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Expression of β-catenin, Ki67 and stroma percentage in Colorectal Cancer

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ABSTRACT

AIMS. The purpose of this study was to explore the expression of β-catenin, Ki67 and stroma percentage in colorectal cancer and investigate their role as biological and prognostic biomarkers.

METHODS. Colorectal cancer samples of approximately 1000 patients were collected, together with the corresponding clinicopathological data. Tissue microarrays were constructed, immunohistochemically stained for β-catenin and Ki67 and scanned into digital images. Nuclear, cytoplasmic and membranous β-catenin staining intensities were manually scored; Ki67 expression was analysed using the Qu Path platform. H-scores and Ki67LI were computed for β-catenin and Ki67 respectively; the stroma percentage was estimated from the content of the TMAs. The statistical analyses were performed using IBM SPSS Statistics version 26. Different cut-offs were used to distinguish population subgroups (i.e. low/high expression), depending on the biological characteristics of the biomarkers and the characteristics of the distributions. A χ^2 test was carried out to investigate the correlation between the biomarkers and clinicopathological parameters. Kaplan-Meier's curves and Log-rank tests were performed to analyse the correlation with survival. The Cox proportional hazard model was used to evaluate the independent effect of the variables on survival. Further analyses were carried out after patients stratification for MMR status, tumour location and final stage. Different parametric and non-parametric tests and relative effect sizes were used to evaluate the correlation between the biomarkers, between the biomarkers in CRC and normal tissue and between the biomarkers in luminal, central and peripheral portions of the tumour. Results were considered significant when showed a p-value less than 0.05.

RESULTS. Nuclear and cytoplasmic β-catenin were found associated with distal CRCs and MMR proficiency, whereas membranous expression was associated with proximal CRCs. Ki67 was significantly correlated with deficient MMR status, conspicuous peritumoral lymphocytes and pushing tumour edge. Stroma-rich tumours displayed advanced T stage, lymph nodes involvement and presence of metastases; they are also associated with recurrence, vascular and perineural invasion and characterized by poor differentiation and infiltrative tumour edge. Survival analyses revealed that nuclear β-catenin is an independent prognostic factor and predictive of good prognosis in stage III CRCs and that high Ki67 is associated with better outcome in dMMR and stage III CRCs but worse outcome in stage II patients. Finally, tumours with high stroma content were associated with poor prognosis. β-catenin changed sub-cellular localization in CRC compared to normal intestinal epithelium, shifting from a predominant membranous localization to a higher nuclear expression; likewise, Ki67 was higher in tumour than normal tissue. Ki67 and β-catenin and β-catenin and stroma were found significantly correlated. Stroma-rich tumours were found associated with low proliferative rate and high nuclear β-catenin. Lastly, we couldn't establish a significant differential expression of these biomarkers between luminal, central and peripheral portions of tumours.

CONCLUSION. In summary, our data show that high β-catenin is a feature of left-sided CRCs, that Ki67 is associated with dMMR tumours and their histological characteristics and that stroma-rich tumours display aspects of advanced disease. High nuclear β-catenin and high Ki67 are associated with good prognosis in stage III CRCs, whereas stroma percentage is predictive of shorter survival.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common cause of cancer-related deaths in both men and women worldwide. Although significant progress has been made in understanding the molecular basis of CRC, the optimal treatments for most patients remain the 'untailored' surgery and chemotherapy.[1]

Relapse is an important factor that influences the survival in colorectal cancer: between 25 and 40% of all patients who undergo primary treatment will develop recurrent disease within 5 years.[2] Cancer relapse occurs when the primary cancer cells survive the initial treatment and eventually will give rise to a new tumour. An explanation to this phenomenon is the extreme heterogeneity that characterizes CRC, which generates a different response to therapy among the tumour cell subpopulations. Tumour heterogeneity is the result of genetic mutations, epigenetic regulation, post-translational modifications and tumour microenvironment, as they all play a role in tumorigenesis and cancer progression.

Cancer progression is a big talking point in cancer research, which for years has been targeting the identification of biomarkers that can be useful for the treatment and follow-up of CRC patients. The molecular characterization of CRC is particularly important for patients with stage II and III disease, who may require adjuvant chemotherapy or targeted therapy.

In this background, cancer research is working to better understand the molecular mechanisms behind tumour development and progression. Increase of cell proliferation, apoptosis inhibition, invasiveness, cell adhesion alterations, angiogenesis and escape of immune surveillance are just a few of the mechanisms that are being investigated in order to develop such knowledge. The identification of biomarkers that can help with treatment decision and monitoring will improve survival rates, avoid over-treatment and strongly contribute to personalised medicine.

1. Colorectal Cancer Epidemiology and Risk Factors

In 2012 Europe registered 447 000 new cases of colorectal cancer, which represents 13.2% and 12.7% of cancer cases in men and women, respectively. [3] The same study reported almost 215 000 deaths from colorectal cancer, designating it as the second most frequent cause of death from cancer. The mortality rate is related to the high frequency of recurrence: 50% of patients develop metastases and 25% of patients have metastases at the time of diagnosis. [4] The 5-year survival ranges from 28.5% to 57% in men and from 30.9% to 60% in women. Overall, the incidence and mortality rates have decreased, most likely due to several factors, such as the screening program, reduction of risk factors e.g. smoking and improved treatments. [5]

There are many risk factors associated with an increased risk to develop CRC, and they can be categorised into lifestyle and genetic aspects.

Physical activity has a known inverse correlation with the risk of cancer and, in respect to gastrointestinal tumours, it is associated with a reduced risk of 25% of developing colon cancer and 15% of developing colonic precancerous lesions, e.g. adenomas. [6] Moreover, sedentary behaviour is associated with approximately 20% increased mortality risk in CRC patients.[7] Being overweight or obese leads to an increased risk of CRC, with a stronger association between BMI and CRC in male population.[8] Diet is an established factor associated with cancer incidence. Several studies reported that a diet low in red and processed meat, saturated fat and sugar and rich in fruits, vegetables, fibres, legumes, wholegrains and low-fat dairy has protective effect on CRC. Alcohol intake is also associated with CRC risk.[9] Smoking is a further determinant of carcinogenesis. Specifically, smokers have been reported to have a higher risk of MSI-high, CIMP-positive and BRAF mutation-positive subtypes.[10]

The majority of CRCs are sporadic, and, in these cases, age is a critical risk factor. CRC is unlikely to be diagnosed before the age of 40 and the incidence rates rise after the age of 50, with significant increments between one decade and the next.[11] However, in recent years, a trend of increased diagnoses among young people has been reported: before the age of 50, the incidence rate increased by approximately 2% annually, and within this population rectal tumours are the most common (37%), followed by distal colon (25%). [12] Familial adenomatous polyposis (FAP) and Hereditary non-polyposis colorectal cancer (HNPCC or Lynch syndrome) are rare (5% of all CRC) but account for most familial colon cancer syndromes. Inherited syndromes are associated with early onset CRC: among patients diagnosed with CRC younger than 50 years, 1 of every 6 patients has at least one pathogenic mutation in cancer susceptibility genes. [13] Even without a determined genetic predisposition, family members with CRC represent a risk factor: having one affected first-degree relative increases the risk of developing CRC, which doubles with two or more affected relatives.[14] Along with family history, also personal history of sporadic CRC or adenomatous polyps increases the likelihood for future disease: 1.5 to 2% of patients who undergo resection of CRC show metachronous primary cancers in the first 5 years post-surgery[11] and the relative risk of CRC for patients with large polyps or with high-grade dysplasia polyps ranges from 3.5 to 6.5[15] .

Inflammatory bowel diseases are an additional non-modifiable risk factors that show a positive correlation with CRC.[8] Ulcerative colitis represents an important risk factor for CRC, depending on the extent, duration and activity of disease: patients with pancolitis see their risk rising up to 30% by the fourth decade of disease. Data about Crohn's disease are less strong, but the extent of colonic mucosa involved appears to be a major determinant for CRC development in these patients as well.[11]

2. Molecular Insights of Colorectal Cancer

The recent insights of the genetics and epigenetics of CRC have contributed to a novel molecular characterization of CRC subtypes. Colorectal cancer is the final step of a process that starts with a benign disease (tubular adenomas or serrated polyps) and progresses into a malignant neoplasm over many years. Genetic and epigenetic mutations and microenvironment modifications are collectively responsible for carcinogenesis. This transformation process is not homogeneous, causing the

formation of tumours subclasses that harbour unique clinical and pathological features which can be exploited to refine patients stratification and develop ad hoc treatments.

Genetic Pathways in CRC

Two major patterns describe the development of CRC, the adenoma-carcinoma sequence and the serrated pathway, which come with different precursor lesions and are characterized by a different mutational landscape, from DNA alterations (e.g. aneuploidy, genome instability, etc.) to epigenetic modifications (e.g. methylation of CpG islands within the promoter region)[16].

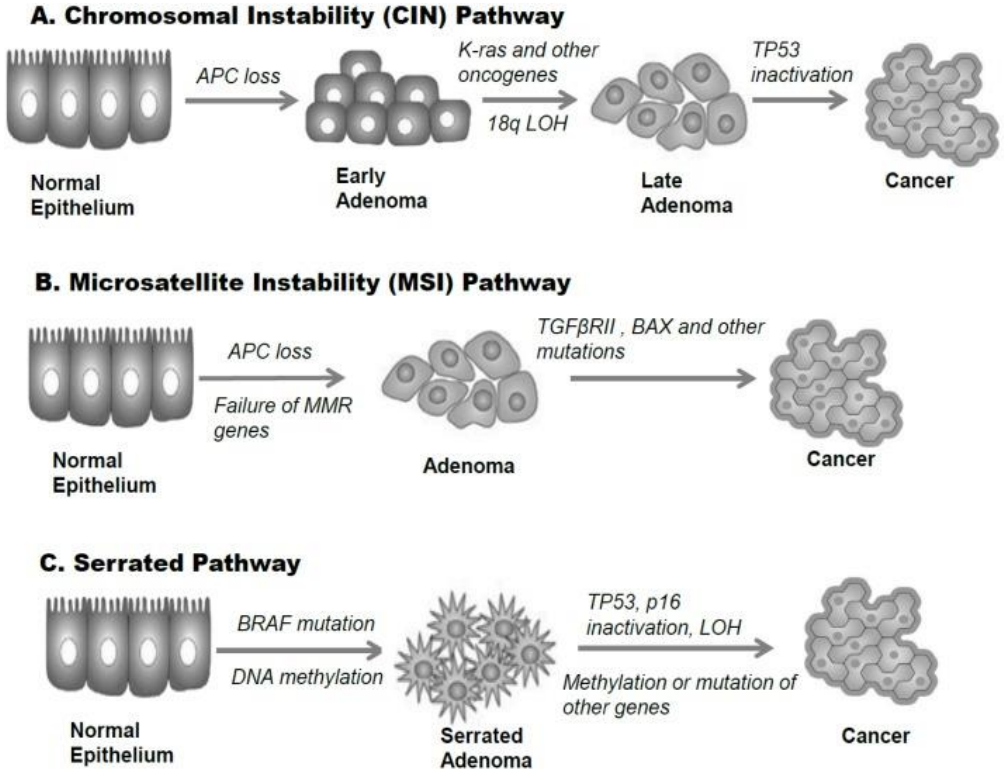


Figure 1. Genetic pathways in colorectal cancer pathogenesis.

(Mundade R et al. Genetic pathways, prevention, and treatment of sporadic colorectal cancer. Oncoscience. 2014 doi:10.18632/oncoscience.59)

Most CRCs are sporadic and approximately 85% of them harbour chromosomal instability (CIN). CIN tumours show changes in chromosome number (aneuploidy) and loss of heterozygosity (LOH) and an accumulation of mutations in specific proto-oncogenes and tumour-suppressor genes. This pathway has been described by Fearon and Vogelstein in 1990 as a multistep model that includes, in order, the biallelic inactivation of the Adenomatous Polyposis Coli (APC), the activation of KRAS, the LOH of long arm of chromosome 18 and inactivation of p53: this set of mutations leads to the transformation of

normal colorectal epithelium into adenoma and ultimately to invasive colorectal cancer (adenoma-carcinoma sequence).[16] Additionally, CIN CRCs seem to be associated with global hypomethylation, which is responsible for cumulative genetic damage and appears to give an early contribution to chromosomal instability.[17]

Another major pathway that accounts for the development of CRC is characterized by the microsatellite instability (MSI). The MSI phenotype is marked by a deficient DNA mismatch repair (MMR) system and subsequent multiple errors during replications, primarily resulting in repetitive DNA sequences (microsatellites) with insertion/deletions of nucleotides that produce longer or shorter alleles. MSI characterises >95% of Lynch Syndrome patients, where germline mutations occur in any of the MMR genes (MLH1, MSH2, MSH6 and PMS2), and ~15% of sporadic CRC, often as a result of aberrant promoter methylation, leading to silencing of MLH1.[18] MSI tumours show a different genetic landscape, with frequent mutations of TGFBR2 and BRAF (except the Lynch syndrome, which doesn't carry BRAF mutations).[19]

MSI is rarely found in polyps and, when it happens, it is mostly in serrated polyps or tubular adenomas that develop in Lynch syndrome; on the other hand, telomere shortening in polyps occurs more often, which suggest that chromosomal rearrangements (CIN) may occur early in the progression to malignancy[20]. Studies on Lynch syndrome also reported that MMR deficiency boosts the polyp transformation, happening in only 2-3 years, which is consistent with the Lynch syndrome being an inherited cancer family syndrome and therefore characterized by an accelerated tumour progression.[21]

A third mechanism of carcinogenesis is the hypermethylation of CpG islands in the promoter regions of tumour suppressor genes and their subsequent inactivation. The CpG island methylator phenotype (CIMP) accounts for approximately 10-20% of CRCs and it is often associated with sessile serrated adenomas, MSI status, BRAF mutations and wild-type KRAS.[19] CRC arising via serrated neoplasia pathway can be broadly classified into two categories: BRAF mutant/CIMP-H (high degree CIMP) with either MSI or microsatellite stability (MSS) and KRAS mutant/CIMP-L (low degree CIMP)/MSS.[22]

Defining the presence of KRAS, NRAS and BRAF and the MSI status of CRC provides important parameters to select patients for the administration of antibodies targeting EGFR or other immunological agents.

Molecular Classification

There are many types of classification that can describe colorectal cancer. From a clinical point of view, CRCs are grouped into proximal or right-sided colon cancers, distal or left-sided colon cancers and rectal cancers. The right-sided tumours originate from the proximal colon (caecum, ascending colon and transverse colon); the distal tumours develop from descending and sigmoid colon; rectal cancer arise within 15cm of the anal sphincter.[23] They show different pathogenesis and genetic background and therefore have different management.

From a histological point of view, the vast majority of CRCs are adenocarcinomas (more than 90%): this type includes adenocarcinomas 'not otherwise specified' ("NOS"), not characterized by specific features, and other subtypes, such as mucinous, signet ring cell, medullary, adenosquamous and micropapillary adenocarcinomas. Other carcinomas, such as squamous cell carcinoma, are rare. Tumours can be well differentiated, when they show glandular structures, or moderately and poorly differentiated, when they progressively lose the ability to form glands: this low- and high-grade tumour classification (grading system) has a great impact on the prognosis.[24]

The histological analysis of CRCs remains very important to predict prognosis and evaluate treatments options: for example, nodal status, degree of differentiation, some histological features such as signet ring morphology, and lymphovascular invasion have a big impact on patients outcome. However, the molecular classification of CRCs has drastically expanded our knowledge about tumours behaviour, and it is important for researchers to continue investigating in this direction.

Molecular Subtypes

With the rise of gene expression profiling studies, the CRC molecular panorama stepped forward. Sadanandam et al. analysed more than 1200 CRCs and correlated the gene expression profiles with survival. The results were grouped into five distinct high-consensus molecular subtypes, which they named by the genes preferentially expressed in each: Goblet-like, Enterocyte, Stem-like, Inflammatory and Transit-amplifying. The stem-like subgroup was associated with shorter disease free-survival, while goblet-like and transit-amplifying types had the best prognosis[25 26].

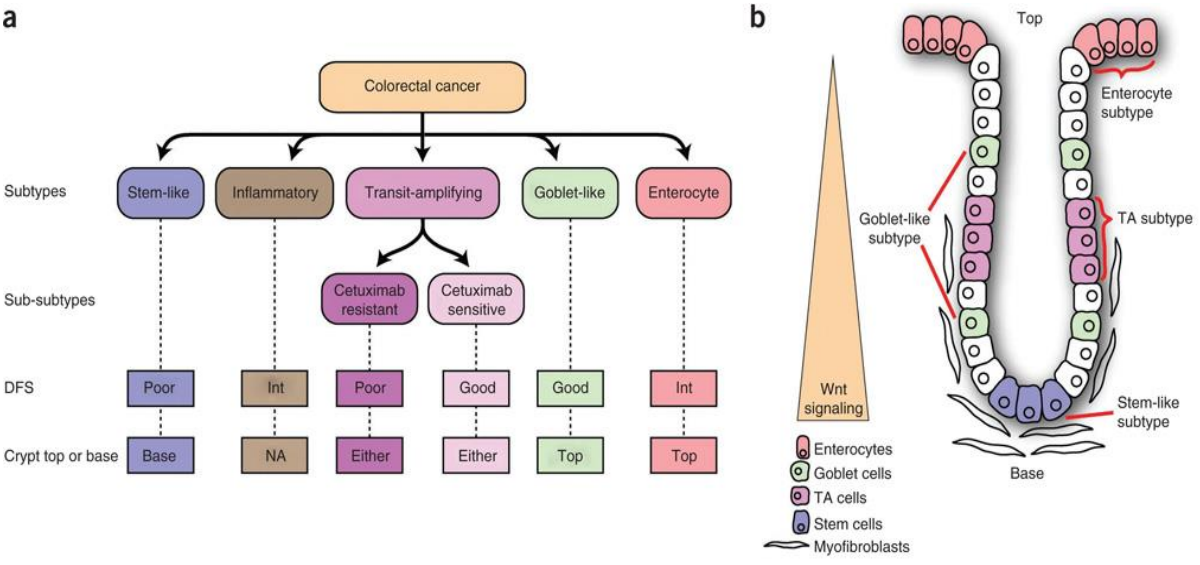


Figure 2. a. The classification system associating cellular phenotype and response to therapy.
b. Colorectal cancer subtypes and corresponding intestinal crypt location.

(Sadanandam A et al. A colorectal cancer classification system that associates cellular phenotype and responses to therapy. Nat Med. 2013 doi:10.1038/nm.3175)

Following the same approach, other studies performing gene expression analyses found similar classification with prognostic meaning. Budinska et al. identified 5 subtypes: surface crypt-like, lower crypt-like, CIMP-like, mesenchymal and mixed: mesenchymal subtype and surprisingly CIMP-like (as they were enriched for MSI) had the worst prognosis[27].

These and other studies[28 29] exploiting molecular profiling, despite the different clustering, agreed on the MSI status to be characterized by a more positive outcome and the stem-like and mesenchymal types by a poorer survival, which will be confirmed just a few years later by the comprehensive consensus molecular subtype (CMS) classification.

The recently described CMS classification[30] was based on both transcriptomic and genetic data. It identifies four major subgroups:

- CMS1, MSI immune (14%): hypermutated, microsatellite unstable and strong immune activation
- CMS2, Canonical (37%): epithelial, characterized by Wnt and Myc signalling activation
- CMS3, Metabolic (13%): epithelial, metabolic dysregulation
- CMS4, Mesenchymal (23%): TGFβ activation, stromal invasion and angiogenesis.

CMS1 MSI immune	CMS2 Canonical	CMS3 Metabolic	CMS4 Mesenchymal
14%	37%	13%	23%
MSI, CIMP high, hypermutation	SCNA high	Mixed MSI status, SCNA low, CIMP low	SCNA high
BRAF mutations		*KRAS* mutations	
Immune infiltration and activation	WNT and MYC activation	Metabolic deregulation	Stromal infiltration, TGF-β activation, angiogenesis
Worse survival after relapse			Worse relapse-free and overall survival

Figure 3. The consensus molecular subtypes of colorectal cancer.

(Guinney J et al. The consensus molecular subtypes of colorectal cancer. Nat Med. 2015 doi:10.1038/nm.3967)

A later study compared these subtypes with survival data on a large population scale and described five different subtypes[31]:

- type 1: MSI-high, CIMP, BRAF mutation (good survival)
- type 2: MSS or MSI-low, CIMP, BRAF mutation (highest disease-specific mortality)
- type 3: MSS or MSI-low, non-CIMP, KRAS mutations
- type 4: MSS or MSI-low, non-CIMP , negative for BRAF and KRAS mutations

- type 5: MSI-high, non-CIMP, negative for BRAF and KRAS mutations (lowest disease-specific mortality).

A study conducted on stage III CRCs, analysed BRAF and KRAS mutations and MMR status and their correlation with clinical features. They identified 5 subtypes[32]:

- MMR proficient:
    - positive for BRAF mutation
    - positive for KRAS mutation
    - negative for both mutations
- MMR deficient:
    - Sporadic type positive for BRAF mutation and/or hypermethylation of MLH1
    - Familial type negative for BRAF mutation and hypermethylation of MLH1.

The 'classic' CRC, that develops following the adenoma sequence and harbours the APC mutation, shows MSS, chromosomal instability and it's negative for BRAF. The prognosis is generally superior to tumours with BRAF mutations but inferior to MSI-high tumours.

