Expression of Colorectal Cancer Metabolic and Angiogenic Markers:
Association with Clinicopathological Characteristics and Impact on Prognosis

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Tese de Doutoramento em Medicina

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Expressão de Marcadores de Metabolismo e de Angiogense no Cancro Colorectal: Associação com Caracteristicas Clinico-patológicas e Impacto no Prognóstico.

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ABSTRACT

Colorectal cancer (CRC) is one of the most common cancers and a leading cause of cancer death worldwide. Several features are common to all cancers, but particularly two aroused of interest to us, namely the capacity of tumour cells to reprogram their energy metabolism and inducing angiogenesis. Therefore, the objective of this study was to understand the role of metabolic and angiogenic markers in CRC, by studying their expression and establish possible correlations with clinicopathological data. To achieve these goals we created a prospective database of CRC patients treated at Braga Hospital in the period 2005-2010, with clinical, pathological and follow-up data. From surgical specimens of CRC patients submitted to surgical treatment, Tissue Microarrays were constructed for subsequent immunohistochemical evaluation.

The metabolic markers selected were the *Monocarboxylate Transporter* (MCTs), particularly MCT1 and MCT4 essential for lactate transport across the plasma membrane, so contributing for intracellular homeostasia. To better characterize the role of MCTs in CRC metabolism we also evaluated the expression of the chaperones CD147 and CD44 and the glycolytic marker GLUT1. The angiogenic markers selected were members of *Vascular Endothelial Growth Factor* (VEGF) family: VEGF-A, VEGF-C and the receptors VEGFR-2 and VEGFR-3 with functions of angiogenesis and lymphangiogenesis. The expression of metabolic markers on CRC Hepatic metastasis was also evaluated in order to assess whether the metabolic profile of CRC was maintained by the metastatic cells. In CRC series and CRC Hepatic metastasis series, the correlation with clinicopathological data and survival curves were evaluated to assess their potential as prognostic biomarkers.

The epidemiological results allowed a better knowledge of our population, since CRC epidemiological data are scarce in Portugal. Our results, consistent to that observed in the literature, clearly demonstrated that CRC is a major problem of public health and that our population can be considered a high-risk population for CRC development.

We have demonstrated that the metabolic markers are overexpressed in human CRC samples, when compared with normal adjacent tissues and the same expression pattern was observed in CRC Hepatic Metastasis. Also, analysis of the association between expression of the MCT isoforms and chaperones and GLUT1 in CRC and CRC Hepatic Metastasis, demonstrated that tumour MCT1 positive cases were associated with CD147 plasma membrane expression and

between MCT4 and CD147, CD44 and GLUT1. Also, CRC Hepatic Metastasis holds the same metabolic profile alterations documented in CRC tissues for MCT4 positive cases. Thus, we can conclude that these metabolic markers contribute to the malignant phenotype of CRC and this phenotype persists in Hepatic Metastasis. Overexpression of these markers in CRC, compared to normal adjacent cells, places them as potential therapeutic targets in CRC and especially in metastatic CRC as most of these proteins were not expressed on normal adjacent tissue. When analyzing correlations of these markers with epidemiological data we documented associations with parameters that reflect a worse prognosis, reflecting the metabolic advantage that these tumour cells have acquired, documented by the survival curves of MCT1 and MCT4 with stage IV and stage III, respectively, for colon cancer.

Assessing the expression of angiogenic markers in CRC series, we observed that all molecules were overexpressed, reflecting their role in tumour development and progression. When we compared CRC tissue and normal adjacent tissue we observed a statistically significant correlation for VEGF-C and a tendency for correlation with VEGFR-2, so contributing for tumour grow and tumour metastization. Expression of these markers in normal adjacent cells was less pronounced for VEGFR-3 than the remaining proteins, making VEGFR-3 an attractive therapeutic target since the lower expression in normal tissues will be associated to fewer side effects. When we evaluated the correlation of these markers with epidemiological data, we found correlations with tumour characteristics that contribute to progression, invasion, metastasis and poorer prognosis, documented by the overall-survival curves of VEGF-C and VEGFR-3 with stage III and stage IV for rectal cancer.

In conclusion, the results observed in this thesis, in addition to documenting the metabolic and angiogenic gain of CRC cells compared to normal adjacent cells thereby contributing to proliferative advantage and metastization capacity, also document that the presence of this metabolic and angiogenic markers are associated with tumour characteristics that reflects a worse prognosis and so worse patient survival. Altogether, these findings support their role as biomarkers and potential therapeutic targets in CRC and metastatic CRC.

RESUMO

O Cancro Colorectal (CCR) é um dos tumores mais frequentes, assim, como uma das principais causas de morte por doença neoplásica, a nível mundial. Várias características são comuns a todos os cancros, mas duas particularmente despertaram o nosso interesse, nomeadamente a capacidade de reprogramação do metabolismo celular e a de angiogénese. Assim, o objetivo deste estudo foi compreender o papel dos marcadores do metabolismo e de angiogénese no CCR, e, estabelecer possíveis correlações com dados clinico-patológicos. De forma a alcançar estes objetivos foi construída uma base de dados prospetiva, de doentes tratados por CCR, no Hospital de Braga, no período de 2005-2010, onde foram reunidos dados clínicos, anatomopatológicos e de follow-up. A partir dos blocos das peças cirúrgicas dos doentes operados, foram realizados "Tissue Microarrays" para posterior avaliação imunohistoquímica.

Os marcadores de metabolismo selecionados foram os *Transportadores de Monocarboxilatos* (MCTs), nomeadamente MCT1 e MCT4, essenciais para o transporte de lactato através da membrana plasmática, contribuindo para a homeostasia intracelular. De forma a melhor caracterizar o papel dos MCTs no metabolismo do CCR também foram avaliados os chaperones CD147 e CD44 e o marcador glicolítico GLUT1. Os marcadores de angiogénese selecionados foram membros da família do *Fator de Crescimento Vascular Endotelial* (VEGF): VEGF-A, VEGF-C e os recetores, VEGFR-2 e VEGFR-3, com funções conhecidas em termos de angiogénese e linfangiogénese. No caso dos marcadores do metabolismo, foram também avaliadas as expressões destes marcadores numa série de Metástases Hepáticas de CRC, com o objetivo de avaliar se o perfil metabólico observado no CCR se mantinha nas respetivas metástases. Em ambas as séries, foram avaliadas as correlações destes marcadores com dados anatomopatológicos e as curvas de sobrevida, de forma a avaliar o seu potencial como marcadores biológicos.

Os resultados epidemiológicos contribuíram para um melhor conhecimento da nossa população, uma vez que estes dados são escassos em Portugal. Os resultados obtidos, concordantes com os observados na literatura, demonstraram que o CCR é um problema importante de saúde pública e que a nossa população pode ser considerada uma população de alto-risco para o seu desenvolvimento.

Demonstramos que os marcadores metabólicos analisados estão sobre-expressos nas amostras do CCR comparativamente com o tecido normal adjacente e que o mesmo padrão de

expressão foi observado nas Metástases Hepáticas de CCR. A análise da correlação da expressão das isoformas dos MCT com os chaperones e o GLUT1, na série de CCR, demonstrou que o MCT1 estava associado à expressão plasmática do CD147 e o MCT4 à expressão plasmática do CD147, CD44 e GLUT1. Na série de Metástases Hepáticas de CCR o mesmo perfil metabólico foi observado para o MCT4. Desta forma podemos concluir que estes marcadores de metabolismo contribuem para o fenótipo maligno do CCR e que este se mantem nas metástases hepáticas. A sobreexpressão destes marcadores no CCR comparativamente com o tecido normal adjacente coloca-os como potenciais alvos terapêuticos no tratamento do CCR em especial no CCR metastizado uma vez que estes marcadores não se encontram expressos no tecido normal adjacente. Ao analisarmos as correlações destes marcadores com os dados epidemiológicos documentamos a associação com características que revelam um pior prognóstico, refletindo a vantagem metabólica que estas células tumorais adquiriram, comprovada pelas curvas de sobrevida do MCT1 e MCT4 para o estadio IV e III, respetivamente, para o cancro do cólon.

Avaliando a expressão dos marcadores de angiogénese, na série de CCR, observamos que todos estão sobre-expressos o que reflete o seu papel no desenvolvimento e progressão tumoral. Quando comparamos o tecido tumoral com o tecido normal adjacente observamos uma correlação para o VEGF-C e uma tendência para a correlação com o VEGFR-2, desta forma contribuindo para o crescimento e para a metastização tumoral. A expressão destes marcadores no tecido normal adjacente foi menos pronunciada para o VEGFR-3, tornando-o um alvo terapêutico atrativo, uma vez que esta menor expressão estará associada a menores efeitos secundários. Quando avaliamos a correlação com os dados epidemiológicos, encontramos correlações com características tumorais que contribuem para a progressão, metastização e pior prognóstico, documentado pelas curvas de sobrevida do VEGF-C e VEGFR-3 para o estadio III e IV, respetivamente, para o cancro do recto.

Em conclusão, os resultados observados nesta tese documentam o ganho em termos metabólicos e de angiogénese das células tumorais de CCR em relação ao tecido normal adjacente, contribuindo assim para a sua vantagem proliferativa e de metastização, assim como o facto de a presença destes marcadores estar associada a características tumorais de pior prognóstico e com impacto na sobrevida dos doentes. Estes factos suportam o possível papel destes marcadores de metabolismo e angiogénese, como biomarcadores e potenciais alvos terapêuticos no CCR e CCR metastizado.

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ABBREVIATION LIST

AJCC - American Joint Committee for Cancer

Ang - Angiopoietin

APC - Adenomatous Polyposis Coli

ASR-W - World Age Standardization

ATP - Adenosine Trifosfato

BV - Bevacizumab

CAIX - Carbonic Anhydrase

CD147 - Cluster of Differentiation 147

CEA - Carcinoembryonic Antigen

CHC - α-Cyano-4-Hydroxycinnamate

CIN - Cromossomic Instability

c-Myc - Cell Cycle Regulating Genes

CRC - Colorectal Cancer

CSF-1R - Colony Stimulating Factor-1 Receptor

CT - Computerized Tomography

DDC - Deleted in Colorectal Cancer

DFS - Survival Free Disease

EGF - Endothelial Growth Factor

EGFR - Epidermal Growth Factor Receptor

EMMPRIN - Extracellular Matrix Metalloproteinase Inducer

EPIC -European Prospective Investigation into Cancer and Nutrition

EUS - Endoscopic Ultrasound

FAP - Familial Adenomatous Polyposis

FDA - Food and Drug Administration

FGF - Fibroblast Growth Factor

Flt-3 - Flt ligand receptor

5-FU - **5-**Fluorouracil

GLUT - Glucose Transporter

HE - Hematoxylin-Eosin

HGF - Hepatocyte Growth Factor

HIF1 - Hypoxia-Inducible Factor 1

HNPCC - Hereditary Non-Polyposis Colorectal Cancer

IGF2 - Insulin-Like Growth Factor 2

IFL - Leucovorin

Kit - Stem Cell Factor Receptor

LPA - Lysophosphatic Acid

mCRC - Metastatic Colorectal Cancer

MCT - Monocarboxylate Transporter

MMPs - Matrix Metalloproteinases

MMR - Mismatch Repair

MRI - Magnetic Resonance Imaging

MSI - Microsatellite Instability

MSS - Microsatellite Stable

NA - Normal Adjacent

NCCN - National Comprehensive Cancer Network

OS - Overall Survival

OXPHOS - Oxidative Phosphorylation

PDGF - Platelet Derived Growth Factor

PET - Positron Emission Tomography

PlGF1 and 2 - Placental Growth Factor 1 and 2

RORENO - Registo Oncológico Regional do Norte

ROS - Reactive Oxygen Species

SDF1 - Stromal Cell-Derived Factor 1

SPSS - Statistical Package for the Social Sciences

TGF-β - Transforming Growth Factor-β

TMA - Tissue Microarray

TP*53* - Tumour Protein 53

UICC - International Union for Cancer Control

VEGF - Vascular Endothelial Growth Factor

WCRF/AICR - World Cancer Research Fund/American Institute for Cancer Research

WHO - World Health Organization

1.1 COLORECTAL CANCER EPIDEMIOLOGY

Colorectal cancer (CRC) is the third most common cancer and the fourth most frequent cause of cancer death worldwide (1–5), accounting for over 9% of all cancer incidence (6,7). Approximately 1 million of new CRC cases are diagnosed every year and about half a million people worldwide die due to this cancer (8). Globally, CRC incidence is very variable, with higher rates in North America, Australia and Western Europe and lower rates in developing countries (4,9), although, in recent years, high CRC rates have also been reported in these countries (10). In terms of mortality, geographic disparities have also been observed (4,11). In Western countries, CRC is the second most common cause of death from cancer, and despite advances in treatment, mortality remains high with metastatic spread to the liver occurring in about 50% of patients (4,12).

European countries presents the highest values in terms of CRC incidence and mortality (9,10). Data from the World Health Organization (WHO) and National Registries, reveal that CRC is the second most common cancer, after lung cancer in males and breast cancer in females (13). From 1998 to 2002, in Europe, the incidence of CRC for men and women was 38.5 and 24.6 (world age standardization (ASR-W)) per 100 000 inhabitants and mortality was, 18.5 and 10.7 (ASR-W) per 100 000 inhabitants, respectively (14). However, over the past twenty-five years, mortality rates among Caucasians have steadily dropped (15). Data from the WHO, between 1997 and 2007 revealed that CRC mortality decreased around 2% per year from 19.7 to 17.4/100 000 for men (world standardized rates), and from 12.5 to 10.5/100 000 for women, and these decreases in CRC mortality rates in several European countries are likely due to improvement in earlier diagnosis and treatment, with a consequent impact in survival (16).

CRC is a growing problem in Portugal, as its mortality rate has been increasing since the 1980s, between 1993 e 2001 the new CRC annual cases grew by 44% in men (from 2,060 to 2,975) and 28% in women (from 1,722 to 2,205) and between 1993 to 2005 total cancer mortality grew 15.8% (17). Data from the "National Statistic Registry", revealed that CRC, in Portugal, is the second most common cancer, after gastric cancer, with an incidence of 5000/year and a leading cause of cancer death (18).

In the North of Portugal, data from RORENO (Northern Regional Oncologic Registry) shows that, in 2005, CRC was the most prevalent cancer, followed by prostate cancer in males and breast

cancer in females (19), and the second cause of cancer death, followed by lung cancer (20). Despite improvement in earlier diagnosis and advances in treatment from 2000 to 2005, the number of CRC deaths increased at an annual average growth rate of 3% (17).

Incidence is generally higher in men, and the risk increases with age, as the majority of cases are diagnosed in patients older than 50 years (1,3,4,14), with only 5% of cases recorded in patients younger than 40 years (1,4). Advanced CRC prevalence, also increases with age and is higher among men than women (4,21). A large study identified CRC as one of the 10 most common cancers, diagnosed in both genders aged 20-49 years (22).

1.2 Colorectal Cancer Risk Factors

Literature data concerning hereditary, experimental and epidemiological issues state that CRC is a result of elaborated interrelationships between genetic and environmental factors (6,23).

1.2.1 Environmental risk factors

Evidence suggests that environmental risk factors are of major importance in the cause of CRC (17,24) and responsible for the increase in CRC cases in the last 30 years (17). Those factors including cultural, social, and lifestyle factors, nutritional practices, physical activity, obesity, cigarette smoking and heavy alcohol consumption are well established environmental risk factors (25). In the 1970s, Burkitt proposed the hypothesis that dietary fiber reduces CRC risk, based on the observation of low rates of CRC among rural Africans who eat a high-fiber diet (25). In 2003, the European Prospective Investigation into Cancer and Nutrition (EPIC) study reported a linear reduction in CRC risk with increasing fiber intake (25,26) and this result was confirmed in subsequent studies (27–30). The lost of Mediterranean diet adoption (especially lower consumption of cereals and olive oil) and higher energy intake (animal fats, red meat and alcohol) are key diet risk factors (17) for CRC. Also, metabolic syndrome, characterized by obesity, insulin resistance and hypertension, and a consequence of western dietary and behavior patterns was been demonstrated

to contribute to CRC risk (31).

Beyond dietary factors, lifestyle factors have also been extensively investigated. The second World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) expert report showed that high levels of body fat (BMI >23 kg/m2) or a large waist circumference and lower physical activity are associated with increased risk (32–35). Jacobs et al. (32,36) pointed obesity as a risk factor for colorectal adenoma development, particularly in men, in short-interval follow up (3 years). In addition, recent evidence has demonstrated that increasing physical activity in men aged over 50, results in a decrease in CRC risk (31,32,35,37).

Alcohol is one of the best known and most preventable CRC risk factors (32,33,35,38,39). Many epidemiological studies (38,40), but not all (41), have reported a positive association between alcohol consumption and CRC risk (32,33,35,38,39).

1.2.2 GENETIC RISK FACTORS

Epidemiological studies suggested that approximately 15% of CRCs arise in individuals with an inherited predisposition to the disease (18,42). A much smaller proportion of cases, fewer than 5%, results from gene mutations that are associated with mendelian syndromes; familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC), in this setting, CRC risk is very high. The remaining ones are sporadic, without a CRC familiar history (18,42).

The morphogenesis of CRC is well understood (**Figure 1**): it develops in a dysplasia-adenoma-carcinoma sequence (43), that was described by Fearon and Vogelstein in 1990 as a linear process from normal mucosa to a small polyp to a large polyp to an invasive cancer (44,45). Nowadays, it is know that a total of 4-5 steps have to occur and that these cumulative events are more important than the sequence that is followed (46) and is responsible for 80-85% of CRCs (43–45,47).

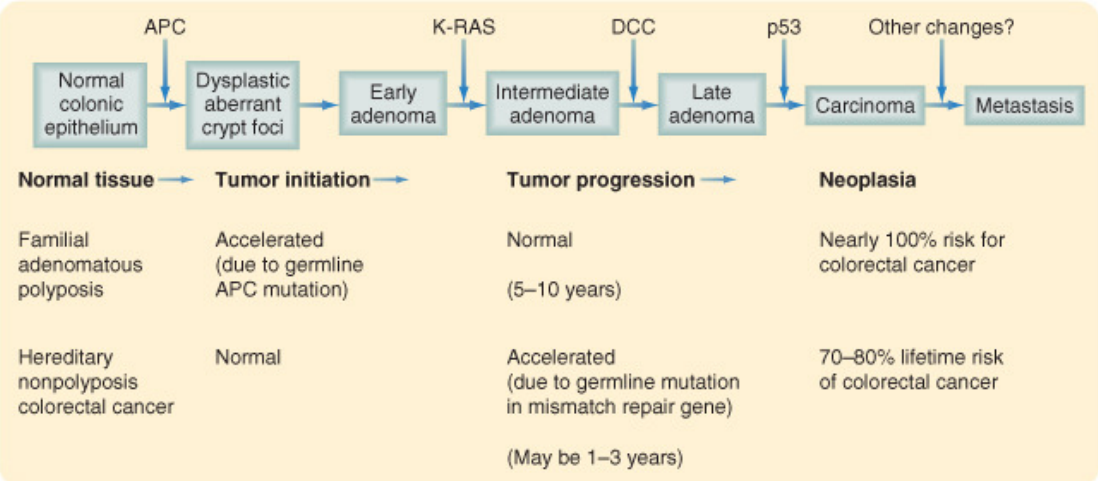


Figure 1: The adenoma-carcinoma sequence in sporadic and hereditary colorectal cancer (48).

In genetic terms, three types of genes are involved in CRC: proto-oncogenes, tumour suppressor genes and mismatch repair genes. In molecular terms, there are two major tumourigenic pathways leading to CRC: cromossomic instability (CIN) and microsatellite instability (MSI), 80% and 15-20% of sporadic colorectal cancer, respectively (43,44,47). In the first pathway, mutations accumulate in the KRAS oncogene and tumour-suppressor genes, leading to a progression from normal mucosa to adenoma and carcinoma. The second pathway is characterized by mutations in mismatch-repair genes. If somatic cells are affected, MSI is responsible for sporadic tumours (43,44,47).

1.2.2.1 CROMOSSOMIC INSTABILITY PATHWAY

This pathway involves chromosomal instability and is characterized by allelic losses on chromosome 5q (APC), 17p (p53), and 18q (DCC/SMAD4), high frequency of allelic imbalance involving chromosomal arms 5q, 8p, 17p, and 18q, chromosomal amplifications, and translocations (49). This model, besides the previously mentioned tumour suppressor genes alterations, is also characterized by alterations in oncogenes such as KRAS and BRAF (50) (**Figure 2**).

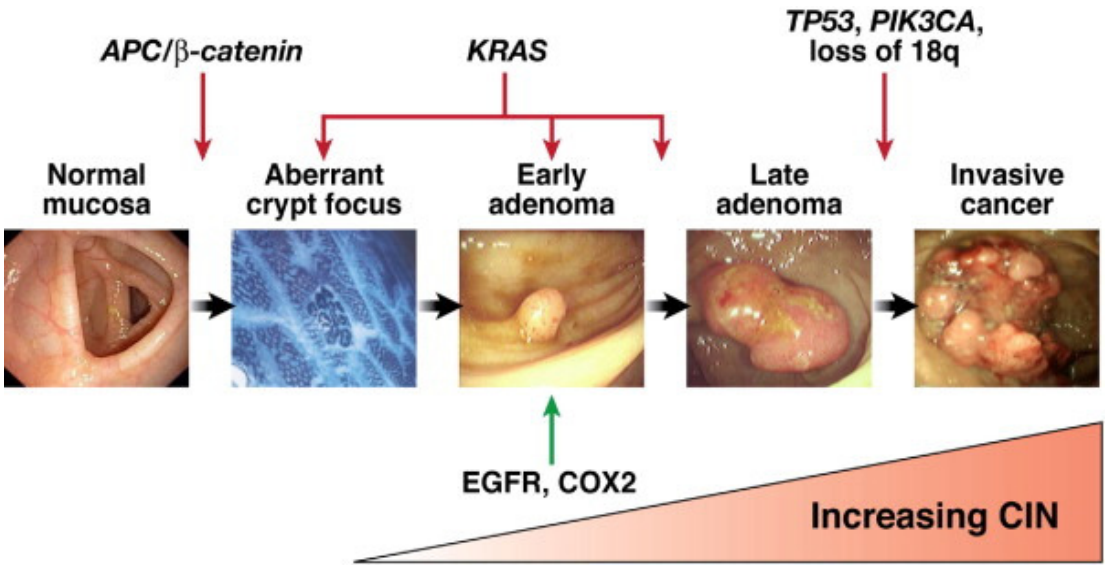


Figure 2: Multistep genetic model of colorectal carcinogenesis (51).
The initial step in colorectal tumourigenesis is the formation of aberrant crypt foci as a result of mutations in the *APC* gene. Progression to larger adenomas and early carcinomas requires activating mutations of the proto-oncogene *KRAS*, in *TP53*, and loss of heterozygosity at chromosome 18q. Mutational activation of *PIK3CA* occurs late in the adenoma–carcinoma sequence in a small proportion of CRC. CIN is observed in benign adenomas and increases with tumour progression.

1.2.2.1.1 Adenomatous Polyposis Coli gene

Mutation on Adenomatous Polyposis Coli (*APC*) gene, a tumour suppressor gene, is present in 50 -70% of sporadic CRC (52). This gene acts as a gatekeeper of intestinal epithelial homeostasis by restricting cytoplasmic levels of β-catenin, the central activator of transcription in the Wnt signaling pathway (50,52–54). At molecular level, *APC* promotes phosphorylation and subsequent degradation of β-catenin by supporting a multiprotein destruction complex, composed of the tumour suppressor Axin and the serine-threonine kinases GSK3b and CK1, which (53) (**Figure 3**).

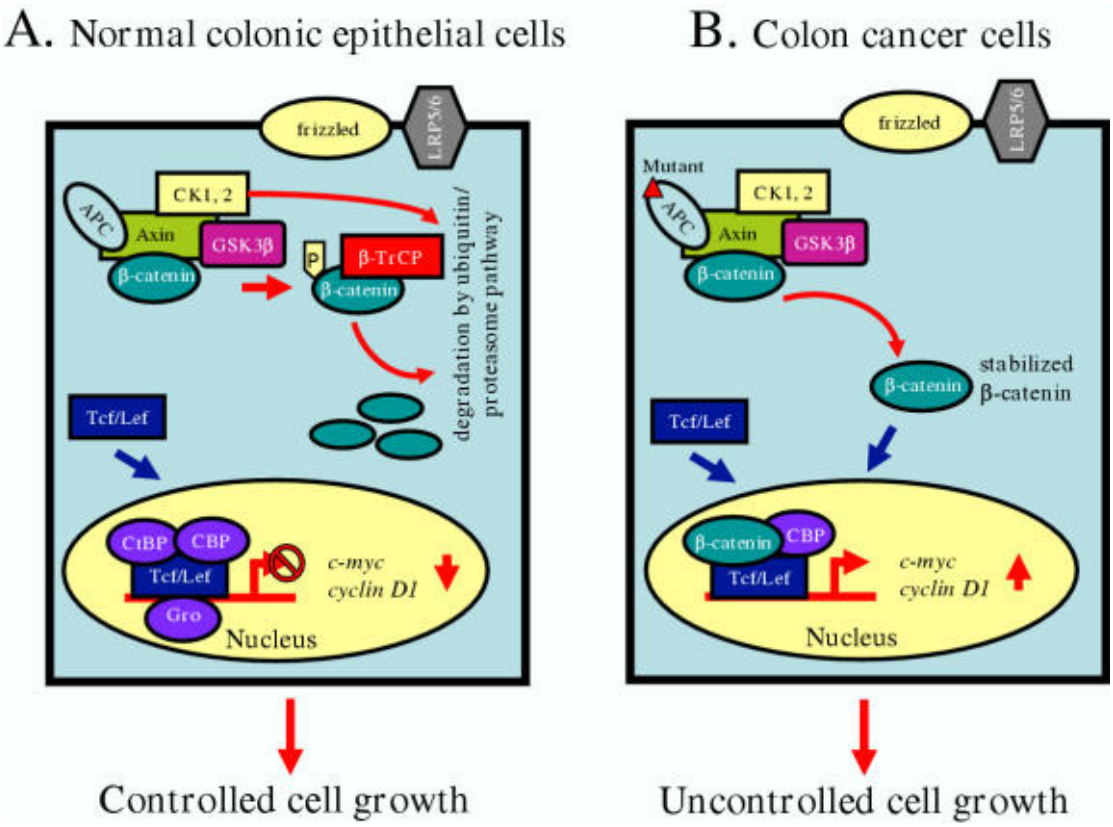


Figure 3: A model for the Wnt-signaling pathway (54).
Panel A depicts the down-regulation of β-catenin transactivation activity in normal colonic epithelial cells. β-catenin remains in a complex of Axin/Axil/conductin, APC, GSK3β kinase and casein kinase 1 or 2 (CK1 or 2). In the absence of Wnt-signaling, GSK3β and CK1 or 2 kinases become active and phosphorylate β-catenin. The phosphorylated β-catenin then binds with F-box protein β-TrCP of the Skp1-Cullin-F-box (SCF) complex of ubiquitin ligases and undergoes proteasomal degradation. Some other known genes which are regulated by β-catenin/Tcf-Lef pathway are given here – *cyclin D1, CDH1, Tcf-1, c-jun, Fra-1, PPARd, Gastrin, uPAR, MMP7, Conductin, CD44, Id2, Siamois, Xbra, Twin* and *Ubx*.
Panel B shows the role of mutations in the APC or β-catenin protein in the regulation of β-catenin level and its transactivation property in colon cancer cells. The mutant β-catenin escapes its degradation through Wnt-pathway and becomes stabilized in the cytoplasm. The stabilized level of β-catenin then heterodimerizes with Tcf-Lef transcription factor and locates into the nucleus, where it actively transcribes cell cycle related genes causing cellular proliferation.

In the case of *APC* mutations, β-catenin is not directed towards degradation, instead it is translocated to the nucleus and is responsible for transcriptional activation of several cell cycle regulating genes (*cyclin D* and *c-Myc*), genes connected to tumour progression (*MMP-7, MMP-26*) and also the peroxisome-proliferator-activated receptor delta gene (53). *APC* gene is connected to carcinogenesis at different levels such as cell migration and adhesion (52,55,56); besides the function on Wnt pathway, it regulates cell migration due to its role in cytoskeletal regulation (52,55) mitosis, by promotion of chromosomal alignment (56) and influencing centrosome duplication (57).

1.2.2.1.2 DELETED IN COLORECTAL CANCER GENE

Deleted in Colorectal Cancer (*DDC)* gene is a tumour suppressor gene (58). Mutation is present in 73% of sporadic CRC (52,58). The protein codified by *DCC* is a transmembrane receptor of the immunoglobulin superfamily for netrins, factors involved in axon guidance in the developing nervous system; besides this function it also has a role in intracellular signaling, apoptosis, cell cycle and cell motility (59,60). There are studies that refer that when mutations are present in this gene, a worst prognosis results (52).

1.2.2.1.3 TUMOUR PROTEIN 53 GENE

Tumour Protein 53 *(TP53)* gene is a tumour suppressor gene that encodes p53. Mutation on *TP53* is present in 60-80% of sporadic CRC (52,61,62). This gene stops cells in G phase until DNA repair occurs; if that repair does not occur, cells enter apoptosis (52,63), so mutations in this gene are involved in malignant transformation, and are associated with a worse prognosis (52).

