be disease-specific immunotherapeutic targets and, offer lower risk of side effects, playing critical roles in cancer cell biology[50]. Many monoclonal antibodies (mAbs) have been and are being produced against these type of antigens[49].

Essential characteristics of mAbs are affinity, which measures the strength of the interaction between an epitope and an antibody's antigen binding site and the avidity, which gives a measure of the overall strength of an antibody-antigen complex. These two parameters are often exploited to selectively target tumours. The IgG mAbs isotype is generally preferred because of their high affinity and antibody-dependent cell cytotoxicity (ADCC), but also IgMs isotype as a strong inducer of complement-dependent cytotoxicity (CDC). ADCC and CDC mechanisms have been proved to be very important in the clinical efficacy of antiganglioside mAbs[50].

Sterner *et al.* (2016) generated a database for Anti-Glycan Reagent (DAGR) where currently, 1120 unique monoclonal antibody entries have been classified[63]. Many mAbs have been produced for Lewis antigen detection, in particular of $Lewis^y$ (Le^y). They have been created with different degrees of specificity and cross reaction with other closely related Lewis antigens and tested either alone or as a targeting agent coupled to a toxin or drugs[64].

Among these are BR96, a mouse or chimeric human anti-$Le^{y/x}$ and B3, a mouse anti-Le^y mAb: the former has been studied coupled either to toxins or chemotherapeutic agents, while the latter has been tested coupled to Pseudomonas aeruginosa exotoxin A. BR96 resulted as a powerful immunoconjugate with high efficiency in CRC xenograft studies[65]. Also, B3, showed significant clinical activity, with responses in colon cancer[66].

Durrant *et al.* (2006) produced SC104, a mouse IgG1 that recognizes sialyltetraosylceramide, that can cause tumour cell death by ADCC, CDC and apoptosis. *In vivo*, it induced potent tumour rejection in colorectal xenograft models[67]. Other two mAbs anti sLe^a (5B1, IgG1 and 7E3, IgM) kill target cells by CDC and/or ADCC, thereby prolonging the survival of mice in a colorectal xenograft model[68].

RAV12, a chimeric IgG1 mAb which recognizes a sugar, RAAG12, overexpressed on adenocarcinomas of colorectal has been taken into account as a targeting approach. RAV12 has demonstrated to be cytotoxic *in vitro* against the colorectal cell line COLO205 and induced antitumour activity *in vivo* in athymic mice bearing human colon[69].

Other immune-conjugates have been created against protein biomarkers of CRC. For instance PR1A3, a humanized IgG1 mAb anti-CEA linked to the immunotoxin n-succinimidyl-3-(2-pyridyldithio)-propionate was demonstrated to be effective in the treatment of CRC[70]. Another promising mAb, Edrecolomab, reacts with EpCAM, a pan-epithelial differentiation antigen that is overexpressed in 90% of CRC cases[71]. CD24 is also expressed in ~ 90% of colorectal adenomas and adenocarcinomas, and an anti-CD24 mAb (SWA11) linked to the immunotoxin ZZ-PE38 showed improved cytotocixity *in vitro*[72].

Loureiro *et al.* (2018) developed a monoclonal antibody against STn-antigen, the LA25, that show high affinity and specificity. The L2A5 showed great

potential as therapeutic and diagnostic tool in clinics, being able to react with CRC and not normal colon[73].

mAbs, specially of murine origin, can be recognized as 'non-self' molecules by the immune system and trigger immune reactions such as acute anaphylaxis and serum sickness. Hence, in order to minimize the possibility of these events, antibody technology researcher have generated single-chain variable fragments: these engineered antibodies conserve the function of the variable part (Fv) but lack the constant Fc region, that is considered to be highly immunogenic[74]. Lutterbuese *et al.* (2009), have constructed a series of bispecific single-chain antibodies (bscAb) that combine various single-chain variable fragments, recognizing human CEA and human CD3, a T-cell co-receptor that helps to activate the cytotoxic T cells. Treatment with CEA/CD3-bscAbs showed killing of tumour by human T cells and prevented the growth of human CRC cell lines in a severe combined immunodeficiency mouse[75].

In this context, existing monoclonal antibodies gained attention for the production of bscAb, where the bi-specificity can be directed simultaneously against both protein biomarkers, such as CEA and TACAs.

10. GLYCOSYLATION AND NEW THERAPIES

The urge for innovative treatments has led many research groups to investigate new ways to exploit aberrant glycosylation in cancer.