On the other hand, the serrated pathway neoplasia evolves in tumours characterized by CIMP phenotype, BRAF mutation and inconstant MS status: the ones with MSS have generally a poorer prognosis, whereas the MSI-high status seems to provide a better outcome.[32] [31] Finally, BRAF mutation is associated with shorter survival and BRAF or KRAS mutations are more likely to be present in distal tumours, whereas MMR deficiency seems to confer better prognosis in proximal tumours.[33]

Intratumor Heterogeneity

Tumour heterogeneity can be classified into inter-tumor heterogeneity, i.e. the same histologic type differs between patients, and intra-tumor heterogeneity, i.e. the same tumour shows differences within the primary site or between the primary site and its metastases (spatial heterogeneity) and/or when the tumour acquires new genetic aberrations over time (temporal heterogeneity). To complicate this picture, genetic heterogeneity doesn't always mean morphologic differences and vice-versa. As expected, tumours characterised by microsatellite instability tend to show higher polyclonality and two or more morphological patterns in more than 50% of cases, compared to microsatellite stable tumours.[34] However, as shown in breast cancer, genetically heterogeneous tumours can consist of only a few major cell subpopulations, as well as diverse tumour subpopulations can share a similar genetic landscape.[35]

Intratumor heterogeneity is the result of genetic mutations, epigenetic modulation, reversible transition among cellular states and tumour microenvironment: these factors contribute to different tumorigenic activity among cancer cells, which ultimately results in differences in prognosis and therapeutic response.

Genesis of intratumor heterogeneity

Cancer genomes are characterized by hundreds of mutations and they can be classified into driver mutations, which are essential for the development of cancer, and passenger mutations, which occur by chance or as consequence of the transformation process. Driver mutations play a pathogenic role and are positively selected during carcinogenesis, whereas passenger mutations confer no selection advantage. This represents the rationale behind the cancer clonal evolution model. The Darwinian approach to carcinogenesis states that genetic and epigenetic aberrations produce tumour cells clones that adapt differently and therefore a sequential selection of cell subpopulations occurs. This perspective has been acknowledged for decades[36] and validated by current cancer research[37] . Only a few driver mutations are required for malignant transformation and they are normally involved in crucial cellular processes, such as proliferation, differentiation, adhesion, migration, cell death and DNA repair. The diverse mutational spectrum is behind tumour heterogeneity, one of the reasons of the limited efficacy of current therapies.

Another major model hypothesising the genesis of tumour heterogeneity is the hierarchical model of cancer stem cells (CSC). The CSC model is often antagonistic to the clonal evolution model, even though they are not necessarily mutually exclusive. According to the hierarchical evolution model, the CSCs represent the few tumour initiating cells, able to drive carcinogenesis. Stem cells are characterized by self-renewal capacity, and therefore capable of long-term clonal growth. Being long-lived, these cells would accumulate numerous genetic and epigenetic alterations that would be passed to both daughters cancer stem cells and cancer cell progenitors.[38] It has been demonstrated that the deletion of APC in Lgr5 cells results in a growing neoplasia and that the transformed stem cells remain at the crypt bottom while the progenitors fuel the adenoma growth, preserving in this way the cell hierarchy. In the same study, the induction of APC in short-lived transit-amplifying cells, the lesions did not expand as efficiently, confirming the unique role of stem cells in carcinogenesis[38]. To support the stem cell hypothesis, recent works reported that most CRCs express intestinal stem cell signatures.[39] [40] With the same purpose of proving cancer stem cells as tumour initiating cells, other experiments focused on the "dedifferentiation" of normal cell and subsequent acquisition of stemness properties. In vivo studies demonstrated that enhancing Wnt signalling, by increasing NF-kB, induces dedifferentiation of non-stem cells and acquisition of tumour-initiating potential.[41] Cell plasticity is a very important concept in cancer biology, not only during carcinogenesis, but also during the epithelial-mesenchymal transition, which anticipates the metastatic process.

A third theory, the "Bing Bang" model, hypothesizes that intratumor heterogeneity is mostly an early process, occurring during cancer development. Sottoriva et al. described how the tumours grow as a single expansion and harbour intermixed subclones from the beginning.[42] In this case, the primary cancer is highly heterogeneous, due to various and early passenger mutations that progresses towards metastasis following a linear evolution, rather than the clonal selection.

Besides the controversial evolutionary pattern, the degree of tumour heterogeneity is a crucial determinant of tumour behaviour, to the extent that it has been suggested as a potential prognostic factor [43].

Temporal and Spatial Heterogeneity

To date, there are no guidelines for the sampling that would exactly map the molecular features of a tumour, nor for the minimum sensitivity of the methods used to detect ITH. Some studies recommend that at least two biopsies from different parts of the tumour are needed in order to investigate tumour heterogeneity.[44]

The mutational concordance of primary CRCs and metastatic lesions has been widely studied, in pancreatic adenocarcinomas[45], clear cell renal cancer[46] and colorectal cancer[47], among others. A pioneer study reported that only 3% of the mutations found in metastases were not present in the primary colorectal tumours[48]. Jones et al.[49] found a decrease of K-Ras and p53 point mutations and a relative stability of LOH on chromosome 5 and 18q during tumour progression [50]. More findings reported that KRAS, TP53, APC, PIK3CA, BRAF and NRAS are concordant between the primary and metastatic lesions [51] and that APC, KRAS and TP53, being universal events, could be the only three driver mutations required for CRC development[52]. This temporally narrowed genetic heterogeneity in metastases could represent the effect of selection of sub-clones; cancer treatment represents a second reason of selection. Other investigators focused on KRAS, NRAS,BRAF and PI3KCA genes and reported a difference between the mutational profiles of primary tumours and lymph node/metastases of 48%, highlighting some degree of spatial and temporal clonal evolution[53].

Spatial heterogeneity can refer to differences between cells in the primary tumour, in different metastatic lesions in the same patient or in the same metastatic lesion. Although there is generally a high degree of concordance between driver mutations in different portions of the primary tumour, KRAS and BRAF status have been found incorrectly assigned in 10-30% of cases due to ITH [54]. A different study, assessing the SNVs and CNVs contribution to CRC heterogeneity, found mutations of APC and TP53 equally spread throughout the tumours, but highly variable DNA copy numbers in many genes (distinct CNVs profiles were found within the same tumour). [55] Ultra-deep sequencing allows the investigation of ITH patterns in different sectors of the same tumour: greater heterogeneity was found in MSI tumours[56] and in biopsy specimens with ≥20% tumor cellularity, both suggesting a role of mutation rate in IHT.

3. Molecular diagnostics and Therapy Overview

The progress made by molecular oncology in the past few decades shaped a new idea of treatment, where therapy is not given based on the success it had on similar patient cohorts, but it's custom made based on the unique molecular features of the disease. Targeted therapy and immunotherapy have shown great results in specific metastatic CRCs subtypes.

Prognostic and Predictive Factors and Molecular Testing

KRAS proto-oncogene encodes for a GTPase protein involved in many pathways, including EGFR[57] , and its mutation is broadly considered predictive of poor response to EGFR inhibitors (even though in some low-prevalence variants such therapy may be effective)[58] . Other tissue biomarkers, like BRAF and PIK3CA, have been assessed as predictive biomarkers, but studies on both have showed inconclusive results[59] [60]. BRAF mutations have been reported by numerous as associated with poorer OS rates [61]; the analysis of BRAF V600, which accounts for more than 90% of BRAF mutations, is now recommended to be performed in selected patients[62]. Microsatellite instability is another parameter that has been investigated for its potential prognostic value: MSI-high tumours consistently show better OS and disease-free survival rates than microsatellite stable CRC[63]. The status of the mismatch repair system, which normally fixes DNA error to avoid genomic instability, is therefore also recommended for prognostic stratification.[64] Other biomarkers, including blood biomarkers like cell-free DNA [65], are being evaluated to help reinforce the current guidelines and establish a standard molecular biomarker testing proceeding.

In 2009, the analysis of KRAS mutation at codons 12 and 13 became part of the recommendation for patients with metastatic CRC to predict the response to anti-EGFR monoclonal antibody therapy[64]. Since then, more KRAS codons and NRAS mutations have been discovered to cause resistance and currently, all patients with metastatic CRC eligible for anti-EGFR antibodies are recommended to be screened for *KRAS* and *NRAS* exons 2 (codons 12 and 13), 3 (codons 59 and 61), and 4 (codons 117 and 146)[62].

Growing literature is also showing the role of BRAF mutation in the resistance to anti-EGFR drugs (cetuximab and panitumumab), thus BRAF p.V600E mutation analysis joined the extended RAS testing recommendation[66]. Besides the prognostic and predictive information, BRAF pV600 mutational analysis is also carried out in dMMR tumours with loss of MLH1 to exclude Lynch syndrome cases[62].

The mutation in exon 20 of PIK3CA was found associated with low response to cetuximab and chemotherapy in CRCs negative for KRAS mutation[66], suggesting room for improvement of the screening of KRAS wild-type population. However, currently PIK3CA and PTEN are both not included in the recommendation for therapy selection outside of a clinical trial, due to insufficient evidence[62].

MMR status can be determined by looking for microsatellite instability (by DNA testing), or variation in protein expression by IHC, and it is indicated in CRC patients to expose a possible Lynch syndrome and/or prognostic stratification. Deficiency in mismatch repair generates a hypermutated genome, characterized by an MSI phenotype and newly generated tumour antigens[67] and respond well to immunotherapy, therefore defining MMR status also has a predictive meaning. A study presented at 2020 ASCO annual meeting showed that the median progression-free survival of patients with stage IV MSI-high CRC was 16.5 months with Pembrolizumab and 8.2 with chemotherapy [68]. The overexpression of checkpoint molecules, including PD-1 and PDL-1, found in MSI-high tumours is an additional reason for the high efficiency of the immune checkpoint inhibitors in these tumours[69].

Therapy Overview

The ideal treatment for CRCs is the complete removal of the tumour achieved by surgery. Unfortunately, almost 25% of patients have metastatic disease when diagnosed and almost 50% will develop metastases[4] , thus curative surgical treatment is not always possible. In these cases, resection of metastatic disease remains an option for an immediate tumour and related symptoms control, and it is followed by medical treatment.

Chemotherapy standard protocols (5-fluorouracil based) are normally used as first- or second-line treatment, in combination with molecular targeted drugs (anti-VEGF and anti-EGFR monoclonal antibodies) in selected cases. Bevacizumab (a recombinant humanised Mab that inhibits VEGF) has shown improved outcome for metastatic CRC patients [70]. Cetuximab and Panitumumab belong to the anti-EGFR Mab family and can be used as single agents or in combination with chemotherapy[71]. As previously mentioned, EGFR inhibitors are used in wtKRAS CRCs, even though they showed similar outcome in patients carrying mutation in KRAS codon 13[72]. In wtKRAS patients, the association FOLFIRI/cetuximab showed a slightly better OS than FOLFIRI/bevacizumab[73]. The addition of anti-EGFR agents to the combination chemotherapy/bevacizumab was found associated with shorter PFS, increased toxicity and general poorer quality of life[74]. Other targeted therapies using different drugs, such as anti HER2 agent, are currently being evaluated[75].

Tumour progression and relapse impose second-line therapy, decided based on which drugs have been used in the first-line. When standard therapies fail, further agents such as Regorafenib and TAS-102 are given, with no predictive biomarkers currently anticipating the outcome[75].

Immune checkpoint therapy has been approved by the Food and Drug Administration (FDA) to treat several tumours (renal cell cancer, melanoma and NSCLC) and showed a significant response in dMMR CRCs[76] [77]. The KEYNOTE-164 study confirmed the efficacy of Pembrolizumab in MSI-high metastatic CRC (responsive rate of 33%)[78]. Nivolumab was investigate in the CheckMate-142 trial and showed an overall response rate of 31.1% and 1-year PFS of 50.4%[79]; the combination Nivolumab/Ipilimumab as second-line treatment reached a PFS of 71% at 1 year[80]. This combined regimen was also tested as first-line treatment, demonstrating even greater response and efficacy (1 year PFS of 77% and disease control rate of 84%)[81] . Thanks to these results, Nivolumab, Ipilimumab and Pembrolizumab, have been approved for first- and second-line treatment of metastatic CRC MMR-D or MSI-H, and other immune checkpoint inhibitors are under investigation.

Collectively, these studies demonstrate the importance of prognostic and predictive factors for patient stratification and incremented treatment response, and future research is going to expand the list of biomarkers that we can exploit for more and more personalised therapy.

4. β-catenin

β-catenin is a protein encoded by the CTNNB1 gene (3p22.1).[82] It has a double function, cell adhesion and signalling, carried out as a component of the cadherin-based adherens junctions and as the nuclear effector of the canonical Wnt pathway.

β-catenin structure and functions

The protein comprises 781 aa in humans, organised in a relatively rigid central region and flexible N- and C-terminal domains (NTD and CTD). The central structure is characterized by twelve Armadillo (ARM) repeats of ~42 aa residues each; together, all ARM repeats are arranged in a positively charged groove that acts as a scaffold and can bind different partners at the membrane, in cytosol and nucleus. The binding sites are overlapping, which results in interactions with only one partner at the time.[83] The major partners that bind β-catenin are E-cadherin at the membrane, APC, to form the destruction complex in the cytosol, and TCF/Lef to regulate transcription in the nucleus.

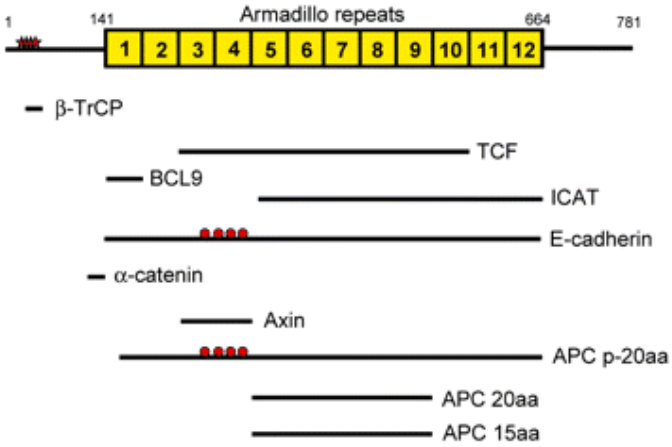


Figure 4. β-catenin functional domains and correspondent binding partners.

(Xu W et al. Mechanistic insights from structural studies of beta-catenin and its binding partners. J Cell Sci. 2007 doi:10.1242/jcs.013771)

β-catenin is mostly located at the cytoplasmic side of the membrane, to cooperate with cadherin proteins to form the polarised epithelial tissue. Epithelial Cadherins (E-cadherin) are transmembrane glycoproteins with a cytoplasmic tail that binds β-catenin[84]. The bond β-catenin/E-cadherin stabilises both proteins and avoids their degradation. In proximity of the NTD, β-catenin also has a binding site for α-catenin: the role of the α-catenin/β-catenin heterodimer is to promote α-catenin/actin interaction and consequent actin filament polymerization. The α-catenin/β-catenin heterodimer also stabilizes β-catenin in a hinged form, which prevents the binding site for BCL9 to be exposed[85]. Another level of regulation of β-catenin stability is by phosphorylation: when Ser684, Ser686, and

Ser692 residues of E-cadherin are phosphorylated, the cadherin–β-catenin bond is strengthened; on the contrary, phosphorylation of β-catenin on Tyr654 weakens the bond at the membrane leading the protein either towards degradation or nuclear translocation.[85]

When not bound to cadherins, β-catenin is exposed in the cytoplasm and is normally incorporated in the destruction complex for degradation. Axin and APC can both directly interact with the protein.[86] The destruction complex acts as a scaffold for other molecules, the kinases: CK1α phosphorylates β-catenin at Ser45, which makes the protein ready for a second phosphorylation wave at Thr41, Ser37, and Ser33 by GSK3 β[87]. Subsequently, β-catenin bound to APC migrates from the destruction complex, so that Ser33 and Ser37 are recognised by E3 ubiquitin ligase and the complex is destroyed by the 26S proteasome[85]. This fate is subverted by Wnt signalling activation.

When β-catenin escapes degradation, it accumulates in the cytoplasm and with a mechanism not yet fully understood, it translocates to the nucleus. Tyr654 in the last ARM repeat and ARM repeats R10-12 interactions with nuclear pore complex components, and Forkhead-box transcription factor FoxM1 were suggested to play a role in β-catenin import[88 89]. β-catenin doesn't directly bind DNA but exploits different complexes to regulate gene expression. The main nuclear partner is TCF/Lef transcription factor, which translates most of the messages carried by Wnt signalling. There are 4 variants of TCF proteins (TCF1, TCF3,TCF 4 and Lef1) and they can all interact with β-catenin. Normally TCFs partner with Groucho/TLE and act as transcriptional repressors. The interaction with β-catenin impairs the repressor complex and activate transcription[90]. The list of Wnt/TCF/β-catenin target genes is long and growing, and they account for the many biological processes in which they are crucial: c-myc, Cyclin D, CD44, MMP7, EphB/Ephrin-b, VEGF, LGR5, Sox17, Sox9, Oct4, Nanog, Wnt3a, EGF receptor, E-cadherin, p16ink4A, FGF4 are only a few[91]. It is important to remember that some promoters recruited by β-catenin repress transcription: an example is the repression of p16ink4A in melanomas[92].

Wnt/β-catenin in Stem Cells

In most CRCs, the earliest event that initiates cancer development is a Wnt/β-catenin signalling activating mutation. Wnt pathway is involved in multiple biological processes, from gut development to adult intestinal epithelial homeostasis, and is a key regulator of intestinal stem cells (ISCs), thus it is not surprising that its dysregulation is associated with malignant transformation. The idea of ISC as cells-of-origin of CRC that fuel tumour growth has gained more and more interest, thanks to the extraordinary progress made by stem cell biology.

The intestinal tract is characterized by crypt-villus structures (in the small intestine) or crypts (in the large intestine). These are lined by a monolayer of specialized cells that interact with the surrounding stroma in a dynamic bidirectional manner.[93] Together the epithelial cells, stromal cells and the extracellular matrix constitute the intestinal crypt niche. There are two postulated stem cell types that lie at the intestinal crypt base. One group comprises fast-cycling crypt base columnar (CBC) stem cells: they express Lgr5, a marker of actively proliferative cells, and are capable of self-renewal and multipotency. [94 95] A second pool of stem cells, known as "position +4" (as they lay four cells up from the base crypt), acts like a quiescent 'reserve' team and is active upon tissue damage or loss of the

Lgr5-population. [96] As ISCs gradually proliferate, they originate daughter cells which enter the so-called transit-amplifying zone (TA): these cells will concurrently migrate towards the apex of the crypt or villus and differentiate in their terminal fate[93]. The intestinal epithelium consists of enterocytes or colonocytes (with absorptive function), goblet cells, Paneth cells in the small intestine and Reg4+ cells in the large intestine (the only differentiated cells that will migrate to the bottom of the crypt), enteroendocrine cells and tuft cells (with secretory function).

As mentioned above, Wnt is crucial for stem cell maintenance. There are several Wnt ligands, secreted by both epithelial and mesenchymal cells, that show a redundant role in governing the crypt homeostasis[97]. The bond between Wnt ligands to their receptors (the Frizzled and LRP5/6 complex) activates the canonical Wnt pathway: Frizzled receptors recruit the cytoplasmic protein Dishevelled, which mediates multiple interactions and phosphorylations that lead to the recruitment of Axin and inactivation of GSK3β, destabilising the destruction complex[98 99]. With the inactivation of the kinase activity of the destruction complex, β-catenin is free to translocate to the nucleus and activate the transcription of Wnt target genes that maintain cell stemness. Among the genes regulated by Wnt, many concur to shape the identity of the stem cell niche, constantly amended by positive and negative feedback loops[97].

Wnt/β-catenin Signalling in CRC

In colorectal cancer, the loss-of-function of APC is the most frequent mutation that leads to the dysregulation of Wnt signalling, translocation of β-catenin to the nucleus and subsequent carcinogenesis.[100] The importance of a constitutive activation of this pathway for CRC has been demonstrated by several studies. For example, modulating APC using doxycycline-regulated shRNA on mice, Lowe et al. showed that restoring the normal function of APC results in tumour regression and re-establishment of normal crypt-villus homeostasis[101].

Besides APC mutations, there are other genetic alterations that lead to the inappropriate stabilization of β-catenin. AXIN1 and AXIN2 act as a scaffold for the assembling of the β-catenin destruction complex; both somatic and germline mutations are found linked to CRC initiation. In addition, point mutations of CTNNB1 result in the removal of the N-terminal Ser/Thr destruction motif and subsequent accumulation of the protein in the cytoplasm.[100]

Inactivation of Ubiquitin E3 ligase ring finger 43 (RNF43) is an additional rare mutation that avoids the destruction of β-catenin and enables cancer development and progression. This particular activation of Wnt signalling seems to be more common in MSI tumours and it's associated with enhanced tumour growth and a high recurrence rate in CRC patients.[102]

R-spondins are proteins that enhance Wnt pathway and are crucial for the intestinal crypt homeostasis. They also bind RNF43 and ZNRF3, that inhibit Wnt signalling by degrading Wnt receptors and avoid aberrant expansion of the intestinal crypt.[103] A study conducted on more than 70 primary tumours identified multiple fusion transcripts involving R-spondin family members RSPO2 and RSPO3. These mutations were found to be mutually exclusive with APC mutations, suggesting their primary role in tumour initiation.[104]

The final step of the Wnt pathway activation is the migration of β-catenin into the nucleus, where it interacts with the N terminus of DNA-binding proteins of the Tcf/Lef family. Studies that investigated TCF-4 gene targets showed that the same program is carried out in CRC and stem-cell crypt: the inhibition of TCF-4 activity in CRC results in cell cycle arrest, inhibition of differentiation and imposes a crypt stem cell-like phenotype on CRC cells.[105] Essentially, the biallelic loss of APC makes the adenoma cells the transformed equivalent of the crypt progenitors: the β-catenin/TCF-4 complex executes the Wnt pathway stemness programme, proposing β-catenin as major maker of stemness.