1.2.2.1.4 KRAS, BRAF AND C-MYC GENE

Besides the previously mentioned genes, mutation on *KRAS* gene, a proto-oncogene, is present in 40-50% of sporadic CRC (43,62) and plays a important role in cell division, cell differentiation and apoptosis (51) (**Figure 4**).

These mutations are generally observed as somatic mutations. The most frequent types of *KRAS* mutations in CRCs are G-to-A transitions (64) and G-to-T transversions (65). KRAS mutations occur in MSI tumours, both in HNPCC and in sporadic CRC, in 40% and 18% of cases respectively (66). This mutation occurs in earlier stages of dysplasia-adenoma-carcinoma sequence, being associated with adenoma growth (43). Several studies support the importance of mutational activation of KRAS in the progression of CRC. KRAS gene codon 12 and codon 13 mutations were associated with a mucinous and a non-mucinous phenotype, respectively, but were characterized as

more aggressive tumours with a greater metastatic potential (67). Moreover, the frequency of associated KRAS and BRAF mutations increased along with the depth of intestinal wall invasion and a higher frequency of KRAS mutations was observed in lymph node metastases as compared to the primary tumours, suggesting that KRAS mutations are responsible for a more invasive tumour cell behavior (66).

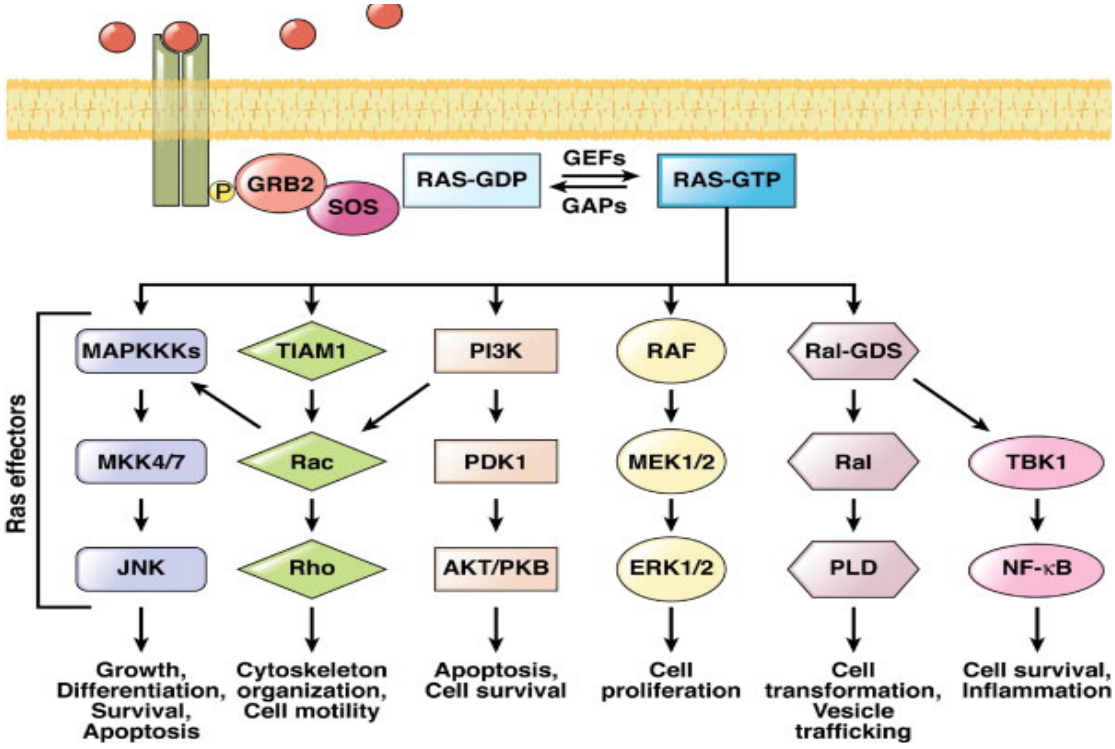


Figure 4: The RAS signaling pathway (51).
Growth factors binding to their cell surface receptors activate guanine exchange factors (GEF), such as SOS (son of sevenless) that are attached by the adaptor protein GRB2 (growth-factor-receptor bound protein 2). SOS stimulates the release of bound guanosine diphosphate (GDP) from RAS, and it is exchanged for guanosine triphosphate (GTP), leading to the active RAS-GTP conformation. The guanosine triphosphatase (GTPase)-activating proteins (GAP) can bind to RAS-GTP and accelerate the conversion of RAS-GTP to RAS-GDP, which terminates signaling. Mutated RAS is constitutively active in the RAS-GTP conformation. Activated RAS regulates multiple cellular functions through effectors including the Raf–MEK–ERK pathway, phosphatidylinositol 3 kinase (PI3K), RALGDS, RALGDS-like gene (RLG), and RGL2.

Mutations of BRAF are associated with increased kinase activity and are present in 9 -11% of CRC especially at Dukes' stage A and B (68). In sporadic CRC with a MSI phenotype, BRAF mutations were found in 31-45% of the cases (68–70). Remarkably, a single glutamic acid for valine substitution at codon 600 (V600E) accounts for approximately 90% of the BRAF mutations found in

human tumours (68), this mutation leads to constitutive kinase activation (71) and with rare exceptions, V600E BRAF mutations are found in a mutually exclusive pattern with KRAS mutations, suggesting that these genetic events activate a set of common effectors of transformation (72).

Mutation on *c-myc* gene is present in one third of sporadic CRC, it is essential for progression of G1 to S phase and regulation of cellular differentiation. It seems to be associated with distal tumours, and discriminate a group of patients who have earlier recurrence after surgery (52).

1.2.2.2 MICROSATELLITE INSTABILITY PATHWAY

During each cell division, DNA polymerase makes errors while copying DNA. These mistakes are more frequent at the level of repeated sequences, known as microsatellites, and are normally repaired by a system called MMR (mismatch repair). Tumours defective in this system accumulate mutations and are called MSI. Microsatellites are numerous and dispersed throughout the genome, in coding and non-coding regions and the instability of non-coding microsatellites is a good indicator of the MSI status (73).

MSI phenotype (defects in the mismatch repair genes hMLH1 and hMSH2) has been found in 10-20% of sporadic CRC (73,74) and in 95% of HNPCC (48). These tumours occur preferentially in the right colon, 30% versus 2% when comparing right and left CRC, respectively (74). MSI tumours were associated with a better prognosis than MSS (Microsatellite Stable) tumours, and respond differently to conventional chemotherapeutic agents used in CRC treatment (73,74).

1.3 DIAGNOSIS AND STAGING

CRCs are usually diagnosed either by direct endoscopic visualization or by a radiological investigation (barium enema, computerized tomography (CT) or CT colonography). For the majority of cases, histological confirmation is obtained through endoscopic biopsy; 85% of CRCs are adenocarcinomas, 10% are mucinous adenocarcinomas and the remainders are rare histological

types such as papillary carcinoma, adenosquamous carcinoma and signet ring cell carcinoma (75).

Pre-operative staging is central in CRC, on the one hand there are a wide range of clinical scenarios and treatment options (75); on the other hand, CRC survival is directly associated with the tumour stage at the time of diagnosis; patients with distant metastasis have a poor 5-year survival (12%), compared with patients with a localized disease (90%) (76–78).

A number of imaging modalities are used in the pre-operative staging of CRCs including CT, Magnetic resonance imaging (MRI), ultrasound imaging and positron emission tomography (PET) (75).

1.3.1 Computerized Tomography (CT)

This exam is capable of identifying the primary tumour, lymph nodes and other organs metastases, but the major limitations of CT is that it does not provide neither histological diagnostic neither functional information and hence cannot discriminate between active cancer and scar tissue (75).

Pre-operative staging with abdominal CT can change the patient treatment plan, by finding liver metastases, peritoneal carcinomatosis and locally advanced colon cancer. Although in the past, these conditions were considered incurable, nowadays various multi-modality treatments can be offered to selected patients (79–81), even in the case of incurable advanced CRC, staging may change the treatment plan towards a palliative treatment plan, avoiding surgery in selected patients (81,82).

Staging with chest CT as a routine procedure before surgery is controversial, mainly due to the low incidence of clinically relevant lung metastases and low specificity of chest CT (83). After the liver, lung is the most common site for distant metastatic in CRC, and about 10% of CRC patients develop pulmonary metastasis (84). However, fewer than 10% of the patients who develop pulmonary metastasis are candidates for surgical resection (84,85). According to the National Comprehensive Cancer Network (NCCN) guidelines, chest CT is recommended for pre-operative staging of CRC patients (84,86,87). On the other hand, Dutch national evidence-based guideline for diagnosis and treatment of patients with colorectal metastases states that in the case of lung

metastasis, imaging exam could be limited to conventional chest X-ray, based on the low prevalence of lung metastases and the occurrence of false-positives at CT (88).

1.3.2 Magnetic Resonance Imaging (MRI)

MRI is ideal for rectum as this bowel segment is relatively fixed and for this reason, the use of MRI to stage rectal cancers by assessing primary tumour and its relationship to the bowel wall is standard and essential in guiding rectal cancer treatment (75).

MRI can also be used in the assessment of metastatic liver disease, not only in cases where there is some doubt about the nature of the liver lesions but also identifying metastases that have not been seen by standard CT and providing a roadmap for surgery in the case of metastatic liver disease candidate for surgical resection (75).

1.3.3 Ultrasound Imaging

Transrectal ultrasound is a exam that is used to the staging of rectal cancers by assessing the tumour, its relationship to the bowel wall and the presence of lymph node metastasis (75,89,90). Like MRI, transrectal ultrasound is essential in guiding rectal cancer identifying patients that are candidates to the use of pre-operative radiotherapy (75).

1.3.4 Positron Emission Tomography (PET)

PET is capable of identifying cancer earlier than other exams such as CT and MRI. Actually, in CRC, the main indications for PET are: assessment of residual mass following treatment and of apparently isolated metastatic disease (75). Depending on the tumour type, it can be highly effective in assessing treatment response or for detecting disease recurrence. However, in histological CRC subtypes, like mucinous carcinoma, due to its low metabolic rate, it is not useful (75).

1.3.5 STAGING

The need of stratification patients with CRC in order to establish an appropriate treatment results in the first clinical staging system proposed by Dukes and Jass, for rectal cancer, based on the extent of the primary tumour and presence/absence of lymph node involvement (91–94). However, this staging system lacks some important tumour characteristics, such as extent of lymph node involvement and tumour grade. Later, in 1987, Jass added two new characteristics, the nature of the expanding front of the tumour and the presence/absence of lymphocytic infiltration at the advancing edge, thus addressing some of those absences (95). In the following years, as new factors became known, the Dukes'classification has been repeatedly modified by others (Kirklin, Astler and Coller, etc.) (91).

Nowadays, TNM staging is the most widely used system, it classifies the extent of cancer based on anatomical information about the size and extent of primary tumour (T), the regional lymph node status (N) and the distant metastases (M), grouping the cases with similar prognostic (91,96). The system is maintained collaboratively by the International Union for Cancer Control (UICC) and the American Joint Committee for Cancer (AJCC), resulting in periodical and simultaneously publication of the *TNM Classification of Malignant Tumours* and the *AJCC Cancer Staging Manual*. The 7th revision of TNM staging was recently published by the AJCC and UICC, and became operational starting on 2010.01.01 (91,97).

The staging system is categorized from Stage 0 through stage IVB (**Table I**) and correlates with patient prognosis (**Table II**). Stage I disease includes tumours with tumour depth penetration into the submucosa (T1) or the muscularis propria (T2). In stage IIA–IIC CRC, tumour penetration can extend from muscularis propria to adhere to other organs however, there is no lymph node involvement. Nodal involvement begins in stage IIIA–IIIC regardless tumour depth penetration. Finally, stage IVA–IVB, incorporates one distant organ involvement (M1a) or greater than 1 organ/peritoneal involvement (M1b), independently of tumour depth penetration and regional lymph nodes involvement (98).

Table I: 7th revision of the TNM Staging of Colorectal carcinoma [Adapted from (97)].

Primary Tumour (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria
T1	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades through the muscularis propria into pericolorectal tissues
T4a	Tumour penetrates to the surface of the visceral peritoneum
T4b	Tumour directly invades or is adherent to other organs or structures

Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1–3 regional lymph nodes
N1a	Metastasis in one regional lymph node
N1b	Metastasis in 2–3 regional lymph nodes
N1c	Tumour deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis
N2	Metastasis in 4 or more regional lymph nodes
N2a	Metastasis in 4–6 regional lymph nodes
N2b	Metastasis in 7 or more regional lymph nodes

Distant Metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Metastasis confined to one organ or site
M1b	Metastases in more than one organ/site or the peritoneum

Stage	T	N	M
0	Tis	N0	M0
I	T1	N0	M0
	T2	N0	M0
IIA	T3	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N0	M0
IIIA	T1-T2	N1/N1cM0	M0
	T1	N2a	M0
IIIB	T3-T4a	N1/N1cM0	M0
	T2-T3	N2a	M0
	T1-T2	N2b	M0
IIIC	T4a	N2a	M0
	T3-T4a	N2b	M0
	T4b	N1-N2	M0
IVA	Any T	Any N	M1a
IVB	Any T	Any N	M1b

Table II: TNM Staging of Colorectal Carcinoma and 5-Year Survival by Stage [Adapted from (98)].

Stage	5-Year Survival
I	93.2%
IIA	84.7%
IIB	72.2%
IIC	
IIIA	83.4%
IIIB	64.1%
IIIC	44.3%
IVA	8.1%
IVB	8.1%

Note: Five year percentages based on data prior to institution of 7th edition, AJCC staging guide (99).

In the last years, there has been a growing interest focusing on the role of non-anatomic markers as prognostic and treatment response in cancer patients (91). These molecules might allow more accurate CRC staging, improving patients selection for multimodal therapy and sparing patients from unnecessary procedures (77,78). However, besides TNM, few stage markers have been validated as diagnosis criteria worldwide (77,78).

1.4 Cancer Metabolic Behavior

Reprogramming of energy metabolism is one of the hallmarks of cancer, which was recently added to sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion, metastasis and evading immune destruction (100).

Normal cells and tumour cells differ markedly in energy metabolism; normal cells use glucose as their primary energy source. In the presence of adequate oxygen supply, normal cells completely oxidize glucose, a process that involves cytoplasmic glycolysis, mitochondrial citric acid cycle and electron transport chain/oxidative phosphorylation (OXPHOS). Consequently, normal cells drive the maximum possible energy from glucose by fully oxidizing the molecule to CO_2 (**Figure 5**).

When the oxygen supply is disrupted, normal cells turn their metabolism to anaerobic glycolysis, due to mitochondrial function suppression, as a consequence of oxygen absence. This metabolic pathway has lactate as the end product and conversion of pyruvate, the glycolytic end product, into lactate is mandatory for continued operation of glycolysis in the absence of oxygen. Consequently, the regeneration of NAD^+, the coenzyme for glyceraldehyde-3-phosphate dehydrogenase, is ensured. Contrary to “aerobic glycolysis”, this pathway only produces a fraction of energy from glucose (Energetic yield: 2 ATPs/glucose molecules). Thus, this less efficient energetic pathway is adopted by normal cells only under anaerobic conditions (102) (**Figure 5**).

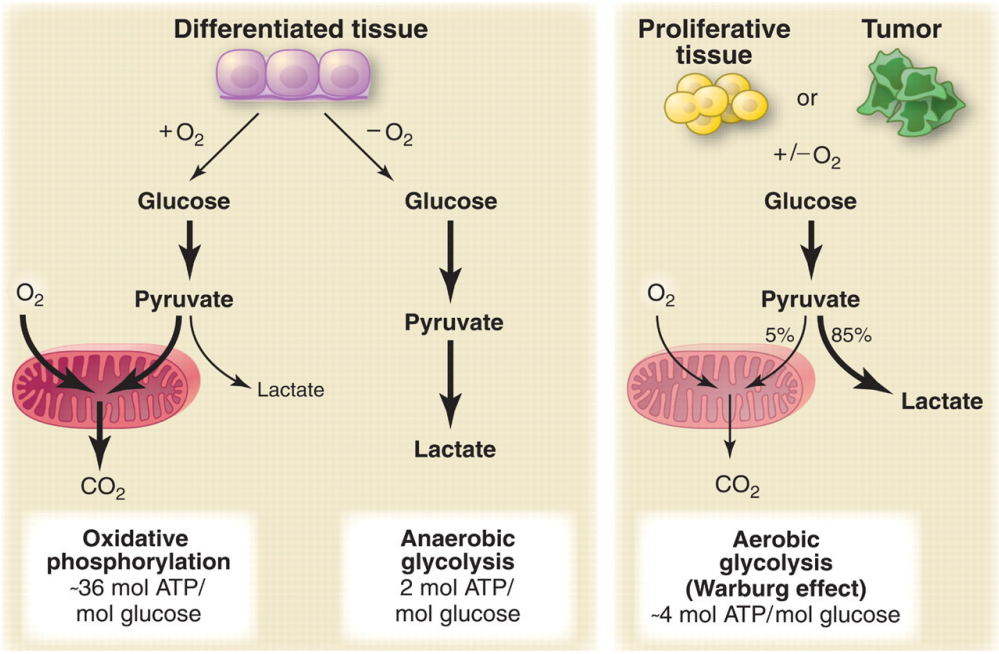


Figure 5: Schematic representation of Warburg effect (101).
Represents the differences between OXPHOS, anaerobic glycolysis, and "aerobic glycolysis".

Unlike normal cells, tumour cell metabolism depends mainly on this metabolic pathway, even in the presence of oxygen. This phenomenon, "aerobic glycolysis" or "Warburg effect", was first described almost one century ago, by the Nobel Prize winner Otto Warburg, it was the first tumour-metabolism specific alteration described and consists of an increase in glycolytic rate that is maintained even in the presence of adequate levels of oxygen. As a consequence, tumour cells are producing lactate at higher levels compared to non-malignant tissue (103–106).

In order to maintain the high rates of glycolysis, cancer cells use elevated amounts of glucose as energetic source (107), with increase in glycolytic flux (103,108–112), mainly caused by upregulation of numerous glycolysis-related genes in the majority of human cancers (104).

There are several reasons why enhanced "aerobic glycolysis" constitutes an advantage for tumour growth (113):

- Tumour cells are able to survive in conditions of low oxygen tension, that would be lethal for cells that depends mostly on aerobic metabolism to generate energy (113,114).

- The acidic tumour microenvironment, resulting by the acids produced by cancer cells, namely lactic acid (115) is associated with tumour aggressiveness features, such as growth,

increased survival, migration, invasion, and angiogenesis (104,116,117), and suppress anticancer immune effectors (118). Moreover, lactate can be taken up by stromal cells to regenerate pyruvate that can be either extruded to refuel the cancer cell or can be used for OXPHOS (115).

- Tumours are able to metabolize glucose, through the pentose phosphate pathway, to generate NADPH thus supplying cell's anti-oxidant defenses against a hostile microenvironment and chemotherapeutic agents (119). NADPH can also contribute to fatty acid synthesis.

- Cancer cells use intermediates of the glycolytic pathway for anabolic reactions: glucose 6-phosphate for glycogen and ribose 5-phosphate synthesis, dihydroxyacetone phosphate for triacylglyceride and phospholipid synthesis, and pyruvate for alanine and malate synthesis (119). Moreover, pyruvate may enter a truncated tricarboxylic acid cycle. The resultant acetyl-CoA is exported from the mitochondrial matrix and becomes available for the synthesis of fatty acids, cholesterol, and isoprenoids (113).

- Reduced ATP generation in mitochondria is a compromise that tumour cells have to make in order to initiate oncogenic transformation by partially inhibiting OXPHOS, consequently, the generation of reactive oxygen species (ROS) increase (120), causing mutations in proto-oncogenes to initiate tumourigenesis (102).

Since enhanced glycolysis in cancer is associated with lactate production and secretion (103,109–112) and despite the large amounts of lactic acid produced only the interstitial pH of tumours is low, while the intracellular pH of tumours is either normal or higher than normal tissues (109–111), tumour cells must find a way to eliminate lactic acid to prevent cellular acidification and apoptosis (103,104,112). This is achieved by specific transporter upregulation like proton pumps, sodium-proton exchangers, bicarbonate transporters, and monocarboxylate transporters (MCTs) (109).

By counteracting intracellular acidification, the export of lactic acid leads to acidification of the extracellular milieu which turns to be advantageous to tumour progression for two reasons; first extracellular acidification may kill adjacent normal cells, allowing tumour cells to spread, second it facilitates angiogenesis and metastization through upregulation of molecules involved in tumours growth, progression and metastization such as Vascular Endothelial Growth Factor (VEGF), Hypoxia-inducible Factor 1, (HIF-1α), and hyaluronan and its receptor CD44 (103,121). Some studies report that elevated lactate levels correlate with increasing incidence of metastases (122), radioresistance

(123) and poor prognosis, particularly poor overall survival and poor disease-free survival (104,123,124) in human cervical cancers (125–127), head and neck cancer (111), brain cancer (128,129) non-small-cell lung cancer (130) adenocarcinoma lung cancer (131) and CRC (132,133).

1.4.1 MONOCARBOXYLATE TRANSPORTERS

The monocarboxylate transporter (MCT) family is presently composed by 14 members, and is encoded by the SLC16 gene family (134,135). Currently, only four members (MCT1-MCT4) of the MCT family have been demonstrated to transport aliphatic monocarboxylates, including lactate, pyruvate and ketone bodies (135,136). Besides the previously mentioned monocarboxylates, MCTs also transport the branched-chain oxo-acids derived from leucine, valine and isoleucine, and the ketone bodies acetoacetate, β-hydroxybutyrate and acetate. Consequently, MCTs play a central role in metabolism and are critical for metabolic communication between cells (136).

MCT1 has a broader distribution and transports a wider range of substrates when compared to other family members. The main function of this transporter has been associated with the uptake or efflux of monocarboxylates through the plasma membrane, according to cell metabolic needs and behaving as a high affinity transporter for L-lactate, but not for D-lactate, as well as for pyruvate, acetate, propionate, D,L-β-hydroxybutyrate and acetoacetate (134,135). It has also been implicated in the transport of butyrate and propionate in human colonocytes, the principal energy substract for these cells (127,135,137).

MCT4 demonstrates several similarities to MCT1, namely tissue distribution, regulation and substrate/inhibitor specificity. The principal difference between these isoforms lies in tissue specific localization and substrate affinities. MCT4 is predominantly expressed in highly glycolytic cells such as white muscle and white blood cells (135,138) and also strongly expressed in placenta, exporting lactic acid rapidly from the fetal to the maternal circulation, thus suggesting that its physiological function is lactate efflux (139). Another difference is that MCT4 shows a lower affinity for substrates, than MCT1 (138,140). In fact, MCT4 will not only be important for the acid-resistant phenotype, but

also for the hyper-glycolytic phenotype, by exporting newly formed lactate, allowing continuous conversion of pyruvate to lactate, so, and, therefore, continuous aerobic glycolysis (135).

In the past few years some studies reported abnormal expression of MCTs, particularly in solid tumours, however, with contradictory conclusions (141). CRC provides intriguing information regarding MCT expression in cancer. Koukourakis et al. demonstrated that both membrane and cytoplasmic MCT1 expression was seen in both normal colonic tissue as well as in colonic tumour cells (127,142,143). In our previous results, Pinheiro et al. have demonstrated an increase in MCT1 and MCT4 in CRC compared with normal colonic epithelium (108,127). On the other side, Lambert et al. described a decrease in MCT1 expression during transition to malignancy (108,127,144).

1.4.2 MCT REGULATION BY CHAPERONES

Functional expression of MCTs is regulated by accessory proteins **(Figure 6)**, such as Cluster of Differentiation 147 (CD147), also known as Basigin (BSG) or Extracellular Matrix Metalloproteinase Inducer (EMMPRIN) that are involved in trafficking and anchoring of plasma membrane proteins (135).

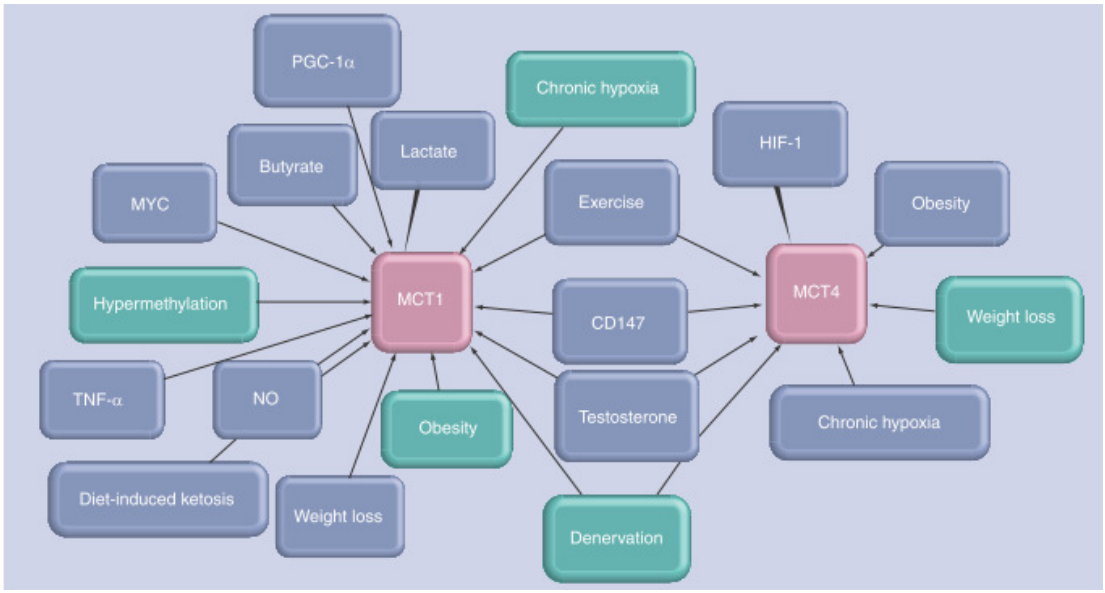


Figure 6: MCT1 and MCT4 regulation
(Blue boxes indicate upregulation of the specific MCT subtype while green boxes indicate a downregulation) (127).

CD147 is a broadly distributed plasma membrane glycoprotein and belongs to the immunoglobulin superfamily (145). This chaperone is ubiquitously expressed on the cell surface, with the highest levels found in metabolically active cells such as lymphoblasts and cancer cells (146,147). CD147 promotes extracellular matrix degradation, tumour growth and metastasis of cancer cells through increasing production of hyaluronan (148), and stimulating the production of multiple matrix metalloproteinases (MMPs) by fibroblasts, endothelial cells and cancer cells and so increasing the invasiveness of tumour cells (149–154). CD147 also stimulates angiogenesis by upregulation VEGF expression (155) as well as its main receptor VEGFR-2 in both cancer cells and endothelial cells (156).

Regulation of MCT1 and MCT4 by CD147, was supported by evidence on human and in vitro studies (104,135,157–161). Besides the role of CD147 as chaperone for MCT1 and MCT4 activity, these MCT isoforms also have been implicated in CD147 membrane expression (135,157,160). Thus, the contribution of MCTs to the malignant phenotype is not limited to their own function as lactate transporters and pH regulators, but through its role in CD147 expression MCTs may also have indirect roles in tumour growth, invasion and angiogenesis (135,162–164).

Like MCT, studies on CD147 expression in CRC are limited. High expression of CD147 has been observed in various carcinomas including colorectal cancers (149,165–167); breast cancers (148,168–170), hepatomas (171), oesophageal (179) and cervical squamous cell carcinomas (172), ovarian carcinomas (173) and gastric cancer (174). On the other hand, van der Jagt et al. observed that CD147 expression was higher in normal tissue compared to tumour tissue (175).

Elevated CD147 expression has also been shown to correlate with the progression of various malignancies (148,150,151,166,168,171–173,176). Zheng et al. (177) documented that CD147 expression was stronger in CRC and metastatic carcinoma than non-neoplastic superficial epithelium. Also, Buergy et al. and Jin et al. reported that a high relative CD147 expression was associated with advanced tumour stage and with metastatic disease (178,179). Baba et al. observed that blocking CD147 led to an increase in cell death (180).

CD44 was originally described as an antigen on red blood cells and platelets, and subsequently recognized as a lymphocyte homing receptor (181,182)**.**

It is a transmembrane glycoprotein that acts mainly as a receptor for hyaluronan but can

also bind to other extracellular matrix ligands like chondroitin sulphate, heparan sulphate, fibronectin, serglycin, osteopontin but with lower affinity (181,182). It's main function is communication of cell-matrix interactions (181,182) but also participates in other cellular processes, including growth, survival, differentiation, and motility (183). Recently it was found that CD44 may also act as a chaperone for MCT expression (162).

CD44 is encoded by a single gene containing 20 exons, 10 of which are variant exons inserted by alternative splicing (181),some of these variant isoforms are up-regulated in cancers (181,184–187) and has been implicated in numerous aspects of cancer progression (184–187).