A promising approach is the carbohydrate-based anticancer vaccine, which has been ongoing under clinical trials. The idea relies on triggering anti-glycan immune responses and breaking the immune self-tolerance since many aberrant glycans are recognized as 'self' molecules[76]. The glycan-based vaccines link multiple aberrant glycans to an immunogenic carrier protein keyhole limpet hemocyanin (KLH)[76]. Theratope, a phase III KLH-STn conjugate, tested in metastatic breast cancer has shown to increase the OS of patients undergoing hormone therapy. However, this vaccine failed when administered alone as it fails to activate T cell-mediated immune response [77].

Further studies on glycan-based vaccines have tried to mimic the cell surface to stimulate B cell and T cell-mediate immune responses. A tri-genic vaccine containing globo H, Le^y and Tn was produced from Danieshefky *et al.* (2000) and showed to recruit helper T cells against each of the aberrant glycan[78]. Finally, higher immunogenic vaccines have been produced by incorporating chemically modified sialic acid residues with unnatural N-acyl side chains[53] into KLH conjugates.

Esko *et al.* (2005) proposed to use decoys as anti-metastatic drugs through metabolic inhibition of sLe^x in metastasis[79]. In a parallel study, Shirota *et al.* investigated the GSC-150 an analogue of sLe^x that binds to selectin, adhesion receptors which promote interactions of tumour cells with host platelets, leukocytes and endothelial cells. The data suggested that the sLe^x –selectin interaction is a contributing factor to metastasis with selectin inhibitors having a potential role in cancer treatment[80]. In addition to this theory the clinically approved anticoagulant drug heparin as a selectin inhibitor resulted in the suppression of tumour metastasis in experimental animal models and has shown beneficial effects in human clinical trials of colon cancer[81].

Posey *et al.* (2016) used genetically modified T cells that express chimeric antigen receptor (CARs) targeting cancer-associated Tn glycoform of MUC1. The CARs displayed target-specific cytotoxicity and could successfully control tumour growth in xenografts models of T cell leukaemia and pancreatic cancer[82].

Finally, significant developments have been made in the field of nano-glycomics. For instance, glyconanoparticles (GNP) mimicking the carbohydrate decorated cells serve as glyco-decoys controlling the cell adhesion and competing with interactions at the host cell surface. Nanoparticles (NPs) could be functionalized to deliver glycan-based galectin inhibitors or glycan ligands to sites

of tumour growth, as already shown in pancreatic cancer tissue[83].

Danhier *et al.* (2015) have performed local administration of chitosan lipid nanocapsules (LNCs) containing the anti-epidermal growth factor receptor (EGFR) and anti-GAL-1 siRNAs, which prolonged survival of nude mice bearing orthotropic U87MG glioblastoma cells[84].

11. CONCLUSION

In the last 10 years, many advances have been made in CRC research and technology development towards an increase on the average of the OS of CRC and mCRC patients. High cancer heterogeneity makes the progression towards a personalized treatment more difficult. Early diagnosis can improve the OS of CRC patients, but the absence of symptoms and of specific early stages liquid biomarkers leads to late detection. Most of the screening program proposed did not result in improved 5 years of OS, suggesting the urge to improve the existing programs with different and more specific test. Moreover, in the last 30 years the clinical trials have been focused to find the most effective treatment, but few efforts have been put in looking for new early diagnostic methods.

Genetic biomarkers have been studied in order to understand their role as diagnostic, prognostic and predictive biomarkers. Except for some established cases such as such the role of KRAS in anti-EGFR therapies, further validation of other biomarkers has to be improved. The CSM classification in CRC, contributed to the understanding of cancer heterogeneity by considering other aspects of host-tumour interaction, i.e. the immune system. Besides the DNA- and protein-based therapies, also post-translational modifications, such as glycosylation, play a crucial role in tumour microenvironment and for this, it has been investigated. Aberrant glycans expressed mainly on protein surfaces suggested high potential as diagnostic tool in liquid biopsy; the same targets could be exploited in future targeted therapies.

Antibodies and related products are the fastest growing class of therapeutic agents. Monoclonal and bispecific short fragments antibodies have been demonstrated to have promising effects in specific cancer cytotoxicity. However, the presence of side effects requires new ideas to combine functionality of monoclonal antibodies, epitopes and stealth 'self' molecular behaviour. As alternative techniques cancer-based vaccines and CAR-T cells have been proposed and studied.

In conclusion, CRC still remains the second deadliest kind of cancer and although the many steps forward were made in understanding cancer heterogeneity and improving personalized treatment, it is still missing a focus on early diagnosis. The field of glycobiology appears interesting for the creation of new therapeutic and diagnostic target, where the challenge is the understanding of the biological context of the glycoprotein and glycolipid targets on cancer cells.

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