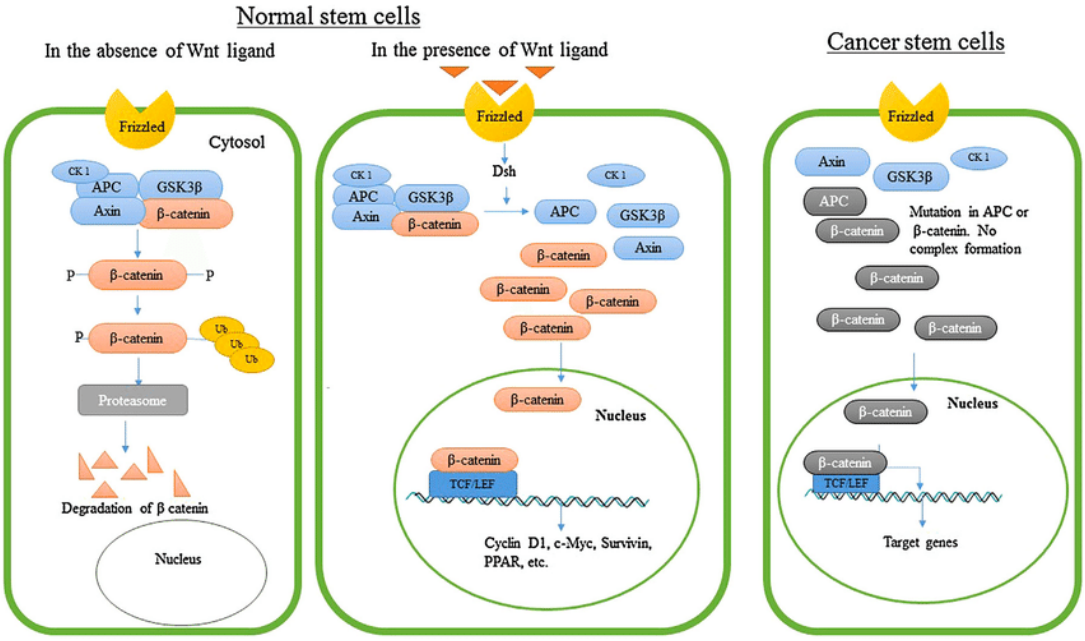


Figure 5. Wnt/β-catenin pathway in intestinal stem cell and cancer stem cells.

(Vadde R et al. Role of hypoxia-inducible factors (HIF) in the maintenance of stemness and malignancy of colorectal cancer. Crit Rev Oncol Hematol. 2017 doi:10.1016/j.critrevonc.2017.02.025)

5. Ki67

Ki67 is a protein encoded by the MKI67 gene (10q26.2). It is a non-histone nuclear protein, first described in 1983 in Hodgkin lymphoma cells, where it was detected in cycling cells but not in G0 and early G1 cells and therefore considered a marker of cell proliferation.[106] A recent work described how it accumulates in the S, G2 and M phases and it's degraded continuously in G1 and G0, suggesting a heterogeneous level of expression (and not binary) during G0 and G1.[107] Currently it is largely used in clinical practice to grade multiple types of cancer and in some cases as prognostic factor. While the clinical relevance has been largely investigated, the biological function is less defined.

KI67 structure and functions

The primary structure of Ki67 comprises a N-terminal forkhead-associated (FHA) domain, a protein Phosphatase 1 (PP1) binding domain, a central region characterized by tandem repeats, and a C-terminal LR (leucine/arginine-rich) chromatin-binding domain[108]. Five different splicing variants have been identified; they share the same C-terminal and central region but not the N-terminal domain[109]. The alternative inclusion of exon 7 showed a different impact on cell cycle, as well as the variable expression of an N-terminal fragment; additionally the variable expression of exon 7 affects the translocation of cyclin B to the nucleolus, which is essential for the G2 progression into M: these few examples show a major structural and effector role of Ki67 during mitotic transition[109 110].

The FHA domain mainly interacts with two proteins: Hklp2, involved in the bipolar spindle assembly, and the nucleolar protein NIFK, which has also been associated with cell proliferation and cancer metastases[110]. The PP1 domain is involved in numerous dephosphorylation events and targets different regulatory proteins to modulate gene transcription and cell cycle progression[111].

The diverse nuclear localization of Ki67 is determined by the phosphorylation of the central tandem repeats by CDK1: in interphase it is dephosphorylated around the perinucleolar heterochromatin; at the beginning of the mitosis, it's over-phosphorylated so that weakly binds DNA and can migrate on the chromosome until anaphase; during mitosis it's dephosphorylated so that the positive charge avoids the hyper aggregation of the arms of the chromosomes[110].

Finally, the C-terminus is needed to bind the chromosomes via heterochromatin protein 1 (HP1)[112].

Ki67 prognostic role

The expression of Ki67 by IHC is generally measured as Ki67 Labelling Index (KI67LI), i.e. the percentage of cancer cell nuclei that are positive for Ki67 over the total cancer cell nuclei). There are many issues that can affect the assessment of KI67, from interlaboratory variation (such as the type of fixative or the different anti-Ki67 antibody clone used) to scoring (for example visual vs automated analysis) to data analysis (lack of standard cut-off).

The role of Ki67 as proliferation marker is widely accepted, but the prognostic value is not as easy to determine. In breast cancer many randomised clinical trials and meta-analyses reported a clear association between high levels of Ki67 and poor outcome[113 114]. Ki67 is routinely used together with other biomarkers in ER-positive/HER2-negative BC to distinguish Luminal A from Luminal B breast cancer. Currently, a cut-off of 20% to stratify patients with high Ki67 expression is used according to the St. Gallen guidelines[115]. Despite the literature discrepancy, ki67 is routinely used to characterize breast cancer. Ki67 prognostic role has been investigated also in other tumours: Non-Small Cell Lung Cancer (NSCLC)[116], Merkel cell carcinoma[117], neuroendocrine tumours [118], gastric cancer[119], melanoma [120], gliomas[121] among others.

6. Stroma

Tumour development is the result of a complex crosstalk between tumor epithelial cells and tumour microenvironment. The predominant focus of cancer research has been the tumour cell, but in the past decades the biological and prognostic role of the tumour surrounding has been increasingly acknowledged[122].

In normal tissue, stroma has the important role of support and remodelling. The components of the stroma are the extracellular matrix (ECM) and tissue-specialized connective cells, such as fibroblasts, myofibroblasts and mesenchymal cells. Other cell types that populate the tissue microenvironment, not strictly considered as stromal cells, are endothelial cells, pericytes, adipocytes and immune cells. The same components are found in the tumour microenvironment (TME), where it provides the same support for tumour growth, metastatic invasion through angiogenesis and epithelial-mesenchymal transition and conceivably resistance to therapy.[123]

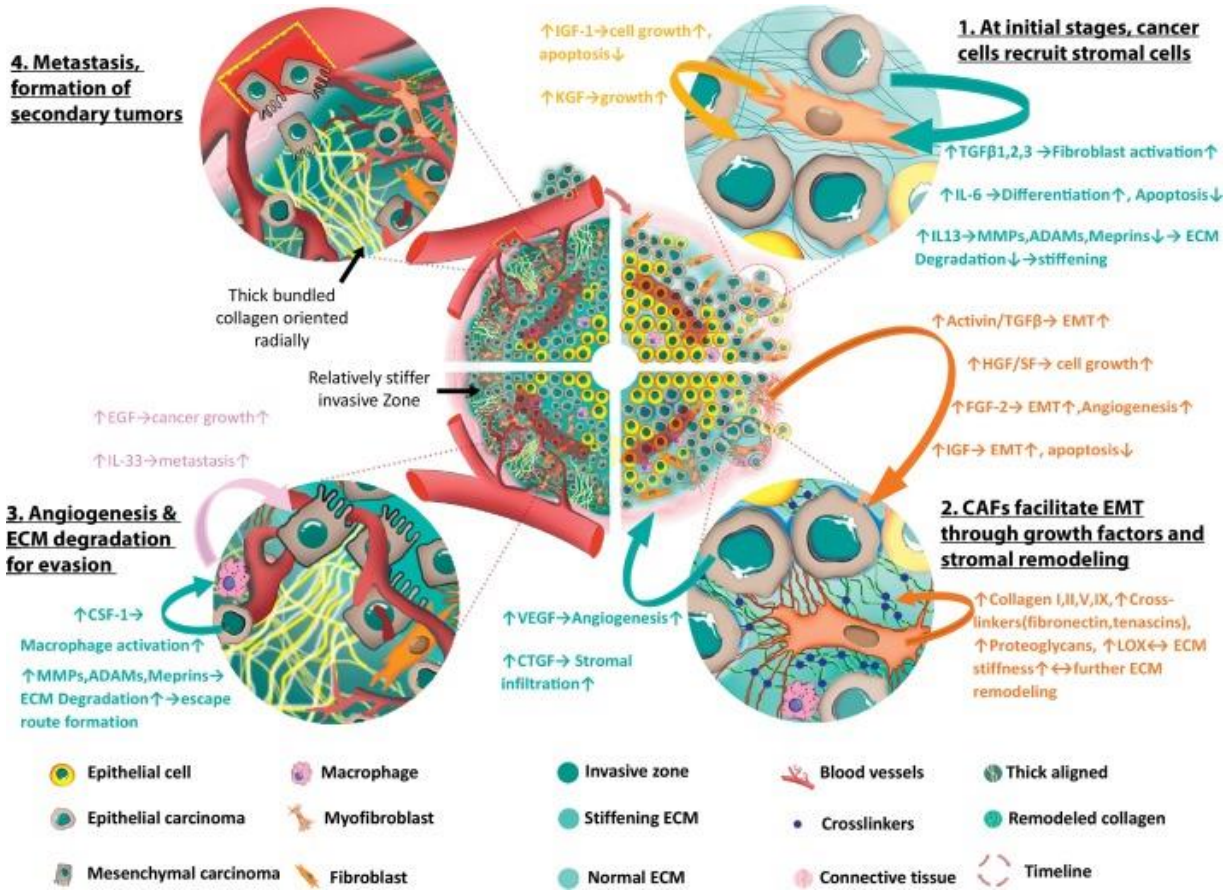


Figure 6. Evolution of stromal architecture, from cancer development to metastatization.

(Emon B et al. Biophysics of Tumor Microenvironment and Cancer Metastasis - A Mini Review. Comput Struct Biotechnol J. 2018 doi:10.1016/j.csbj.2018.07.003)

In the same way normal cells undergo tumorigenesis, also TME sustains a degree of evolution. Some of the changes are: an increment of vascular permeability through increased production of VEGF, to initiate angiogenesis; extravasation of plasma components, such as fibrinogen and plasminogen, developing a temporary oedematous area that will attract mesenchymal stromal cells and inflammatory cells to develop the mature tumor stroma; degradation and remodelling of the 'provisional' highly vascularised stroma into a dense fibrotic stromal matrix, characterized by desmoplasia and remarkably active ECM, mesenchymal stromal cells (MSCs) and cancer-associated fibroblasts (CAFs).[124]

The tumour stroma is characterized by a denser ECM, with alternative components, i.e. tenascin and fibronectin, that shape a rigid connective structure but by no means static. Indeed, it is a highly dynamic structure and comprises two elements: the interstitial connective tissue matrix and the basement membrane, which separates the epithelium from the surrounding stroma. The main components are collagens, proteoglycans, glycoproteins (such as laminins, elastin, fibronectins, thrombospondins, tenascins and nidogen) and various ECM-associated proteins such as cytokines. They have different specific role and all together contribute to the ECM dual function of structural support and signalling. It communicates with epithelial cells and provides them ligands for receptors such as integrins: these interactions mediate adhesion, migration, proliferation and differentiation. It also storages growth factors and other crucial signalling molecules such as EGF, FGF, WNTs, TGF-β. [124]

Fibroblasts are generally resting in normal tissue and are activated in response to wound healing, inflammation and fibrosis. In cancer, they become highly secretory and are referred as Cancer-associated fibroblasts (CAFs). They are more metabolically active, show increased proliferative and migratory abilities and release collagens and proteolytic enzymes such as MMPs to regulate ECM homeostasis, and also cytokines and chemokines to favour angiogenesis and immune evasion. [125]

Mesenchymal stromal cells (MSCs) were first characterized as fibroblastic colony-forming units in bone marrow[126], which were later found to be multipotent and be able to differentiate into specialised mesenchymal cells such as osteoblasts, chondrocytes or adipocyte.[127] As the CAFs, MSCs mediate processes as migration, differentiation, local immune response and angiogenesis and therefore have been investigated for their role in tumorigenesis and cancer growth[128].

The stroma dynamically cooperates with the tumour. While the tumour epithelial cells acquire mutations which cause the cancer to develop, the stroma can have both tumour-promoting and tumour-suppressing functions. Indeed, in the premalignant context, it has been shown to compete with tumour promotion. [129] The overexpression of metalloproteinases inhibitors, such as the tissue inhibitors of metalloproteinases (TIMPs), is one way to contain tumour growth, fight angiogenesis and invasion[130]. Conversely, CAFs have an increased production of MMPs and other tumour-promoting factors compared to inert fibroblasts, and they have shown to be directly involved in malignant transformation[131].

The tumour stroma can also influence therapy outcome. For example, it can restrict the drug access in many ways: a dense ECM can act as a physical barrier around the tumour or with the cell adhesion-

mediated drug resistance (CAM-DR) mechanism, by which cancer cells vigorously adhere to ECM and escape the vessels[128]. Another way exploited by the tumour stroma to counteract therapy is producing specific factors after the drug administration, as it has been seen in a prostate cancer model, where the level of GDNF increased after docetaxel or mitoxantrone to favour cell proliferation and chemotherapy resistance[132]. Other studies reported increased secretion of hepatocyte growth factor (HGF) and reactivation of angiogenesis[133 134]: depending on the therapeutic agent, stroma secretions have been demonstrated to play an essential role in acquired resistance to therapy.

AIMS

This study addresses the validity of specific biomarkers in CRC and the extent of CRC heterogeneity, with the purpose of identifying markers that can easily be applied into clinical practice, that could improve the diagnostic process and help select patients who most benefit from certain treatments.

Specifically, we analyse the expression of β-catenin, Ki67 and stroma percentage and investigate their biological and prognostic role.

The main research questions are:

- Is there a correlation between the biomarkers and clinicopathological criteria and do these correlations match the current knowledge of CRC biology?
- Do the biomarkers correlate with survival data and can they be considered as prognostic biomarkers?
- How does the expression of the biomarkers change between normal intestinal epithelium and tumoral tissue?
- How does the expression of the biomarkers change among luminal, central and peripheral side of the tumour?
- Is there a correlation between the three biomarkers?

We think that addressing these questions can expand the knowledge of the mechanisms behind tumour development, highlight the processes that characterise tumour progression and help with the development of a biomarkers panel for patient stratification and ultimately contribute to precision medicine.

METHODS

For the purpose of this study, the tissue microarrays (TMAs) technology represents an outstanding technique, offering the possibility to study molecular aspects of cancer in hundreds of tissue samples. TMAs procedure and IHC staining allow us to assess the protein expression pattern in normal and tumor samples, from which we can obtain important data to further correlate with clinicopathological factors of the patients.

Population Data

This study was performed using data collected from one thousand patients consecutively diagnosed with colorectal cancer in the Nottingham University Hospitals between 2008 and 2014. Specimens were obtained from the Histopathology department in the Queen's Medical Centre, and the following data about the patients were retrieved: sex, age at diagnosis, survival data, cause of death, tumour site, tumour size, lymph node involvement, metastases, final stage, Dukes stage, grade, recurrence, vascular invasion, perineural invasion, lymphovascular invasion, peritumoral lymphocytes, tumour edge, tumour budding and MMR status. The collection of the clinicopathological data and the construction of the TMA (see below) were undertaken by a senior research scientist.

TMAs and Immunohistochemistry

Colorectal cancer tissue microarrays (TMAs) were prepared by standard protocol[135] and kindly provided by senior research assistants. The concept behind TMAs technology is to take several core samples from a "donor" tissue block and transfer them to an empty "recipient" block using a Tissue Microarrayer. Step by step, formalin-fixed, paraffin-embedded colorectal cancer tissue blocks, and relative H&E sections are reviewed and marked by a pathologist. The number, core size and sampling size are chosen according to the study research question: in this case, a total of 1017 cases of CRC were arrayed into cores of 0.6 mm diameter. For each case, cores were taken from the luminal side of the tumour, from the middle areas of the tumour, from the advancing edge and from the adjacent normal colonic epithelium. The region-specific TMA grid allows for the cores to be more representative of the whole tumour and for intra-tumour heterogeneity to be assessed. Finally, the TMA blocks are cut into 4-µm sections and transferred onto slides.

Standard IHC was performed on the slides by the Pathology department of the Queen's Medical Centre. The NHS GRADE system has been used for the assessment of the protocols and antibodies used to ensure a high standard quality of evidence. Roche (Mannheim, Germany) kits were used as primary antibodies (RTU mouse monoclonal antibody (product code 05973945001) for β-catenin and RTU rabbit monoclonal antibody (product code 05278384001) for Ki67). Positive and negative controls were used as follow: normal colorectal tissue as internal positive control for β-catenin and Ki67; liver, pancreas, kidney, urothelium, spleen and skin tissues as external positive control for β-catenin and external negative control for Ki67; lymph nodes tissue was used as negative control for β-catenin and positive control for Ki67.

Lastly, the TMAs were scanned in using the 3D Histech slide scanner to create digital images ready to be scored. Images were scanned in at X20 magnification.

Evaluation of Immunostaining

Every single core was evaluated unless it failed to pass the quality requirements. Failure was caused by missing cores, insufficient presence of tumour (<15%), folded cores, cores overstained, not in focus or containing major artefacts. These were excluded from the scoring process.

The expression of β-catenin was manually scored. There is expression in the nucleus, cytoplasm and membrane and each cellular compartment was separately scored for each core. Scoring was undertaken using the Histoscore (H-score). This is a commonly used method which allows for heterogeneity of staining within a tissue section. It depends on quantification of intensity of staining using a four point score. The staining intensity was scored as follows:

- 0 = no expression
- 1+ = weak expression
- 2+ = moderate expression
- 3+ = strong expression.

The final H-score depends on the proportion of cells showing the different levels of expression and is given by the formula: H-score= [(percentage of weakly stained cells X1) + (percentage of moderate stained cells X2) + (percentage of strongly stained cells X3)]. This gives a possible of range of 0 (no staining at all) to 300 (every cell shows strong expression).

The intensities of β-catenin expression were defined after observing over 250 cores and approved by two senior pathologists. In order to ensure consistent scoring, 5% of the cores were re-scored and a kappa value of at least 0.8 was always obtained for both inter-observer and intra-observer concordance. In cases of uncertain cores, mostly because overstained, the cases were individually discussed and recorded either with the agreed score or as not-scorable.

Scoring of each individual core allows assessment of heterogeneity but, for the purposes of analysis of the cases, a mean value was calculated for the three cores unless heterogeneity was seen. In this case, analysis based of regional expression was also undertaken.

The representative expressions in the different cell compartments are reported below.

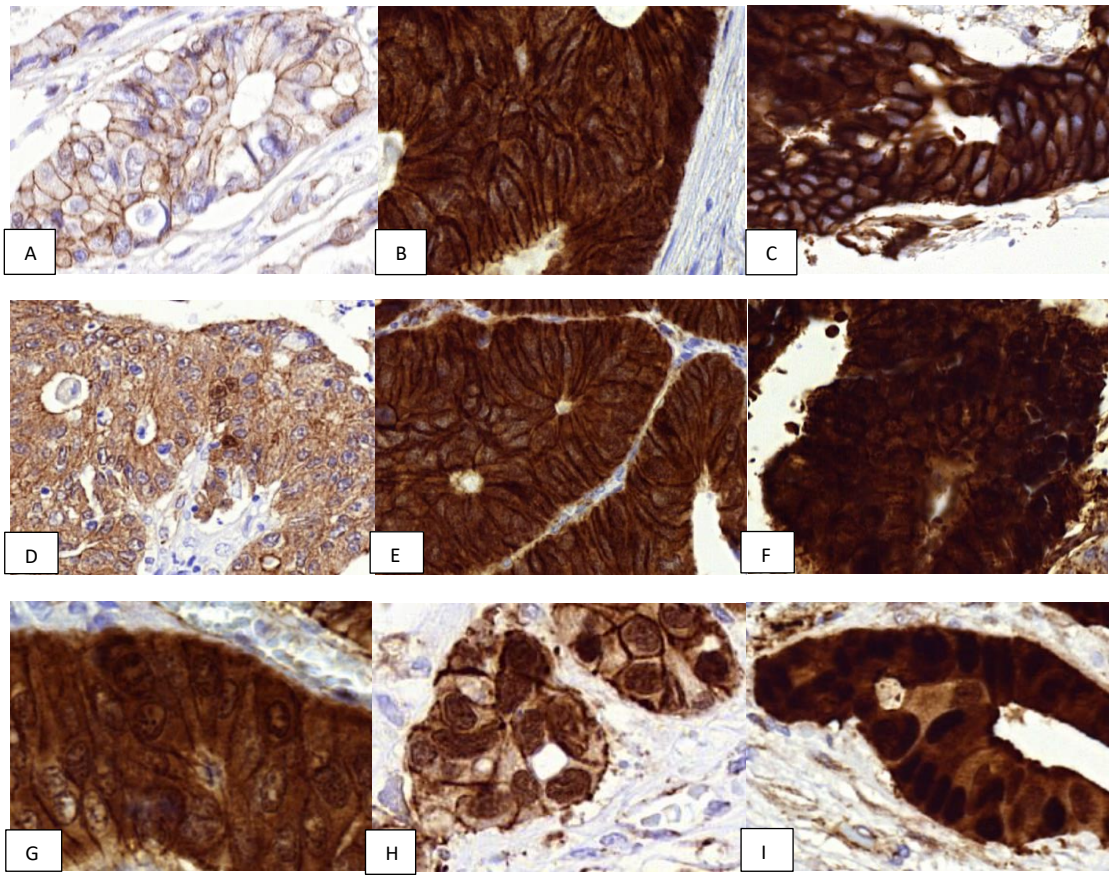


Figure 7. Staining intensities of β-catenin expression. A. Membranous weak expression. B. Membranous moderate expression. C. Membranous strong expression. D. Cytoplasmic weak expression. E. Cytoplasmic moderate expression. F. Cytoplasmic strong expression. G. Nuclear weak expression. H. Nuclear moderate expression. I. Nuclear strong expression.