Additionally, parallel analysis of CD44 and MCTs expressions in human cancer, show that CD44 is associated with MCT1 in lung cancer (104) and both MCT1 and MCT4 in prostate cancer (188). Several studies have suggested an important biological role for CD44 in tumour progression, metastasis and as a potential clinicopathological marker of disease progression for colorectal cancer (189–194) breast cancer (195), pancreatic cancer (196) gastric cancer (197) and esophageal carcinoma (198,199).

Some studies correlates variant isoforms of CD44 expression with a poor prognosis in colon cancer (200–202) and that can be a molecular marker for colorectal cancer and for the presence of micrometastasis in regional normal lymph node (202), but different conclusions have been achieved about an potential relationship between variant CD44 expression and the prognosis of patients with CRC (181,203–205) and more recent results suggest either no role or a worse clinical outcome for CD44 variant isoforms expression (192,206–208).

1.4.3 Glucose transporters

Cancer cells, in order to continue their uncontrolled growth and proliferation, must compensate the inefficient extraction of energy from glucose, this is achieved by overexpression of glucose transport through plasma membrane (209–211), that is mediated by a family of facilitated glucose transporter proteins named (GLUT 1–14) (209,212). This up-regulation may be a constitutive feature of the malignant phenotype in many cancers or may result from an adaptative

increase in GLUT1 expression, a hypoxia-responsive transporter, due to local hypoxia in the tumour microenvironment (213,214).

The GLUT family is expressed in the membrane of nearly all cell types; GLUT1, a high-affinity glucose transporter, is restricted to erythrocytes and blood-tissue barriers such as the blood-brain and blood-nerve barriers (210,212,213).

Overexpression of GLUTs has been observed in various cancers (209,210), namely breast, lung, kidney, urinary bladder, stomach, colorectum, endometrium, thyroid, head and neck, liver, ovary, salivary gland, and prostate cancer (210,212,215) due to a high metabolic rate and fast growth environment. The lack of GLUTs expression in benign epithelial tissues makes it a potential marker for malignant transformation (210,214,216).

Other studies revealed a correlation between GLUT1 expression level and the grade of tumour aggressiveness (209,212,213,217,218), increased proliferative activity and energy requirements (212) suggesting that GLUT1 expression may correlate with prognostic (209,213,219).

1.4.4 MCT TARGETING THERAPY IN CANCER

Tumour cells intracellular pH homeostasis and subsequent extracellular acidosis have been considered a key factor essential for both cell transformation and progression of the neoplastic process (220). MCT inhibition, by affecting pH homeostasis, will have a direct impact in cellular and extracellular balance with an important effect on cell viability. MCT inhibition not only induces apoptosis due to cellular acidosis, but would also lead to reduction in tumour angiogenesis (221), invasion (222), and metastization (223) (**Figure 7**).

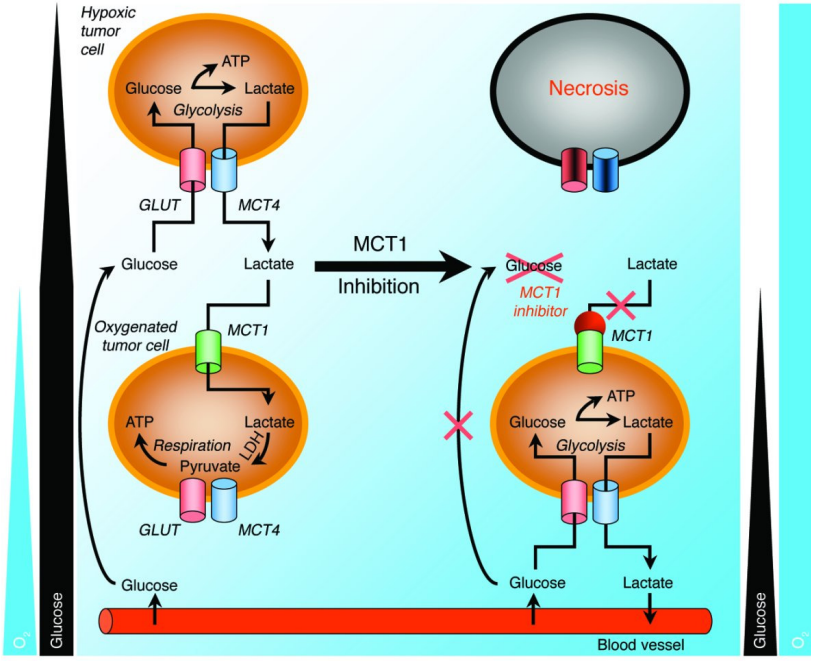


Figure 7: Model for therapeutic targeting of lactate-based metabolic symbiosis in tumours (124).
Hypoxic tumour cells depend on glycolysis to produce energy. Lactate, diffuses along its concentration gradient toward blood vessels. By contrast, oxygenated tumour cells import lactate (mediated by MCT1) and oxidize it to produce energy. Upon MCT1 inhibition, oxidative tumour cells switch from lactate oxidation to glycolysis, thereby preventing adequate glucose delivery to glycolytic cells, which die from glucose starvation.

This hypothesis was already proven both *in vitro* and *in vivo* in various cancers models, namely in gliomas (224,225) and neuroblastomas (226). In order to investigate a novel method to enhance radiosensitivity of gliomas, namely by modulating the metabolite flux immediately before radiotherapy, Colen et al. (224) disrupted cell metabolic balance with α-cyano-4-hydroxycinnamate (CHC) concluding that this inhibitor of MCT activity supported the usefulness of metabolic remodeling before low-dose radiation-based glioma therapy. Also Mathupala et al. (225) in malignant gliomas, demonstrated that small interfering ribonucleic acid (siRNA) specific for MCT1 and MCT2, in U-87 MG cells, reduced lactate efflux by 30% individually and 85% in combination, with a concomitant decrease of intracellular pH. Additionally, with prolonged silencing, cell viability was reduced by 75% individually and 92% in combination. Fang et al. (226) also pointed MCT1 as a therapeutic target in neuroblastoma.

Also, inhibition studies on CD147 with RNAi have demonstrated significant decreases in invasiveness, MMP secretion, multidrug resistance and increased cell death. Inhibition by a mouse

monoclonal antibody, who disrupts CD147–MCT1 association, led to specific cancer cell death while sparing normal fibroblast (227).

Since MCT inhibitors have the potential for altering metabolism, intracellular pH, and angiogenic response, they are promising therapeutics targets but we cannot forget the deleterious whole-body effects that they can cause and so it is mandatory to evaluate toxicity to normal tissue (227). Currently a clinical trial is ongoing based on the antitumoural effect of CHC as MCT1 inhibitor, a related orally administered compound, AZD3965, is currently entering Phasel/II clinical trials for advanced solid tumours (228).

1.5 Tumour Angiogenesis

Angiogenesis plays a key role in tumourigenesis and metastatic processes (4,229–234). It consists in the formation of new blood vessels from the endothelium of pre-existing vasculature (232,235) but recruitment and *in situ* differentiation of bone marrow-derived endothelial progenitor cells are also involved (232); it includes proliferation and migration of activated endothelial cells to reach remote targets, assembly of these cells into new capillary tubes, followed by synthesis of a new basement membrane and maturation with formation of a vascular lumen (232).

During tumourigenesis, the appropriate balance between proangiogenic and antiangiogenic molecules which arise from cancer cells and stromal cells is lost (4,232,235–239). This “angiogenic switch” is triggered by several factors including: (a) oncogene-mediated tumour expression of angiogenic proteins including VEGF, hepatocyte growth factor (HGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), endothelial growth factor (EGF), lysophosphatic acid (LPA), and angiopoietin (Ang), (b) metabolic and/or mechanical stress, (c) genetic mutations, (d) the immune response, and (e) hypoxia, maybe the most prominent. Tumour-angiogenesis therefore depends on tumour type, localization, growth and stage of disease and contributes to tumour growth, invasion, and metastization (4,235,238,240–244).

Oxygen tension is the key regulator of VEGF expression, predominantly via the hypoxia-inducible factor/von Hippel-Lindau tumour suppressor gene pathway. As a result of tumour growth

and insufficient vascularization, tumours often are accompanied by a decrease in oxygen tension (238) and under these hypoxic conditions, non-hydroxylated HIF accumulates, translocates to the nucleus initiating transcription of various genes that play a central role in angiogenesis. Genes induced by HIF include: VEGF, PDGF, transforming growth factor-β (TGF-β), TGFα, epidermal growth factor receptor (EGFR), insulin-like growth factor 2 (IGF2), MMP1, stromal cell-derived factor 1 (SDF1), GLUT 1, carbonic anhydrase 9 (CAIX), and activin B (238,245,246).

Tumour angiogenesis is essential to allow neoplastic mass development favoring access to the blood components, and also strengthening the vascular routes in the metastatic process (4,241,242,244,247,248). Neovascularization as a whole promotes tumour growth by supplying nutrients, oxygen and releasing growth factors that promote tumour cell proliferation (4,232,239,244,249,250). Hypoxia in solid tumours occurs at a distance of $\geq$ 70 μm from functional blood vessels and tumours do not exceed a volume of 1-2 mm^3 without induction of angiogenesis (4,250). The onset of angiogenesis precedes an exponential phase of tumour growth and local organ invasion. The velocity of angiogenic capillary growth ranges from 0.223 to 0.8 mm/day (248,251). During this expansion, cancer cells grow as a cuff around each new microvessel with a thickness of 50-200 μm. In this configuration, one endothelial cell supports the metabolic needs of 5-100 cancer cells (248,252). Eventually, invading blood vessels occupy 1.5% of the tumour volume (248).

Intratumoural vasculature density is associated directly with cancer cell entrance into the systemic blood circulation, with the ability of cancer cells to invade locally normal anatomic structures and metastasize in distant organs (4,240).

VEGF, a key mediator of angiogenesis, is overexpressed in many tumour types, and has been associated with poor prognosis (233,253), although the role of angiogenesis as a prognostic factor remains controversial (4,254,255). An association between increased angiogenesis and an increased incidence of metastases and a subsequent decrease in survival curve rates was observed for the vast majority of solid tumours (2,4,12,240,244,249).

Several studies revealed that high angiogenic activity in CRC was correlated with aggressive histopathological features such as: parietal invasion, tumour stage, tumour differentiation, metastatic potential and poor patient survival (1,4,254,256). This data were confirmed by Tanigawa et al. (249) that also have document a inverse relationship between tumour vascularity and patient survival.

Gurzu et al. (254) added that augmented angiogenesis in CRC was higher in early-stages of tumour proliferation but was not a progressively increasing process, having rather an oscillating character. However, other studies revealed that angiogenesis does not provide any significant information (4,231,232,254). These controversial findings may be credited to the lack of standardization of the different methods of counting tumour blood vessels and to the different cut-offs used to define relevant parameters to consolidate the results and, lastly, to the different antibodies used (4,231,232,254). Despite the debates, assessment of tumour angiogenesis may be particularly useful in prognostic classification of patients with apparent early cancer by conventional tumour staging, although some may still develop early recurrence or metastasis (4,232). De Vita et al. (240) observed that highly angiogenic tumours were associated with the presence of lymph node invasion. Nevertheless, a higher percentage of patients with node-positive colon cancer than those without will experience recurrence and might benefit from anti-angiogenic adjuvant therapy. Thus, angiogenesis can be used to identify a subset of patients at high risk for recurrence regardless of their lymph node involvement (249) and so the most important clinical implication of tumour angiogenesis is probably the development of anti-angiogenic therapy (4,232).

1.5.1 The Vascular Endothelial growth Factor Family

In mammals, VEGF family consists of VEGF-A, B, C, D (**Figure 8**) and placental growth factor 1 and 2 (PlGF1 and 2).

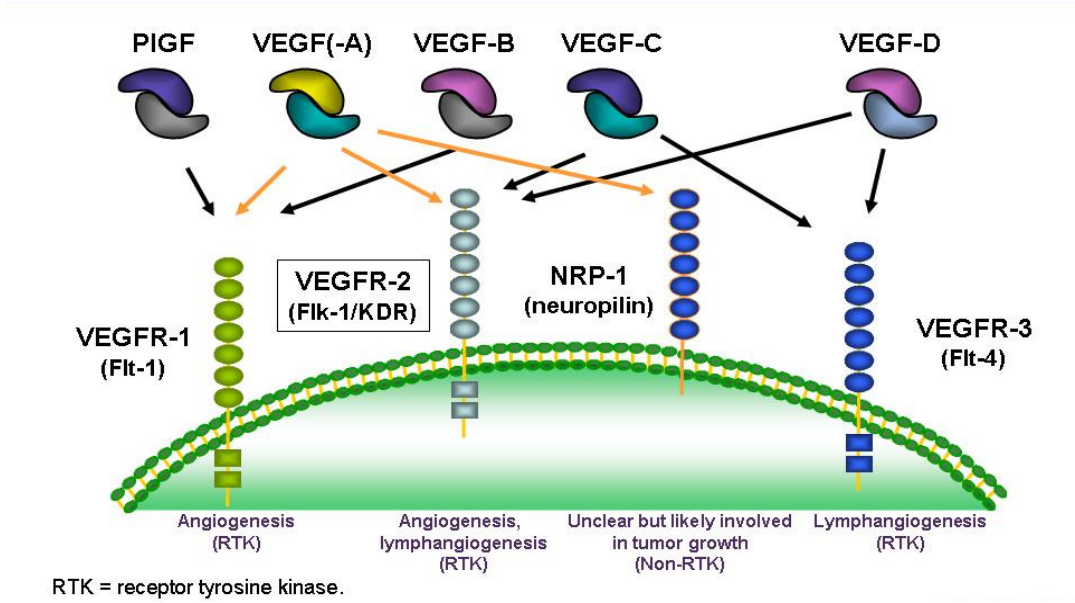


Figure 8: VEGF Family and their Receptors (257).

VEGF-A, is a key inducer of tumour angiogenesis (234,258,259), it belongs to a subfamily of secreted, dimeric glycoproteins of approximately 40 kDa, which in turn belongs to the PDGF superfamily (259–261). VEGF-A exists as multiple isoforms; $VEGF_{121}$, $VEGF_{145}$, $VEGF_{162}$, $VEGF_{165}$, $VEGF_{165b}$, VEGF183, $VEGF_{189}$ and $VEGF_{206}$, that results from alternative splicing (259,260,262,263). The isoforms $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ are preferentially expressed in VEGF producing cells (264), being $VEGF_{165}$ the most predominant isoform (259–261,265) and represents the major angiogenic form (261). $VEGF_{121}$ is readily diffusible but apparently has no important physiological role and $VEGF_{189}$ is tightly matrix-bound due to interactions with heparin sulfate proteoglycans (261).

VEGF-B, which is similar to VEGF-A in its amino acid sequence (approximately 43% identical), is mitogenic for endothelial cells and can form heterodimers with VEGF-A, being involved in angiogenesis in muscle and heart (266).

VEGF-C and **VEGF-D** affect primarily the development of the lymphatic vasculature and PlGF is primarily expressed in the placenta and its levels are inversely correlated with preeclampsia, but it is also detected in non negligible amounts in the heart and lungs (267–270).

All VEGF molecules/ligands transduce their signal through their binding to VEGF receptor -1, -2 and -3 on vascular endothelial cells (**Figure 8**). This distribution on endothelial cells accounts for the selectivity and specificity of action of VEGF. The three VEGF receptors are related to the PDGFR (α and β), the FGF receptors (1–4), the stem cell factor receptor (Kit), the Flt ligand receptor (Flt-3), and the colony stimulating factor-1 receptor (CSF-1R), all of which contain extracellular immunoglobulin domains and a kinase insert (271).

VEGFR-1 plays a negative role in angiogenesis in the embryo, most likely by trapping VEGF, but a positive role in adulthood. VEGFR-1 is expressed not only in endothelial cells but also in macrophage-lineage cells, and promotes tumour growth, metastases, and inflammation (272). Activation of VEGFR-1 is implicated in the increased expression of urokinase type of plasminogen activator and plasminogen activator inhibitor-1 in endothelial cells, that plays a role in extracellular matrix degradation and cell migration (271), although no direct proliferative or cytoskeletal effects was recognized (271,273).

VEGFR-2 is the key molecule for VEGF signaling in tumour micro-environment, as several cascades emanating from the VEGF/VEGFR-2 complex regulate critical angiogenic responses of endothelial cells (259), namely proliferative and increase of vascular permeability (259,260).

VEGFR-3 plays a key role in remodeling the primary capillary plexus in the embryo and contributes to angiogenesis and lymphangiogenesis in the adult. This receptor occurs in embryonic vascular endothelial cells but is restricted to lymphatic vessels in the adult (271,274). Inactivating mutations in the catalytic loop of the kinase domain of VEGFR-3 lead to a human hereditary lymphedema, the Milroy's disease, that is characterized by a chronic and disfiguring swelling of the extremities owing to defective cutaneous lymphatic vessels (271).

1.5.2 ANTIANGIOGENIC THERAPY

As previously mentioned one important clinical implication of tumour angiogenesis is probably the development of anti-angiogenic therapy (4,232). The participation of angiogenesis in

the pathogenesis of neoplastic disease has been described in many papers (275–278); the presence of VEGF has been found in cancers of the thyroid (279,280), bronchus, stomach, colon, breast, ovary, kidney, and urinary bladder (280). VEGF mRNA expression has been demonstrated in malignant tumours of the brain, esophagus, stomach, CRC, liver, breast, ovary, kidney, and urinary bladder (281,282). High VEGF concentrations in the blood have been found in patients with esophageal cancer (283), CRC, breast cancer (284), ovary (285), uterus (286), bone (287), and hormone-resistant prostate cancer (288). Also, several studies reports the connections between the degree of VEGF expression with tumour aggression and prognosis in patients with cancer of the uterus, ovary (289), breast (289,290), stomach (291), melanoma (292), head and neck neoplasms (289), and small cell lung cancer (290). Similarly, high VEGF expression coexists with worse survival time and an increased probability of recurrence of malignant CRC and kidney neoplasms (289).

Antiangiogenic therapy is based on: (a) inhibitory effects of proangiogenic ligands and their receptors; (b) Stimulation or delivery of angiogenesis inhibitors; and (c) direct destruction of neoplastic tumour vasculature (275) (**Figure 9**).

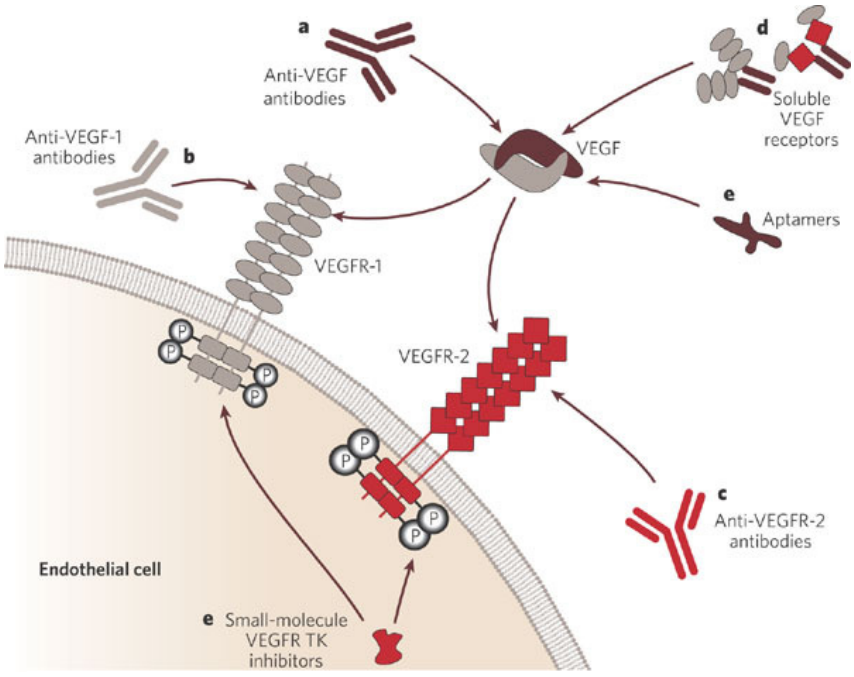


Figure 9: Strategies to inhibit VEGF signaling (293).
These include monoclonal antibodies targeting VEGF-A (a) or the VEGF receptors (b, c). Chimaeric soluble receptors such as the 'VEGF-trap' (domain 2 of VEGFR-1 and domain 3 of VEGFR-2 fused to a Fc fragment of an antibody) (d). Additional extracellular inhibitors are aptamers (e) that bind the heparin-binding domain of VEGF165. Additional strategies to inhibit VEGF signaling include antisense and siRNA targeting VEGF-A or its receptors.

Practical applications of monoclonal antibodies anti-VEGF (bevacizumab, ranibizumab) have already been found, for example in CRC patients with hepatic metastases (275,294,295). Through the development of anti-angiogenic therapy, CRC prognosis is improving (4,232,296–298), the median survival of patients with metastatic CRC (mCRC) is approximately 6 months. Palliative chemotherapy considerably improves treatment outcome, with 5-fluorouracil plus irinotecan and/or oxaliplatin extending median overall survival to approximately 20 months (4,299). Thus, in the past decade, the median overall survival of patients with mCRC has increased from 12 months to approximately 20 months mainly due to the development of new combinations with standard chemotherapy (4,300). Currently, anti-angiogenic treatment can prolong the survival time by some months, however, the results are not reproducible for all cases (4,254). There have been clinical trials that show as many as 94% of invasive carcinomas and 88% of *in situ* carcinomas having a complete response (4,301). Unfortunately, there are no tumour characteristics or molecular markers, at present, that help to identify patients who are likely to benefit from anti-angiogenic treatment (4,302).

Bevacizumab (BV) is a monoclonal antibody against VEGF with anti-angiogenic properties, and several clinical trials supported the use of BV in the first-line treatment of mCRC (4,303,304).

BV is typically used in combination with other chemotherapeutic agents such as oxaliplatin, irinotecan, leucovorin, and 5-fluorouracil (5-FU) for treatment of patients with mCRC (4,303,305). In addition to its direct anti-angiogenic effects, BV may also improve the delivery of chemotherapy by changing tumour vasculature and decreasing the elevated interstitial pressure in tumours (4,302). When combined with standard chemotherapy regimens, it has been associated with significant improvements, compared with chemotherapy alone, in the efficacy end points of overall survival, progression-free survival, and response rates in patients with mCRC, and for some facilitates secondary resections (4,306). Jubb et al. (307) demonstrated that in patients with mCRC, the addition of BV to irinotecan, 5-FU/leucovorin (IFL) improves survival regardless of the level of VEGF expression. The addition of BV to IFL resulted in a statistically significant increase in median overall survival of 4.7 months, and in a median progression-free survival of 4.4 months (308).

BV ultimately achieved “Food and Drug Administration” (FDA) approval in 2004 as a first-line treatment for mCRC in combination with chemotherapy, based on its statistically and clinically

meaningful benefits on progression-free survival and overall survival (309).

Ranibizumab (which binds to and inhibits a number of subtypes of VEGF-A) received FDA approval in 2006 for the treatment of diabetic macular oedema (310).

Apart from monoclonal antibody, antagonists of VEGF receptors have been used with great success in regulating the angiogenic process, as they are administered orally they present a better patient treatment compliance (310). Sunitinib is an orally active antagonist of VEGFR-1, PDGFR and c-Kit, received FDA approval in 2006 for treatment of renal cell advanced carcinoma and Gastrointestinal stroma tumours resistant to imatinib (310) and Vandetanib is an orally active antagonist of VEGFR-2, epidermal growth factor receptor (EGFR or HER1 or ErbB1) and RET kinase, and is available for the treatment of metastatic medullary thyroid cancer (275).

CRC is a major public health problem; on the one hand it presents a high incidence and prevalence, on the other hand the elevated cost associated with diagnostic and treatment measures. Despite recent advances in both, earlier diagnosis and treatment options, that resulted in a reduction of CRC mortality, this remains considerably.

Nowadays research in CRC has turned to the attempt to identify new biological markers that can be used as potential therapeutic targets that selectively operate in cancers cells and that along with TNM staging system, can be used to identify subgroups of patients that will have a worse prognostic and so offer those more aggressive therapeutics and follow-up measures. The assessment of metabolic and angiogenic markers fulfil these two goals, so with this work we intend to identify the prevalence of selected Metabolic and Angiogenenic markers of Colorectal Cancer and determine possible associations with clinicopathological characteristics and impact on prognosis by:

- Elaborating a clinicopathological data base of patients with CRC diagnosis treated at Braga Hospital between 1 January 2005 and 1 January 2010.

- Evaluating the role of MCTs in the carcinogenesis of CRC by assessing the immunohistochemical expression of the MCT isoforms 1 and 4, chaperones CD147 and CD44 and glycolytic metabolic marker GLUT1.

- Investigating the role of VEGF family in the carcinogenesis of CRC by assessing the immunohistochemical expression of VEGF-A, VEGF-B, VEGFR-2 and VEGFR-3.

- Correlating the expression of the protein markers with clinicopathological parameters.

The beginning of the development of this thesis coincided with the creation of the Coloproctology Unit of Braga Hospital, responsible, among others diseases, by the treatment of patients with diagnosis of colorectal cancer.

In order to standardize the diagnosis, staging, treatment and follow-up of these patients we have elaborated several protocols that were discussed with the Oncology team and approved by the "Conselho de Administração of Braga Hospital". (approved protocols are in appendix 1-8:"Protocolo de estudo de Cancro do Colon"; "Protocolo de estudo de Cancro do Recto"; "Protocolo de Registo de Cancro Colorectal"; "Protocolo Terapêutico de Cancro Recto"; "Protocolo de Follow-up de Cancro Colorectal"; "Protocolo de Registo de recidiva de Cancro Colorectal"; "Protocolo de Antibioprofilaxia para Cirurgia Colorectal" and "Protocolo de Processamento da peça operatória").

Most patients (except emergent cases) were discussed preoperatively by a multidisciplinary team involving surgeons, oncologists and sometimes a pathologist.

3.1 EPIDEMIOLOGICAL DATA

We conducted an observational, prospective and descriptive study between 1 January 2005 and 1 January 2010. The population covered consisted in all patients with histological CRC diagnosis, treated at Braga Hospital.

Data from 672 patients, with CRC diagnosis, were collected prospectively in two excel databases – Colon Cancer and Rectal Cancer – data regarding clinical, preoperative diagnostic examinations, operative reports by the surgeons, histopathological and follow-up were collected.

Clinical and preoperative diagnostic examinations included: age, gender, past oncologic history, clinical presentation, tumour localization, tumour mobility (for rectal cancer), histological type, macroscopic appearance, carcinoembryonic antigen (CEA) level and preoperative staging.

Tumour localization was recorded and classified as right sided (caecum, ascending colon, hepatic flexure and transverse colon), left sided (splenic flexure, descending colon, sigmoid colon) and rectum (between anal verge and 15 cm at rigid rectoscopy). Rectal cancer localization was subdivided as superior, middle and lower third (≤15 and > 10 cm; ≤10 and > 5 cm and ≤ 5cm from anal verge, respectively). Except for emergent cases (defined as a surgery performed for obstruction

or perforation of the colon or rectum) all patients were preoperatively staged for local and distant metastases by chest x-ray and abdominal CT in colon cancer, and toraco-abdominal CT, pelvic magnetic resonance and rectal ultrasonography in rectal cancer.

Operative reports by surgeons like presence of perforation, tumour mobility and type of surgery were also collected. All patients received antibiotic and thrombosis prophylaxis and all operations were performed by or under supervision of a senior surgeon.

The histopathological reports included: tumour extent (T), extent of spread to lymph nodes (N), presence of distant metastasis (M), tumour differentiation, resection margin involvement and lymphatic and blood vessel invasion. The level of positive lymph nodes was not described in all specimens. The histological type of CRC was determined by two experienced pathologists and tumour staging was graded according to TNM classification, sixth edition (311).

All patients were followed up periodically, and their outcomes were investigated.

All cases in this study were identified using a series of unified Code and the study protocol was approved by the Ethics Committee of Braga Hospital. All patients provided written consent.

3.2 Tumour Block selection

At Pathology Department of Braga Hospital, we proceeded to the selection of the surgical specimens blocks of the patients submitted to colorectal cancer resection, ideally with “tumour” and “normal adjacent epithelium” in the same block. This block selection was confirmed by a pathologist and corresponded to 580 cases of the 672 patients with CRC diagnosis, since there were patients who did not undergo surgical intervention, patients that have been operated in other institutions and patients for who was not possible to retrieve the paraffin block. Another series of 45 patients with histological diagnosis of CRC Hepatic Metastasis operated between 1 January 2003 and 1 de January 2011 was also collected.

3.3 Hematoxylin-Eosin Staining slides preparation

After tumour block selection, Hematoxylin-eosin (HE) slides of all cases, CRC and Hepatic metastasis, were made at "Life and Health Sciences Research Institute" (ICVS) laboratory. In all this slides we proceed to the selection of "tumour" and "normal adjacent epithelium" in both series. This selection was confirmed by a pathologist.

3.4 Construction of Tissue microarrays

In CRC series, after identification, in HE slides, of "tumour" and "normal adjacent epithelium", new slides with 80 cases of "tumour" and "normal adjacent epithelium" were made at ICVS laboratory. In the Tissue Microarray (TMAs) technique, a hollow needle is used to remove tissue cores as small as 0.6 mm in diameter from regions of interest in paraffin-embedded tissues. These tissue cores are then inserted in a recipient paraffin block in a precisely spaced, array pattern. Sections from this block are cut using a microtome, mounted on a microscope slide.