The expression of ki67 was assessed by automated scoring, in the nucleus of the cells for each specimen. The TMAs analysis was performed using the open source software Qu Path version 0.2.2.

The workflow comprised TMA dearraying (cores are detected and matched with the TMA map, so that each core has a unique ID); manual quality check of the cores (removing of the not-scorable cores, if missing, with insufficient tissue (<15% tumour tissue and/or <500 tumour cells) or with major artefacts); estimate of stain vectors for each digitized slide (this is a key step that uses colour deconvolution to detect the stains in a tissue image, separating them from the normal RGB background). Subsequently, cells were detected with the following settings: detection image, optical density sum; requested pixel size, 0.5 µm; background radius, 8 µm; median filter radius, 0 µm; sigma, 1.5 µm; minimum cell area, 10 μm^2; maximum cell area, 400 μm^2; threshold, 0.1; maximum background intensity, 2. An additional feature (i.e. smoothed features, radius 50µm) was added to the default cell morphometry aspects calculated by the Cell Detection command, to reduce the 'noisiness' of the measurements. A random trees classifier[136] was trained to identify tumour cells, stromal cells, immune cells and necrosis, annotating regions of each subclass across multiple cores.

The classifier was then applied to each slide and the Nucleus: DAB OD mean, 0.2 was used as single threshold within the cell intensity classification to separate positive and negative stained tumour cells.

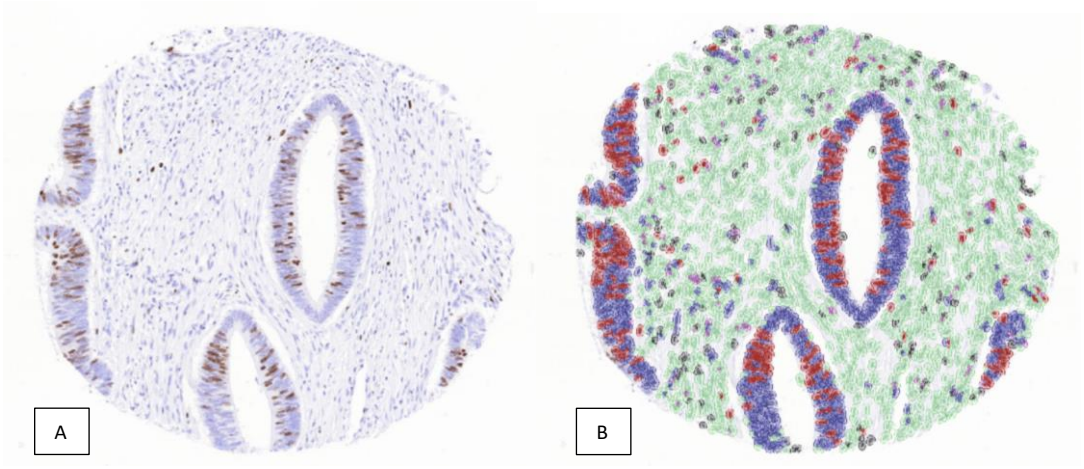


Figure 8. Ki67 analysis with QuPath cell classification. A. Core containing tumour tissue stained for Ki67. B. Same core after the classifier and the cell intensity classification are applied, showing the positive (red) and negative (blue) cells.

A second classifier, for the sole purpose of identifying the epithelial tissue in the core, was developed for normal colon samples stained for ki67; to distinguish positive and negative stained nuclei, a single threshold of 0.2 was set as Nucleus: DAB OD mean in the cell intensity classification command.

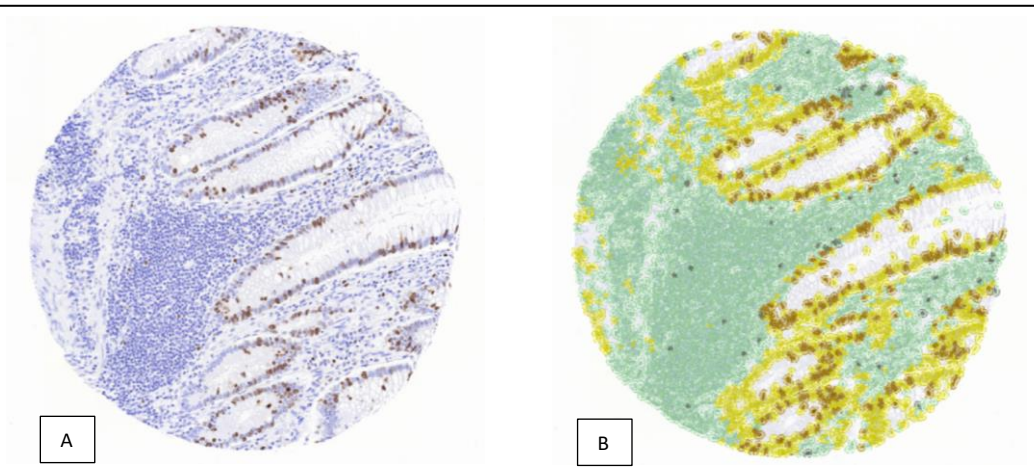


Figure 9. Ki67 analysis with QuPath cell classification. A. Core containing normal colon tissue stained for Ki67. B. Same core after the classifier and the cell intensity classification are applied, showing the positive (brown) and negative (yellow) cells.

The Ki67 index was computed as the percentage of nuclear positive tumoral cells in each core (KI67LI= n of positive tumour cells/total n of tumour cell x100).

The stroma content was mathematically computed. Using the data generated by QuPath, namely the percentage of tumour present in each core, the stroma percentage was determined by subtracting the percentage of the tumour present in the TMA from the total content of the TMA (percentage of stroma = 100 - percentage of tumour). In this way the stroma content included in various degree connective tissue cells and non-stromal cells (such as endothelial cells and immune cells). Additionally, as this analysis was planned at a later stage of the study, and the cores with less than 15% had been already excluded, the maximum value for the stroma percentage was 85%: in other words, cores with more than 85% of stroma (and less than 15% of tumour) were not scored and therefore a core with 85% stroma was considered as entirely containing stroma.

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics version 26.

The distributions of β-catenin H-scores, ki67 index and Stroma percentage were first assessed. When the distribution was not normal, a transformation to reduce the skewness was attempted; when not successful, non-parametric tests were performed for these variables.

To distinguish subgroups in the biomarkers expression (i.e. low/high), different cut-offs were used, depending on the biological characteristic of the biomarkers and the characteristic of the distributions.

The χ^2 test was carried out to investigate the correlation between the expression of the biomarkers with clinicopathological parameters. Kaplan-Meier's curves were performed to estimate the overall survival and the log-rank test was used for the comparison between the groups. The Cox proportional hazard model was used to evaluate the independent effect of the variables on survival. Results were considered significant when showed a p-value less than 0.05 and to account for the variation in sample size, the adjusted residuals were computed as a measure of strength for each correlation.

The same analyses were also carried out after patients stratification for MMR status, location and final stage.

To investigate tumour heterogeneity and assess the difference between the variables (Nuclear, Cytoplasmic and Membranous expression in Luminal, Middle and Advancing Edge tumour sides), Repeated measures Anova, Friedman and Paired sample t-test were performed. Pairwise comparisons and effect sizes were estimated to assess which pairs were truly correlated and the strength of the correlation.

Finally, Paired sample t-test and Wilcoxon signed-rank test were used for the comparisons between normal and tumour tissues and estimate the size of the global effect of the biomarkers on tumour development and progression.

RESULTS

1. Population and clinicopathological parameters

A total of 1000 patients of the original 1017 were included in this study. 43% were women and 57% men; the median age was 70 (IQR 15) and only 6.3% of population was under 50 years old; the youngest patient was 16 years old. 490 tumours developed in the right colon (defined as caecum, ascending and transverse colon), 363 in the left colon (descending and sigmoid colon) and 14.7% in the rectum. Most of the tumours were stage II or III (40.2% and 31.9% respectively) and showed moderate differentiation (G2, 88.7%). 28.7% of patients experienced recurrent disease; metastases were reported in 119 patients, 31% of the patients presented lymphovascular invasion and 18.9% perineural invasion. Survival data were available for all patients with a median of 55 months.

Demographic and Clinicopathological Features		N	%
Sex	Female	432	43.2
	Male	568	56.8
Age	Less than 50	63	6.3
	Between 50 and 69	426	42.6
	70 or more	511	51.1
Tumour Location	Right Colon	490	49.0
	Left Colon	363	36.3
	Rectum	147	14.7
TNM staging	Stage I	161	16.1
	Stage II	402	40.2
	Stage III	319	31.9
	Stage IV	118	11.8
Primary tumour stage	1	74	7.4
	2	106	10.6
	3	526	52.6
	4	294	29.4
Lymph node Involvement	Uninvolved	570	58.5
	Involved	404	41.5
Distant Metastasis	Absent	881	88.1
	Present	119	11.9
Histological Grade	Well differentiated	20	2.0
	Moderately differentiated	886	88.7
	Poorly differentiated	93	9.3
Lymphovascular Invasion	Absent	641	69.1
	Present	287	30.9

Table 1. Clinicopathological characteristics of the study population.

The following parameters were correlated with survival: tumour pT stage (*p* 3.8653e-20), lymph nodes involvement (*p* 1.3052e-18), metastases (*p* 8.0841E-32), tumour grade (*p* 3.6733e-8), intramural lymphovascular invasion (*p* .002) and tumour location (*p* .022) were correlated with survival.

The clinicopathological data and the outcome with some of these parameters were consistent with published literature and show that there was no bias in the cohort.

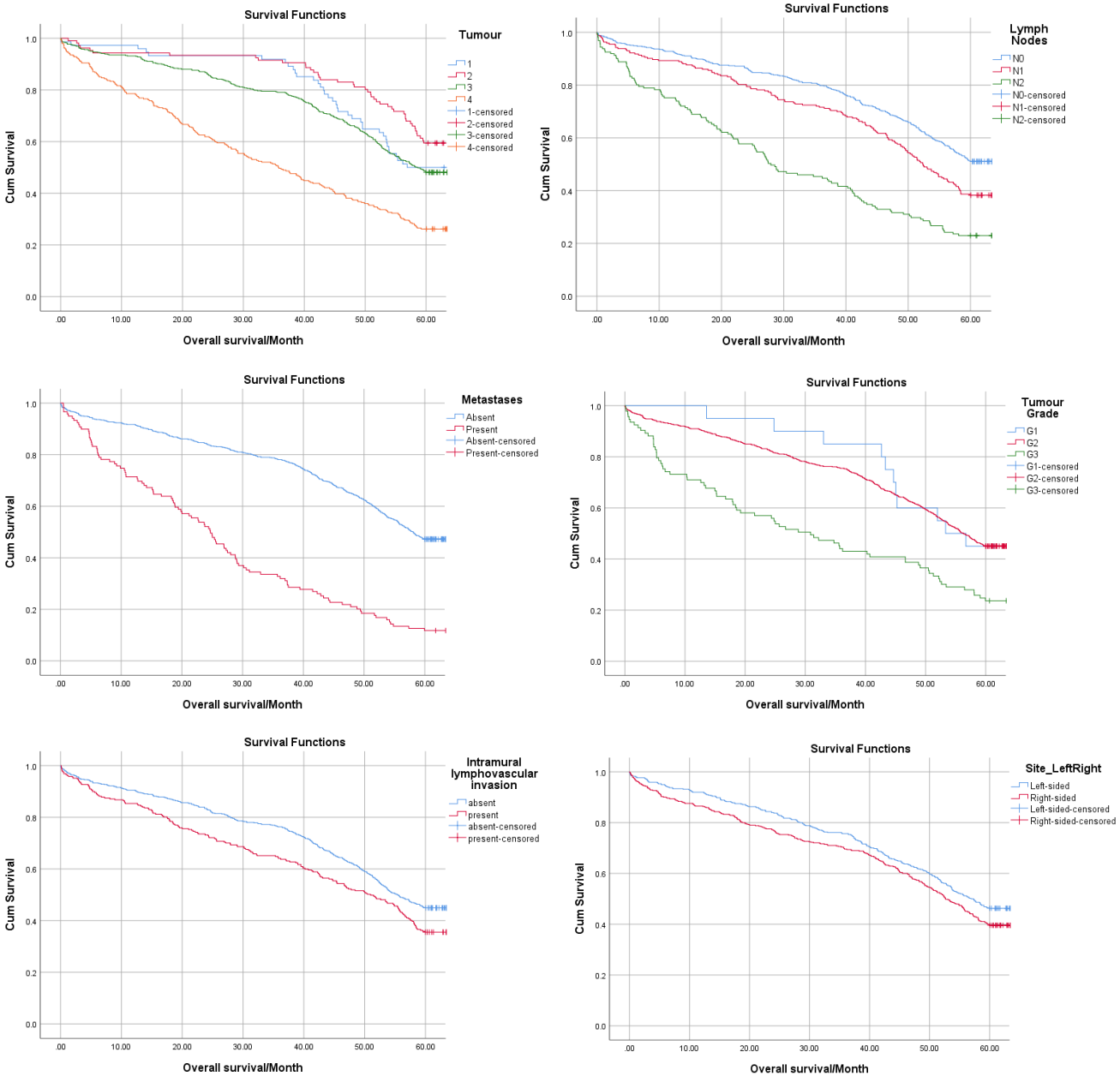


Figure 10. Association between survival and primary tumour stage, lymph nodes involvement, metastases, tumour grade, intramural lymphovascular invasion and tumour location.

2. β-catenin in Colorectal Cancer

A total of 933 cases were scored for nuclear, cytoplasmic and membranous expression of β-catenin and used for the statistical analysis. One TMA slide (67 cases) was scored but not used for further analysis due to poor immunohistochemical staining. Of these 933 cases, 783, 813 and 794 were actually used to analyse β-catenin nuclear, cytoplasmic and membranous expressions, respectively, in relation to the clinicopathological parameters; the reasons for the exclusions are listed in the methods section.

As an initial evaluation, a mean of the H-scores of the 3 subcellular compartments was computed to investigate any correlation between the "overall" expression of the biomarker and clinicopathological parameters. The new variable "overall β-catenin" presented 775 valid cases (M=141.73 SD=32.51).

The distribution was normal and therefore was dichotomized into high- and low- expression by the median (142.50). High β-catenin showed a significant correlation with left-sided CRC (*p* .000007) and MMR proficiency (*p* 6.3301e-8). There was no significant difference in the survival curves between the two groups (*p* .443), and the variable didn't show an independent prognostic potential (*p* .273) (data not shown).

Unless specified otherwise, nuclear, cytoplasmic and membranous β-catenin will now be described individually and scores refer to the mean of the H-scores in the luminal, central and peripheral tumour portions (for example, nuclear β-catenin= (nuclear β-catenin in luminal area + nuclear β-catenin in central area + nuclear β-catenin in peripheral area)/3). Only the significant results are reported.

Nuclear β-catenin and clinicopathological parameters

The nuclear expression showed a positively skewed distribution and couldn't be transformed into a normal distribution. The median was 45.00 (IQR 94.17), the minimum value reported was 0.00 and the maximum 300.00, covering the entire H score range (0-300). Given the high percentage of unstained nuclei (8.9%) and the severity of the skewness (1.12 ± .087), the lowest tertile (H-score = 20.00) was chosen as cut-off to divide the distribution into low-expression (n= 242) and high-expression (n= 541).

Clinicopathological Criteria		β-catenin Nuclear Expression				χ^2 (p-value)
		Low (%)	A.R	High (%)	A.R.	
3Y Survival						
	Alive	165 (29.0)	-1.8	403 (71.0)	1.8	3.342 (.068)
	Dead	77 (35.8)	1.8	138 (64.2)	-1.8	
5Y Survival						
	Alive	89 (27.1)	-1.9	239 (72.9)	1.9	3.762 (.052)
	Dead	153 (33.6)	1.9	302 (66.4)	-1.9	
Site						
	Right colon	153 (41.1)	5.9	219 (58.9)	-5.9	
	Left Colon	55 (19.5)	-5.2	227 (80.5)	5.2	**38.340 (2.3944e-8)**
	Rectum	25 (23.8)	-1.7	80 (76.2)	1.7	
	Transverse and Ascending Colon	9 (37.5)	.7	15 (62.5)	-.7	

Grade						
	1	7 (53.8)	1.8	6 (46.2)	-1.8	**48.458 (3.0024e-11)**
	2	195 (27.4)	-6.9	517 (72.6)	6.9	
	3	40 (70.2)	6.7	17 (29.8)	-6.7	
Perineural Invasion						
	No	180 (29.2)	-2.1	437 (70.8)	2.1	**4.505 (.034)**
	Yes	53 (38.4)	2.1	85 (61.6)	-2.1	
MMR Status						
	Proficient	174 (26.6)	-5.9	481 (73.4)	5.9	**38.057 (5.4447e-9)**
	Deficient	68 (54.0)	6.1	58 (46.0)	-6.1	

Table 2. Association between nuclear β-catenin and clinicopathological variables

High nuclear β-catenin was associated with left-sided CRC (*p* 2.3944e-8), lower grade (*p* 3.0024e-11), absence of perineural invasion (*p* .034) and proficiency of the MMR system (*p* 5.4447e-9). There was no association with TNM parameters nor vascular or lymphovascular invasion.

The overexpression of β-catenin in the nucleus was also associated with a better outcome (χ^2 4.32, *p* .038), as shown in the 5year survival curve below (the mean for the low expression was 61.67 months (95%CI 56.45 – 66.89) and 72.67 months (95%CI 68.81 – 76.53).

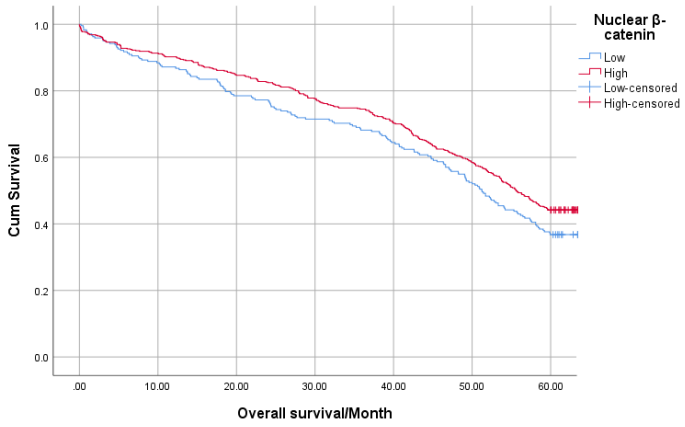


Figure 11. Association between survival and nuclear β-catenin.

Cytoplasmic β-catenin and clinicopathological parameters

The values of cytoplasmic β-catenin displayed a symmetric curve; the mean was 185.07 (SD 41.40). The median (190.00) is more "robust" against outliers, therefore, given the considerable range of the distribution (min 21.67 – max 300.00), it was preferred over the mean as cut-off to dichotomise the variable. The cytoplasmic expression was found high in 410 patients (50.4%) and low in 403 patients (49.6%).

Clinicopathological Criteria		β-catenin Cytoplasmic Expression				χ^2 (p-value)
		Low (%)	A.R.	High (%)	A.R.	
3Y Survival						
	Alive	280 (47.2)	-2.2	313 (52.8)	2.2	**4.849 (.028)**
	Dead	123 (55.9)	2.2	97 (44.1)	-2.2	
Site						
	Right colon	225 (59.2)	5.2	155 (40.8)	-5.2	
	Left Colon	120 (41.0)	-3.7	173 (59.0)	3.7	**27.898 (0.000004)**
	Rectum	45 (39.1)	-2.4	70 (60.9)	2.4	
	Transverse and Ascending Colon	13 (52.0)	.2	12 (48.0)	-.2	
T						
	1	28 (46.7)	-.5	32 (53.3)	.5	
	2	43 (47.3)	-.5	48 (52.7)	.5	**12.983 (.005)**
	3	195 (45.1)	-2.7	237 (54.9)	2.7	
	4	137 (59.6)	3.6	93 (40.4)	-3.6	
Grade						
	1	8 (53.3)	.3	7 (46.7)	-.3	
	2	347 (47.1)	-4.3	390 (52.9)	4.3	**21.765 (0.000019)**
	3	47 (78.3)	4.6	13 (21.7)	-4.6	
MMR Status						
	Proficient	313 (46.0)	-4.6	367 (54.0)	4.6	**22.286 (0.000014)**
	Deficient	89 (68.5)	4.7	41 (31.5)	-4.7	

Table 3. Association between cytoplasmic β-catenin and clinicopathological variables

High cytoplasmic β-catenin was reported in cases with better outcome: 52.8% patients with high cytoplasmic β-catenin are still alive 3 years after diagnosis (*p* .028); however, no significant correlation was reported for 5years survival nor overall survival. High cytoplasmic β-catenin has shown significant correlation with younger age (<70 years old) (*p* .015), distal colon and rectal cancers (*p* .000004), lower T stages (*p* .005), lower grade (*p* 0.000019) and MMR proficiency (*p* 0.000014).

High expression was confirmed to be associated with a better survival by the Log Rank test (χ^2 4.82, *p* .028) (M=96.75, 95%CI 92.36 – 101.13 vs M=82.80, 95%CI 78.43 – 87.17).

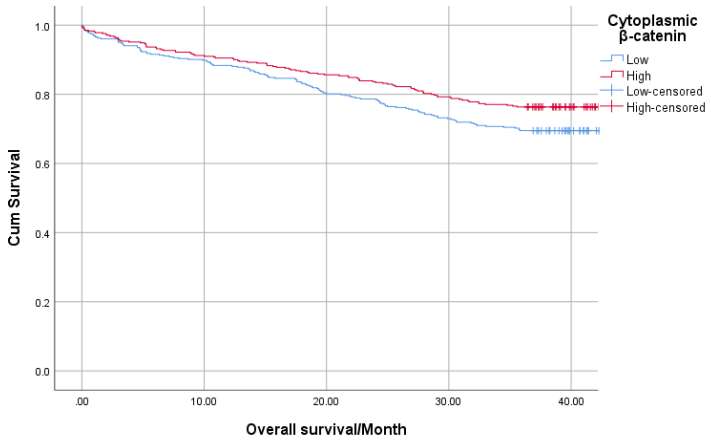


Figure 12. Association between survival and cytoplasmic β-catenin.

Membranous β-catenin and clinicopathological parameters

Cell membrane was generally strongly stained for β-catenin (36.6% of cases were spread around an H-score range of 180 and 230, despite the values covering the entire H-score range 0-300). The mean was 171.71 (SD 57.11). As cut-off, the lowest tertile (H score = 150.00) seemed a reasonable value to valorise the biological meaning of a true weak expression (even though 150 is still quite an intense stain) and, at the same time, to have the patients split in an acceptable proportion (low-expression n=192 and high-expression n=390).

When assessed in relation with clinicopathological parameters, high membranous β-catenin was found significantly associated only with better survival (*p* .022) and right-sided CRC (*p* .025).

The membranous expression didn't show a conclusive survival plot (the mean values for 5y survival in months were 62.84 (95% CI 57.88 – 67.79) and 72.53 (95% CI 68.61 – 76.45) for low- and high-expression respectively; χ^2 4.27, *p* .039).

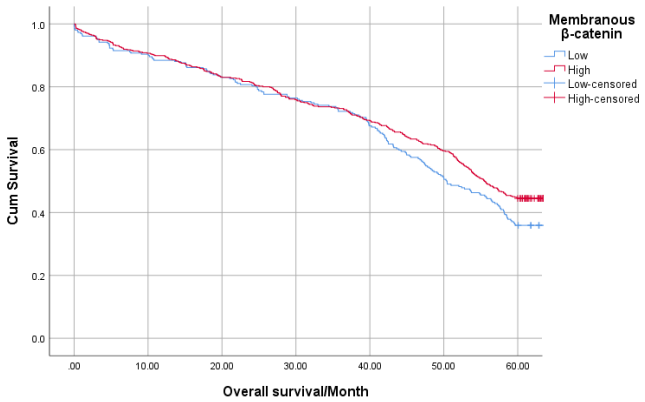


Figure 13. Association between survival and cytoplasmic β-catenin.

Multivariable Cox regression analysis

To better investigate the prognostic significance of β-catenin, a multivariable Cox regression model was computed with the extent of the primary tumour (T), lymph nodes involvement (N), distant metastasis (M), histologic grading (G) and the different subcellular localizations.

The overall model was significant (*p* 1.5507e-30), and nuclear and membranous β-catenin were found to be independent markers of good prognosis (HR <1).

Variables		5y SURVIVAL	
		HR (95% CI)	P value
T			
	T1	.85 (.48 – 1.49)	**0.000161**
	T2	1.07 (.65 – 1.74)	
	T3	1.66 (.99 – 2.76)	
N			
	N1	1.11 (.88 – 1.40)	**0.000284**
	N2	1.73 (1.32 – 2.27)	
M		2.58 (1.98 – 3.37)	**2.5306e-12**
G			
	G2	.91 (.43 – 1.94)	**0.099113**
	G3	1.35 (.60 – 3.05)	
Nuclear β-catenin		.78 (.63 - .98)	**0.028684**
Cytoplasmic β-catenin		1.20 (.98 – 1.46)	0.080437
Membranous β-catenin		.77 (.63 - .94)	**0.010082**

Table 4. Multivariable Cox regression model.

Nuclear β-catenin at the invasive margin of the tumour

The same analyses were conducted to investigate the role of β-catenin in the nucleus at the invasive margin of the tumour. The distribution was dichotomized using 0.00 as cut-off: the cases with no nuclear stain were 76 (13.3%) and the cases that expressed nuclear β-catenin were 497 (86.7%).

At the advancing edge, nuclear β-catenin was found associated with left-sided CRC (*p* .010), lower grade (*p* .000002), MMR proficiency (*p* .000006) and infiltrative tumour edge (*p* .008). 90.2% of the tumours that presented vascular invasion had nuclear expression (*p* .025).

Clinicopathological Criteria		ADVANCING EDGE				χ^2 (p-value)
		No Expr (%)	AR	Nuclear Expr (%)	AR	
Site						
	Right colon	50 (17.7)	3.1	233 (82.3)	-3.1	
	Left Colon	16 (7.7)	-3.0	192 (92.3)	3.0	**11.44 (.010)**
	Rectum	7 (10.4)	-.7	60 (89.6)	.7	
	Transverse and Ascending Colon	3 (20.0)	.8	12 (80.0)	.-8	
V						
	No	48 (16.2)	2.2	249 (83.8)	-2.2	**5.01 (.025)**
	Yes	26 (9.8)	-2.2	240 (90.2)	2.2	
Grade						
	1	5 (45.5)	3.2	6 (54.5)	-3.2	**26.08 (0.000002)**
	2	57 (11.0)	-5.0	461 (89.0)	5.0	
	3	14 (32.6)	3.9	29 (67.4)	-3.9	
Tumour edge						
	Infiltrative	24 (10.0)	-2.7	217 (90.0)	2.7	**7.04 (.008)**
	Pushing	41 (18.6)	2.7	180 (81.4)	-2.7	
MMR Status						
	Proficient	48 (10.1)	-4.8	426 (89.9)	4.8	**24.17 (0.000006)**
	Deficient	28 (28.6)	4.9	70 (71.4)	-4.9	

Table 5. Association between nuclear β-catenin at advancing edge and clinicopathological variables.

No association was found between nuclear expression at advancing edge and survival status; the Log Rank test was not significant (χ^2 .620, p value .431).

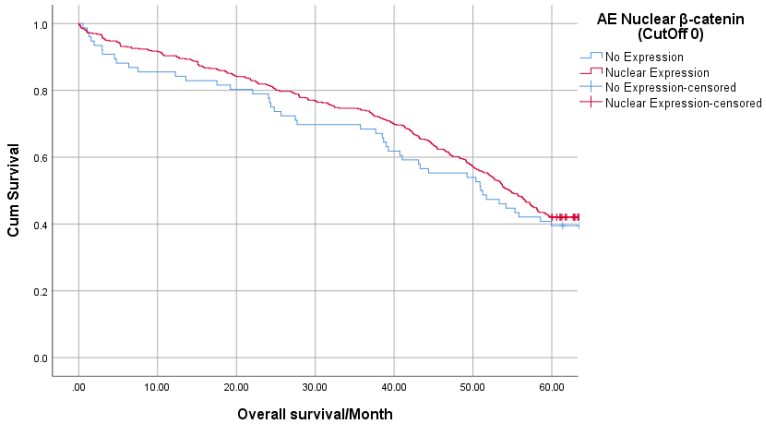


Figure 14. Association between survival and nuclear β-catenin at advancing edge.

β-catenin in CRC and normal colorectal mucosa

The overall β-catenin expression in the three compartments of the cell in the tumour samples was compared to the equivalent in the normal colon samples. The paired sample t-test showed a small statistically significant difference between the two variables, being the mean H-score for the tumour tissue 142.68 (SD 32.13) and for the normal tissue 132.92 (SD 21.88) (*p* 5.4454e-9).

The analysis was extended to the nuclear, cytoplasmic and membranous expression in CRC and normal tissue. Cytoplasmic β-catenin was found to be higher in tumour than in normal tissue (t 23.13, *p* 1.9413e-58); likewise, nuclear expression was higher in CRC than normal epithelium (Z -17.04, *p* 4.4271e-65). The average cytoplasmic expression was 186.59 (SD 42.05) for the tumour and 129.79 (SD 36.20) for normal tissue (Cohen's d 1.07) and the average nuclear expression was 70.68 (SD 71.38) for the tumour and 4.11 (SD 10.31) for normal tissue (r 0.80). Conversely, membranous β-catenin was higher in normal tissue (M 265.23, SD 43.60) compared to tumour tissue (M 171.01, SD 56.48) (t -31.03, *p* 6.9513e-115, Cohen's d 1.44).

3. KI67 in Colorectal Cancer

A total of 932 samples were scored to compute KI67 Labelling Index (Ki67 LI). The missing values were excluded for the reasons listed in previous sections. The mean of the Ki67 LI for luminal, middle and advancing edge was computed to perform analyses of correlation between the biomarker and multiple clinicopathological parameters and investigate its prognostic value.

KI67 and clinicopathological parameters

The histogram for the mean of Ki67 showed a normal distribution, with the following characteristics: M= 44.92 (CI 95% 43.67-46.18); range: min .00 max 92.82; skewness -.004 (SE .080). Given the large amount of literature about Ki67 and its use in the characterization of many types of cancers, a low cut-off was the destined choice to dichotomise the distribution.

Using the lowest quartile (Ki67LI=31%), it was divided into low- and high- expression groups, counting 233 and 767 values, respectively.

Clinicopathological Criteria		Ki67LI				χ^2 (p-value)
		<31% (%)	AR	>31% (%)	AR	
5Y Survival						
	Alive	117 (27.2)	2.5	313 (72.8)	-2.5	**6.451 (.011)**
	Dead	116 (20.4)	-2.5	454 (79.6)	2.5	
Peritumoral Lymphocytes						
	Inconspicuous	132 (24.0)	2.4	417 (76.0)	-2.4	**5.715 (.017)**
	Conspicuous	35 (16.1)	-2.4	182 (83.9)	2.4	
Tumour edge						
	Infiltrative	113 (28.6)	4.3	282 (71.4)	-4.3	**18.768 (0.000015)**
	Pushing	57 (15.5)	-4.3	310 (84.5)	4.3	
MMR Status						
	Proficient	210 (25.7)	3.8	608 (74.3)	-3.8	**17.154 (0.000188)**
	Deficient	17 (10.6)	-4.1	143 (89.4)	4.1	

Table 6. Association between Ki67 and clinicopathological variables

High Ki67 was significantly associated with conspicuous peritumoral lymphocytes (*p* .017), pushing tumour edge (*p* 0.000015) and dMMR status (*p* 0.000188). Low ki67 showed a better 5y survival (*p* .011), but the survival plot wasn't conclusive and the Log Rank test barely significant (*p* .049).

The multiple regression model used for the cox regression was overall significant (*p* 1.205e-47) and ki67 was found to have an independent prognostic value of poor outcome (*p* .022).

Covariates		5y SURVIVAL	
		HR (95% CI)	P value
T			
	T1	0.89 (.53 – 1.49)	**0.000004**
	T2	1.10 (.71 – 1.71)	
	T3	1.76 (1.11 – 2.79)	
N			
	N1	1.15 (.94 – 1.42)	**0.000208**
	N2	1.66 (1.30 – 2.11)	
M		2.60 (2.07 – 3.26)	**2.8669e-16**
G			
	G2	1.23 (.63 – 2.40)	**0.011**
	G3	1.82 (.90 – 3.70)	
Ki67LI		1.28 (1.04 – 1-57)	**0.022**

Table 7. Multivariable Cox regression model.

The same analyses were performed with a cut-off of Ki67LI = 25% [low-expression group (n=156) and high-expression group (n=775)] and , following the guidelines for breast cancer, also KI67LI =20% [low-expression group (n=107) and high-expression group (n=825)]. From cut-off 31% to 25% to 20% , the groups became quite uneven in numbers, the p values for the association with survival increased (*p* .011, .027, .046 respectively) and, in agreement, the survival plots lost significance (data not shown).

Clinico-pathological Criteria	Ki67LI				χ^2 (p-value)	Clinico-pathological Criteria	Ki67LI				χ^2 (p-value)
	<25% (%)	AR	>25% (%)	AR			<20% (%)	AR	>20% (%)	AR	
5Y Survival						**5Y Survival**					
Alive	80 (19.9)	2.2	323 (80.1)	-2.2	**4.880**	**Alive**	56 (13.9)	2.0	348 (86.1)	-2.0	**3.98**
Dead	76 (14.4)	-2.2	452 (85.6)	2.2	**(.027)**	**Dead**	51 (9.7)	-2.0	477 (90.3)	2.0	**(.046)**
T						**Perineural Invasion**					
1	4 (5.7)	-2.6	66 (94.3)	2.6	**10.029**						**3.89**
2	22 (21.4)	1.3	81 (78.6)	-1.3	**(.018)**	**No**	78 (10.7)	-2.0	654 (89.3)	2.0	**(.049)**
3	77 (15.7)	-1.0	415 (84.3)	1.0		**Yes**	27 (16.1)	2.0	141 (83.9)	-2.0	
4	53 (19.9)	1.6	213 (80.1)	-1.6							
Tumour edge						**Tumour edge**					
Infiltrative	81 (22.1)	4.6	285 (77.9)	-4.6	**21.12**	**Infiltrative**	57 (15.5)	4.4	310 (84.5)	-4.4	**18.96**
Pushing	33 (9.5)	-4.6	314 (90.5)	4.6	**(0.000004)**	**Pushing**	19 (5.5)	-4.4	328 (94.5)	4.4	**(0.000013)**
MMR Status						**MMR Status**					
Proficient	140 (18.1)	2.5	632 (81.9)	-2.5	**15.79**	**Proficient**	95 (12.3)	1.7	678 (87.7)	-1.7	**17.86**
Deficient	12 (7.9)	-3.2	139 (92.1)	3.2	**(0.000372)**	**Deficient**	8 (5.3)	-2.6	143 (94.7)	2.6	**(0.000133)**

Table 8. Association between Ki67 (cut-offs 25% and 20%) and clinicopathological variables

KI67 in CRC and normal colorectal mucosa

The expression of Ki67 LI in normal tissue was positively skewed. The range was .13 – 57.74 and presented three evident outliers; however, they were not excluded from the analyses as the cores didn't show any histological artifact.

Ki67 was higher in tumour than in normal tissue; the difference observed was 27.66 (SD 22.45) (*p* 4.5737e-107) and the effect size for this correlation was large (Cohen's d= 1.23)

4. Further analyses with population stratification

Colorectal cancer actually refers to many different neoplasms, rather than just one disease, due to the high degree of heterogeneity. Thus, the distributions of the two biomarkers have been investigated in multiple patients subgroups. Below are reported the differential distributions of the biomarkers in multiple patients subgroups.

	Nuclear β-catenin Median (IQR)	Cytoplasmic β-catenin Mean (SD)	Membranous β-catenin Mean (SD)	Ki67LI Mean (SD)
Whole Population	45.00 (94.17)	185.07 (41.40)	171.72 (57.11)	44.92 (19.56)
pMMR	52.50 (100.00)	188.76 (40.84)	172.28 (57.85)	43.45 (19.11)
dMMR	15.00 (54.17)	165.93 (39.28)	169.68 (51.90)	53.61 (19.34)
Left-sided CRC	67.50 (110.00)	193.43 (39.54)	166.39 (58.71)	43.87 (19.45)
Right-sided CRC	26.67 (67.29)	176.64 (41.57)	177.04 (55.03)	46.03 (19.64)
Stage I	55.00 (105.42)	192.55 (38.90)	172.70 (61.37)	44.97 (19.26)
Stage II	41.67 (110.00)	184.71 (41.04)	168.52 (55.13)	45.92 (19.81)
Stage III	45.00 (85.00)	188.33 (41.65)	174.06 (56.16)	45.08 (19.94)
Stage IV	35.00 (92.50)	183.72 (41.34)	174.83 (60.70)	40.61 (17.57)

Table 9. Mean and Standard Deviation and Median and Interquartile Range of β-catenin and Ki67 stratified for MMR status, location and stage.

While the cytoplasmic and membranous expressions of β-catenin seemed to not diverge much from the respective ones in the original cohort, the nuclear values considerably decrease in dMMR population and on the right side of the colon. dMMR also showed higher levels of Ki67. Further analyses have been carried out to explore these differences and also focus the attention on Stage II and III CRC. Only the significant results are reported.

β-catenin

1. *Stratification for MMR status*

81.6% of the CRC samples were MMR proficient and 16.4% were MMR deficient. The mean overall survival was 54 months (SD 29) for pMMR tumours and 50 months (SD29) for dMMR; however, there was no significant association between MMR status and survival (*p* .496). In our cohort, dMMR strongly correlated with pushing tumour edge and pMMR with infiltrative tumour edge (*p* 1.2781e-8).

The cut-offs to dichotomize β-catenin into low- and high- expression were the lowest tertile for nuclear H-score (20.00), the median for cytoplasmic H-score (190.00) and the lowest tertile for the membranous H-score (150.00).

In the pMMR population, both nuclear and cytoplasmic β-catenin were associated with left-sided (*p* .000108 and .001 respectively) and moderately differentiated tumours (*p* 0.000070 and .023 respectively). Membranous location was found correlated only with right-sided CRCs (*p* .038).

MMR Proficient											
Clinico-pathological Criteria	*Nuclear β-catenin*				χ^2 (p-value)	Clinico-pathological Criteria	*Cytoplasmic β-catenin*				χ^2 (p-value)
	Low (%)	AR	*High (%)*	AR			*Low (%)*	AR	*High (%)*	AR	
Gender						**3Y Survival**					
Female	90 (30.5)	2.1	205 (69.5)	-2.1	**4.28**	**Alive**	219 (43.7)	-2.0	282 (56.3)	2.0	**4.11**
Male	84 (23.3)	-2.1	276 (76.7)	2.1	**(.039)**	**Dead**	94 (52.5)	2.0	85 (47.5)	-2.0	**(.043)**
Site						**Site**					
Right	95 (35.4)	4.3	173 (64.6)	-4.3		**Right**	150 (55.1)	3.9	122 (44.9)	-3.9	
Left	48 (18.3)	-3.9	215 (81.7)	3.9	**20.95**	**Left**	109 (39.6)	-2.8	165 (60.4)	2.8	**15.78**
Rectum	24 (23.5)	-.8	78 (76.5)	.8	**(0.000108)**	**Rectum**	44 (39.3)	-1.6	68 (60.7)	1.6	**(.001)**
Transv/Asc	7 (31.8)	.6	15 (68.2)	-.6		**Transv/Asc**	11 (47.8)	.2	12 (52.2)	-.2	
Grade						**Grade**					
1	5 (55.6)	2.0	4 (44.4)	-2.0	**19.13**	**1**	6 (54.5)	.6	5 (45.5)	-.6	**7.51**
2	154 (24.8)	-4.4	466 (75.2)	4.4	**(0.000070)**	**2**	288 (44.8)	-2.6	355 (55.2)	2.6	**(.023)**
3	15 (60.0)	3.9	10 (40.0)	-3.9		**3**	18 (72.0)	2.7	7 (28.0)	-2.7	
Perineural						**T**					
Invasion					**5.45**	**1**	25 (44.6)	-.2	31 (55.4)	.2	
No	123 (24.3)	-2.3	383 (75.7)	2.3	**(.020)**	**2**	39 (47.6)	.3	43 (52.4)	-.3	**14.87**
Yes	42 (34.7)	2.3	79 (65.3)	-2.3		**3**	139 (39.8)	-3.3	210 (60.2)	3.3	**(.002)**
						4	110 (57.0)	3.6	83 (43.0)	-3.6	

Table 10. Association between nuclear and cytoplasmic β-catenin and clinicopathological variables in pMMR tumours

Cytoplasmic β-catenin was also found associated with better outcome (log rank test $\chi^2(1)$ 4.08, *p* .043).

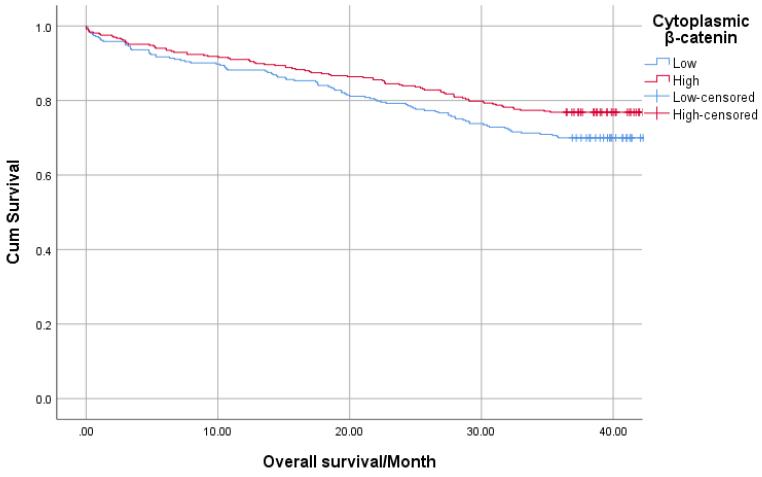


Figure 15. Association between survival and cytoplasmic β-catenin in pMMR tumours.

No valuable association were found in the deficient MMR subpopulation, except for moderately differentiated CRCs, which were correlated with high nuclear and membranous β-catenin (*p* .005 and .055 respectively) (data not shown).

2. Stratification for Location

The original cohort was balanced between left- (n=50.1%) and right- sided (49.9%) CRCs.

In left-sided CRC, Nuclear expression showed significant correlation only with G2 (*p* .002) and membranous expression only with lymphnode involvement (*p* .037). Cytoplasmic β-catenin showed an association with younger patients (<50, n=7%): 81% of them showed high cytoplasmic expression. Younger patients also showed high nuclear expression when the cut-off was set at the highest tertile of the distribution (H-score=81.66) (*p* .039; data not shown).