3.5 Immunohistochemistry

In CRC series, TMA protein expression of metabolic markers (MCT1, MCT4, CD147, CD44 and GLUT1) and angiogenic markers (VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3) was evaluated by immunohistochemistry.

In Colorectal cancer Hepatic Metastasis series, protein expression of metabolic markers (MCT4, CD147, CD44 and GLUT1) was evaluated by immunohistochemistry. MCT1 immunohistochemical reaction was not performed, in this series, due to problems with the "detection system".

Detailed information is given in **Table III and IV**. Briefly, after deparaffinization and rehydration, 4μm cytoblock sections were immersed in 0.01M citrate buffer (pH 6.0) and heated at 98 °C for 20 minutes for epitope antigen retrieval. Subsequently, endogenous peroxidase was

blocked with 0.3% hydrogen peroxide in methanol. Slides were then incubated with the respective primary antibodies and 3,3'-diamino-benzidine (DAB+ Substrate System, Dako) was used for detection. Cytoblock sections were counterstained with haematoxylin and permanently mounted. Negative controls were obtained by omitting the primary antibody incubation step.

After immunohistochemical procedure, the slides were evaluated.

Table III: Detailed aspects of the immunohistochemical procedure used to visualize the different metabolic proteins.

Protein Marker	Antigen retrieval	Positive Control	Peroxidase inactivation	Detection system	Antibody		
					Company	**Dilution**	**Incubation period**
MCT1	Citrate buffer 10mM pH=6.0	Colon carcinoma	0.3% H_2O_2 in methanol, 30 min.	R.T.U. VECTORSTAIN ® Elite® ABC Kit	CHEMICON	1:300	Overnight
MCT4	Citrate buffer 10mM pH=6.0	Colon carcinoma	0.3% H_2O_2 in methanol, 30 min.	R.T.U. VECTORSTAIN ® Elite® ABC Kit	Santa Cruz Biotechnology	1:200	Overnight
CD147	EDTA 1mM pH=8	Colon carcinoma	3% H_2O_2 in methanol, 10 min.	LabVision	Zymed	1:500	2 hours
CD44	Citrate buffer 10mM pH=6.0	Duodenum	3% H_2O_2 in methanol, 10 min.	LabVision	Serotec	1:400	2 hours
GLUT-1	Citrate buffer 10mM pH=6.0	Skin	3% H_2O_2 in methanol, 10 min.	LabVision	Abcam	1:500	2 hours

Table IV: Detailed aspects of the immunohistochemical procedure used to visualize the different angiogenic proteins.

Protein Marker	Antigen retrieval	Positive Control	Peroxidase inactivation	Detection system	Antibody		
					Company	**Dilution**	**Incubation period**
VEGF-A	EDTA Buffer 1X pH=8.0	Tonsil	3% H_2O_2 in methanol, 10 min.	LabVision	Abcam	1:100	Overnight
VEGF-C	EDTA Buffer 1X pH=8.0	Tonsil	3% H_2O_2 in methanol, 10 min.	LabVision	Invitrogen	1:200	Overnight
VEGFR-2	Citrate Buffer 0.01M pH=6.0	Tonsil	3% H_2O_2 in methanol, 10 min.	LabVision	Abcam	1:100	Overnight
VEGFR-3	Citrate Buffer 0.01M pH=6.0	Tonsil	3% H_2O_2 in methanol, 10 min.	LabVision	Abcam	1:100	Overnight

3.6 IMMUNOHISTOCHEMICAL EVALUATION

Sections were evaluated for immunoreaction, which included both cytoplasmic and membrane-positive staining. MCT1, MCT4, CD147, CD44, GLUT, VEGF-A, VEGF-B, VEGFR-2 and VEGFR-3 immunohistochemical reactions were scored semi-quantitatively for immunoreaction extension as follows (**Table V)**: 0: 0% of immunoreactive cells; 1: <5% of immunoreactive cells; 2: 5–50% of immunoreactive cells; and 3: >50% of immunoreactive cells. Also, intensity of staining was scored semi-qualitatively as 0: negative; 1: weak; 2: moderate; and 3: strong. Immunoreaction final score was defined as the sum of both parameters (extension and intensity), and grouped as negative (0), weak (2), moderate (3), and strong (4–6). For statistical purposes, only moderate and strong immunoreaction final scores were considered as positive. Positive plasma membrane staining was also assessed. Immunohistochemical expression evaluation was performed blindly by two independent observers and discordant cases were discussed in order to determine a final score.

Table V: Criteria for evaluation immunoreaction depth and the intensity staining.

Extension		Intensity	
Scored	**Immunoreactive cells (%)**	**Scored**	**Staining**
0	0%	0	Negative
1	<5%	1	Weak
2	5 a 50%	2	Moderate
3	>50%	3	Strong

3.7 Statistical Analysis

All data were collected and stored in an Excel PC database and statistically analyzed using the Statistical Package for the Social Sciences, version 19.0 (SPSS Inc., Chicago, Illinois, USA). All comparisons were examined for statistical significance using Pearson's chi-square ($\chi 2$) test and Fisher's exact test (when $n < 5$), with the threshold for significance P values <0.05.

Overall survival (OS) was defined as time from disease diagnosis until death from any cause and *Survival free disease (DFS)* was defined as time from disease diagnosis until disease relapse, both were assessed using the Kaplan-Meier method.

4.1 Epidemiological Characterization

Data from 672 patients treated between 1 January 2005 and 1 January 2010 at Braga Hospital, with CRC diagnosis was collected prospectively in two excel databases – Colon Cancer and Rectal Cancer. Clinical, preoperative diagnostic examinations, operative reports by the surgeons, histopathological and follow-up data were collected.

4.1.1 General characterization

4.1.1.1 Age and Gender

The casuistic included 672 patients, 419 (62.4%) males and 253 (37.6%) females; the age range of most patients (61%) was 61-80 years old, 20.4% (n=137) 41-60 years old; 16.1% (n=108) older than 81 and 2.5% (n=17) younger than 40 years old (**Figure 10**). Except for the group older than 81 years old, CRC incidence was more frequent in men.

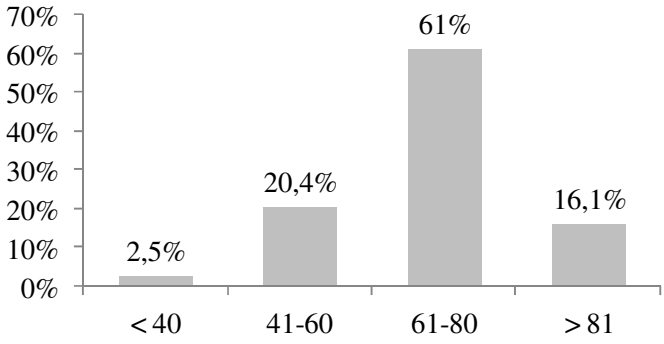


Figure 10: Age distribution of CRC patients.

4.1.1.2 Anatomic distribution of tumours

Among the 672 patients, 439 tumours (65.3%) arouse from colon and 233 (34.7%) from rectum (**Figure 11**).

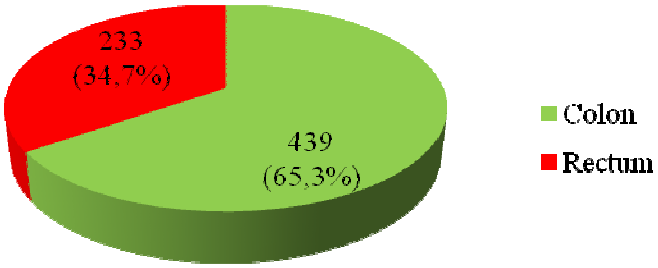


Figure 11: Anatomic distribution of CRC.

4.1.1.3 PAST PERSONAL AND FAMILIAR HISTORY

In patients with colon and rectum cancer, n=672, analysis of past personal history of presence of polyps, colorectal or other cancers and familiar CRC history showed that 94.8% (n=637) of patients had no history of previous colorectal polyps; from the patients with polyps, 4.3% (n=29) were tubular, 0.4% (n=3) adenomatosos, 0.3% (n=2) tubulo-viloso and 1 was non-classified.

From overall patients, 4.1% (n= 28) had previous personal history of CRC and 7.7 % (n=52) had personal history of other cancer. 9.7% (n=65) had a positive CRC familial story.

4.1.1.4 CLINICAL PRESENTATION

Most of patients, 81.3 % (n=546), with CRC were symptomatic at diagnosis, the remainder 18.8% (n=126) were asymptomatic and detected by routine colonoscopies (**Figure 12**). From the symptomatic patients, 82.1% (n= 450) of patients presented symptoms 6 months prior to colonoscopy and 14.6% (n=98) symptoms beyond 6 months.

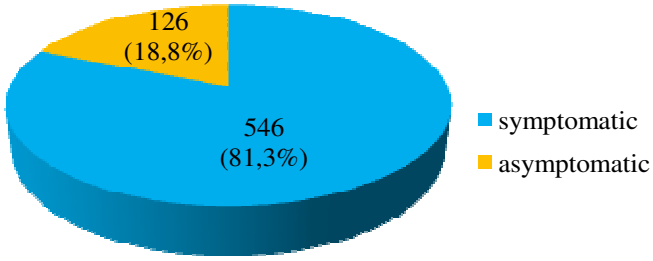


Figure 12: CRC presentation at diagnosis.

4.1.2 COLON CANCER

4.1.2.1 CLINICAL AND PREOPERATIVE DIAGNOSTIC AND STAGING EXAMINATION

4.1.2.1.1 CLINICAL PRESENTATION

Most colon cancer patients (77.4%, n=340 patients), were symptomatic at diagnosis. The most frequent symptom was digestive bleeding, 17.1% (n= 75), followed by large bowel obstruction, 15% (n= 66). Other frequent symptoms observed were: change in bowel habits (8.9%), change in bowel habits with digestive bleeding (8.6%), constitutional symptoms (6.6%), change in bowel habits with abdominal pain (6.4%) and abdominal pain (4.8%) (**Table VI**).

Table VI: Summary of colon cancer symptoms.

Symptom	n (%)
Digestive bleeding	75 (17.1)
Large bowel obstruction	66 (15.0)
Change in bowel habit	39 (8.9)
Digestive bleeding + changes in bowel habit	38 (8.6)
Constitutional symptoms	29 (6.6)
Abdominal pain + changes in bowel habit	28 (6.4)
Study (ascites; anemia, deep venous thrombosis, hepatic metastasis; occult blood losses, colonvesical fistula)	23 (5.2)
Abdominal pain	21 (4.8)
Parcial large bowel obstruction	13 (3.0)
Large bowel perfuration	8 (1.8)

4.1.2.1.2 LOCALIZATION

Most cancers were left-colon, 64.7% (n=284): 6.8% (n=30) were in the splenic flexure; 4.3% (n=19) in the descending colon, 49.2% (n=216) in the sigmoid colon, and 4.3% (n=19) in the rectosigmoid transition. Right-sided tumours comprised 35.3% (n=155) of patients: 8.4 % (n=37) were localized in the caecum, 8.2% (n=36) in the ascending colon and 13.7% (n=60) in the hepatic flexure. 5.0% (n=22) of cancers were localized in the transverse colon.

4.1.2.1.3 DIAGNOSIS AND STAGING

Imaging diagnosis was made by total colonoscopy in 76.1% (n=334) of cases and rectosigmoidoscopy in 13% (n=57). In 10.9% (n=48), diagnosis was made by other imagiological exams and patients did not have a preoperative colonoscopy.

Most lesions (47.2 %, n=207) were polypoid/vegetant cancers. The remaining 21.0% (n=92) were ulcerated, 8.4% (n=37) infiltrative and 11.2% (n=49) exofitic cancers (**Figure 13**). In 54 patients (12.3%) there was no cancer macroscopic appearance information. In 19.1% (n=84) of patients, synchronous lesions were observed.

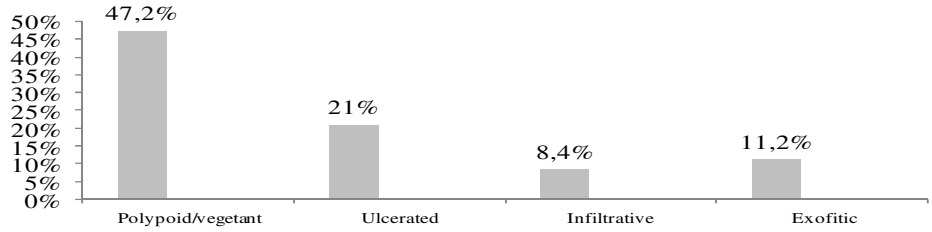


Figure 13: Frequency of macroscopic colon cancer appearance.

Pre-operative colon biopsy revealed colon adenocarcinoma in 83.8% (n=368) of the patients, 3.9% (n=17) there was no preoperatory information. 85.7% (n=376) of patients were staged by computerized axial tomography and most patients (79.1%; n=347) with colon cancer had a localized cancer at diagnosis. Most patients with disseminated disease had hepatic metastasis, followed by lymph node metastasis (**Table VII**).

Table VII: Summary of colon cancer metastasis localization.

Metastasis	n (%)
Lymph node	24 (5.5)
Lymph node + Hepatic	7 (1.6)
Lymph node + Hepatic + pulmonary	3 (0. 7)
Hepatic	44 (10.0)
Hepatic + pulmonary	4 (0.9)
Hepatic + pulmonary + bone	3 (0.7)
Hepatic +spleen+ bone	1 (0.2)
Pulmonary	3 (0.7)
Peritoneal	3 (0.7)

4.1.2.2 OPERATIVE REPORTS BY SURGEONS

Of the 439 patients with colon cancer diagnosis, 422 (96.1%) were submitted to surgical treatment in this period; 334 (79.1%) and 88 (20.9%) were submitted to a scheduled and urgent surgery, respectively. At exploration, 32 patients (7.6%) presented tumour perforation, including not only the patients with clinical perforation, but also the patients with buffered tumour perforation and iatrogenic perforation during surgery.

Also at surgical exploration, 347 (82.2%) had a mobile tumour, 65 (17.8%) a fixed tumour and no information was available for 10 patients.

4.1.2.3 HISTOPATHOLOGICAL REPORTS

Histopathological reports were determined by two experienced pathologists at the Pathology Department of Braga Hospital.

4.1.2.3.1 TUMOUR SIZE

Most patients, 207 patients (49.0%), presented with tumours smaller than or equal to 4.5 cm, 165 (39.0%) patients presented with tumour bigger than 4.5 cm and in the remainder no size information was referred.

4.1.2.3.2 MACROSCOPIC SEROSAL INVOLVEMENT

Macroscopic serosal involvement was observed in 295 patients (69.9%). In 103 (24.4%) this was not observed and not referred in the remainder 24 patients.

4.1.2.3.3 TUMOUR DIFFERENTIATION

Most patients, 172 (40.8%), presented a moderately-differentiated tumour, followed by well and poorly-diferentiated tumour (168 and 41 patients, respectively). 1 patient presented an undifferentiated tumour (**Figure 14**).

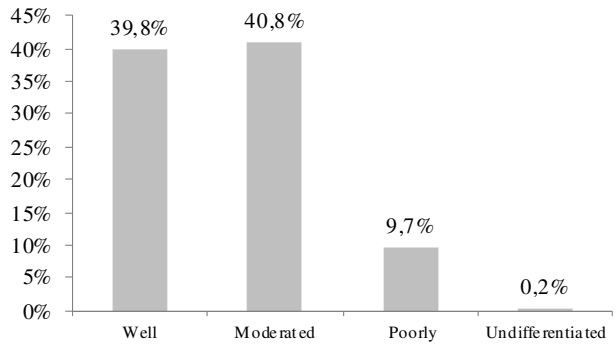


Figure 14: Distribuition of colon cancer differentiation.

4.1.2.3.4 RESSECTION MARGINS INVOLVEMENT

Ressection Margins examination did not reveal involvement in 392 patients, this was observed in 6 patients and in the remainder 24 this was not mentioned.

4.1.2.3.5 VASCULAR INVASION

Although no specific marker of lymphatic or hematogeneous vessels was been used, it was documented that 229 (54.2%) patients had venous vessel invasion and 166 (39.3%) lymphatic vessel invasion. In 156 (36.9%) and 209 (49.5%) patients, respectively, no invasion was documented and in the remainder there was no information.

4.1.2.3.6 HISTOLOGICAL STAGING

Histological staging was determined by two experienced pathologists and tumour staging was graded according to the TNM classification, sixth edition (American Joint Commitence on Cancer) (311). In the majority of patients (33.7%; n=142) colon cancer was stage IIA, followed by stage IIIB (22.5%; n=95). In 7 patients post-operative histological stage was not determined because the patients underwent surgery without resection (ex. derivative colostomy) (**Table VIII**).

4.1.2.4 FOLLOW-UP

A total of 137 patients (31.2%) died from all causes, 27.8% (122 patients) had a colorectal cancer-related cause and the remaining 3.4% (15 patients) died in the post-operative period (mortality within 30 days of surgery). Follow-up time ranged between 2 and 7 years; 14.6% (62 patients) had recurrence during follow-up. Stage IIIB was the stage most frequently associated with tumour recurrence (**Table IX**).

Table VIII: Summary of colon cancer histological staging.

Stage	n (%)
0	9 (2.1)
I	55 (13.0)
IIA	142 (33.7)
IIB	11 (2.6)
IIIA	6 (1.4)
IIIB	95 (22.5)
IIIC	18 (4.3)
IV	79 (18.7)

Table IX: Summary of histopathogical tumour staging of colon cancer recurrence.

Stage	n (%)
I	1 (1.6)
IIA	12 (19.4)
IIB	6 (9.7)
IIIA	1 (1.6)
IIIB	22 (35.4)
IIIC	4 (6.5)
IV	16 (25.8)

Most metastasis occurred in liver, followed by lymph node and lung. Local recurrence occurred in nine cases (**Table X**).

Most patients wih metastasis and recurrence were asymptomatic (79.0%; n=49), of that 29.0% (n=18) of patients presented asymptomatic elevation of tumour markers. The remaining

cases were patients with abdominal pain (4.8%; n=3), abdominal mass (4.8%; n=3), intestinal obstruction (3.2%; n=2), bone pain (3.2%; n=2), supraclavicular mass (1.6%; n=1), enterocutaneous fistula (1.6%; n=1) and pathological fracture (1.6%; n=1) (**Table XI**).

Table X: Summary of colon cancer metastasis localization and recurrence.

Metastasis localization and Recurrence	n (%)
Hepatic	32 (51.6)
Local recurrence*	9 (14.5)
Lymph node	5 (8.1)
Pulmonary	5 (8.1)
Peritoneal carcinomatosis	4 (6.5)
Hepatic + Pulmonary	3 (4.8)
Hepatic + Pulmonar + Peritoneal carcinomatosis	1 (1.6)
Hepatic + adrenal glands	1 (1.6)
Hepatic + Peritoneal carcinomatosis	1 (1.6)
Bone	1 (1.6)

*Local recurrence refers to anastomotic, para-anastomotic and abdominal mass

Table XI: Summary of symptoms/signs in colon cancer metastasis and recurrence.

Metastasis and Recurrence Colon Cancer symptoms/signs	n (%)
Abdominal mass	3 (4.8)
Abdominal pain	3 (4.8)
Intestinal obstruction	2 (3.2)
Bone pain	2 (3.2)
Supraclavicular mass	1 (1.6)
Pathological fracture	1 (1.6)
Enterocutaneous fistula	1 (1.6)

4.1.3 RECTAL CANCER

4.1.3.1 CLINICAL AND PREOPERATIVE DIAGNOSTIC AND STAGING EXAMINATION

4.1.3.1.1 CLINICAL PRESENTATION

Most rectal cancer patients (88.5%, n=206 patients), were symptomatic at diagnosis. 23% (n= 54) presented digestive bleeding, followed by digestive bleeding with change in bowel habits, 17.4% (n= 41). Other frequent symptoms observed were: change in bowel habits (14.5%; n= 34) and large bowel obstruction (4.7%; n= 11) (**Table XII**).

Table XII: Summary of rectal cancer symptoms.

Symptom	n (%)
Digestive bleeding	54 (23.0)
Digestive bleeding + change in bowel habit	41 (17.4)
Change in bowel habit	34 (14.5)
Large bowel obstruction	11 (4.7)
Incomplete stool evacuation sensation	11 (4.7)
Tenesmus	10 (4.2)
Tenesmus + Digestive bleeding	10 (4.2)
Tenesmus + changes in bowel habit	9 (3.8)
Abdominal pain	7 (3.0)
Constitutional symptoms	6 (2.6)
Abdominal pain + digestive bleeding	5 (2.1)
Study (hepatic metastasis, pelvic mass)	4 (1.7)
Large bowel perfuration	2 (0.9)
Urgency	1 (0.4)
Anal pain	1 (0.4)

4.1.3.1.2 LOCALIZATION

From the 233 rectal cancers, most (50.6%, n=118) were localized in the middle third, followed by distal rectum in 28.3% (n=66) and proximal rectum in 21% (n=49).

4.1.3.1.3 DIAGNOSIS AND STAGING

In rectal cancer patients, diagnosis was made by total colonoscopy in 79.8% (n=186) and rectosigmoidoscopy in 18.9% (n=44). In 1.3% (n=3) of cases, it was impossible to perform an endoscopic exam (rectal stenosis).

Most lesions (55.8%, n=130) were polypoid/vegetant cancers. The remaining 21.0% (n=49) were ulcerated, 10.7% (n=25) were infiltrative; 9.0% (n=21) exofitic cancers; 0.4% (n=1) were vilosous and for the reminder 7 patients (3%) there was no cancer macroscopic appearance information (**Figure 15**). Synchronous lesions were observed in 10.3% (n=24) of patients.

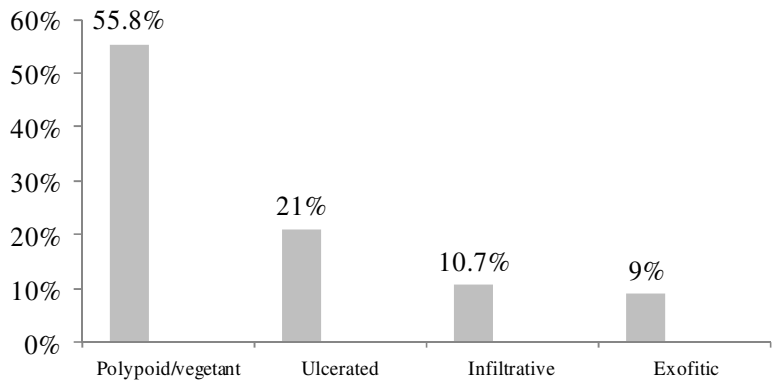


Figure 15: Frequency of macroscopic rectal cancer appearance.

Pre-operative biopsy revealed rectal adenocarcinoma in 91.4% (n=213) of the patients, invasive adenocarcinoma in 2.1% (n=5), adenomatosous dysplasic lesions in 4.7% (n=11); villous lesions in 1.3% (n=3) and mucinous adenocarcinoma in one patient (0.4%). From the 233 patients, 27.0% (n=63) had synchronic metastasis at diagnosis, more frequently lymph node and hepatic metastasis (**Table XIII**).

Table XIII: Summary of rectal cancer metastasis localization.

Metastasis	n (%)
Lymph node	24 (10.2)
Hepatic	20 (8.5)
Peritoneal	6 (2.6)
Pulmonary	4 (1.7)
Lymph node + Hepatic + pulmonary	4 (1.7)
Lymph node + pulmonary	2 (0.8)
Hepatic + pulmonary	1 (0.4)
Hepatic + pulmonary + adrenal	1 (0.4)
Bone	1 (0.4)

Pelvic magnetic resonance (MR) and rectal endoscopic ultrasound (EUS) were used in combination for local staging. After staging, 26% (61 patients) had indication for neoadjuvant therapy; 21% (49 patients) underwent chemotherapy and radiotherapy, the remaining had not done neoadjuvant therapy due to comorbidities (2 patients) or underwent chemotherapy or radiotherapy alone due to specific contra-indications (**Table XIV**).

Table XIV: Summary of neoadjuvant treatment.

Neoadjuvant Treatment	n (%)
None	172 (73.8)
With indication for neoadjuvant treatment but comorbilities	2 (0.9)
Neoadjuvant chemotherapy and radiotherapy	49 (21.0)
Neoadjuvant chemotherapy	8 (3.4)
Neoadjuvant radiotherapy	2 (0.9)

4.1.3.2 Operative reports by surgeons

From the 233 patients with rectal cancer diagnosis, 203 (87.1%) were submitted to surgical treatment in this period; 193 (95.1%) and 10 (4.9%) were submitted to a scheduled and urgent surgery, respectively. At exploration, 3 patients (1.5%) presented tumour perforation, including not only the patients with clinical perforation, but also the patients with buffered tumour perforation and iatrogenic tumour perforation during surgery. In 197 (97.0%) patients no perforation was documented and in 3 patients this data was not referred.

At surgery, mobility exploration was documented in 136 (66.9%) patients, 50 (24.6%) patients had a fixed tumour and in 17 patients this data was not referred.

4.1.3.3 Histopathological reports

4.1.3.3.1 Tumour size

Most patients, 107 patients (52.7%), presented tumours smaller than or equal to 4.5 cm, 48 (23.6%) patients presented tumours bigger than 4.5 cm and in the remainder 48 patients no size information was referred.

4.1.3.3.2 Macroscopic serosal involvement

From the patients examinated, 109 (53.7%) presented macroscopic serosal involvement and 70 (34.5%) without. No information was referred in the remainder 24 patients.

4.1.3.3.3 Tumour differentiation

Most patients, 80 (39.4%) presented a moderately-differentiated tumours, followed by well and poorly-diferentiated tumours (73 (36.0%) and 9 (4.4%) patients, respectively). 1.0% of patients (2 patients) presented undifferentiated tumours and in 40 patients this data was not mentioned (**Figure 16**).

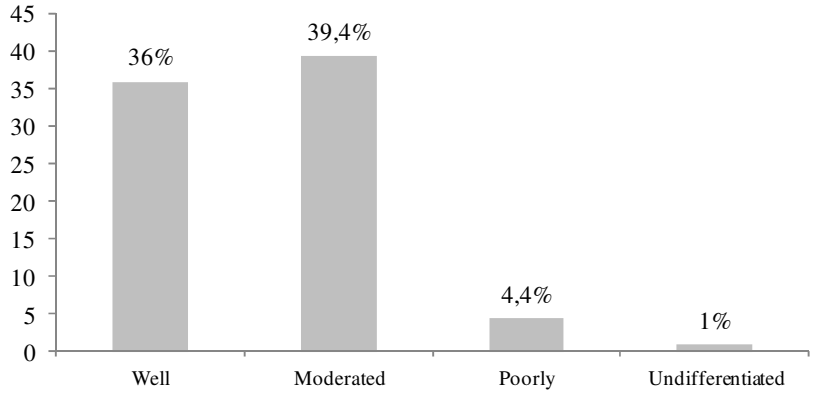


Figure 16: Distribution of rectal cancer differentiation.

4.1.3.3.4 RESSECTION MARGIN INVOLVEMENT

Ressection Margins examination did not reveal involvement in 155 patients (76.4%), this was observed in 20 patients (9.6%) and for the remainder 28 this data was not mentioned.

4.1.3.3.5 VASCULAR INVASION

As previously mentioned, despite no specific marker of lymphatic or hematogeneous vessels being used, it was documented that 113 (55.6%) patients had venous vessel invasion and 90 (44.3%) lymphatic vessel invasion. In 59 (29.0%) and 81 (39.9%), respectively, no invasion was documented and for the remainder patients no information was mentioned.

4.1.3.3.6 HISTOLOGICAL STAGING

Post-operative histological staging was determined by two experienced pathologists and tumour staging was graded according to the TNM classification, sixth edition (American Joint Commitence on Cancer) (311). Most patients with rectal cancer were stage IIA (21.2%) and stage I (18.7%), followed by stage IV (18.2% patients). In 8 patients, post-operative histological stage was not determined because the patients have realized surgery without resection (ex. derivative colostomy) (**Table XV**).

Table XV: Summary of rectal cancer histopathological staging.

Stage	n (%)
0	21 (10.3)
I	38 (18.7)
IIA	43 (21.2)
IIIA	12 (5.9)
IIIB	31 (15.3)
IIIC	13 (6.4)
IV	37 (18.2)

4.1.3.4 FOLLOW-UP

A total of 52 patients (22.3%) died from all causes, 28.0% (42 patients) had a colorectal cancer-related cause and the remaining 4.3% (10 patients) died in the post-operative period (mortality within 30 days of surgery).Follow-up time ranged from 2 to 7 years; 18.0% (42 patients) had recurrence during follow-up. Stage IV was the stage most often associated with tumour recurrence (**Table XVI**).

Table XVI: Summary of histopathological tumour staging of rectal cancer recurrence.

Stage	n (%)
I	4 (9.5)
IIA	12 (28.6)
IIIB	7 (16.7)
IIIC	3 (7.1)
IV	16 (38.1)

Most metastasis occurred in liver, followed by lung, while local recurrence occurred in 9 patients (**Table XVII**). Most patients with metastasis and recurrence (73.8%; n=31) were asymptomatic and 14.2% (n=6) of those presented with asymptomatic elevation of tumour markers. In the case of symptomatic patients, the most frequent symptoms/signs was a rectal mass (9.5%; n=4), and intestinal obstruction 4.7% (n=2) (**Table XVIII**).