Clinicopathological Criteria		Left-sided CRC				χ^2 (p-value)
		Cytoplasmic β-catenin				
		Low (%)	AR	High (%)	AR	
Age						
	<50	5 (19.2)	-2.3	21 (80.8)	2.3	**5.19 (.023)**
	>50	150 (41.9)	2.3	222 (58.1)	-2.3	
Age						
	<70	80 (34.9)	-2.6	149 (65.1)	2.6	**6.57 (.010)**
	>70	85 (47.5)	2.6	94 (52.5)	-2.6	
3Y Survival						
	Alive	116 (37.1)	-2.5	197 (62.9)	2.5	**6.38 (.012)**
	Dead	49 (51.6)	2.5	46 (48.4)	-2.5	
Overall Survival						
	Alive	101 (36.7)	-2.2	174 (63.3)	2.2	**4.83 (.028)**
	Dead	64 (48.1)	2.2	69 (51.9)	-2.2	
T						
	1	19 (42.2)	.3	26 (57.8)	-.3	
	2	24 (41.4)	.2	34 (58.6)	-.2	**8.60 (.035)**
	3	72 (34.4)	-2.5	137 (65.6)	2.5	
	4	50 (52.1)	2.7	46 (47.9)	-2.7	
Grade						
	1	1 (20.0)	-.9	4 (80.0)	.9	**12.79 (.002)**
	2	153 (39.1)	-2.4	238 (60.9)	2.4	
	3	10 (90.9)	3.5	1 (9.1)	-3.5	
Tumour budding						
	Low	73 (33.3)	-2.2	146 (66.7)	2.2	**5.02 (.025)**
	High	46 (46.5)	2.2	53 (53.5)	-2.2	

Table 11. Association between cytoplasmic β-catenin and clinicopathological variables in left-sided tumours.

On the right sided CRCs, high nuclear expression correlated with moderately differentiated tumours and MMR proficiency (*p* 0.000007 and 0.000123 respectively), as on the left side; membranous expression showed a significant association with G2 (*p* . 013). Cytoplasmic β-catenin displayed more significant associations.

Clinicopathological Criteria		Right-sided CRC				χ^2 (p-value)
		Cytoplasmic β-catenin				
		Low (%)	AR	High (%)	AR	
5Y Survival						
	Alive	104 (66.7)	2.6	52 (33.3)	-2.6	**6.54 (.011)**
	Dead	134 (53.8)	-2.6	115 (46.2)	2.6	
Grade						
	1	7 (70.0)	.7	3 (30.0)	-.7	
	2	194 (56.1)	-2.7	152 (43.9)	2.7	**7.23 (.027)**
	3	37 (75.5)	2.5	12 (24.5)	-2.5	
Tumour edge						
	Infiltrative	89 (51.7)	-2.0	83 (48.3)	2.0	**3.83 (.050)**
	Pushing	104 (62.3)	2.0	63 (37.7)	-2.0	
MMR Status						
	Proficient	161 (54.6)	-2.8	295 (45.4)	2.8	**8.24 (.016)**
	Deficient	76 (69.7)	2.7	109 (30.3)	-2.7	

Table 12. Association between cytoplasmic β-catenin and clinicopathological variables in right-sided tumours

Only the cytoplasmic location of β-catenin correlated with survival. In distal tumours, high expression was correlated with better survval (χ^2 (1) 6.37, *p* .012), and in proximal tumours with worse survival (log rank test χ^2 (1) 4.21, *p* .040). However, as you can see from the survival curves below, the right-sided plot was inconclusive.

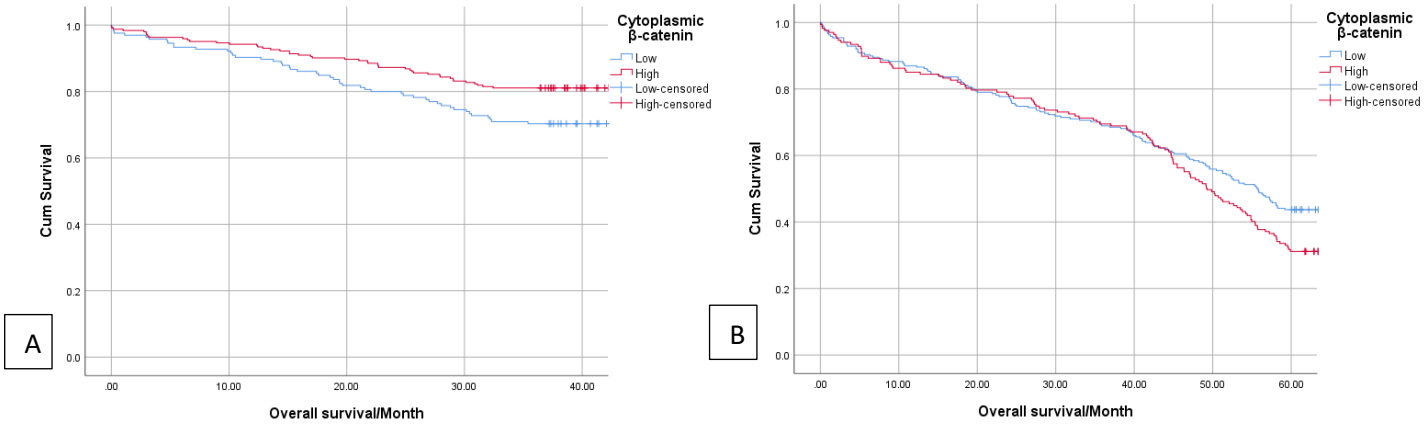


Figure 16. Association between survival and cytoplasmic β-catenin in left-sided tumours (A) and right-sided tumours (B).

3. *Stratification for Stage*

The prognostic value of nuclear β-catenin and Ki67 was tested in stage II and stage III CRCs.

β-catenin displayed significant association with survival in stage III CRC, where a high expression was associated with a better outcome.

Clinicopathological Criteria	Stage II CRC					Stage III CRC				
	β-catenin Nuclear Expression					β-catenin Nuclear Expression				
	Low (%)	AR	High (%)	AR	χ^2 (p-value)	Low (%)	AR	High (%)	AR	χ^2 (p-value)
3Y Survival										
Alive	79 (31.9)	.1	169 (68.1)	-.1	**.006**	44 (25.4)	-2.5	129 (74.6)	2.5	**6.19**
Dead	21 (31.3)	-.1	46 (68.7)	.1	**(.936)**	32 (41.0)	2.5	46 (59.0)	-2.5	**(.013)**
5Y Survival										
Alive	48 (31.8)	.0	103 (68.2)	.0	**0.000237**	19 (19.6)	-2.9	78 (80.4)	2.9	**8.56**
Dead	52 (31.7)	.0	112 (68.3)	.0	**(.988)**	57 (37.0)	2.9	97 (63.0)	-2.9	**(.003)**
Overall Survival Status										
Alive	70 (31.7)	.0	151 (68.3)	.0	**.002**	42 (28.0)	-1.0	108 (72.0)	1.0	**.917**
Dead	30 (31.9)	.0	64 (68.1)	.0	**(.967)**	34 (33.7)	1.0	67 (66.3)	-1.0	**(.338)**

Table 13. Association between nuclear β-catenin and clinicopathological variables at stage II and III.

The log rank tests were χ^2 (1)= .028 (*p* .868) and χ^2 (1)= .017 (*p* .896) for 3y and 5y survival for stage II, respectively, and χ^2 (1)= 6.64 (*p* .010) and χ^2 (1)= 10.04 (*p* .002) for 3y and 5y survival for stage III, respectively.

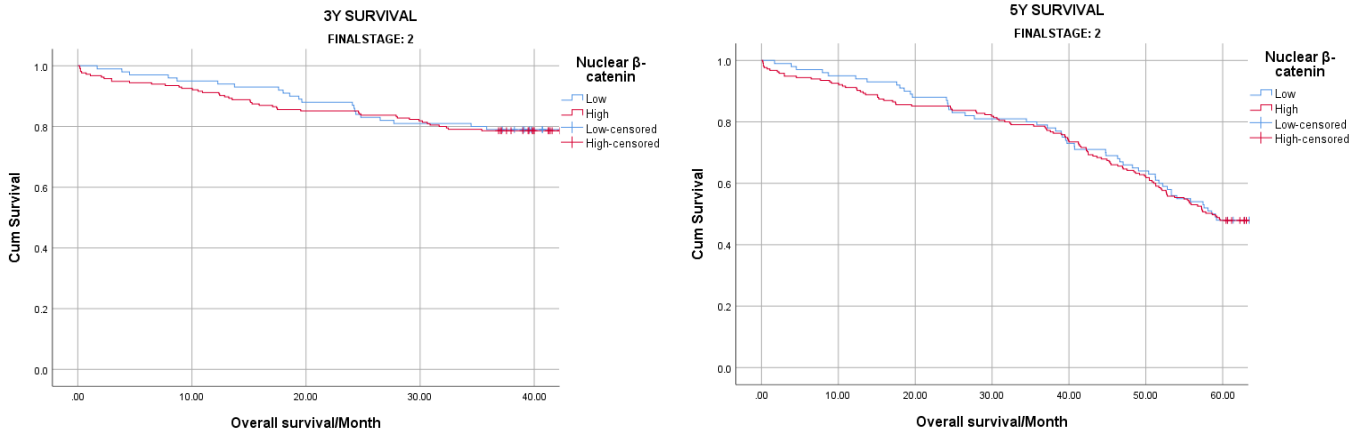


Figure 17a. Association between survival and nuclear β-catenin at 3 years and 5 years from diagnosis for stage II patients.

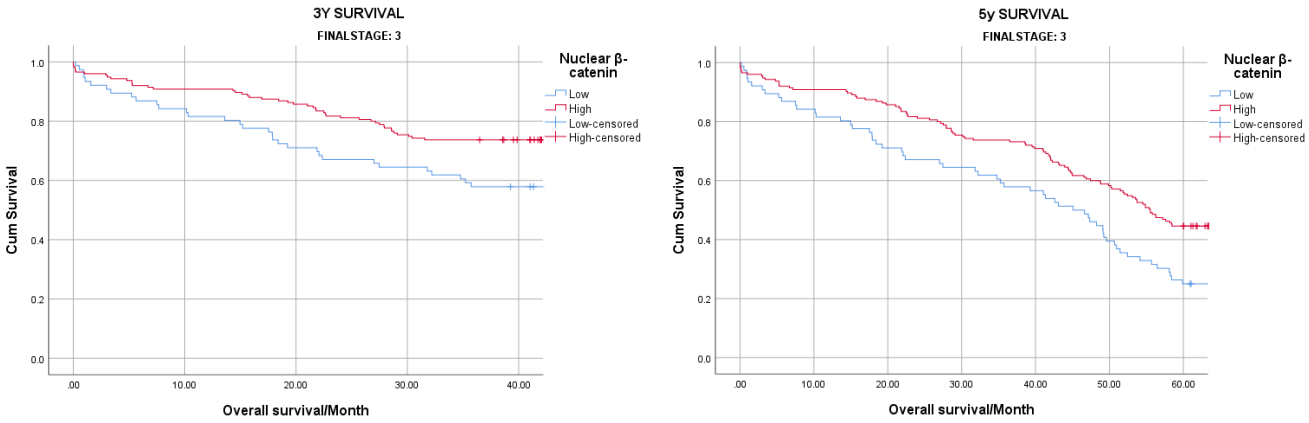


Figure 17b. Association between survival and nuclear β-catenin at 3 years and 5 years from diagnosis for stage III patients.

In stage III patients, high nuclear β-catenin showed to be a significant independent prognostic factor of good outcome in both single-variable and multi-variable analyses (HR .59 95%CI .43 - .82; *p* .002 and HR .60 95%CI .43 - .86; *p* .005 respectively).

Membranous β-catenin showed only marginally significant correlation with survival. High expression was found associated with a better outcome in stage II (*p* .058) and stage III (*p* .069). The log rank tests were not significant (*p* .100 and *p* .052 for stage II and stage III respectively).

Ki67

1. *Stratification for MSI status*

In the Ki67 dataset, 82% of the population presented CRCs with proficient MMR. Different cut-offs of KI67 LI were tested (31% and 25%).

In the pMMR subpopulation, when the lowest quartile of the distribution was used (31%), Ki67 showed association with a better survival (*p* .007), pushing tumour edge (*p* .001) and conspicuous peritumoural lymphocytes (*p* .047). The log rank test was significant (χ^2 (1) 4.54, *p* .033), but the survival plot didn't show any substantial differences between the survival distributions of low- and high-Ki67 (not shown).

When the cut-off was moved to KI67 LI = 25%, similar results were obtained. Ki67<25% also correlated with T4 and KI67>25% with T1. The log rank test was not significant and the Kaplan Meier plot inconclusive.

Clinicopathological Criteria	MMR Proficient									
	KI67 LI					KI67 LI				
	<31% (%)	AR	>31% (%)	AR	χ^2 (p-value)	<25% (%)	AR	>25% (%)	AR	χ^2 (p-value)
5Y Survival										
Alive	108 (30.3)	2.7	248 (69.7)	-2.7	**7.19 (.007)**	74 (21.8)	2.4	265 (78.2)	-2.4	**5.56 (.018)**
Dead	102 (22.1)	-2.7	360 (77.9)	2.7		66 (15.2)	-2.4	367 (84.8)	2.4	
Tumour edge										
Infiltrative	104 (29.8)	3.3	245 (70.2)	-3.3	**11.04 (.001)**	74 (22.8)	3.7	251 (77.2)	-3.7	**13.70 (0.000214)**
Pushing	48 (18.1)	-3.3	217 (81.9)	3.3		28 (11.0)	-3.7	227 (89.0)	3.7	
Peritumoral Lymphocytes										
Inconspicuous	120 (26.1)	2.0	340 (73.9)	-2.0	**3.94 (.047)**	120 (26.1)	2.0	340 (73.9)	-2.0	**3.94 (.047)**
Conspicuous	28 (18.2)	-2.0	126 (81.8)	2.0		28 (18.2)	-2.0	126 (81.8)	2.0	

Table 14. Association between Ki67 and clinicopathological variables in pMMR tumours.

With respect to the dMMR subgroup, both variables spit by KI67 LI= 31% and 25% presented significant association with survival and significant but very modest association with grade.

MMR Deficient										
Clinico-pathological Criteria	KI67 LI					Clinico-pathological Criteria	KI67 LI				
	<31% (%)	AR	>31% (%)	AR	χ^2 (p-value)		<25% (%)	AR	>25% (%)		χ^2 (p-value)
OS Status						**3Y Survival**					
Alive	6 (6.3)	-2.1	89 (93.7)	2.1	**4.57 (.032)**	**Alive**	5 (4.9)	-2.0	97 (95.1)	2.0	**4.16 (.041)**
Dead	11 (16.9)	2.1	54 (83.1)	-2.1		**Dead**	7 (14.6)	2.0	41 (85.4)	-2.0	
Grade						**Grade**					
1	2 (66.7)	3.2	1 (33.3)	-3.2	**10.17 (.006)**	**1**	2 (66.7)	3.8	1 (33.3)	-3.8	**14.89 (.001)**
2	10 (9.2)	-.9	99 (90.8)	.9		**2**	6 (5.8)	-1.5	98 (94.2)	1.5	
3	5 (10.4)	-.1	43 (89.6)	.1		**3**	4 (9.1)	.3	40 (90.9)	-.3	

Table 15. Association between Ki67 and clinicopathological variables in dMMR tumours.

The log rank test showed marginally significant difference between the two groups with cut-off 31% (χ^2 (1) 3.27, *p* .071) and statistically significant difference with cut-off 25% (χ^2 (1) 5.20, *p* .023).

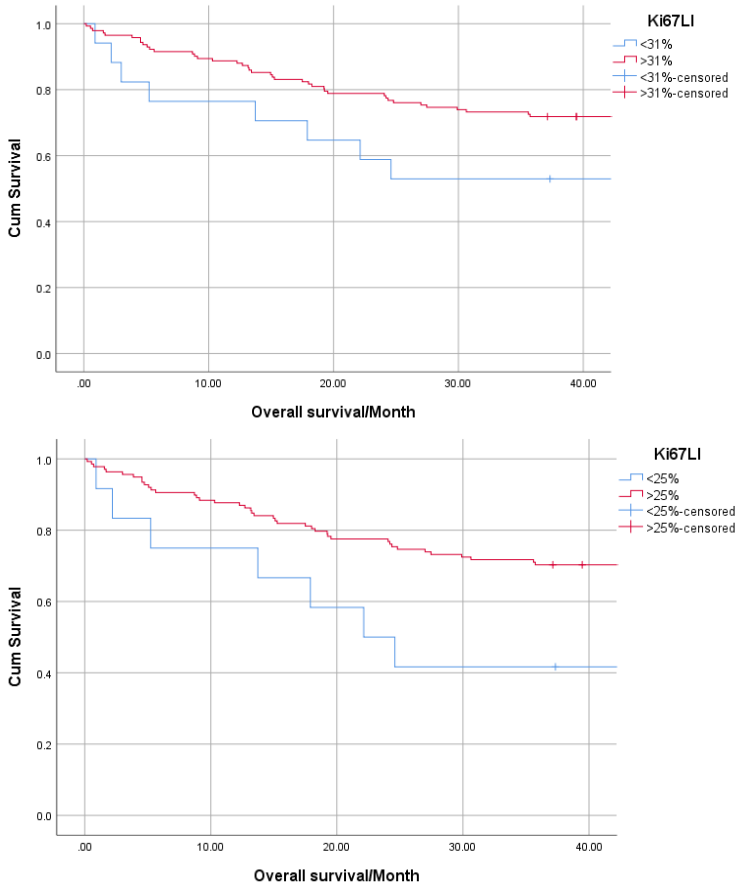


Figure 18. Association between survival and Ki67 in dMMR tumours.

2. Stratification for Location

The original population counted 51% patients with left-sided CRCs and 49% patients with right-sided CRCs.

The 5y survival rate was significantly associated with high Ki67 when the distribution was split by 31% and 25% (*p* .007 and .005 respectively).

Clinicopathological Criteria	Left-sided CRC									
	KI67 LI					KI67 LI				
	<31% (%)	AR	>31% (%)	AR	χ^2 (p-value)	<25% (%)	AR	>25% (%)	AR	χ^2 (p-value)
5Y Survival										
Alive	73 (30.9)	2.7	163 (69.1)	-2.7	**7.39 (.007)**	50 (22.4)	2.8	173 (77.6)	-2.8	**7.83 (.005)**
Dead	56 (20.4)	-2.7	218 (79.6)	2.7		32 (12.7)	-2.8	220 (87.3)	2.8	
Tumour edge										
Infiltrative	59 (29.8)	2.9	139 (70.2)	-2.9	**8.33 (.004)**	39 (21.2)	2.7	145 (78.8)	-2.7	**7.40 (.007)**
Pushing	29 (17.0)	-2.9	142 (83.0)	2.9		17 (10.4)	-2.7	146 (89.6)	2.7	

Table 16. Association between Ki67 and clinicopathological variables in left-sided tumours.

The log rank tests were (χ^2 (1) 5.89, *p* .015), χ^2 (1) 6.08 *p* .014 for 31% and 25% as cut-offs, respectively.

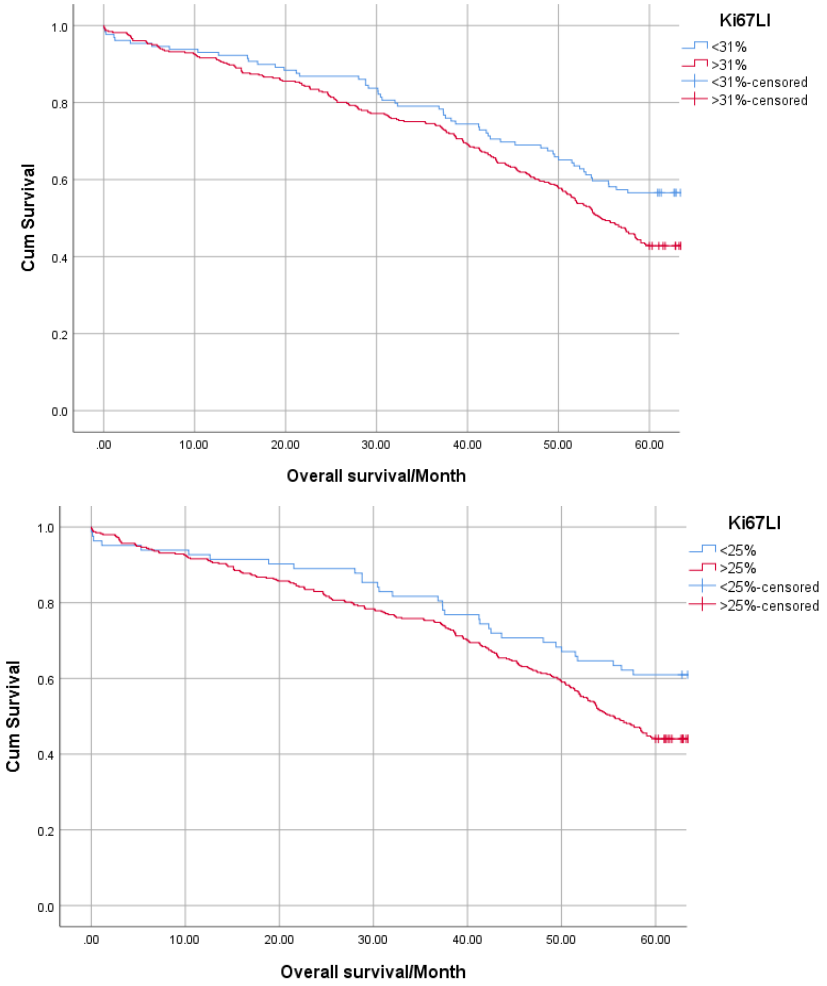


Figure 19. Association between survival and Ki67 in left-sided tumours.

On the right side, more significant correlations were detected compared to the left side.