Table XVII: Summary of rectal cancer metastasis localization and recurrence.

Metastasis localization and Recurrence	n (%)
Hepatic	17 (40.5)
Local recurrence	9 (21.3)
Pulmonary	5 (11.9)
Hepatic + Pulmonary	4 (9.5)
Carcinomatosis	1 (2.4)
Bone	1 (2.4)
Hepatic + Pulmonar + adrenal glands	1 (2.4)
Hepatic + Pulmonary + Bone	1 (2.4)
Hepatic + Pulmonary + Lymph node	1 (2.4)
Pulmonar and Bone	1 (2.4)
Hepatic + Lymph node	1 (2.4)

Table XVIII: Summary of symptoms/signs in rectal cancer metastasis and recurrence.

Metastasis and Recurrence Rectal Cancer symptoms/signs	n (%)
Rectal mass	4 (9.5)
Intestinal obstruction	2 (4.7)
Bone pain	1 (2.4)
Metrorrhagia	1 (2.4)
Anal pain	1 (2.4)
Pleural effusion	1 (2.4)
Rectal blood loss	1 (2.4)

4.1.4 COLORECTAL CANCER OVERALL SURVIVAL

Overall survival (OS) was defined as the time from disease diagnosis until death from any *cause and Survival free disease (DFS)* was defined as the time from disease diagnosis until disease relapse, both were assessed using the Kaplan-Meier method (**Figure 17 and 18**). When patients were divided into two groups by location, colon and rectum, no significant difference was found in the survival rate between the colon cancer group and rectal cancer group; assessed by log-rank test (**Figure 19**).

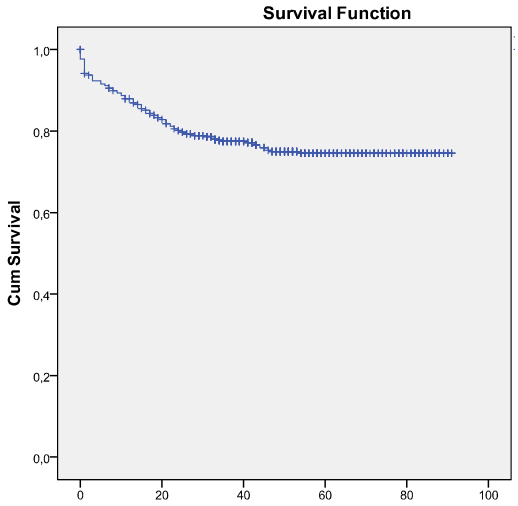


Figure 17: Kaplan-Meier curve depicting overall survival CRC curve.

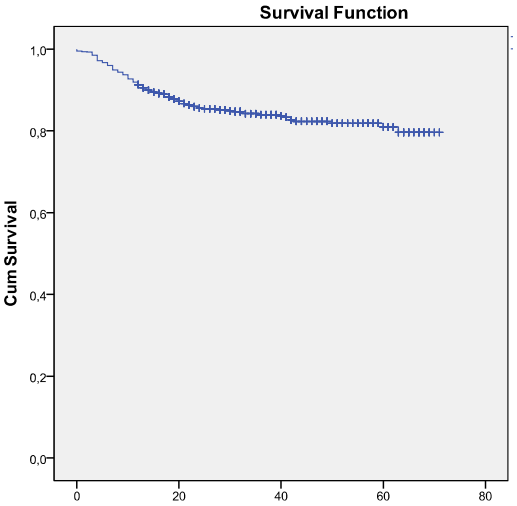
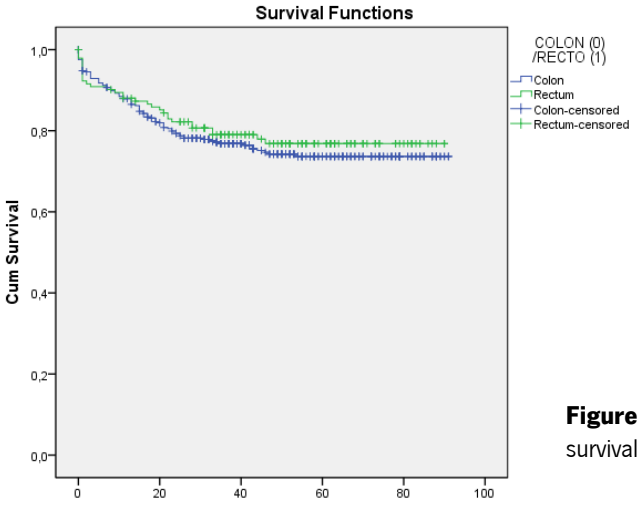


Figure 18: Kaplan-Meier curve depicting disease-free survival CRC curve.



p =0.518

Figure 19: Comparison between colon and rectum cancer survival assessed by log-rank test.

4.2 Analysis of the Associations of MCTs, Chaperones and Glycolytic Metabolic Markers in Primary Colorectal Cancers and Normal Adjacent Tissues

Our previous study analyzed the expressions of MCT1, 2, and 4 in a series of 126 CRC (109) and we reported that the expression of the MCT isoforms in tumour cells was significantly increased when compared to normal adjacent epithelium. Remarkably, there was a significant gain in membrane expression for MCT1 and MCT4 and loss of plasma membrane expression for MCT2 in tumour cells. However, the tumour series analyzed at that time was relatively small. To reinforce the results obtained, we evaluated MCT1, MCT4 immunohistochemical expression in this series of 580 cases, adding evaluation of immunohistochemical expression of the MCT chaperones CD147, CD44 and the glycolytic metabolic marker GLUT1, besides the advantage of the possibility of correlation with epidemiological patients' data. Sections were evaluated for immunoreaction, which included both cytoplasmic and membrane-positive staining.

4.2.1 MCT1, MCT4, CD147, CD44 and GLUT1 Immunohistochemical Expression in CRCs and Normal Adjacent Tissues

The results obtained are described in **Table XIX**, which summarizes the frequency of MCT isoforms 1 and 4, chaperones CD147 and CD44 and glycolytic metabolic marker GLUT1 expressions, in tumour and normal adjacent (NA) epithelium.

Figure 20 shows representative cases of MCT1, MCT4, CD147, CD44 and GLUT1 positive staining in tumour cells and in normal adjacent epithelium.

Table XIX: Pattern of protein staining in CRC vs. normal adjacent epithelium.

Protein		***Immunoreaction***		***Plasma membrane***	
	n	Positive (%)	*p*	Positive (%)	*p*
MCT1			**<0.001**		**<0.001**
NA	135	106 (78.5%)		104 (77.0%)	
Tumour	501	469 (93.6%)		464 (92.6%)	
MCT4			**<0.001**		**<0.001**
NA	108	42 (38.9%)		6 (5.6%)	
Tumour	484	368 (76.0%)		275 (56.8%)	
CD147			**<0.001**		**<0.001**
NA	139	19 (13.7%)		17 (12.2%)	
Tumour	495	179 (36.2%)		162 (32.7%)	
CD44			**<0.001**		**<0.001**
NA	103	1 (1.0%)		1 (1.0%)	
Tumour	486	138 (28.4%)		123 (25.3%)	
GLUT1			**<0.001**		**<0.001**
NA	108	7 (6.5%)		4 (3.7%)	
Tumour	464	156 (33.6%)		132 (28.4%)	

Analyzing the results of **Table XIX**, it is possible to observe that all the proteins studied are overexpressed in tumours when comparing with normal-adjacent tissue and in plasma membrane expression pattern ($p<0.001$). We detect a significant increase in both MCT1 and MCT4 expressions when comparing normal adjacent epithelium to tumour tissues ($p < 0.001$, for both), corresponding to 93.6% and 76.0%, respectively and similar results were observed when analyzing membrane expression. Percentage of positive cases decreased for the chaperones CD147 and CD44 as well as in the glycolytic metabolic marker GLUT1.

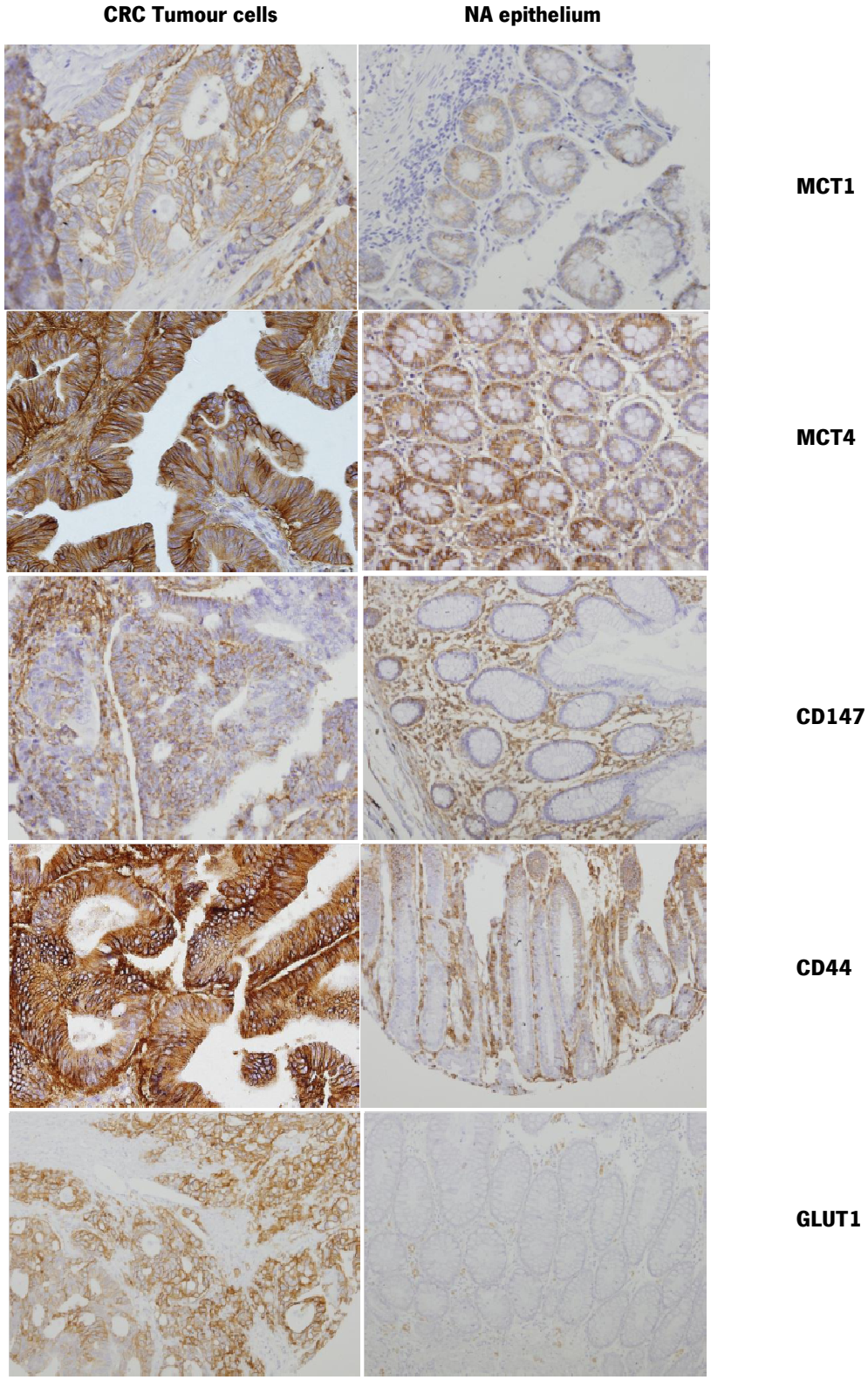


Figure 20: MCT1, MCT4, CD147, CD44 and GLUT1 immunohistochemical expression in CRC samples (200x magnification).

4.2.2 Evaluation of associations between MCTs, CD147, CD44 and GLUT1 expression in CRC

Functional expression of MCTs is regulated by accessory proteins, such as CD147, that are involved in trafficking and anchoring of plasma membrane proteins (135). Regulation of MCT1 and MCT4 by CD147, was supported by evidence on human and *in vitro* studies (104,135,157–161). CD44 is a transmembrane glycoprotein that plays an important role in communication of cell-matrix interactions (181,182) and also function as a chaperone for MCT expression (162).

Moreover, as a consequence of high energetic demands, CRC cells show an increase in glucose uptake. Upregulation of glucose transport across the plasma membrane is mediated by a family of facilitated glucose transporter proteins named (GLUT 1–14) (209,212); thus GLUT1, is expected to be upregulated in tumour cells.

We analyzed the associations between MCTs, CD147, CD44 and GLUT1 Expression in CRC tissues, the results obtained are summarized in **Table XX**.

Table XX: Assessment of associations between MCTs and CD147, CD44, and GLUT1 plasma membrane expression in tumour cases.

		CD147			**CD44**			**GLUT1**	
		Plasma membrane			***Plasma membrane***			***Plasma membrane***	
Tumour	n	Positive (%)	*p*	n	Positive (%)	*p*	n	Positive (%)	*p*
MCT1									
Positive	452	157 (34.7%)	**0.003**	438	116 (26.5%)	0.111	425	126 (29.6%)	0.076
MCT4									
Positive	269	100 (37.2%)	**0.050**	270	98 (36.3%)	**<0.001**	262	90 (34.4%)	**0.001**

We observed that in tumour samples, MCT1 positive cases were associated with CD147 plasma membrane expression (p=0.003) and between MCT4 and both chaperones plasma membrane expression; CD147 (p=0.05), CD44 (p<0.001) and GLUT1 (p=0.001); while association between MCT1 isoform with the chaperone CD44 and the metabolic marker GLUT1 was not achieved (**Table XX**).

4.2.3 Evaluation of associations between MCTs, CD147, CD44, GLUT1 expression in CRC tissues and epidemiological data

The results obtained are described in **Table XXI, XXII** and **XXIII** which summarizes the correlation between MCTs, chaperones, metabolic marker GLUT1 plasma membrane expression and the epidemiological data.

Figure 21 – 25 describes MCT1, MCT4, CD147, CD44 and GLUT plasma membrane expression, respectively, by stage, colon and rectal cancer survival curve assessed by log-rank test.

Table XXI: Assessment of correlation between MCTs, CD147, CD44, and GLUT1 plasma membrane expression and clinical data.
*Comparisons were examined for statistical significance using Fisher's exact test (when n < 5).

	MCT1			MCT4			CD147			CD44			GLUT1		
	n	Positive (%)	*p*	n	Positive (%)	*p*	n	Positive (%)	*p*	n	Positive (%)	*p*	n	Positive (%)	*p*
Sex															
Male	314	92.7	0.934	302	57.3	0.801	312	31.4	0.391	302	25.5	0.933	294	28.6	0.969
Female	186	92.5		180	56.1		182	35.2		178	25.8		169	28.4	
Age															
<=71.5	253	91.3	0.263	242	52.5	**0.052**	250	28.8	**0.056**	242	23.6	0.295	231	28.6	0.977
> 71.5	247	93.9		240	61.3		244	36.9		238	27.7		232	28.4	
Personal history - Polyps															
Negative	435	92.4	0.681	417	55.9	0.276	431	32.5	0.700	416	25.5	0.854	402	28.6	0.905
Positive	65	93.8		65	63.1		63	24.9		64	26.6		61	27.9	
Personal history - CCR															
Negative	487	92.6	0.967	469	56.1	**0.040**	481	33.1	0.560*	467	25.5	0.748*	451	28.6	1.000*
Positive	13	92.3		13	84.6		13	23.1		13	30-8		12	25	
Personal history - cancer															
Negative	462	92.4	0.601	444	56.8	0.892	458	32.5	0.660	443	25.3	0.552	428	28.5	0.993
Positive	38	94.7		38	57.9		36	36.1		37	29.7		35	28.6	

Table XXII: Assessment of correlation between MCTs, CD147, CD44, and GLUT1 plasma membrane expression and diagnosis/surgery data.
*Comparisons were examined for statistical significance using Fisher's exact test (when n < 5).

	MCT1			MCT4			CD147			CD44			GLUT1		
	n	Positive (%)	*p*	n	Positive (%)	*p*	n	Positive (%)	*p*	n	Positive (%)	*p*	n	Positive (%)	*p*
Presentation															
Asymptomatic	87	93.1	0.844	84	48.8	0.102	87	36.8	0.383	85	18.8	0.113	83	28.9	0.928
Symptomatic	413	92.5		398	58.5		407	31.9		395	27.1		380	28.4	
Rectal Examination															
Mobile cancer	41	57.1	**0.059**	40	43.3	**0.003**	40	65.2	0.575	39	46.2	0.221	38	46.7	0.122
Fixed cancer	27	42.9		24	56.7		26	34.8		25	53.8		22	53.3	
Localization															
Colon	360	92.5	0,891	351	59.3	0.080	359	33.4	0.625	349	27.5	0.123	338	29.3	0.541
Rectum	140	92.9		131	50.4		135	31.1		131	20.6		125	26.4	
Macroscopic Cancer type															
Polypoid	254	92.9		247	54.7		249	33.3		246	26.0		239	23.8	
Ulcerative	116	91.4		115	54.8		118	32.3		112	25.0		111	29.7	
Infiltrative	42	85.7	0.492	40	62.5	0.245	40	27.5	0.798	39	12.8	0.294	35	25.7	**0.023**
Exophytic	42	95.2		37	70.3		41	29.3		37	32.4		34	50.0	
Vilosous	2	100		2	100		2	0.0		2	50.0		2	0.0	

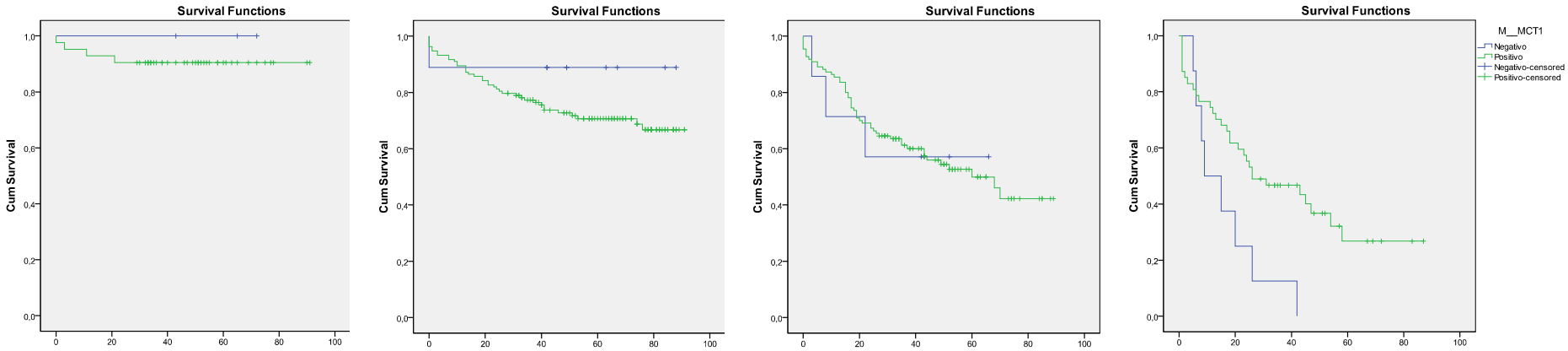
CEA (ng/mL)	122	90.2	0.568	115	60.0	0.665	118	33.1	0.455	115	30.4	0.116	111	36.9	**0.05**
>5	272	91.9		269	57.6		270	29.3		263	22.8		256	22.7	
≥5															
Metastasis															
Hepatic															
Absent	428	93.5	0.083	415	55.7	0.405	427	31.9	0.536	413	23.7	**0.055**	399	26.1	**0.046**
Present	44	86.4		40	62.5		41	36.6		40	37.5		39	41.0	
Lymph Node															
Absent	439	93.4	**0.067**	422	56.9	0.350	436	32.2	0.892	420	24.8	0.748	409	28.1	0.204
Present	33	84.8		33	48.5		33	33.3		33	27.3		29	17.2	
Pulmonar	461	93.3		444	56.1	0.618									
Absent	11	72.7	**0.009**	11	63.6		458	32.3	1.000*	442	24.9	1.000*	427	27.4	1.000*
Present							10	30.0		11	27.3		11	27.3	
Tumour Mobility															
Mobile	433	92.1	0.340	419	56.3	0.619	428	31.3	0.101	419	25.5	0851	405	27.4	0.140
Fixed	66	95.5		62	59.7		65	41.5		60	26.7		57	36.8	
Tumour Perforation															
Absent	475	93.1	0.092	460	56.5	0.510	469	32.6	0.726	457	25.8	0.662	441	27.9	0.187
Present	25	84.0		22	63.6		25	36.0		23	21.7		22	40.9	

Table XXIII: Assessment of correlation between MCTs, CD147, CD44, and GLUT1 plasma membrane expression and pathological data.
*Comparisons were examined for statistical significance using Fisher's exact test (when n < 5).

	MCT1			MCT4			CD147			CD44			GLUT1		
	n	Positive (%)	*p*	n	Positive (%)	*p*	n	Positive (%)	*p*	n	Positive (%)	*p*	n	Positive (%)	*p*
Tumour size															
≤ 4.5 cm	286	93.4	0.389	278	54.7	0.265	283	27.9	**0.003**	278	23.7	0.278	267	29.6	0.466
> 4.5 cm	182	91.2		175	60.0		180	41.1		173	28.3		167	26.3	
Macrosc. serosal involv.															
Absent	124	91.9	0.756	119	56.3	0.926	120	30.0	0.465	121	22.3	0.320	115	22.6	0.111
Present	374	92.8		361	56.8		372	33.6		357	26.9		346	30.3	
Synchronous tumours															
Absent	482	92.5	0.855	463	56.6	0.855	476	32.8	0.898	463	25.7	1.000*	445	28.8	0.574*
Present	16	93.8		17	58.8		16	31.3		15	26.7		16	18.8	
Histological Type															
Adenocarcinoma	417	92.8		402	57.0		411	33.6		399	26.6		386	28.2	
Mucinous	51	90.2	0.456	49	57.1	0.862	52	28.8	0.787	49	16.5	0.463	46	26.1	0.389
Invasive Adenocarc.	24	95.8		24	54.2		23	26.1		24	16.7		23	39.1	
Signet ring and mucinou	4	75.0		3	33.3		4	25.0		4	0.0		4	0.0	

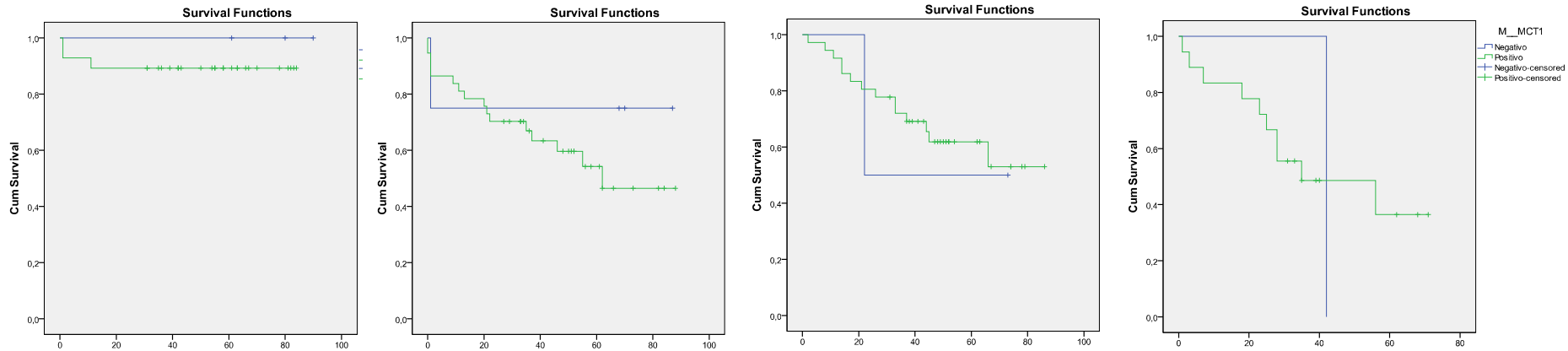
Differentiation														
Well-differentiated	219	93.2		213	56.8		217	34.6		211	24.6		202	21.3	
Moderately-diff.	209	93.3	0.271	204	55.4	0.070	206	32.5	0.875	202	27.2	0.399	197	35.0	**0.009**
Poorly-diff.	49	85.7		43	69.8		48	29.2		45	33.3		43	39.5	
Undifferentiated	4	100.0		3	0.0		4	25.0		4	0.0		3	33.3	
Tumour Penetration															
Tis	5	100.0		6	16.7		4	25.0		5	0.0		5	0	
T1/T2	89	92.1	0.810	86	54.7	0.123	22	24.7	0.179	87	24.1	0.355	82	24.4	0.218
T3/T4	395	92.4		380	57.6		391	34.8		379	26.9		367	30.0	
Spread to lymph nodes															
Absent	280	92.5	0.888	272	54.0	0.269	277	32.5	0.876	274	25.9	0.975	263	25.5	**0.058**
Present	204	92.2		196	59.2		202	33.2		192	26.0		187	33.7	
Vessel invasion															
Absent	159	94.3	0.255	159	58.5	0.541	156	33.3	0.817	158	31.6	**0.031**	150	25.3	0.194
Present	314	91.4		299	55.5		313	32.3		299	22.4		291	31.3	
Surgical margin invasion															
Absent	473	92.6	0.284	456	55.7	0.128	468	32.7	0.309	455	25.5	0.094	441	28.8	0.486*
Present	13	84.6		13	76.9		13	46.2		13	46.2		10	40.0	
TNM															
Stage 0	1	100.0		2	0.0		0	0.0		1	0.0		1	0.0	
Stage I	76	92.1		75	52.0		77	22.1		76	21.1		73	23.3	
Stage II	183	92.9	0.566	179	57.0	0.464	181	36.5	0.147	178	28.1	0.649	173	26.0	0.206
Stage III	155	94.2		151	57.6		154	34.4		147	24.5		142	30.3	
Stage IV	75	88.0		67	59.7		73	31.5		70	30.0		66	39.4	

COLON CANCER



Stage I (***p=0.586***) Stage II (***p=0.294***) Stage III (***p=0.986***) Stage IV (***p=0.017***)

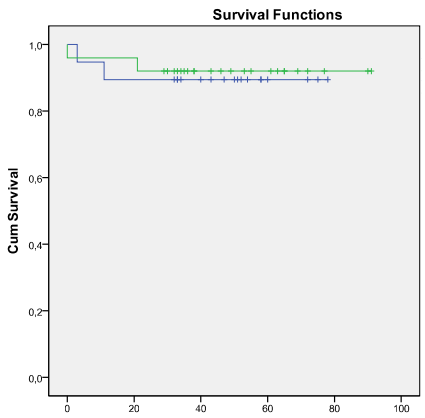
RECTAL CANCER



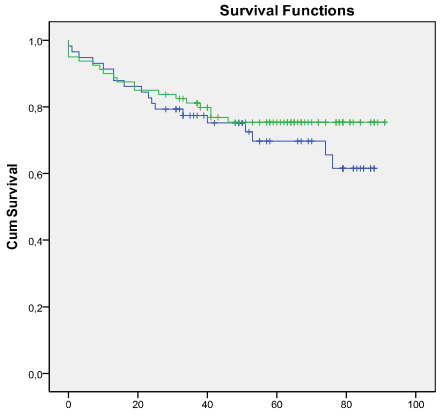
Stage I (***p=0.562***) Stage II (***p=0.411***) Stage III (***p=0.851***) Stage IV (***p=0.845***)

Figure 21: Kaplan-Meyer survival curves of MCT1 plasma membrane expression in colon and rectum, by stage.