Clinicopathological Criteria	Right-sided CRC									
	KI67 LI					KI67 LI				
	<31% (%)	AR	>31% (%)	AR	χ^2 (p-value)	<25% (%)	AR	>25% (%)	AR	χ^2 (p-value)
3Y Survival										
Alive	63 (18.5)	-2.3	278 (81.5)	2.3	**5.25**	43 (13.6)	-2.4	274 (86.4)	2.4	**5.59**
Dead	41 (27.7)	2.3	107 (72.3)	-2.3	**(.022)**	31 (22.5)	2.4	107 (77.5)	-2.4	**(.018)**
Vascular Invasion	38 (15.6)	-3.1	206 (84.4)	3.1	**9.58**	26 (11.2)	-3.0	207 (88.8)	3.0	**9.27**
No	66 (27.0)	3.1	178 (73.0)	-3.1	**(.002)**	48 (21.7)	3.0	221 (78.3)	-3.0	**(.002)**
Yes										

Perineural										
Invasion	76 (19.2)	-2.1	320 (80.8)	2.1	**4.39**	53 (14.3)	-2.2	317 (85.7)	2.2	**5.02**
No	26 (29.2)	2.1	63 (70.8)	-2.1	**(.036)**	20 (24.4)	2.2	62 (75.6)	-2.2	**(.025)**
Yes										
Tumour edge										
Infiltrative	54 (27.4)	3.2	143 (72.6)	-3.2	**10.251**	42 (23.1)	3.8	140 (76.9)	-3.8	**14.19**
Pushing	28 (14.3)	-3.2	168 (85.7)	3.2	**(.001)**	16 (8.7)	-3.8	168 (91.3)	3.8	**(0.000165)**
Peritumoral										
Lymphocytes	65 (23.2)	2.3	215 (76.8)	-2.3	**5.38**	46 (17.8)	2.1	213 (82.2)	-2.1	**4.51**
Inconspicuous	15 (12.9)	-2.3	101 (87.1)	2.3	**(.020)**	10 (9.1)	-2.1	100 (90.9)	2.1	**(.034)**
Conspicuous										
MMR Status										
Proficient	89 (25.8)	3.8	256 (74.2)	-3.8	**15.39**	63 (19.4)	2.9	262 (80.6)	-2.9	**10.88**
Deficient	13 (9.6)	-3.9	123 (90.4)	3.9	**(0.000455)**	10 (7.8)	-3.1	119 (92.2)	3.1	**(.004)**

Table 17. Association between Ki67 and clinicopathological variables in right-sided tumours.

The log rank tests results were χ^2 (1) 4.49 (*p* .034) and χ^2 (1) 5.38 (*p* .020) for the cut-offs 31% and 25% respectively.

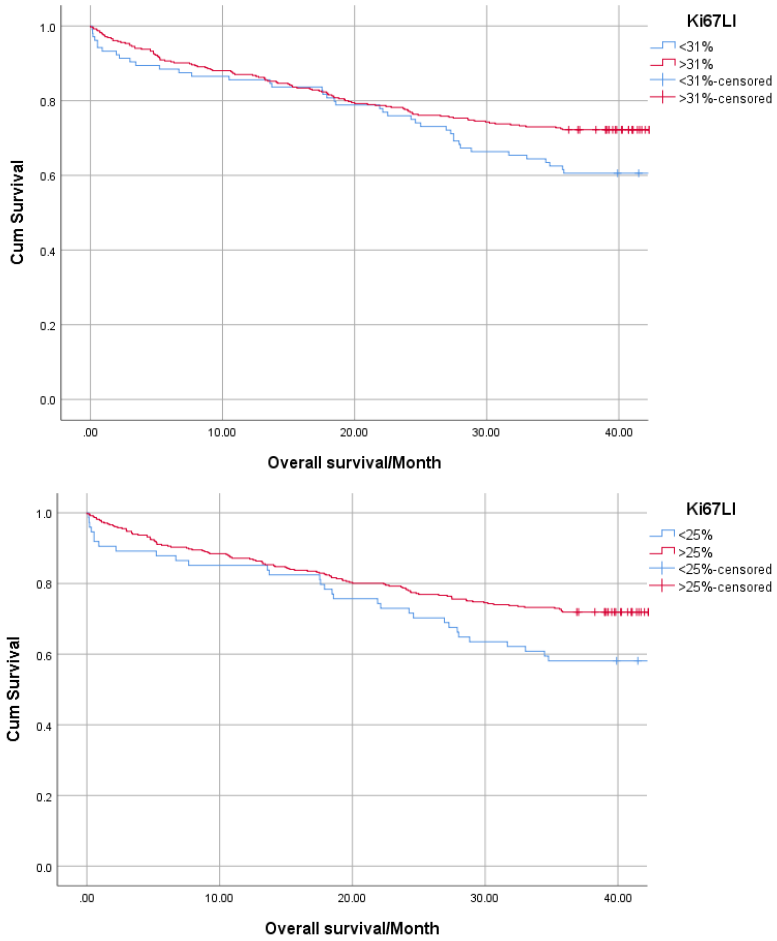


Figure 20. Association between survival and Ki67 in right-sided tumours.

3. Stratification for Stage

The prognostic value was tested for Ki67 in both stage II and III for both cut-offs (31% and 25%).

Clinicopathological Criteria	Stage II CRC					Stage III CRC				
	KI67LI					KI67LI				
	<31% (%)	AR	>31% (%)	AR	χ^2 (p-value)	<31% (%)	AR	>31% (%)	AR	χ^2 (p-value)
3Y Survival									2.4	
Alive	74 (23.4)	.9	242 (76.6)	-.9	**.812**	44 (19.6)	-2.4	181 (80.4)	-2.4	**5.69**
Dead	16 (18.8)	-.9	69 (81.2)	.9	**(.367)**	30 (31.9)	2.4	64 (68.1)		**(.017)**
5Y Survival										
Alive	61 (30.3)	3.8	140 (69.7)	-3.8	**14.66**	31 (24.8)	.5	94 (75.2)	-.5	**.296**
Dead	29 (14.4)	-3.8	172 (85.6)	3.8	**(0.000129)**	43 (22.2)	-.5	151 (77.8)	.5	**(.586)**

Table 18. Association between Ki67 (cut-off 31%) and clinicopathological variables at stage II and III.

Clinicopathological Criteria	Stage II CRC					Stage III CRC				
	KI67LI					KI67LI				
	<25% (%)	AR	>25% (%)	AR	χ^2 (p-value)	<25% (%)	AR	>25% (%)	AR	χ^2 (p-value)
3Y Survival									2.5	
Alive	53 (17.8)	.7	244 (82.2)	-.7	**.525**	26 (12.6)	-2.5	181 (87.4)	-2.5	**6.11**
Dead	12 (14.5)	-.7	71 (85.5)	.7	**(.469)**	21 (24.1)	2.5	64 (75.9)		**(.013)**
5Y Survival								95 (81.9)		
Alive	43 (23.0)	3.0	144 (77.0)	-3.0	**9.14**	21 (18.1)	.8	152 (85.4)	-.8	**.639**
Dead	22 (11.3)	-3.0	172 (88.7)	3.0	**(.003)**	26 (14.6)	-.8		.8	**(.424)**

Table 19. Association between Ki67 (cut-off 25%) and clinicopathological variables at stage II and III.

High KI67LI showed a significant association with worse 5y survival in stage II and better 3y survival in stage III. This opposite nature is valid when 31% and 25% were taken as cut off.

	STAGE II – 5YSURVIVAL		STAGE III – 3Y SURVIVAL	
KI67LI	**MEAN (95% CI)**	**LOG RANK TEST χ^2 (p value)**	**MEAN (95% CI)**	**LOG RANK TEST χ^2 (p value)**
<31%	85.70 (77.82 – 93.57)	**11.31 (.001)**	70.27 (59.43 – 81.10)	**6.74 (.009)**
>31%	69.92 (65.41 – 74.43)		87.24 (81.94 – 92.54)	
<25%	84.24 (74.80 – 93.68)	**6.84 (.009)**	65.69 (51.72 – 79.66)	**7.96 (.005)**
>25%	70.23 (65.72 – 74.73)		86.62 (81.29 – 91.95)	

Table 20. Means, 95%CI and Log rank tests for Ki67 (cut-offs 31% and 25%) in stage II and stage III tumours.

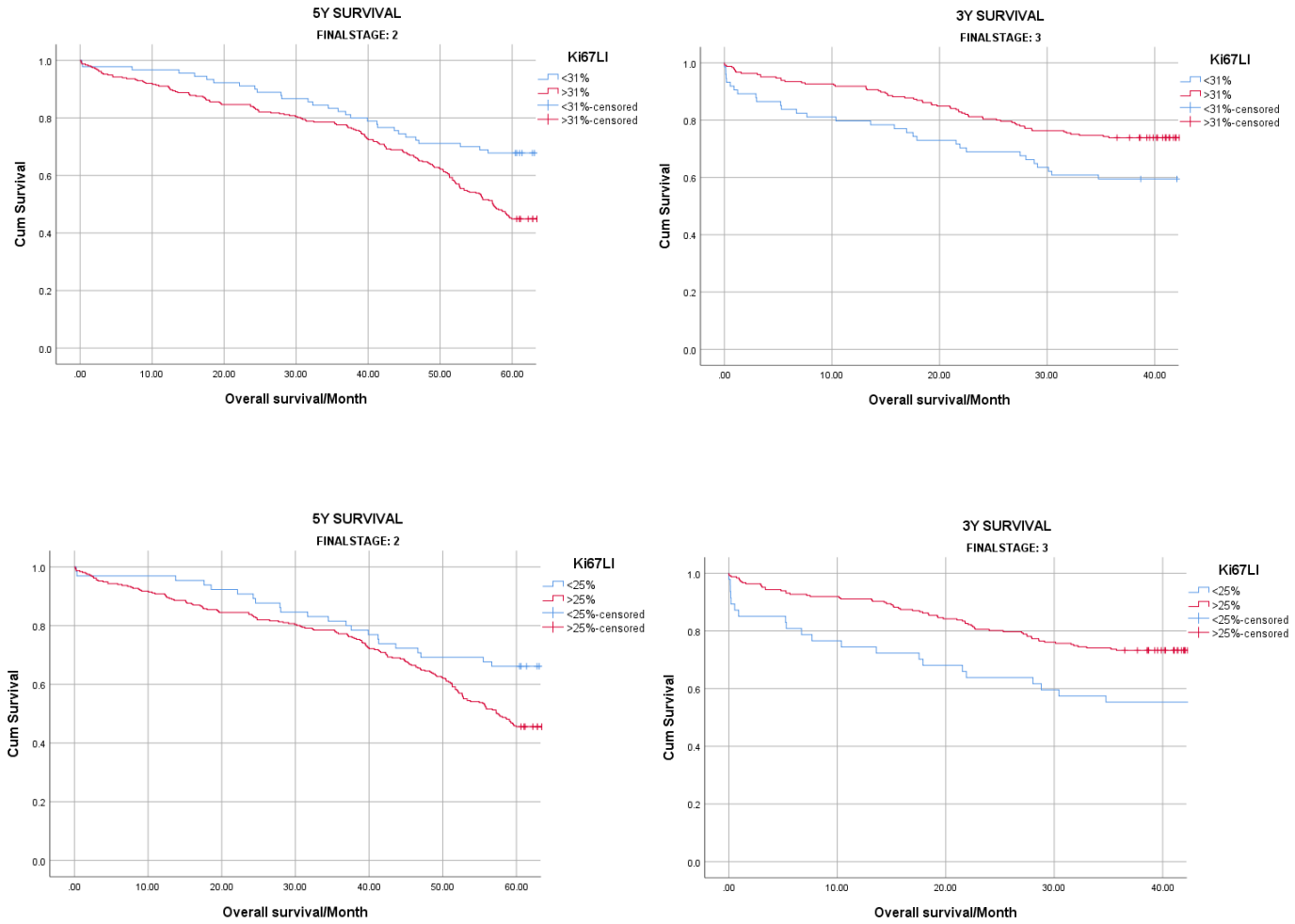


Figure 21. Association between 5 years and 3 years survival and Ki67 in stage II and stage III tumours.

5. Analysis of Stroma Percentage

974 cases were included in the analysis between the tumor stroma and the clinical features.

The stroma percentage showed a symmetric histogram (M=46.51, SD=17.21). In line with the literature, we chose 53% (which in our dataset matched the 2/3 of the distribution) as cut-off to separate the specimens in stroma-poor (n=643) and stroma-rich (n=331).

High stroma content was found significantly associated with worse 3year survival and overall survival (*p* .002 and .041 respectively). Rectal cancer (*p* .034) and poorly differentiated tumours (*p* .012) also correlated with a higher stromal content. Other features of advanced disease that correlated with a high stromal content were recurrence, T4, lymph node involvement, metastases, vascular invasion and perineural invasion. Finally, strong correlation was found with low proliferation rate and high nuclear expression of β-catenin (*p* 4.6176e-20 and .045 respectively).

Clinicopathological Criteria		Stroma				χ^2 (p-value)
		Low (%)	A.R.	High (%)	A.R.	
3Y Survival						
	Alive	490 (69.0)	3.2	220 (31.0)	-3.2	**10.06 (.002)**
	Dead	153 (58.2)	-3.2	110 (33.3)	3.2	
Overall Survival Status						
	Alive	416 (68.4)	2.0	192 (31.6)	-2.0	**4.17 (.041)**
	Dead	227 (62.0)	-2.0	139 (38.0)	2.0	
Site						
	Right colon	295 (66.6)	.3	148 (33.4)	-.3	
	Left Colon	244 (68.5)	1.3	112 (31.5)	-1.3	**8.65 (.034)**
	Rectum	82 (56.2)	-2.7	64 (43.8)	2.7	
	Transverse and Ascending Colon	22 (75.9)	1.1	7 (24.1)	-1.1	
T						
	1	59 (80.8)	2.8	14 (19.2)	-2.8	
	2	74 (70.5)	1.0	31 (29.5)	-1.0	**26.03 (0.000009)**
	3	356 (69.1)	2.2	159 (30.9)	-2.2	
	4	154 (54.8)	-4.7	127 (45.2)	4.7	
N						
	N0	388 (69.3)	2.9	172 (30.7)	-2.9	**11.75 (.003)**
	N1	148 (63.5)	-.7	85 (36.5)	.7	
	N2	85 (54.8)	-3.1	70 (45.2)	3.1	
Lymph node Involvement						
	Uninvolved	388 (69.3)	2.9	172 (30.7)	-2.9	**8.65 (.003)**
	Involved	233 (60.1)	-2.9	155 (39.9)	2.9	
Metastases						
	No	582 (67.7)	3.0	278 (32.3)	-3.0	**9.00 (.003)**
	Yes	61 (53.5)	-3.0	53 (46.5)	3.0	
Recurrence						
	No	472 68.0	2.1	222 32.0	-2.1	**4.28 (.038)**
	Yes	171 61.1	-2.1	109 38.9	2.1	
Vascular Invasion						
	No	354 (71.7)	3.9	140 (28.3)	-3.9	**15.56 (0.000080)**
	Yes	277 (59.6)	-3.9	188 (40.4)	3.9	
Grade						
	1	11 (61.1)	-.4	7 (38.9)	.4	**8.85 (.012)**
	2	588 (67.6)	2.9	282 (32.4)	-2.9	
	3	44 (51.8)	-2.9	41 (48.2)	2.9	
Perineural Invasion						
	No	521 (68.4)	3.6	241 (31.6)	-3.6	**12.93 (0.000323)**
	Yes	97 (54.2)	-3.6	82 (45.8)	3.6	
Tumour edge						
	Infiltrative	233 (60.8)	-4.6	150 (39.2)	4.6	**20.89 (0.000005)**
	Pushing	273 (76.5)	4.6	84 (23.5)	-4.6	
KI67LI						
	<25%	59 (37.8)	-9.2	97 (62.2)	9.2	**84.14 (4.6176E-20)**
	>25%	582 (75.1)	9.2	193 (24.9)	-9.2	
Nuclear β-catenin						
	Low	169 (71.0)	2.0	69 (29.0)	-2.0	**4.03 (.045)**
	High	337 (63.6)	-2.0	193 (36.4)	2.0	

Table 21. Association between stroma percentage and clinicopathological variables

The mean 3y survival was 96.46 (95%CI 92.93 – 99.99) for low stroma content and 82.49 (95%CI 77.37 – 87.61) for high stroma content (*p* .002).

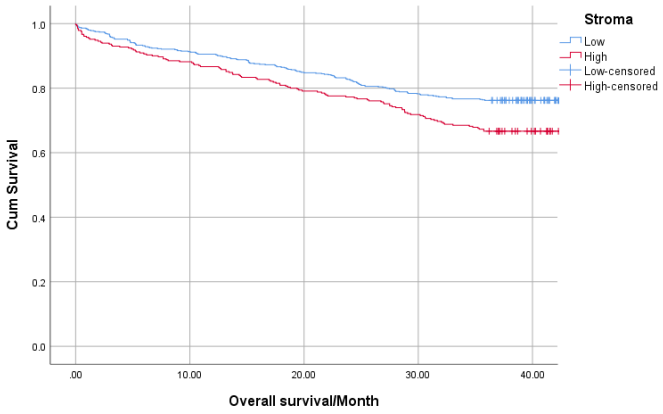


Figure 22. Association between survival and stroma percentage.

The tumour-stroma proportion showed significant prognostic value in the single-variable regression analysis (HR 1.48 95%CI 1.16 – 1.89; *p* .002) but not in the multivariable model (*p* .906) (data not shown).

6. β-catenin, Ki67 and Stroma Percentage Association

Finally, we looked at the association between the three biomarkers. We found a correlation between Ki67 and β-catenin (t(840)= -60.48, *p* 2.6337e-308) and between β-catenin and stroma percentage (t(856)= 61.08, *p* 0.0e0), but not between Ki67 and stroma (t(931)= -.022, *p* .983).

7. Analysis of intratumor heterogeneity

To investigate any intratumor heterogeneity, β-catenin and Ki67 were analysed in relation to three tumour portions: luminal side, middle portion and invasive front.

In regard to β-catenin, the cytoplasmic mean expression was 183.63 (SD 43.41), 186.46 (SD 41.82) and 186.27 (SD 45.23) in the luminal, middle and invasive portion of the tumour respectively, displaying an identical ubiquitous expression. In fact, the cytoplasmic β-catenin did not differ significantly (*p* .136).

The nuclear β-catenin mean values were 66.80 (SD 72.36), 67.83 (SD 72.41) and 74.05 (SD 74.264) in the luminal, middle and invasive portion of the tumour respectively. The Friedman test showed an

overall statistically significant difference between the related groups (χ^2(2) 28.10, *p* 7.8959e-7). The effect size was really small (Kendall's W .036). The pairwise comparison displayed a statistically significant difference between luminal and advancing edge expression (*p* .000004) and the middle and advancing edge expression (*p* .002) but not between the luminal and nuclear expression (*p* .418). Significance values have been adjusted by the Bonferroni correction for multiple tests.

The membranous β-catenin mean expression were 180.12 (SD 62.18), 173.78 (SD 62.59) and 167.93 (SD 63.91) in the luminal, middle and invasive portion of the tumour respectively. The repeated measure anova with a Greenhouse-Geisser correction showed that these differences are statistically significant (*p* .000002), but the magnitude of the variance was small (η_p^2=0.04) . Finally, the pairwise comparison showed a small but highly significant difference between each set of expression: on average, membranous β-catenin is 6.4 higher in the middle of the tumour than the luminal side (p value 0.002), 5.8 lower in the middle than the advancing edge (*p* .027) and 12.2 higher in the advancing edge than the luminal side (*p* .000012).

When Ki67 expression was analysed among the luminal, middle and advancing edge portions of the tumour, a significant difference was found among the three compartments (*p* .001) (Mauchly's Test indicated that sphericity could not be assumed (χ^2 (2) 8.24, *p* .016 so a Greenhouse-Geisser correction was used). The mean values for luminal, central and invasive sides are, respectively, 47.61 (SD 21.97), 45.18 (SD 22.24) and 44.21 (SD 21.67). The difference was significant between middle and luminal side (*p* .027) and invasive and luminal side (p value .002) but not between middle and invasive front (*p* .864): on average Ki67 was found 2.43 higher in luminal than middle portion and 3.40 higher in luminal than invasive front. However, the difference was not relevant (η_p^2=1).

DISCUSSION

Keeping in mind the dual role of β-catenin, the implication of the proliferation rate in cancer and the recently discovered prognostic value of tumour stroma, the aim of this study was to investigate a potential association of these biomarkers with clinicopathological features and further explore their clinical application.

In the tumoral specimens, β-catenin was found localised at the membrane, cytoplasm and in the nucleus, whereas normal epithelium was mostly characterized by an intense membranous stain and showed a low cytoplasmic and approximately null nuclear expression. This is in line with previous works, as many have reported a shift of β-catenin from the membrane to the nucleus during cancer development: it's known that in early stages of carcinogenesis, the loss of APC ultimately causes β-catenin nuclear translocation. Conversely, in normal intestinal tissue, β-catenin is mostly located at the membrane to guarantee the integrity of the epithelium [23]. Normal tissue also showed a lower proliferation rate compared to tumoral tissue, suggesting the association of KI67 with neoplastic progression described in the literature[137].

We conducted analyses to investigate the correlation of β-catenin, Ki67 and stroma percentage with multiple clinicopathological parameters.

A constant association was found between β-catenin and tumour location. The molecular mechanisms behind cancer development in proximal and distal colon are different and subsequently their phenotypes differ as well. Tumours arising from right colon commonly harbour mutations in the mismatch repair system, whereas the classical chromosomal instability traits are observed in the left-sided CRCs (APC, KRAS, PIK3CA and p53 mutations)[23]. The association between β-catenin and aneuploidy has been reported in previous works, as well as the inverse correlation between the nuclear localization and MSI tumours[138]. In our study, nuclear, cytoplasmic and overall β-catenin were found highly expressed in distal CRC and rectal cancer and pMMR. In confirmation of the strong association between CIN/left-sided CRCs/β-catenin, from the analyses stratified for MMR status, dMMR tumours didn't show any significant association with β-catenin.