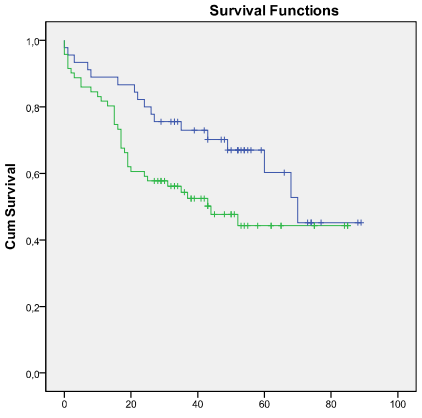
COLON CANCER



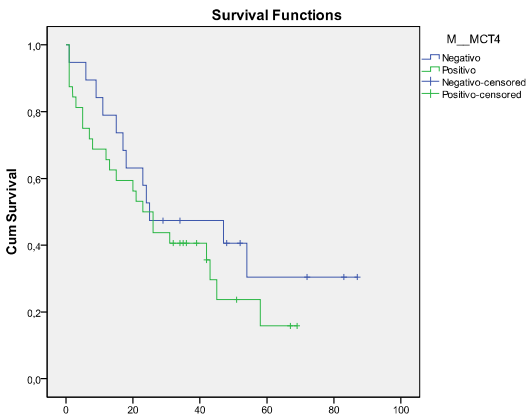
StageI (*p=0.775*)



Stage II (*p=0.350*)

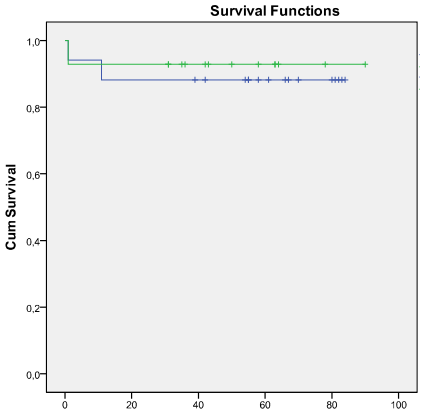


Stage III (*p=0.060*)

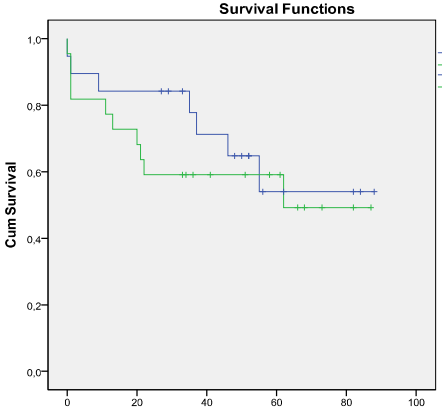


Stage IV (*p=0.314*)

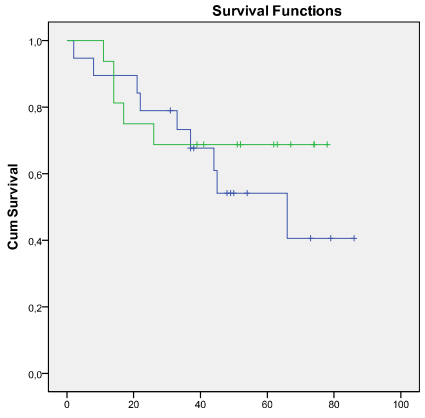
RECTAL CANCER



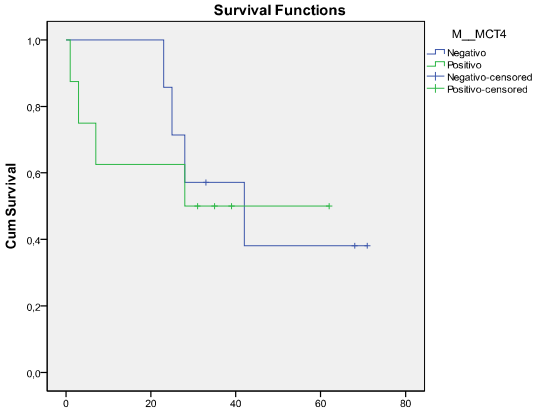
Stage I (*p=0.680*)



Stage II (*p=0.545*)



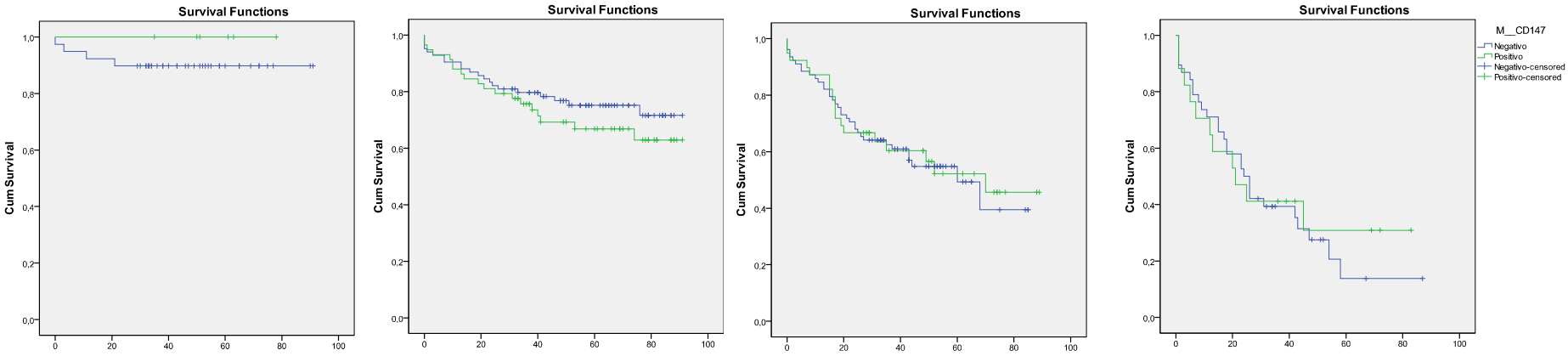
Stage III (*p=0.438*)



Stage IV (*p=0.779*)

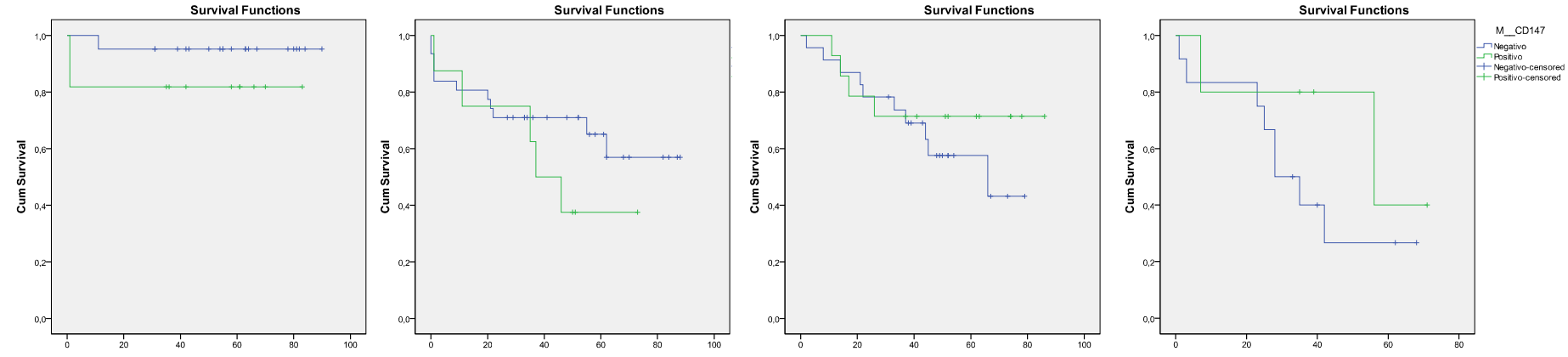
Figure 22: Kaplan-Meyer survival curves of MCT4 plasma membrane expression in colon and rectum, by stage.

COLON CANCER



Stage I (*p=0.424*) Stage II (*p=0.340*) Stage III (*p=0.850*) Stage IV (*p=0.761*)

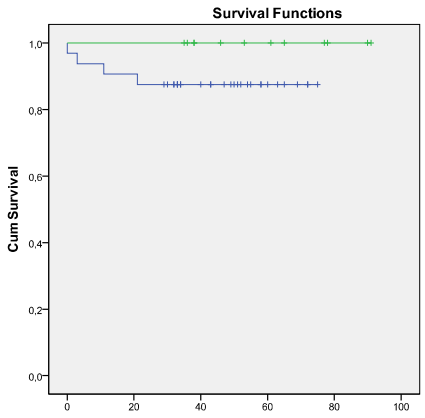
RECTAL CANCER



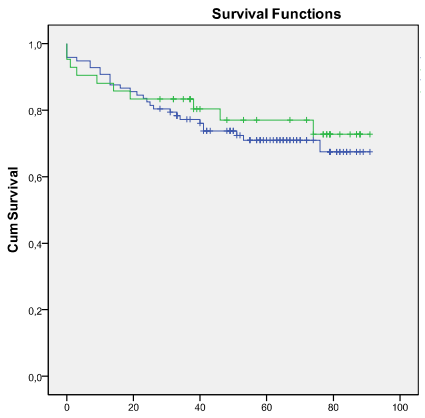
Stage I (*p=0.208*) Stage II (*p=0.253*) Stage III (*p=0.414*) Stage IV (*p=0.298*)

Figure 23: Kaplan-Meyer survival curves of CD147 plasma membrane expression in colon and rectum, by stage.

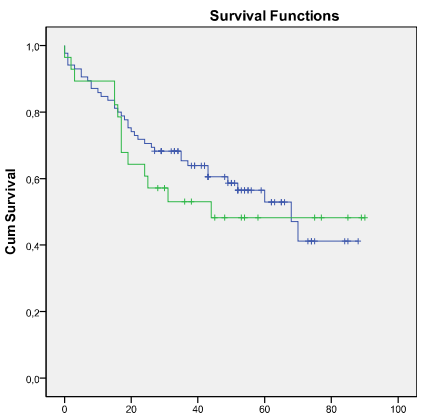
COLON CANCER



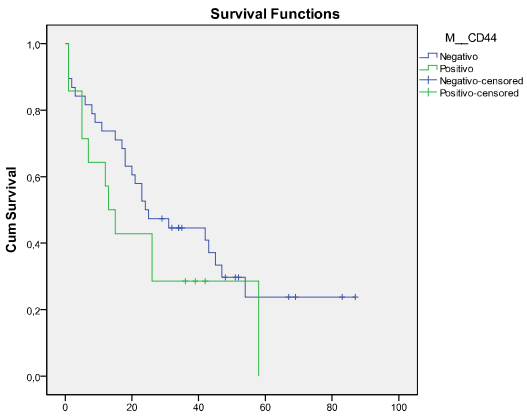
Stage I (*p=0.209*)



Stage II (*p=0.580*)

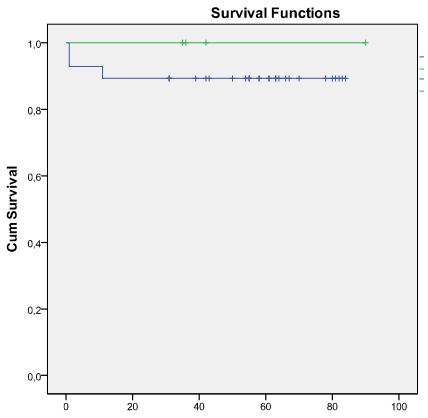


Stage III (*p=0.580*)

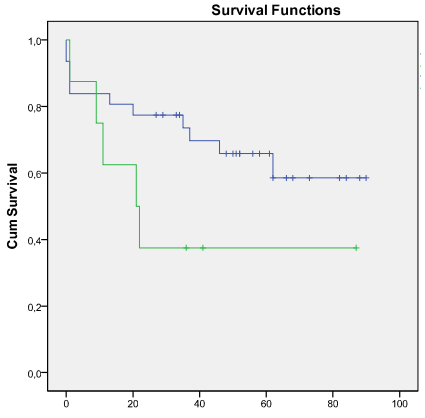


Stage IV (*p=0.233*)

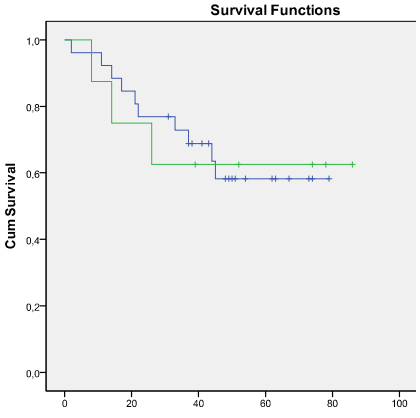
RECTAL CANCER



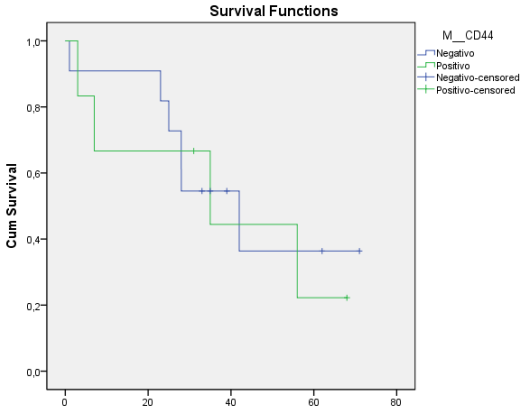
Stage I (*p=0.503*)



Stage II (*p=0.115*)



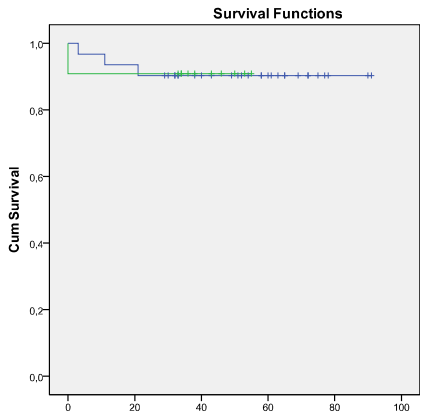
Stage III (*p=0.965*)



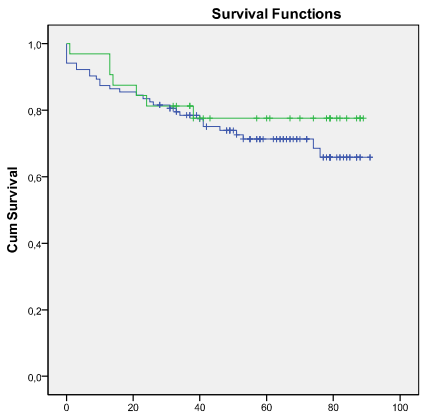
Stage IV (*p=0.787*)

Figure 24: Kaplan-Meyer survival curves of CD44 plasma membrane expression in colon and rectum, by stage.

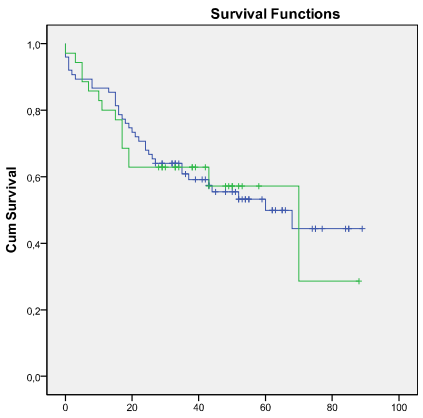
COLON CANCER



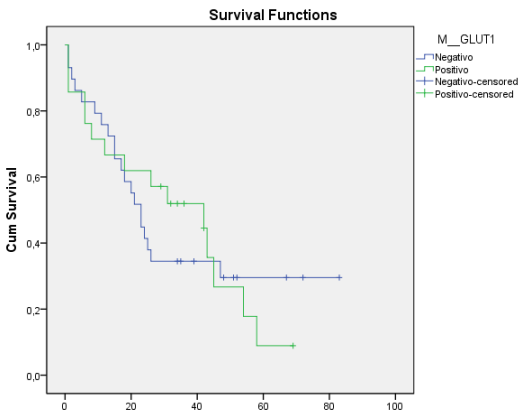
Stage I (*p=0.989*)



Stage II (*p=0.441*)

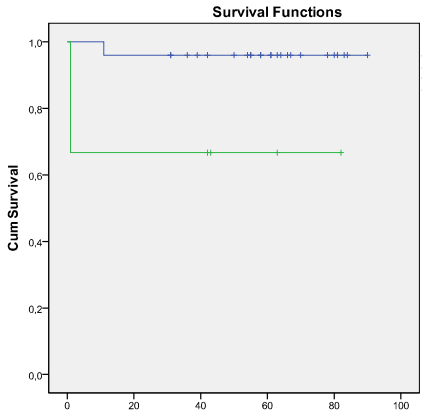


Stage III (*p=0.901*)

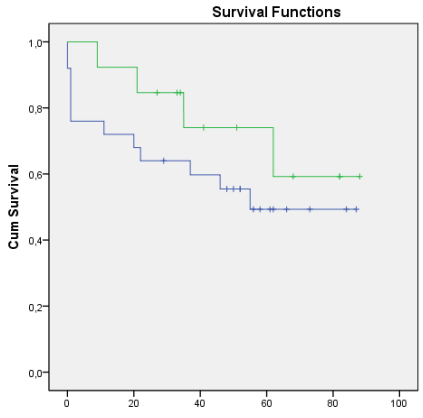


Stage IV (*p=0.952*)

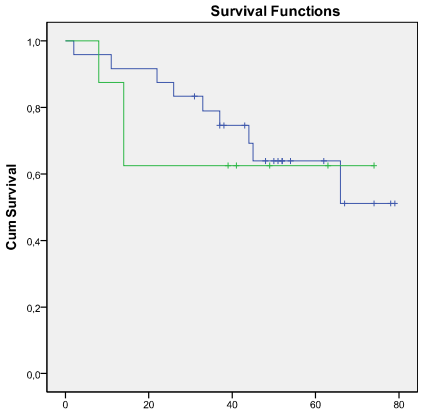
RECTAL CANCER



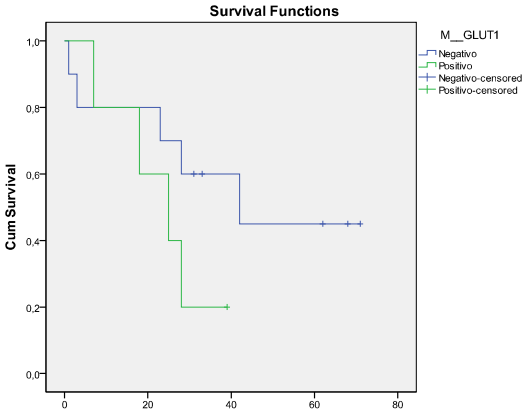
Stage I (*p=0.023*)



Stage II (*p=0.305*)



Stage III (*p=0.754*)



Stage IV (*p=0.216*)

Figure 25: Kaplan-Meyer survival curves of GLUT1 plasma membrane expression in colon and rectum, by stage.

Assessment of correlation between MCTs, chaperones, metabolic marker GLUT1 plasma membrane expression and clinical data revealed MCT4 positive cases were associated with "Personal History of CRC" (p=0.040) and a tendency for association between MCT4 and CD147 with "Age" (p=0.052 and p=0.056, respectively) (**Table XXI**).

When analyzing correlation between plasma membrane expression and data from diagnosis/surgery data we found association between MCT1 plasma membrane expression and with "Pulmonary Metastasis" (p=0.009) and a tendency to association with "Rectal Examination" (p=0.059) "Hepatic and Ganglionar Metastasis" (respectively p=0.083 and p=0.067). MCT4 plasma membrane expression showed association with "Rectal Examination" (p=0.003). CD44 showed a tendency to associate with "Hepatic Metastasis" (p=0.055) and GLUT1 plasma membrane expression showed association with "Macroscopic cancer type" (p=0.023); "CEA" (p=0.05) and "Hepatic Metastasis" (p=0.046) (**Table XXII**).

When analyzing the correlation between plasma membrane expression and pathological data we find association between CD147 plasma membrane and "Tumour size" (p=0.003); CD44 plasma membrane expression and "Vessel Invasion" (p=0.031) and GLUT1 plasma membrane expression and "Tumour Differentiation" (p=0.009) (**Table XXIII**).

Observing colon and rectal cancer survival curves assessed by log-rank test, of MCTs, chaperones and GLUT1, (**Figures 21-25**), we found a statistically significant association for MCT1 expression and stage IV for colon cancer (p=0.017); GLUT1 expression and stage I for rectal cancer (p=0.023) and a tendency to association between MCT4 expression and stage III for colon cancer (p=0.060).

4.3 Analysis of the Associations of MCTs, Chaperones and Glycolytic Metabolic Markers in Colorectal Cancer Hepatic Metastasis and Normal Adjacent Tissues

Liver is the most common site of CRC metastasis (50-60% of the cases). Close to one third of patients have liver metastases either at the time of diagnosis (synchronous in 1/3 of the cases) or during the disease course (metachronous in 2/3 of the cases) (312–314) and about 66% had liver metastases at death time (315,316). Despite recent advances in terms of early diagnosis and therapy which led to improvement in survival (five years survival has increased from <8%, using palliative chemotherapy to 25-40% using multimodal management including palliative chemotherapy and surgery (312,313,317), the prognosis remains reserved (312–316), with a five years survival of 15-50% and 17-33% ten years survival after hepatic metastases resection (315,316).

Surgical resection of liver metastases is considered the only curative treatment option for patients with resectable liver metastases and no extrahepatic disease (312–314) but liver metastases are resectable in only 15% of the cases. The remaining 85% are ineligible to surgery because of the location, size, number, residual normal liver, and the extra hepatic disease (312,313,318). Recently, other new modalities have become available that allow safe ablation of liver metastases without the need for surgical intervention.

Once documented the increases expression of MCTs, CD147 and CD44 chaperones and glycolytic metabolic marker GLUT1 in CRC tissues remains the question if that metabolic profile is maintained in CRC hepatic metastasis. Our initial aim was to evaluate the expression of these proteins in the patients with liver metastasis of our series, but due to the few number of patients that have been submitted to hepatic resection during this period, this was not possible. Thus, we increased the research period of patients submitted to CRC hepatic metastasis resection from January 2003 to January 2011 and analyzed the expression of MCT4, CD147, CD44 and GLUT1 in CRC hepatic metastasis and normal adjacent tissue.

No data exists in the literature about the expression of these proteins in CRC hepatic metastasis, being this the first study to be performed in this direction.

4.3.1 Characterization of MCT4, CD147, CD44 and GLUT1 Immunohistochemical Expression in CRC Hepatic Metastasis and Normal Adjacent Tissue

A total of 45 samples of hepatic metastasis of CRC patients were analyzed, including tumour and normal adjacent tissue. Sections were evaluated for immunoreaction, which included both cytoplasmic and membrane-positive staining. The results obtained are described in **Table XXIV,** which summarizes the frequency of MCT 4, chaperones CD147 and CD44 and glycolytic metabolic marker GLUT1 expressions, in tumour cells and normal adjacent epithelium.

MCT1 immunohistochemical reaction was not performed due to problems with the "detection system".

Figure 26 shows representative pictures of MCT4, CD147, CD44 and GLUT1 positive staining in CRC Hepatic Metastasis and in normal adjacent epithelium.

Table XXIV: Pattern of protein staining in CRC Hepatic metastasis vs. normal adjacent epithelium.

Protein		***Immunoreaction***		***Plasma membrane***	
	n	Positive (%)	*p*	Positive (%)	*p*
MCT4			0.749		**<0.001**
NA	40	15 (37.5%)		0 (0%)	
Tumour	44	18 (40.9%)		275 (40.9%)	
CD147			0.616		**0.001**
NA	40	29 (72.5%)		12 (30.0 %)	
Tumour	43	29 (67.4%)		29 (67.4%)	
CD44			**<0.001**		**<0.001**
NA	41	0 (0.0%)		0 (0.0%)	
Tumour	41	12 (27.3%)		12 (27.3%)	
GLUT1			**<0.001**		**<0.001**
NA	43	0 (0.0%)		0 (0.0%)	
Tumour	44	25 (56.8%)		25 (56.8%)	

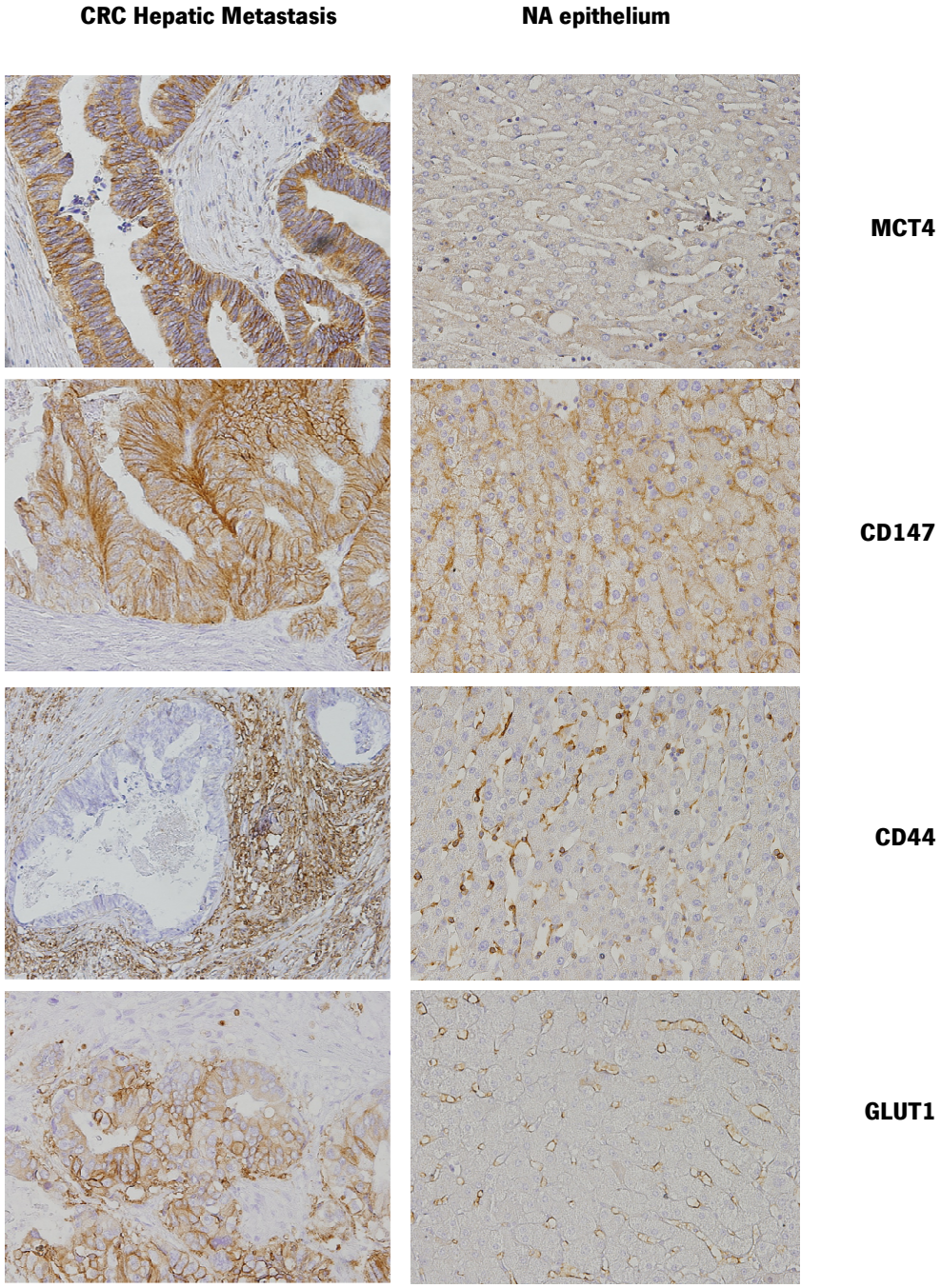


Figure 26: MCT4, CD147, CD44 and GLUT1 immunohistochemical expression in CRC Hepatic Metastasis samples (200x magnification).

Observing the results of **Table XXIV**, in tumour positive cases, immunoreaction and plasma membrane shows similar results. All the proteins studied are overexpressed in CRC hepatic metastasis when comparing with normal-adjacent tissue in plasma membrane expression pattern (p<0.001). The values were lower in normal adjacent tissue and no reaction was observed for MCT4, CD44 and GLUT1.

4.3.2 Evaluation of Associations between MCT4, CD147, CD44 and GLUT1 expression in CRC Hepatic Metastasis

We analyzed the associations between MCT4, CD147, CD44 and GLUT1 expression in CRC hepatic metastasis, the results obtained are summarized in **Table XXV.**

Table XXV: Assessment of associations between MCTs and CD147, CD44, and GLUT1 plasma membrane expression in CRC Hepatic metastases.

		CD147			**CD44**			**GLUT1**	
		Plasma membrane			***Plasma membrane***			***Plasma membrane***	
Tumour	n	Positive (%)	*p*	n	Positive (%)	*p*	n	Positive (%)	*p*
MCT4			**<0.001**			**0.003***			**<0.001**
Positive	18	16 (88.9%)		18	7 (38.9%)		18	18 (100%)	

* Comparisons were examined for statistical significance using Fisher's exact test (when n < 5).

We observed that in tumour samples, MCT4 positive cases were associated with CD147 plasma membrane expression (p <0.001) CD44 plasma membrane expression (p =0.003) and GLUT1 plasma membrane expression (p <0.001) (**Table XXVI**).

4.3.3 Evaluation of associations between MCT4, CD147, CD44, GLUT1 expression in CRC Hepatic Metastasis and epidemiological data

Data from these 45 patients with CRC Hepatic metastasis were retrospectively collected namely anatomopathological data from primary tumour (CRC localization, stage, differentiation, lymphatic and blood vessel invasion) and anatomopathological data from hepatic metastasis (presence of synchronous or metachronous hepatic metastasis, localization, size). Other data that were also collected was CEA level at CRC diagnosis and Hepatic metastasis diagnosis.

The results obtained are described in **Table XXVI** and **XXVII** which summarizes the correlation between MCT4, chaperones and metabolic marker GLUT1 plasma membrane expression and anatomopathological data from primary tumour and hepatic metastasis.

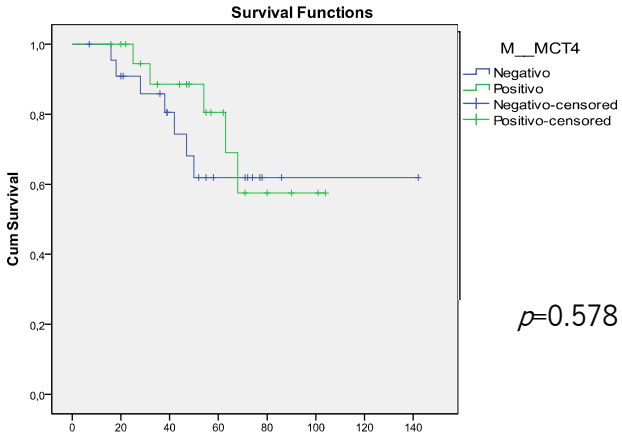
Figures 27 – 30 outline MCT4, CD147, CD44 and GLUT1 plasma membrane expression CRC Hepatic metastasis survival curves assessed by log-rank test, respectively.

Table XXVI: Assessment of correlation between MCT4, CD147, CD44, and GLUT1 plasma membrane expression and anatomopathological data from primary tumour.
*Comparisons were examined for statistical significance using Fisher's exact test (when n < 5).

	n	MCT4		CD147		CD44		GLUT1	
		Positive (%)	*p*	Positive (%)	*p*	Positive (%)	*p*	Positive (%)	*p*
Localization									
Colon	7	28.6	0.682	42.9	0.190	42.9	0.369	42.9	0.443
Rectum	37	43.2		72.2		24.3		59.5	
CRC stage									
I+II	8	62.5	0.250	75.0	1.000	25.0	1.000	62.5	1.000
III+IV	32	37.5		67.7		28.1		56.3	
Differentiation									
Well/ Moderately-diff.	20	35.0	0.457	60.0	0.277	30.0	0.969	45.0	0.117
Poorly/ Undifferentia.	17	47.1		81.3		29.4		70.6	
Venous Vessel invasion									
Absent	20	45.0	0.452	84.2	**0.042**	20.0	0.217	55.0	0.784
Present	11	27.3		45.5		45.5		50.0	
Lymph Vessel invasion									
Absent	23	30.4	0.109	72.7	1.000	17.4	0.075	43.5	0.070
Present	9	66.7		66.7		55.6		80.0	
CEA									
≤ 200ng/ml	24	41.7	0.274*	62.5	1.000*	25.0	1.000*	58.3	0.569*
> 200ng/ml	3	0.0		66.7		33.3		33.3	

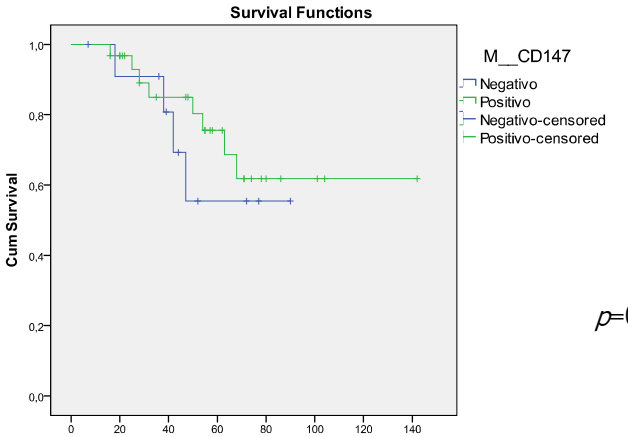
Table XXVII: Assessment of correlation between MCT4, CD147, CD44, and GLUT1 plasma membrane expression and anatomopathological data from hepatic metastasis.
*Comparisons were examined for statistical significance using Fisher's exact test (when n < 5).

	n	MCT4		CD147		CD44		GLUT1	
		Positive (%)	*p*	Positive (%)	*p*	Positive (%)	*p*	Positive (%)	*p*
Localization									
One hepatic lobe	30	50.0	0.251	73.3	0.129	26.7	0.693	60.0	1.000
Both hepatic lobe	9	22.2		44.4		33.3		62.5	
Size									
≤ 5 cm	37	43.2	1.000	70.3	0.373	27.0	1.000	58.3	1.000
> 5 cm	6	33.3		50.0		33.3		50.0	
CEA									
≤ 200ng/ml	35	45.7	0.618*	64.7	1.000*	25.7	1.000*	57.1	1.000*
> 200ng/ml	4	25.0		75.0		25.0		50.0	



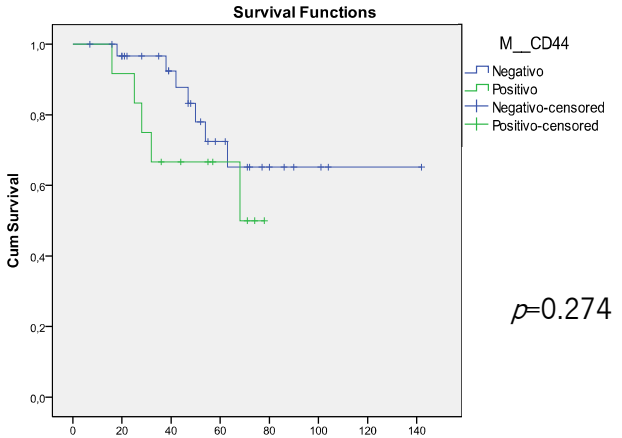
p=0.578

Figure 27: MCT4 plasma membrane expression CRC Hepatic metastasis survival curve assessed by log-rank test.



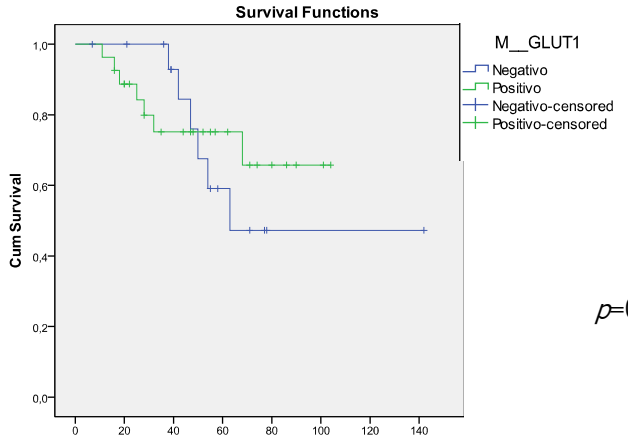
p=0.491

Figure 28: CD147 plasma membrane expression CRC Hepatic metastasis survival curve assessed by log-rank test.



p=0.274

Figure 30: CD44 plasma membrane expression CRC Hepatic metastasis survival curve assessed by log-rank test.



p=0.727

Figure 29: GLUT1 plasma membrane expression CRC Hepatic metastasis survival curve assessed by log-rank

Assessment of correlation between MCT4, chaperones and the metabolic marker GLUT1 plasma membrane expression and anatomopathological data from primary tumour and Hepatic metastasis, revealed CD147 positive cases were associated with "Venous vessel invasion" of CRC (p=0.042, **Table XXVI**) and no correlation was observed with anatomopathological data from Hepatic metastasis (**Table XXVII**).

No statistic significant associations were found for MCT4, CD147, CD44 and GLUT1 plasma membrane expression in CRC Hepatic metastasis survival curve assessed by log-rank test (**Figures 27 – 30**).

4.4 Analysis of the Associations of VEGF's family in Primary Colorectal Tumours and Normal Adjacent Tissues

Angiogenesis plays a key role in tumourigenesis and metastatic processes (4,229–234) and VEGF represents a critical inducer of tumour angiogenesis (234,258,259). In mammals, VEGF family consists of VEGF-A, B, C, D and PlGF1 and 2. All VEGF molecules/ligands transduce their signal through their binding to VEGF receptor -1, -2 and -3. VEGFR-2 is the key molecule for VEGF signaling in the tumour micro-environment including vascular permeability and endothelial cell proliferation (259,260), VEGFR-3 is restricted to lymphatic vessels after their formation (271,274).

We evaluated VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 immunohistochemical expression in CRCs and Normal Adjacent Tissue, in this series of 580 cases and also the correlation with clinical data.

4.4.1 Characterization of VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 Immunohistochemical Expression in CRCs and Normal Adjacent Tissues

The results obtained are described in **Table XXVIII** which summarizes the frequency of VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 expressions, in tumour cells and normal adjacent epithelium.

Analyzing the results of **Table XXVIII**, it is possible to observe that only VEGF-C are overexpressed in tumours when comparing tumour cell with normal-adjacent tissue (p=0.004), and VEGFR-2 shows a tendency to that association (p=0.064).

Figure 31 shows representative cases of VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 positive staining in tumour cells and in normal adjacent epithelium.

Table XXVIII: Pattern of protein staining in CRC vs. normal adjacent epithelium.

Protein		***Immunoreaction***	
	n	Positive (%)	*p*
VEGF-A			1.000*
NA	132	130 (98.5%)	
Tumour	500	490 (98.0%)	
VEGF-C			**0.004**
NA	138	115 (83.3%)	
Tumour	508	466 (91.7%)	
VEGFR-2			**0.064**
NA	142	133 (93.7%)	
Tumour	501	486 (97.0%)	
VEGFR-3			0.903
NA	139	34 (24.5%)	
Tumour	505	121 (24.0%)	

Comparisons were examined for statistical significance using Fisher's exact test (when n < 5).

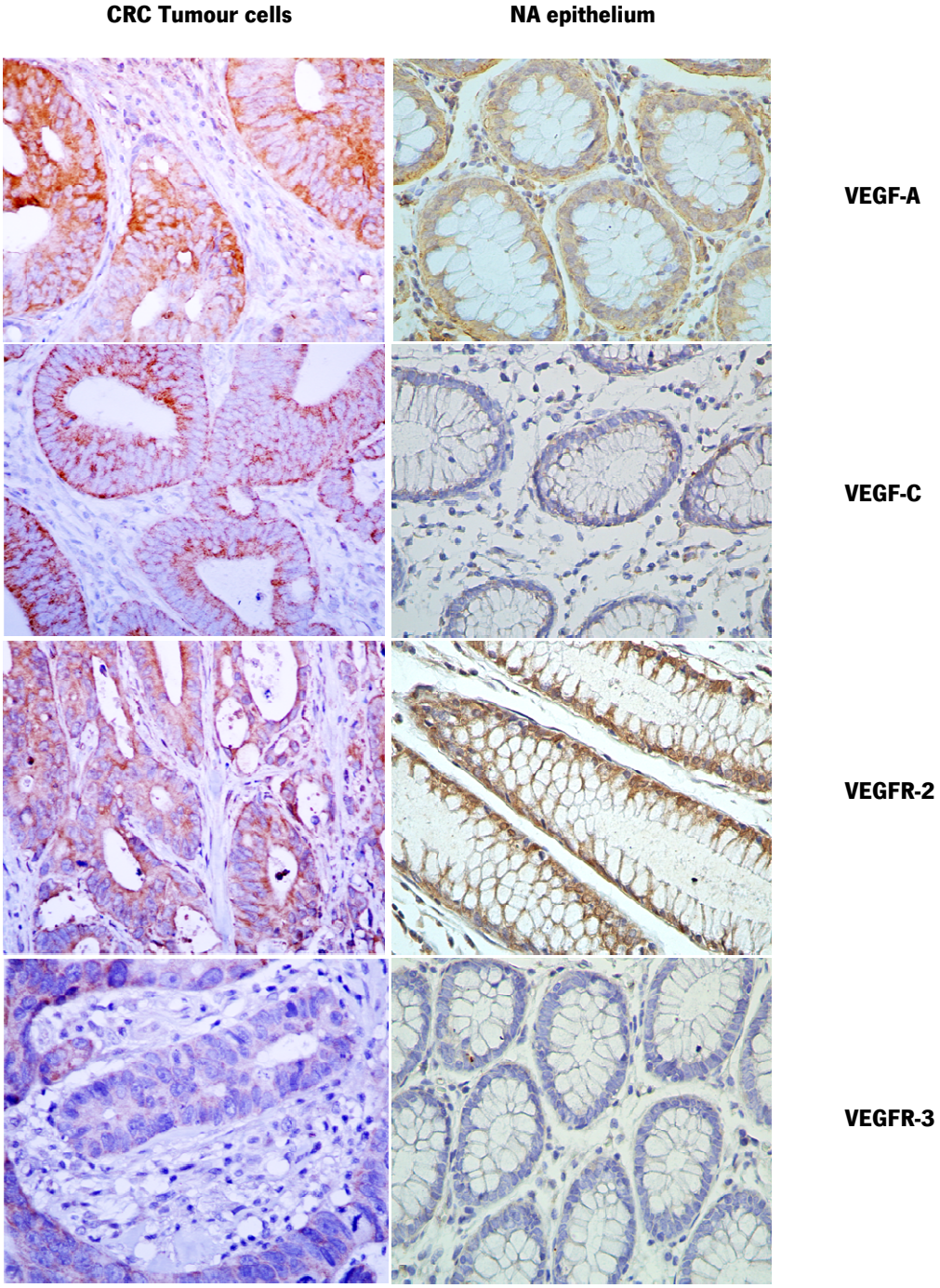


Figure 31: VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 immunohistochemical expression in CRC samples (40x magnification).

4.4.2 Evaluation of Associations between VEGF-A, VEGF-C, and VEGFR-2, VEGFR-3 Expression in CRC tissues

VEGF molecules transduce their signal through their binding to VEGF receptor -1, -2 and -3 (259,260). VEGFR-2 is considered the primary signaling receptor for VEGF during angiogenesis (259,319) and although VEGFR-3 is restricted to lymphatic and some fenestrated vascular endothelium in the adult, it is upregulated in angiogenic blood vessels in tumours, and blocking VEGFR-3 inhibits angiogenesis and growth in some tumours (320).

We analyzed the associations between VEGF-A, VEGF-C and the receptors VEGFR-2, VEGFR-3 expression in CRC tissues, the results obtained are summarized in **Table XXIX**.

Table XXIX: Assessment of associations between VEGF-A, VEGF-C and the receptors VEGFR-2 and VEGFR-3 expression in tumour cases.

		VEGFR-2			**VEGFR-3**	
		Immunoreaction			***Immunoreaction***	
Tumour	n	Positive (%)	*p*	n	Positive (%)	*p*
VEGF-A			1.000*			0.210*
Positive	464	453 (97.6%)		471	120 (25.5%)	
VEGF-C			1.000*			**0.047***
Positive	446	434 (97.3%)		451	117 (25.9%)	

* Comparisons were examined for statistical significance using Fisher's exact test (when n < 5).

We observed that in tumour samples, VEGF-C positive cases were associated with VEGFR-3 expression (p=0.047) (**Table XXIX**).

4.4.3 Evaluation of associations between VEGF-A, VEGF-C, and VEGFR-2, VEGFR-3 expression in CRC tissues and epidemiological data

The results obtained are described in **Table XXX, XXXI** and **XXXII** which summarizes the correlation between VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 expression and epidemiological data.

Figure 34, 35, 36 and **37** describes VEGF-A, VEGF-C, and VEGFR-2, VEGFR-3 plasma expression, respectively, by stage, colon and rectal cancer survival curve assessed by log-rank test.

Table XXX: Assessment of correlation between VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 expression and clinical data.
*Comparisons were examined for statistical significance using Fisher's exact test (when n < 5).

	VEGFA			VEGFC			VEGFR-2			VEGFR-3		
	n	Positive (%)	*p*	n	Positive (%)	*p*	n	Positive (%)	*p*	n	Positive (%)	*p*
Gender												
Male	304	99.3	**0.016***	309	91.6	0.446	306	97.1	0.776*	307	24.4	0.731
Female	181	96.1		184	93.5		179	97.8		182	25.8	
Age												
<=71.5	242	97.9	1.000*	249	90.4	0.107	244	97.1	0.802	247	23.9	0.565
> 71.5	242	98.3		229	94.2		240	97.5		241	26.1	
Personal history-Polyps												
Negative	419	98.3	0.352*	427	92.0	0.804*	420	97.4	0.689*	423	25.8	0.289
Positive	66	97.0		66	93.9		65	96.9		66	19.7	
Personal history - CCR												
Negative	472	98.3	0.219*	480	92.3	1.000*	472	97.2	1.000*	476	25.2	0.533*
Positive	13	92.3		13	92.3		13	100.0		13	15.4	
Personal history of Cancer												
Negative	446	98.2	0.533*	454	91.6	**0.060**	447	97.1	0.612*	450	25.6	0.292
Positive	39	97.4		39	100.0		38	100.0		39	17.9	

Table XXXI: Assessment of correlation between VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 expression and diagnosis/surgery data.
*Comparisons were examined for statistical significance using Fisher's exact test (when n < 5).

	VEGFA			VEGFC			VEGFR-2			VEGFR-3		
	n	Positive (%)	*p*	n	Positive (%)	*p*	n	Positive (%)	*p*	n	Positive (%)	*p*
Presentation												
Asymptomatic	87	96.6	0.207*	89	88.8	0.168	87	97.7	1.000*	88	23.9	0.795
Symptomatic	398	98.5		404	93.1		398	97.2		401	25.2	
Rectal Examination												
Mobile cancer	40	97.5	1.000*	42	81.0	0.300*	40	90.0	1.000*	40	25.0	0.339*
Fixed cancer	25	100.0		25	92.0		25	92.0		24	12.5	
Localization												
Colon	352	98.3	0.711*	357	93.8	**0.037**	354	98.0	0.115	358	24.6	0.756
Rectum	133	97.7		136	88.2		131	95.4		131	26.0	
Macroscopic Cancer type												
Polypoid	244	98.0		255	89.8		246	98.0		252	25.4	
Ulcerative	112	97.4		115	94.8		115	98.3		114	25.4	
Infiltrative	38	97.4	0.896	39	94.9	**0.048**	40	97.5	0.278	38	13.2	0.439
Exophytic	38	100.0		37	97.4		35	92.1		39	28.2	
Vilosous	2	100.0		1	50.0		2	100.0		2	50.0	

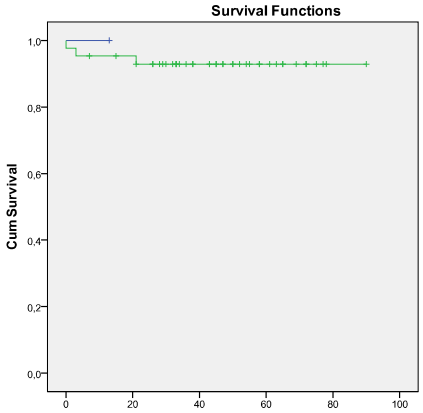
CEA (ng/mL)												
<5	314	94.9	1.000*	314	90.1	0.869	314	93.9	0.756	313	24.6	0.779
≥5	78	93.6		78	89.7		78	94.9		78	23.1	
Metastasis												
Hepatic												
Absent	443	98.2	0.535*	450	92.2	1.000*	443	97.1	0.613*	445	23.8	**0.032**
Present	39	97.4		40	92.5		39	100		41	39.0	
Lymph Node												
Absent	437	98.2	0.589*	442	92.3	0.779*	436	97.5	0.357*	438	25.8	0.285
Present	45	97.8		48	91.7		46	95.7		48	18.8	
Tumour Mobility												
Mobile	418	97.8	0.240	426	91.8	0.158	419	97.4	0.786	423	24.6	0.630
Fixed	63	100.0		63	96.8		62	96.8		62	27.4	
Tumour Perforation												
Absent	460	98.0	0.480	468	92.3	1.000*	461	97.6	0.079	464	24.6	0.403
Present	25	100.0		25	92.0		24	91.7		25	32.0	

Table XXXII: Assessment of correlation between VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 expression and pathological data.
*Comparisons were examined for statistical significance using Fisher's exact test (when n < 5).

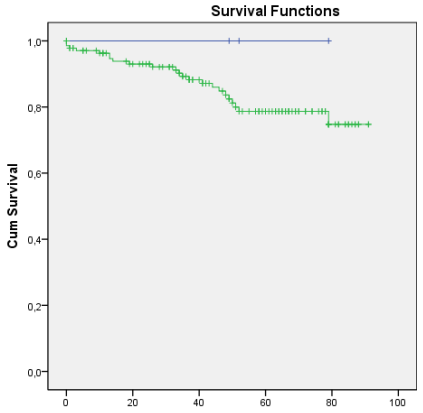
	VEGFA			VEGFC			VEGFR-2			VEGFR-3		
	n	Positive (%)	*p*	n	Positive (%)	*p*	n	Positive (%)	*p*	n	Positive (%)	*p*
Tumour size												
≤ 4.5 cm	281	97.5	0.161*	281	94.3	0.088	276	98.2	0.291	277	27.1	0.287
> 4.5 cm	175	99.4		181	90.1		178	96.6		181	22.7	
Macrosc. serosal involv.												
Absent	121	97.5	0.697*	122	89.3	0.164	119	99.2	0.202*	119	25.2	0.868
Present	362	98.3		369	93.2		364	96.7		368	24.5	
Synchronous tumours												
Absent	467	98.1	1.000*	475	92.2	1.000*	467	97.2	1.000*	471	24.6	1.000*
Present	16	100.0		16	93.8		16	100.0		16	25.0	
Histological Type												
Adenocarcinoma	403	98.3		408	92.6		404	98.0		403	26.1	
Mucinous	50	98.0	0.869	52	90.4	0.470	49	93.9	**0.007**	53	15.1	0.214
Invasive Adenocarc.	25	96.0		25	96.0		24	100.0		25	28.0	
Signet ring and mucinous	3	100.0		4	75.0		4	75.0		4	0.0	

Differentiation	209	99.0		215	92.6		210	97.1		212	23.6	
Well-differentiated	208	97.6	**0.001**	208	94.2	**0.007**	205	97.6	0.973	206	27.7	0.474
Moderately-diff.	48	97.9		48	89.6		47	97.9		49	22.4	
Poorly-diff.	2	66.7		4	50.0		4	100.0		4	0.0	
Undifferentiated												
Tumour Penetration												
Tis	5	100.0		5	80.0		4	100.0		5	20.0	
T1/T2	87	96.6	0.476	88	85.2	**0.010**	87	97.7	0.939	85	27.1	0.830
T3/T4	387	98.4		394	94.2		388	97.4		393	24.2	
Spread to nearby lymphnodes												
Absent	277	97.8	0.742*	276	92.4	0.767	273	97.4	0.782*	273	24.2	0.696
Present	194	98.5		203	93.1		198	97.0		202	25.7	
Vessel invasion												
Absent	166	98.2	0.889	166	92.2	0.988	163	97.5	0.747	164	23.2	0.578
Present	301	98.0		308	92.2		303	97.0		306	25.5	
Surgical margin invasion												
Absent	460	98.0	1.000*	468	92.5	0.264*	459	97.2	1.000*	465	24.3	0.500*
Present	13	100.0		13	84.6		13	100.0		12	33.3	
TNM												
Stage 0	1	100.0		1	100.0		1	100.0		1	0.0	
Stage I	76	96.1		76	86.8		75	98.7		74	28.4	
Stage II	183	98.4	0.713	181	94.5	0.336	180	97.2	0.940	180	21.7	0.550
Stage III	147	98.6		156	92.3		150	96.7		154	24.7	
Stage IV	70	98.6		71	93.0		70	97.1		72	30.6	

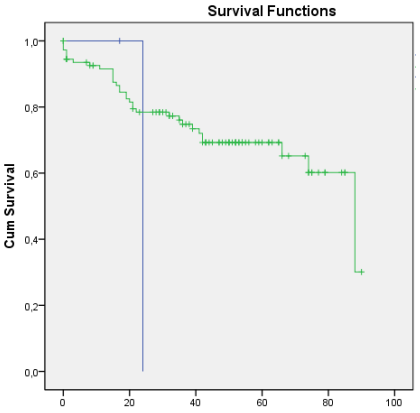
COLON CANCER



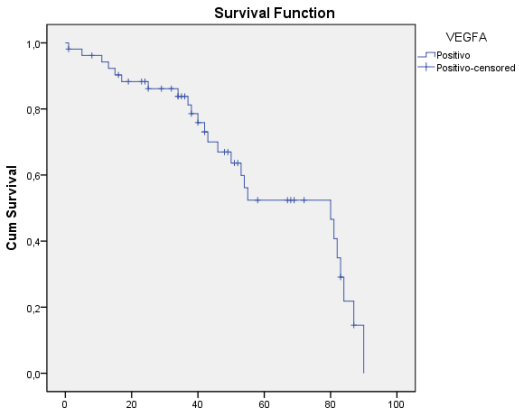
Stage I (*p=0.828)*



Stage II (*p=0.397)*

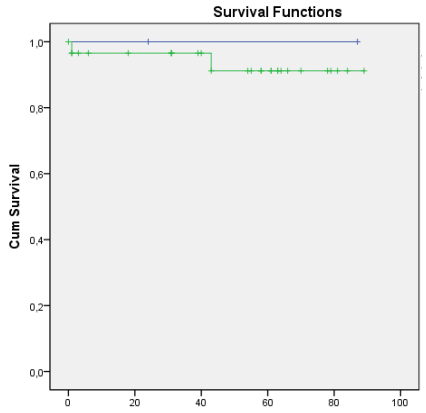


Stage III (*p=0.351)*

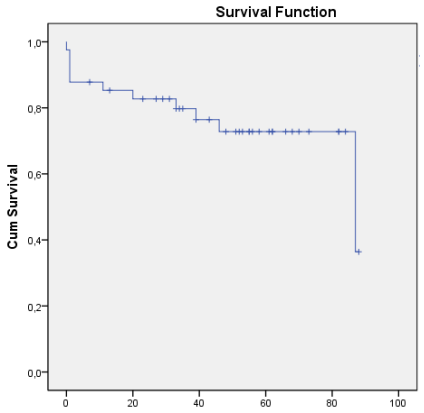


Stage IV*

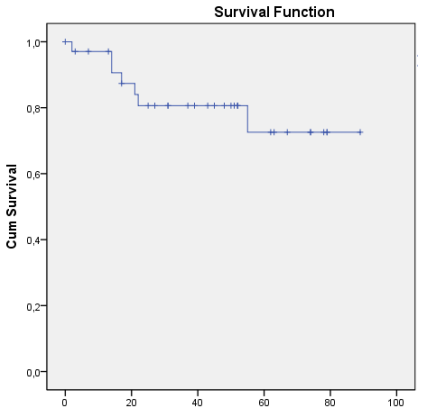
RECTAL CANCER



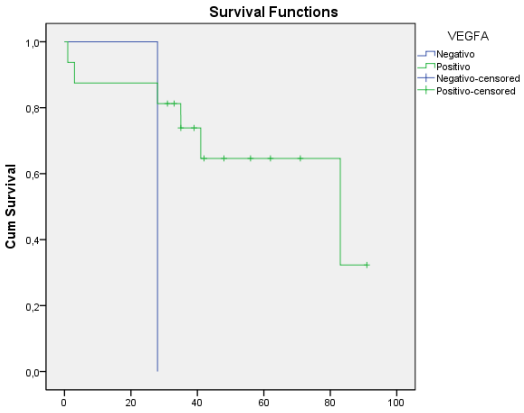
Stage I (*p=0.724)*



Stage II*



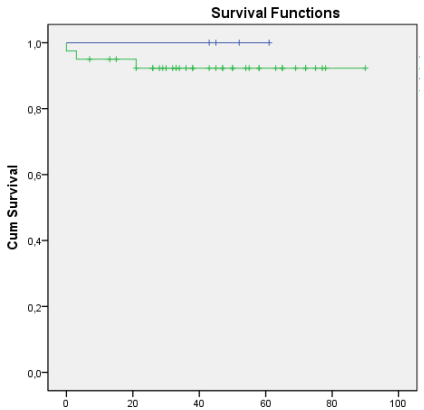
Stage III*



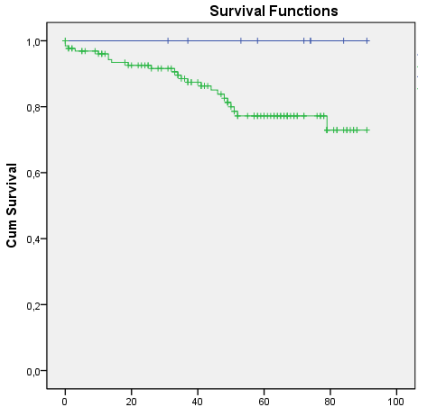
Stage IV (*p=0.120)*

Figure 32: Kaplan-Meyer survival curves of VEGF-A plasma membrane expression in colon and rectum, by stage.
*no comparasion was realized, because all cases are VEGF-A+

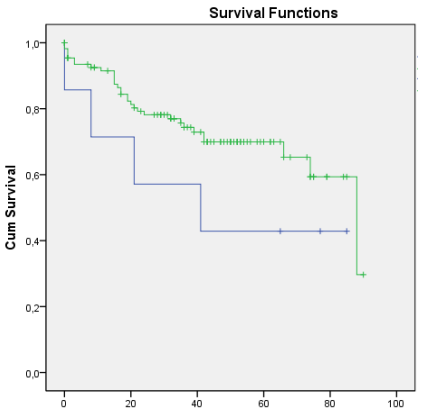
COLON CANCER



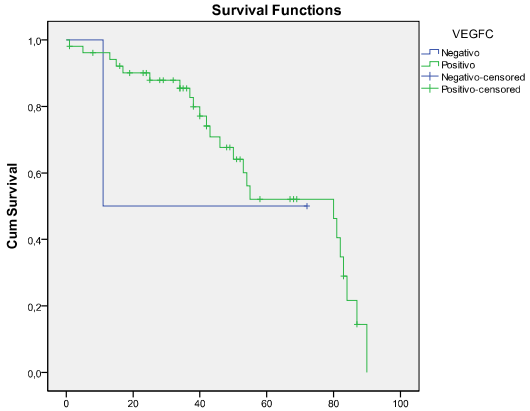
Stage I (*p=0.574*)



Stage II (*p=0.144*)

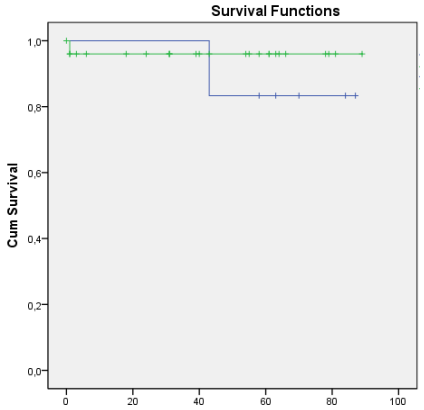


Stage III (*p=0.176*)

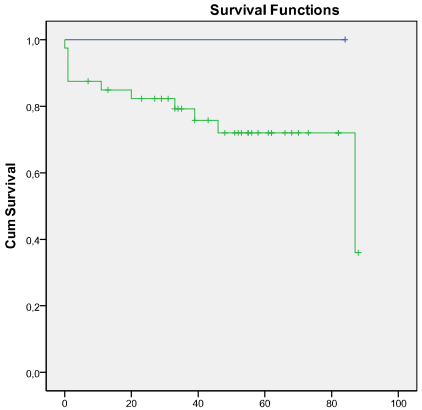


Stage IV (*p=0.700*)

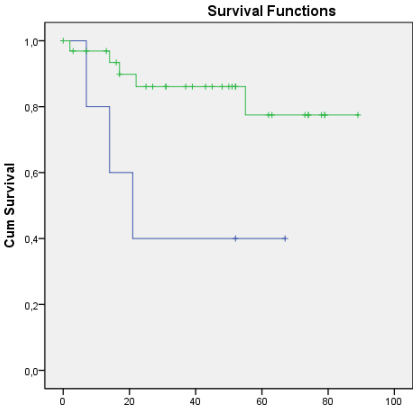
RECTAL CANCER



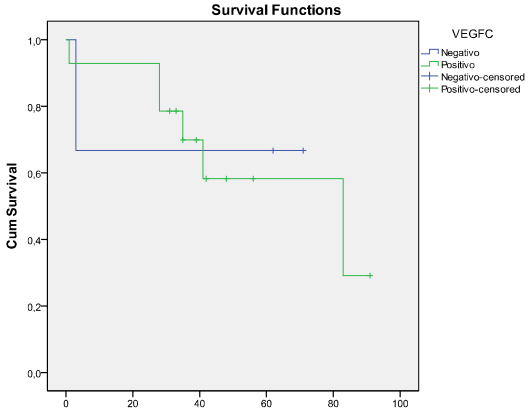
Stage I (*p=0.421*)



Stage II (*p=0.567*)



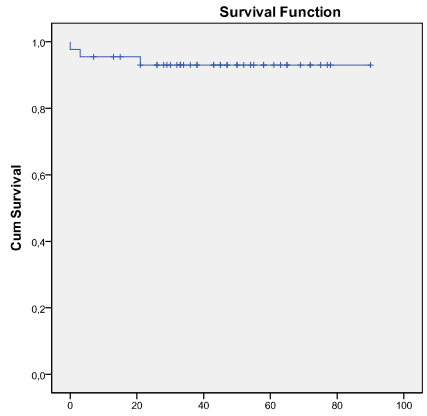
Stage III (*p=0.019*)



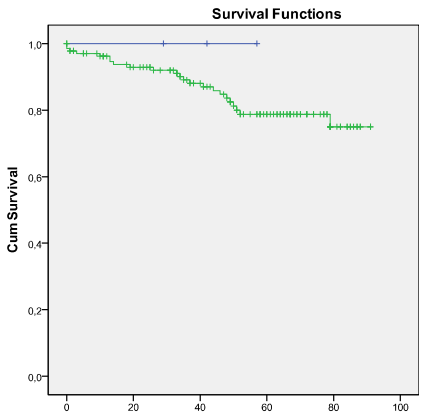
Stage IV (*p=0.946*)

Figure 33: Kaplan-Meyer survival curves of VEGF-C plasma membrane expression in colon and rectum, by stage.