Membranous expression was more intense in right-sided CRCs: this result is difficult to interpret. Right-sided CRCs are often dMMR and have better prognosis, therefore one would expect a high membranous stain. However, in our cohort proximal tumours had worse survival. A reason that might explain this inconsistency is that right-sided tumours develop with a flat morphology, thus they're more difficult to detect: this makes proximal tumours normally diagnosed at advanced stages and more likely to have a bad prognosis[23]. The stage is an additional aspect to take into account: it appears the MSI status has a positive prognostic impact on stage II patients.[139] The literature lacks results about the membranous subcellular localization and its relationship with tumour site. It is known that membranous localization of β-catenin is secondary to cell-cell interactions led by E-cadherin, but several attempts failed to find an association between the levels of E-cadherin and tumour location[140]. Investigating the Wnt pathway in serrated adenomas, Li et al. found that membranous expression was

higher in sessile serrated adenomas (generally right-sided) than in traditional adenomas (generally left-sided)[141]. We don't know if the high membranous expression in proximal CRCs is secondary to the state of E-cadherin or due to specific genetic pathways occurring in right colon or it simply antagonizes the alter-ego nuclear β-catenin in left CRCs.

Among the other clinical features available in this study, β-catenin was found strongly associated with a moderate grade of differentiation, independently from the subcellular localization. In previous works, a higher nuclear β-catenin was observed in poor differentiated tumours, but a significant association was found in some studies[142] and not in others[143]. However, it must be said that our result might be biased by the population composition: over 85% of CRCs were G2 and only 9.5% were G3.

Nuclear β-catenin correlated with absence of perineural invasion and the cytoplasmic expression with smaller pT (T2 and T3). These inverse associations with advanced disease features reveal a protective effect. However, most studies failed to find an association with perineural invasion in CRC and other cancer types, nor (thinking about a destabilization of β-catenin at the membrane) an association between perineural invasion and E-cadherin[144] [145] [146].

Although we haven't found any considerable heterogeneity between the central and the peripheral areas of the tumour, we still carried out an analysis to look at the 'absolute' role of nuclear β-catenin at the peripheral side of the tumour (no expression vs nuclear expression). This in consideration of previous works, that showed an increased expression of nuclear β-catenin at the invasive front compared to the central portion of the tumour.[147] The advancing edge revealed significant correlations between positive nuclear β-catenin and infiltrative tumour edge and vascular invasion. Although discordant with β-catenin protective role previously described, these associations may not be random, but rather indicative of a "functional heterogeneity".

CD44 is a target gene of Wnt/ β-catenin pathway and a common surface marker used to identify CSC. It is involved in many signalling pathways, such as cell-to-ECM adhesion and cell motility, tumour growth, angiogenesis and inflammation [148]. Specifically, the isoforms CD44v can stimulate cytoskeletal changes and interact with VEGFR, contributing to angiogenesis and EMT, so much so that the switch between CD44s to VD44v are found in metastasised tumours and may contribute to angiogenesis and EMT.[149] Moreover, the combined low expression of E-cadherin and CD44 was found associated with decreased PFS and OS[150].

The presence of β-catenin in the nuclei of the cells at the advancing edge was also correlated with infiltrative tumour edge. The infiltrative tumour edge is generally more aggressive and correlates with worse prognosis, often in BRAF-mutated CRCs; conversely, the pushing edge frequently characterizes dMMR tumours, especially in Lynch Syndrome cases[151] . In our cohort dMMR CRCs strongly correlated with pushing tumour border. This association is particularly important in the molecular testing decision[152]: when a proximal tumour is characterized by a pushing margin, in addition with other clinical features such as mucinous pattern and intraepithelial lymphocytes, most likely will show MMR deficiency.

Ki67 consistently showed association with pushing edge and dMMR tumours (but not tumour location). Previous research established a correlation between MSI-H tumours and high proliferation

rate[153]. Pushing edge has been extensively associated with peritumoral inflammatory infiltrates[154], and we can confirm this feature as Ki67 also correlated with conspicuous peritumoral lymphocytes. Together, the inflammatory infiltrates[155], which relate to an efficient host response, the pushing tumour border[156] and MMR-deficiency shape a better prognostic scenario for CRC patients. It remains to be seen if a high proliferation rate can be an independent prognostic factor or just a confounding factor, as so far it has collectively shown controversial results.

Besides conspicuous peritumoral lymphocytes, pushing tumour edge and dMMR, Ki67 didn't show many other significant associations. In dMMR patients, low proliferation rate strongly correlated with well differentiated CRC, but this correlation loses significance when it's contextualized: the well, moderate and poor differentiated patients were n= 1, 15 and 6 respectively.

The stroma content has demonstrated to be a valid biomarker, showing significant association with several features characterising an advanced disease. A high stromal content correlated with T4, lymph node involvement, metastases, poor differentiation, recurrent disease, perineural invasion, vascular invasion and infiltrative margin. These results are in line with previous works, that reported the tumour-stroma ratio as a strong and independent prognostic factor.[157 158]

It's worth remembering that our stroma content comprised stromal and non-stromal cells. A recent work, looking more in detail at the stroma composition, determined that a high presence of CAFs is predictive of tumour recurrence[159]. Another study on CAFs computed an immune-histochemical score, based on two proteins expressed by the fibroblasts, that could predict the response to neoadjuvant therapy in rectal cancer.[160]

We also observed a specific correlation between stroma percentage and rectal cancer. Other studies did not include rectal cancer in their analysis for various reason.[161] 15% of our population was diagnosed with rectal cancer; additionally, most of these patients are frequently treated with neoadjuvant therapy but we don't have any data about treatment. If this was the case for our cohort, prior treatment could explain this association, as neoadjuvant chemotherapy has been found to induce mesenchymal phenotype in residual tumour cells[162].

We further looked at the association between the three biomarkers. We found a correlation between Ki67 and β-catenin and between β-catenin and stroma percentage like others did[163], but not between stroma and Ki67. However, stroma-rich tumours cells showed low KI67 and high nuclear β-catenin. A recent study explored the relationship between the tumour-stroma ratio with the intrinsic subtypes and revealed that stroma-rich feature is strongly associated with stem-like subtype[164]. A low proliferative state with high nuclear β-catenin is evocative of a stem-like phenotype.

Moving to the prognostic analysis, in our population, tumour local stage, lymph node involvement, metastases, tumour grade, tumour location, but not MMR status, were strong independent factors associated with survival.

Our initial survival assessment of "overall β catenin" failed to prove a prognostic value of the cellular expression of the biomarker.

Nuclear β-catenin showed only a marginal association with survival, whereas cytoplasmic expression was significantly correlated. The Kaplan Meier curves showed a better outcome with higher cytoplasmic and nuclear β-catenin. As for membranous expression, patients with high β-catenin significantly correlated with better survival, compared to patients with low-stain intensity; however, the Kaplan Meier curve was inconclusive. Lastly, membranous and nuclear sub-localizations were found to be an independent good prognostic factor in the Cox regression. These results reiterate a protective role of high β-catenin.

The literature is quite controversial about the prognostic value of nuclear β-catenin. Some studies showed no correlation between nuclear localization and survival[165] . At the same time, a few others reported good prognostic value[166] and marked it as predictor of better DSS and DFS. [167] Phospho-β-catenin (a subset of nuclear β-catenin) was found associated with an improved survival and to be an independent predictive factor of overall survival[168]. On the other hand, many others reported bad prognostic value[169 170 147]. With stage stratification, a study found that the protein accumulation in the nucleus (and concurrent loss of membranous localization) only correlated with bad prognosis in Dukes' stage B patients. However, the same research revealed that nuclear β-catenin is also associated with a greater risk of metastases and that metastatic disease tends to show loss of membranous β-catenin and E-cadherin [171]. When looked at different tumour portions, nuclear β-catenin, as well as its co-expression with nuclear MUC1 (Mucin 1, Cell Surface Associated), correlated with worse survival at the invasion front, even though only MUC1 (and not nuclear β-catenin) was confirmed as independent prognostic factor [172] .

The reasons for such inconsistency may be multiple; intrinsic tumour heterogeneity, heterogenous population (stage, age and postoperative treatment), lack of standardization in IHC protocols (different source and dilution of primary antibodies), scoring methods (intra- and interobserver variability and automated vs manual scoring), variation in statistical methods (different cut-offs and number of patients) probably contribute to the contradictory prognostic significance of nuclear β-catenin.

Ki67 also presents discordant results about prognosis. Melling et al. analysed Ki67LI on TMA comprising 1800 CRCs[163]: high KI67 expression was found associated with low tumour stage and nodal status, but not tumour grade or tumour site. They concluded that Ki67 was an independent prognostic factor of favourable outcome. High expression was found associated with survival improvement also in rectal cancer, independently of histopathological tumour grade[173]. When the invasive margin of the tumour was assessed, low proliferation rate showed significantly poorer prognosis[174]. On the other hand, other studies have found ki67 expression correlating with worse outcome[175] , including a meta-analysis: Luo et al. investigated 34 studies and a total of 6180 CRCs patients and concluded that high Ki67 is predictive of poor OS and DFS [176]. A study using the 25% as cut-off reported Ki67 as an indiependent risk factor for poor prognosis[177]. Finally, a high proliferative activity seems to favour the adjuvant chemotherapy sensitivity and therefore in these patients, ki67 was considered a marker of good prognosis [178 179].

To date, there is no explanation for such disagreement. Duchrow et al. highlighted a discrepancy between the expression of Ki67 mRNA and Ki67 protein in CRC: cells with low mRNA and high protein

level seem to proliferate more slowly. Approximately 30% of their cohort showed this pattern and these patients had a better prognosis[180].

We analysed different scenarios, performing the same tests with different cut-offs. Ki67LI >31% was significantly correlated with worse survival and showed an independent prognostic value, but Kaplan Meier plot was not conclusive. KI67>25% was also associated with survival but the Log rank test and the Cox regression didn't confirm its prognostic effect. KI>20% showed significant association with 5y survival and significant independent prognostic value, but not a significant log rank test. Based on these results we can't confirm Ki67 as valid prognostic factor.

However, among the dMMR CRCs Ki67 showed strong associations with survival. With both 31% and 25% as cut-offs, high KI67LI was predictive of good prognosis. The reason for the protective role of KI67 in regard to survival may be due to the balance between apoptosis and proliferation rate, as they have been found linked by a positive linear correlation[153]: MSI-H tumours have high proliferation rate and high apoptosis rate, which would confer the advantage in survival. Conversely, in the left-sided CRCs, mostly MMR proficient, patients with high Ki67 showed worse survival and patients with high cytoplasmic β-catenin showed better survival.

We conducted further analyses to explore a potential prognostic value of the two biomarkers in stage II and stage III CRCs. It is not certain whether stage II CRCs will inevitably progress to stage III CRCs and thus sub-group analysis by stage is worth considering.

In stage III, nuclear β-catenin showed a strong significant association with better outcome. Patients with high nuclear expression had a mean survival of 68.4 months, whereas patients with low nuclear expression only 50.2 months. β-catenin was also found to be an independent prognostic factor. These results reinforce the protective trend seen with the clinical parameters and validate nuclear β-catenin as good prognostic factor for stage III patients.

As for ki67, the analyses showed an association between high expression and worse survival in stage II patients and low expression and worse survival in stage III patients. The reasons for these unexecped results may lay in the role of the low proliferation status at the invasion front of the tumour and the fact that our samples didn't show any heterogeneity in regards to proliferation rate. In stage II, a high proliferation rate might imply that the tumour is growing fast and therefore Ki67 would acquire the negative connotation of progression marker. On the contrary, stage III CRCs may lower their proliferation status so that dedifferentiation and subsequent EMT can occurr and in this picture a high Ki67 means better outcome. This interpretation would also be consistent with a mutual exclusive function of proliferation and stemness, with ki67 and β-catenin being the respective master regulators. Indeed, the two biomarkers were found correlated in our analysis, and there are other related markers that can be explored, alone and in combination with Ki67/β-catenin, to prove this hypothesis.

The stromal compartment of the tumour is linked to important predictive information about outcome, as demonstrated in different types of cancer, including breast[181], oesophageal [182], cervical [183] and NSCLC[184] . Several studies have investigated the prognostic role of the stroma content in CRC [185 186 187]: a high percentage was predictive of poor outcome. A sub-analysis of the VICTOR trial found that it can be considered an independent prognostic factor[157]. They also showed that the assessment

of stroma percentage to identify high-risk stage II patients, who may benefit from adjuvant chemotherapy, improved the current ASCO guidelines at stratifying the trial cohort.

In line with the literature, our results showed a convincing association between tumor-stroma percentage and survival: a high stromal content was found correlated with worse outcome and the mean 3y survival were 96 and 82 months for stroma-poor and stroma-rich CRC, respectively. It also showed prognostic significance as a single variable, but not in the multivariable model.

Our results did not show a consistent difference in proliferation rate between luminal, central and peripheral areas, nor a substantial differential expression of β-catenin: the effect sizes of these correlation were all small.

Whilst the contribution of β-catenin to tumorigenesis is quite clear, its role in tumour progression is more complicated. The differential intracellular expression of the protein is suggestive of EMT, a major aspect during metastasis. Among the genes that are downregulated during this process there is E-cadherin, which partners with β-catenin to stabilize adherens junctions. The loss of E-cadherin at the invasive margin has been investigated in several studies, and it has been reported correlated to invasiveness and prognostic significance[188]. Simultaneously, as the loss of E-cadherin destabilises β-catenin, a decrease of membranous β-catenin and increase of nuclear localization occurs [189]. We observed the same pattern: the membranous stain progressively weakened from luminal to peripheral section while the nuclear stain became more intense. This phenomenon is known as "β-catenin paradox" and highlights an additional level of regulation of Wnt operated by extrinsic cues. Indeed, factors like hepatocyte growth factor, secreted by the myofibroblasts surrounding the tumour, can activate the stem-like phenotype through inhibition of GSK3b.[190] Brabletz et al. described the magnitude of the tumour microenvironment in the invasiveness process. [191]

The main extracellular pathways involved in the EMT process are TGF-β and Wnt/ β-catenin pathways[192]. They are also well known to be part of the fine regulation that characterises the intestinal crypt homeostasis, where multiple pathways work together in a gradient expression. For example, Wnt is most predominant at the base and decreases up the crypt-villus axis, while BMP (a group of extracellular signalling molecules belonging to the TGF-β family) is most active at top: this is consistent with their function, as Wnt stimulate stemness and self-renewal while BMP mediates differentiation.[93] The balance between proliferation and differentiation is regulated by these pathways and many others and multiple extracellular inputs, provided by the neighborough cells and microenvironment. The intestinal crypt is a fantastic model to highlight the communication between the cells and microenvironment.

It has been demonstrated that Wnt/β catenin and TGF-β signalling are involved in the dedifferentiation process of EMT at the peripheral side of t the tumour [193] [194]. The idea of a dedifferentiation program, that would allow a shift first to a stemness phenotype and then to a mesenchymal phenotype in invading tumor cells is quite compelling. It has been shown that the induction of EMT in immortalized human mammary epithelial cells stimulated the expression of stem cell marker[195] . Similar studies in pancreatic cancer cells reported that ZEB1 (transcription factor that regulates EMT) induces stem cell properties and tumorigenic capacity.[196] Twist, an EMT activator, was also found to promote stemness. [197]

Alongside stemness, there is another process that is being investigated in relation to metastasis: senescence. Senescence is a condition of inactive cell cycle in proliferative-competent cells. Through

this process, the tumour seems to recruit stem-like cells to promote epithelial-mesenchymal transition.[198] The main marker of senescence is p16INK4A, a cyclin-dependent kinase that prevent the progression of G1 into S phase. [199] At the invasive front of the tumour, deletion of p16INK4A gene or hypermethylation of its promoter inactivates p16INK4A and restore cell cycle, balancing the cancer at a proliferation or differentiation status. Indeed, the hypermethylation of p16INK4A was found associated with markers of advanced disease[200 201].

When the expression of nuclear β catenin, cycling D1 and p16INK4A was assessed at the invasion front of CRC, some surprising results were obtained.[202] B-catenin strongly correlated with cyclin D1 expression, confirming that the second is a target gene of the first, and they were both overexpressed at the invasion front. However, the same area didn't show high proliferation rate, but rather low Ki67. Cyclin D1 is known to induce proliferation and yet, it was found in not-proliferating cell at the invasion front. Likewise, the cell-cycle inhibitor p16INK4A was observed highly expressed in the nuclei of the cells at the invasion front. Another study revealed that p16INK4A is itself a β catenin/TCF4 target gene[203]: the low proliferation rate of the cells that undergo EMT at the invasion front would be therefore mediated by cell proliferation promoting forces (c-MYC, Cyclin D1) and cell proliferation inhibiting forces (p16INK4A).

In conclusion, our analysis of heterogeneity, even if timidly, confirmed the background for the events occurring at the advancing edge. The mechanisms just mentioned are by no means exhaustive: stemness, proliferation, senescence, dormancy, quiescence and cell cycle re-entry are all cancer cell states described in tumours that lead to different outcomes.[204] The invasion front as one entity, comprising tumour and microenvironment, is a topic that is just starting to be unravelled.

Collectively, our results showed that β-catenin and Ki67 were analysed in the whole cohort have poor prognostic power, which however emerged when subgroups analyses were performed. This alone explains the high degree of complexity of CRC and the different approach that patients need according to their specific stage and disease. On the other hand, the stroma content was found to provide valuable information and confirmed to be a powerful prognostic marker.

Our study comes with several limitations. First, the scoring process, both manual and automated, was performed by one person and although the accuracy was assured by constant supervision and a good kappa value, a second scorer would have added more precision to the data. Some cores were missing, which subtracts power to the analysis conducted with the means: some cases had three tumoral cores which clearly makes the mean more trustworthy than a case with only one valid core. The classifiers built for Ki67 analyses were not perfect: as reported in the images in the methods section, some degree of inaccuracy was present, especially in the detection of normal epithelium. To counteract this issue, a second classifier was built specifically for the "normal tissue" cores and more attention was lavished in the final quality check before exporting the data from Qu Path (even though a one-by-one core check wasn't performed). Finally, the biggest obstacle in the statistical analysis was the choice of the right cut-off for the biomarkers dichotomization. A balance between biology, literature and actual data distribution guided the choice, and when in doubt, results from more cut-offs have been reported. Corrections as adjusted residual and effect size were used to add accuracy to the results.

CONCLUSION

The purpose of this study was to explore the relationship between β-catenin, Ki67 and stroma percentage in colorectal cancer.

Since Vogelstein and colleagues listed the key driver mutations for CRC carcinogenesis, research has made incredible progress in understanding the molecular mechanism of cancer. The activation of Wnt/β-catenin pathway is a pillar for CRC pathogenesis, but the dominance of APC mutations over other Wnt activating alterations and the "just-right" level of Wnt activation to drive tumorigenesis are just two examples of the extreme complicated mechanisms behind CRC development. Moreover, the dual role of β-catenin makes it a key factor in both carcinogenesis and cancer progression. Likewise, since its discovery in 1983, Ki67 has been used as marker of proliferation that could define tumours more or less aggressive. Nowadays, it is also investigated as predictive and prognostic factor in many types of cancer, but so far it has shown considerable contradictions for both roles. On the other hand, there is a good level of agreement on the significance of the tumour stroma: it is involved in tumour development and has shown to predict poor outcome in solid tumours, including colorectal cancer.

We observed that β-catenin changes sub-cellular localization in CRC, compared to normal intestinal epithelium. Nuclear and cytoplasmic expression are associated with distal CRCs and MMR proficiency, in line with the CIN pathway that characterises these tumours. Ki67 is consistently associated with conspicuous peritumoral lymphocytes and pushing tumour edge, histological features typical of dMMR, with which Ki67 was also strongly correlated. We found that stroma-rich tumours display advanced T stage, lymph nodes involvement and metastasis; they're also associated with recurrence, vascular and perineural invasion and characterized by poor differentiation and infiltrative tumour edge. We found that nuclear β-catenin is an independent prognostic factor and predictive of good prognosis in stage III patients. This protective role seems to be shared with membranous and cytoplasmic β-catenin, even though given the inconstant results, more research is needed to validate them as valid prognostic factor for CRC. Ki67 demonstrated its value in two subgroups, dMMR and stage III CRCs: in both populations, high Ki67 is associated with better outcome. However, in stage II patients it correlated with shorter survival. Moreover, a cut-off of 25% for Ki67 in CRC seems to perform well and to provide valid clinical data. Finally, tumours with high stroma content were associated with bad prognosis.

Clinical practice is in need of prognostic tools that can help decide the optimal treatment for patients. The Dukes' staging system has been used for decades and TNM, supported by specific histological features, still is superior in this context. However, in the past few decades, given the high degree of heterogeneity of CRC, molecular markers have shown a great potential. Some markers are already in use for patient stratification and management, others clearly need optimization. We tried to shed some light on the controversial picture present in the literature. The stroma content is so far the only biomarker that showed consistency in our data and in previous investigations. Further research appears necessary to establish the prognostic role of β-catenin and Ki67. Standardization of methods and multiple stratification might be really useful given their differential expression and the complexity of the disease. Moreover, the combination with other markers with similar biological functions is an analysis worth to pursue. In this regard, a bioinformatic approach to investigate

pathogenic and prognostic differentially expressed genes (patheway enrichment analyses) would robustly contribute to our knowledge of the biology of carcinogenesis and caner progression.

There are several precision treatment available for cancer patients, who however are not always responsive, because tumour heterogeneity wasn't taken into account when they were developed. Future reasearch should focus on filling this gap with new technlogy, such as single-cell transcriptomics, that can study the expression levels of individual cancer cells in a single tumour.

Finally, the characterization of cancer stem-cell (CSC) is another hot topic: the role of CSC in tumour development and metastases is being deeply explored but not without obstacles. The lack of cancer stem-cell models and specific biomarkers to identify this population are a major issue. Further knowledge on cell plasticity can definitely help detangle the complex balance between stemness, proliferation and dormancy that cause therapy resistance and cancer progression.

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