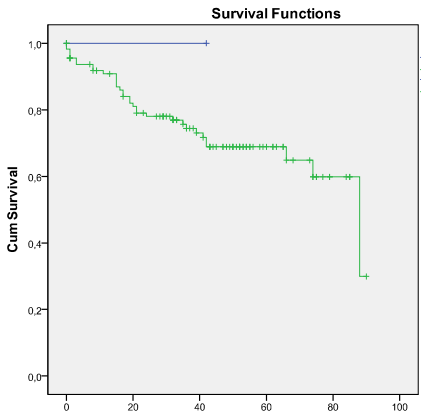
COLON CANCER



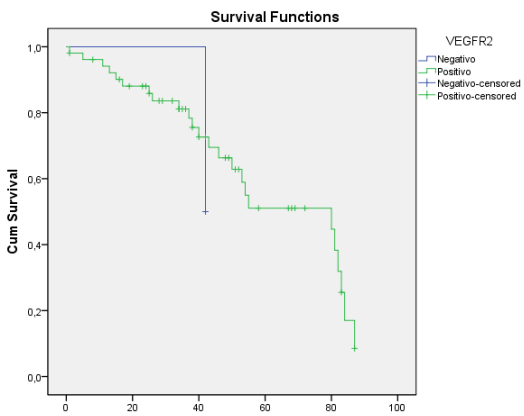
Stage I*



Stage II (***p=0.498***)

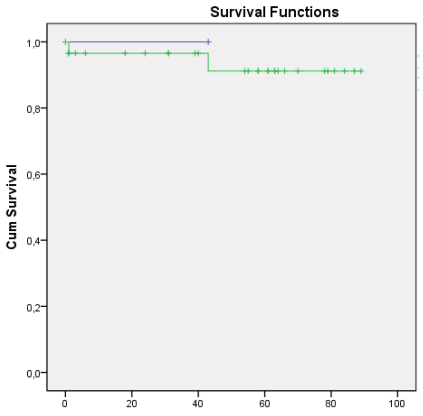


Stage III (***p=0.532***)

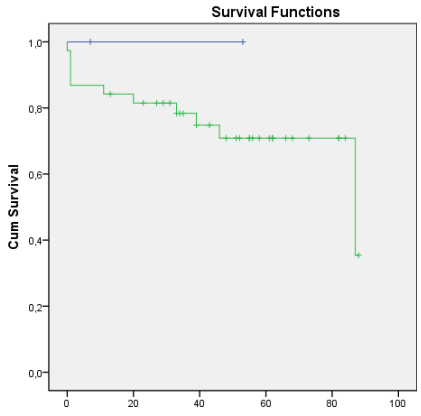


Stage IV (***p=0.683***)

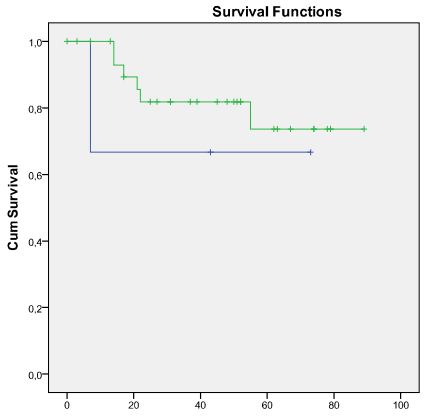
RECTAL CANCER



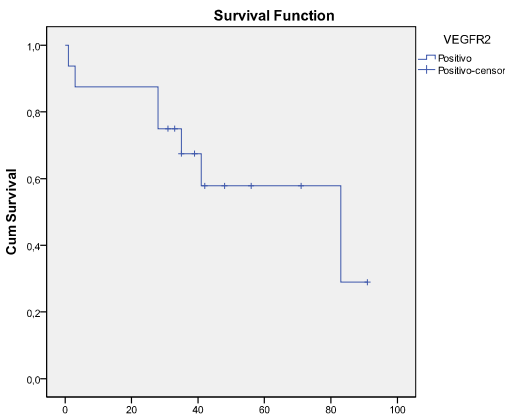
Stage I (***p=0.764***)



Stage II (***p=0.486***)



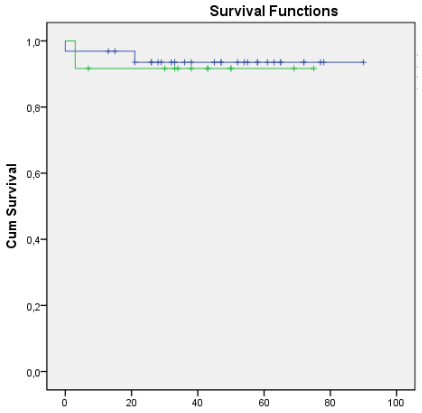
Stage III (***p=0.515***)



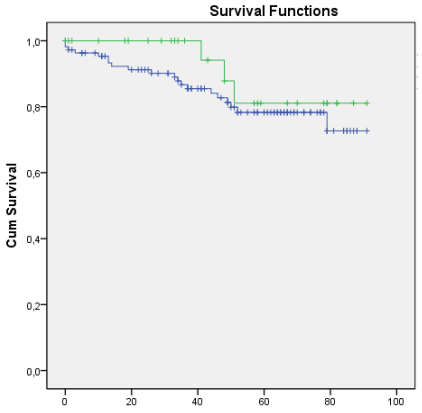
Stage IV*

Figure 34: Kaplan-Meyer survival curves of VEGFR-2 plasma membrane expression in colon and rectum, by stage.
* no comparasion was realized, because all cases are VEGFR-2+

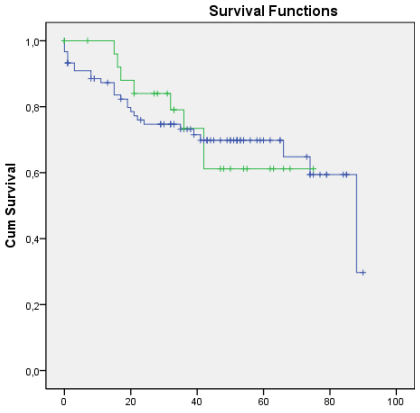
COLON CANCER



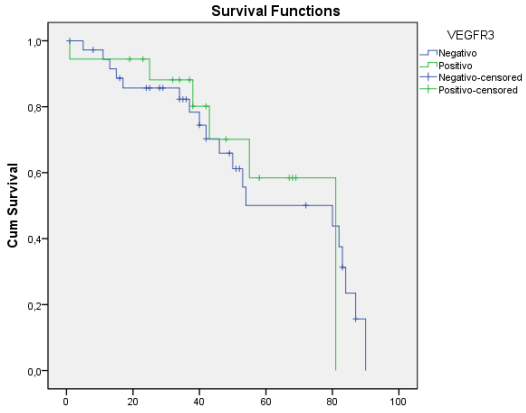
Stage I (***p=0.803)***



Stage II (***p=0.352)***

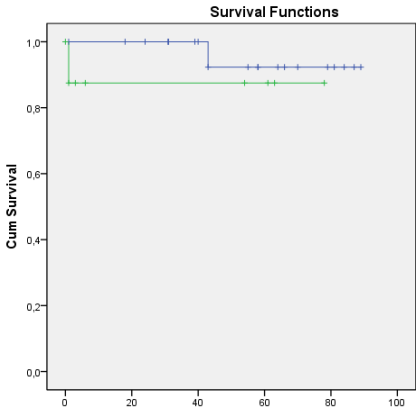


Stage III (***p=0.962)***

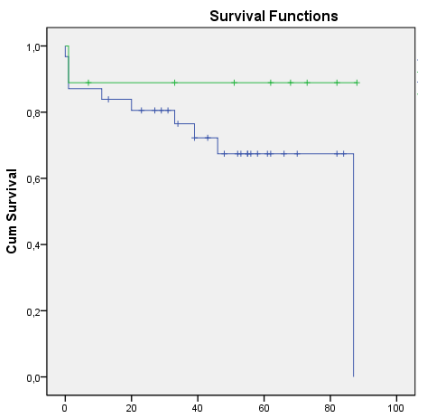


Stage IV (***p=0.852)***

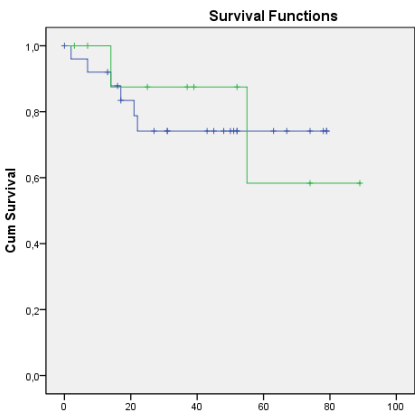
RECTAL CANCER



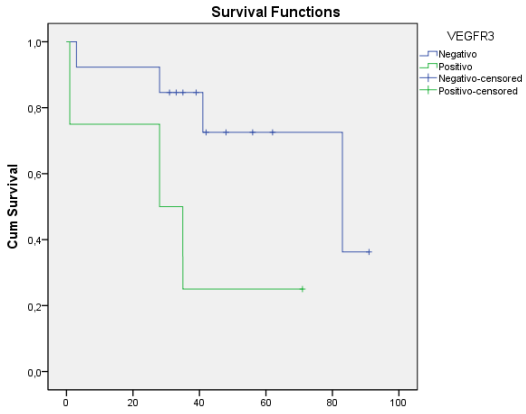
Stage I (***p=0.428)***



Stage II (***p=0.186)***



Stage III (***p=0.840)***



Stage IV (***p=0.047)***

Figure 35: Kaplan-Meyer survival curves of VEGFR-3 plasma membrane expression in colon and rectum, by stage.

Assessment of correlation between VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 expressions and the clinical data revealed that VEGF-A positive cases were associated with "Patient gender" (p=0.016) and VEGF-C shows a tendency to association with "Personal History of CRC" (p=0.060) (**Table XXX**).

When analyzing correlation with data from diagnosis/surgery we find association between VEGF-C expression with "Tumour Localization" (p=0.037), and "Macroscopic Cancer type" (p=0.048). VEGFR-3 shows association with "Hepatic Metastasis" (p=0.032) (**Table XXXI**).

When analyzing correlation with pathological data we find association between VEGF-A and VEGF-C expression and "Differentiation" (p=0.001 and p=0.007, respectively); VEGF-C expression and "Tumour penetration" (p=0.010); VEGFR-2 expression and "Histological type" (p=0.007) (**Table XXXII**).

Observing colon and rectal cancer overall-survival curves assessed by log-rank test, of VEGFA, VEGFC, and VEGFR-2, VEGFR-3, **Figure 32 – 35** we find a statistically significant association in VEGF-C expression and stage III for rectal cancer (p=0.019) and VEGFR-3 expression and stage IV for rectal cancer (p=0.047).

5.1 Epidemiological Characterization

CRC epidemiological data abounds in the worldwide literature, but in the case of the Portuguese population this data are scarce and the existing studies are retrospective studies based on cancer registries but with few data that permits to characterize the affected population.

In the developed world, CRC represents a major public health problem (321) and in Portugal, it is the second most frequent cancer and the second cause of death by cancer (18,20).

The North of Portugal is traditionally considered to be an area of high CRC incidence. Braga Hospital, in the North of Portugal, has an area of reference of 300000 patients, so with this first task we intended to characterize the patients treated at this hospital and also comparing the results with the literature data. In the future and with the extension to other regions this will permit a better adjustment of screening programs. Our results clearly demonstrated that CRC is a major problem of public health impact due to the high incidence and the degree of advanced stage of the tumours at moment of diagnosis.

5.1.1 General characterization

5.1.1.1 Age and Gender

In this study most of the 672 patients, 419 (62.4%), were male patients and the age range of most patients (61%) was 61-80 years old. Except for the group older than 81 years old, CRC incidence was more frequent in men. Similar results were found in literature with CRC, being more frequent at advanced age and in men (1,3,4,14).

Advanced age is the most significant risk factor for diagnosis of CRC which is defined as a disease of elderly people, with the majority of cases arising after 65-70 years of age and with an incidence relatively lower under 40 years. Still, 15% of cases will occur in people ≤ 50 years old (3,13,22,230,322–326), although another study suggests a lower value (7%) (327) and a large study identifies it as one of the 10 most commonly diagnosed cancers among men and women aged 20-49 years (22). Early onset of CRC is assumed to be indicative of genetic susceptibility (323), often associated with a positive family history (328). In some studies, such younger patients

presented more advanced disease and more aggressive tumour grades at diagnosis and had less favourable prognosis (22,327,329). Also advanced CRC prevalence increases with age and is higher among men than women (21,321,326,329,330) and cross-sectional analyses estimated that men reach an equivalent prevalence at a much younger age than women (21).

5.1.1.2 ANATOMIC DISTRIBUTION OF TUMOURS

Among the 672 patients, colon cancer was more frequent than rectal cancer (65.3% versus 34.7%) and most colon cancers were left-sided (64.7% of all colon cancers). In the case of rectal cancer most (50.6%, n=118) were localized in the middle third. Similar results are documented in literature (13,329,331–333).

Tumour distribution throughout colon and rectum depends on genetic and environmental factors involved in colorectal carcinogenesis and on gender, race and patient´s age (13,329). In general, almost two-thirds of all bowel cancers are colon cancers and over one-third are rectal cancers (331–333). Recently, other studies reported a shift of CRC distribution to the right colon in the high risk population for unknown reasons (334–338), and other have suggested that the frequency of right-sided colon cancer increases in elderly patients (13). This shift of CRC distribution implies that arguments used to recommend full colonoscopy instead of flexible sigmoidoscopy in CRC screening can be applied in high risk countries and that this is an issue that deserves further attention in future years, to document if that shift is also occurring in the population of Braga Hospital.

5.1.1.3 PAST PERSONAL AND FAMILIAR HISTORY

Epidemiological studies suggest that at least 15% of colorectal cancers arise in individuals with an inherited predisposition for the disease (18,339). The literature also reveals that positive familiar story is strongly associated with CCR (13,326) although it is considered a high specific association with low sensitivity (326).

In our study, 94.8% of patients had no history of previous colorectal polyps; 4.1% of patients had a previous personal history of CRC; 7.7 % had a personal history of other cancers and 9.7% of patients had a positive familiar story for CRC.

Knowing CRC natural history, we would expect a higher incidence of previous colorectal polyps history. This lower value could be the result of the low adherence of patients to colonoscopy without symptoms. Also the value of familiar story is underestimated since a significant number of patients do not know ignore their relative's cause of death.

5.1.1.4 Clinical presentation

Most patients (81.3%) from our study were symptomatic at diagnosis. Analysing colon and rectal cancer, 77.4% (n=340) and 88.5% (n=206), were symptomatic at diagnosis, respectively. Digestive bleeding was the most frequent symptom for both (17.1% and 20% respectively), followed by large bowel obstruction in colon cancer (15.0%) and digestive bleeding associated with change in bowel habits (17.4%) and change in bowel habits (14.5%) in rectal cancer.

Symptoms of CRC can be nonspecific or quite fulminant (340). Signs and symptoms of colon and rectal cancers are varied, nonspecific, and somewhat dependent on the localization of the tumour (48). Traditionally right-side colon cancers bleeds asymptomatic and are detected by anemia discovered by a routine haemoglobin determination or when studying constitutional symptoms. Cancers located in the left colon are often constrictive in nature, so patients more frequently notice a change in bowel habit. In rectal cancer, the most frequent symptom is hematochezia, other frequent symptoms are tenesmus and change in bowel habits (48). In a meta-analysis Jellema et al. (326) analysed various symptoms of CRC and concluded that the symptoms most commonly investigated included abdominal pain, rectal bleeding, change in bowel habits, and perianal symptoms. Of the typical symptoms of CRC, only weight loss had some diagnostic value, with a fairly high specificity (326).

5.1.2 Operative reports by surgeons

Operative reports by surgeons like type of surgery, presence of perforation and tumour mobility were collected.

Emergency situations are most commonly related to the complications of tumour obstruction (341) or tumour perforation (341,342), both with a poor prognosis and high risk of recurrence (341,343).

Data from literature are variable regarding the emergency operation incidence, but overall approximately 20% of patients with colorectal cancer present as an emergency (343). Cuffy et al. (344) reported that over 15% of all cases with CRC present acutely as obstruction or perforation, with a mortality rate reaching 8.2% after an emergency operation. A lower value was documented by Lane Smothers et al. (345), 15.7% in a study with 184 CRC patients, and by Pavlidis et al. (346), 12%, in a study realized with 1009 patients with CRC.

In this study, 422 (96.1%) of colon and 203 (87.1%) of rectal cancer patients have been submitted to surgical treatment, and of this, 20.9% and 4.9% have been submitted to a urgent operation, respectively.

Perforation was more frequently associated with colon than rectal cancer (7.6% *vs.* 1.5%) and in both cases cancers were presented at laparotomy as mobile masses (82.2% and 66.9% respectively).

5.1.3 Histopathological reports

When pathologists examine a CRC specimen, they are taking a single fragment of the tumour at a given time, thereby providing information on the extent of tumour diffusion. A quantitative assessment of tumour extension, however, is insufficient to provide additional diagnostic, prognostic, and possibly predictive information required to plan the best therapeutic strategy (347).

Histopathological reports like tumour size, macroscopic serosal involvement, tumour differentiation, margin resection and blood and lymph node involvement was determined by two

experienced pathologists at Pathology Department of Braga Hospital. Some of these data will be reflected in the final pathological stage, pTNM.

5.1.3.1 TUMOUR SIZE

Most of the cancers analyzed, 49.0% of colon cancer and 52.7% of rectal cancer, have a maximum tumour diameter smaller than or equal to 4.5 cm. Tumour size should be reported as part of permanent record of tumour description. Although the size of the tumour is of no prognostic significance, it may be important for quality control of tumour size determined by nonpathologic means (eg, imaging modalities) (348).

5.1.3.2 MACROSCOPIC SEROSAL INVOLVEMENT

Macroscopic serosal involvement corresponds to a pT3 in TNM classification; in our series, 69.9% of colon cancers and 53.7% of rectal cancer, presented with macroscopic serosal involvement. When Macroscopic serosal involvement is present, even in the absence of lymph node involvement (AJCC/UICC stage IIB classification) it also identifies high-risk disease requiring adjuvant therapy (347,349,350).

5.1.3.3 TUMOUR DIFFERENTIATION

Tumour differentiation is consistently recognized as an important prognostic parameter (347,351). In our series, most of the cancers analysed were moderately-differentiated, 40.8% of colon cancer and 39.4% of rectal cancer.

5.1.3.4 RESSECTION MARGINS INVOLVEMENT

For colon cancer the primary determinant of the extent of bowel resection is the need for adequate removal of lymph nodes and arterial supply that is consistent with the creation of a well-vascularized anastomosis. An adequate minimum length for proximal and distal colon resection margin is 5 cm, although they are generally much greater. Radial, non-peritonealized negative margins resection of the colon should be obtained and must be histologically free of disease to achieve a curative resection (352).

For rectal cancer the primary determinant of the extent of resection of proximal rectum is determined by technical considerations for obtaining adequate lymphadenectomy and reconstruction. The margin resection length should be a minimum of 5 cm (352). The current recommendation for a adequate distal margin of resection is 2cm, and this is adequate for preventing local recurrence (353). In the case of the circumferential margin, 1 mm of margin is the current accepted margin, but if 2 mm were obtained instead of the 1 mm, local recurrences rates decreases from 16% to 5.8% (353).

In our study we only observed “Margins resection involvement” in 6 patients of colon cancer and in 20 of rectal cancer patients.

5.1.3.5 VASCULAR INVASION

In our study, it was reported 54.2% and 55.6% of venous vessel invasion and 39.3% and 44.3 of lymphatic vessels invasion, for colon and rectal cancer, respectively.

CRC exploits the lymphatic and venous drainage sistem for dissemination to regional lymph nodes and distant organs and vascular invasion is an independent adverse prognostic factor in CRC (347,354,355). The diagnosis of vascular invasion in CRC specimens may be exceedingly difficult with conventional hematoxylin-eosin staining alone (356). Literature data reported a CRC vascular invasion in ranges from 10% to 89% (355) most likely due to the different criteria used for its identification or because of patient selection. To note that in some studies no distinction was made between venous and lymphatic vessels or intramural and extramural venous invasion (347).

5.1.3.6 HISTOLOGICAL STAGING AND FOLLOW-UP

Stage at diagnosis plays a significant role in CRC survival (15,254,324–326,340,357) and is actually the main prognostic factor in CRC (3,15,235,324,325) but it is difficult to accurately determine the stage prior to surgical treatment (358).

Staging has evolved over time, and TNM system is used currently. This is an evaluation system based on 3 variables: primary tumour (T), regional nodes (N), and metastasis (M) (340,359). In the past, patients presenting the same stage of CRC were considered similar in terms of prognosis. The new staging criteria recognize that they are usually quite different and subsets of patients with varying survival statistics can be found (340,358). Less than one quarter of the patients present early disease (Stage I) that is curable by surgical resection (15,324,340) and more than 20% of CRC patients present stage IV disease at diagnosis (340). This has an impact in five year survival rates and we can expect a five-year survival rate greater than 90% for stage I (15,324,326) and less than 10% for stage IV (326). On the other hand, around 40% of patients diagnosed with CRC eventually develop metastatic disease (325) and about two-thirds of the patients undergo resection with curative intent, but 50% of patients still die of the disease within five years (357,360).

As we stated above, most colon and rectal cancer patients from our study were stage IIA (33.7% ans 21.2%, respectively), followed by stage IIIB (22.5%) for colon cancer patients and stage IV (18.2%) for rectal cancer patients.

Despite expecting a worse prognosis in rectal cancer patients, we observed that 27.8% of colon cancer and 18.0% of rectal cancer patients died from a colorectal cancer-related cause. Follow-up time ranged from 2 to 7 years and in that period 14.6% of patients with colon cancer and 19.3% with rectal cancer had recurrence, mostly in liver.

These data are consistent with the literature (15,324,340), with low percentage of patients diagnosed at stage I, 13.0% for colon cancer and 18.7% for rectal cancer. Also, the percentage of stage VI diagnosed patients was very close to that observed in literature, with 18.2% for rectal cancer and 18.7% for colon cancer (340). From these data, we would expect a higher mortality in rectal cancer patients compared to colon cancer, but we observed very similar results, documented by the

log-rank test, when comparing between colon and rectum cancer survival (p=0.518). In the literature, studies have shown conflicting results when comparing prognosis and localization (360). Reduced survival in left colon cancer compared to right colon was reported in a Norwegian study from 1987 and Aldrige et al. (360–362) reported similar results, but no differences were detected in other studies (360,363–365). We also observed a lower value of 5 years disease recurrence, 14.6 % and 19.3% for colon and rectal cancer respectively, when compared with values of 40% found in the literature. These data may reveal a different biological behaviour or be the result of the follow-up time, however, other studies with larger series must be done.

5.2 MCTs, Chaperones and Glycolytic Metabolic Markers Immunohistochemical expression in CRCs and Normal Adjacent Tissues and correlation with epidemiological data

Our group has previously analyzed the expressions of MCT1, 2, and 4 in a series of 126 CRC (109) and we document that the expression of the MCT isoforms in tumour cells was significantly increased when compared to normal adjacent epithelium and we also observe a significant gain in membrane expression for MCT1 and MCT4 and loss of plasma membrane expression for MCT2 in tumour cells (109).

With this work we hypothesize to reinforce the results obtained, by evaluating MCT1, MCT4 immunohistochemical expression in this larger series of 580 cases, adding immunohistochemical expression evaluation of chaperones CD147, CD44 and glycolytic metabolic marker GLUT1 and correlation with MCTs expression to further understand the role of MCTs in CRC glycolytic metabolism, besides the advantage of the correlation with epidemiological data.

In this study, we evaluated MCT1, MCT4, CD147, CD44 and GLUT1 immunohistochemical expression in a CRC series of 580 cases and we observed that all the proteins studied are overexpressed in tumours when comparing with normal-adjacent tissue and in plasma membrane expression pattern (p<0.001). MCTs were the proteins most frequently expressed, followed by CD147, GLUT1 and CD44.

MCT1 results are consistent with the previous results of our group (104,109), also documented by Koukourakis et al. (115) who document a strong membranous expression in cancer cells of CRC but not in the adjacent stroma or the normal colonic mucosa.

Similar results were obtained with MCT4, we observed that MCT4 expression and plasma membrane staining was higher in tumour cells than in normal adjacent cells. These results are consistent with the previous results of our group (104,109), although Koukourakis et al. (115) and Lambert et al. (144), observed only a weakly and no expression of MCT4 in tumour cells, respectively, suggesting a minimal role in the metabolic intratumoural communication (115).

As stated before, cancer is associated with an increase in glycolytic flux (102,108–110,112,122) with consequent increase in lactic acid production (103,109–112). The maintenance of intracellular pH is achieved by upregulation of MCTs (109) namely; MCT1 with a ubiquitous tissue

expression (109,127) and participating in the bidirectional transmembrane exchange of lactic acid (115) and MCT4 with a localization more restricted to the glycolytic cells (109,366) and with a low-affinity lactate (105,124,138,366). So we might predict that its expression would increase in CRC cell to enable to export the increased quantities of lactic acid and so prevent apoptosis.

The lower expression in normal adjacent cells is in accordance to what is known on normal colon metabolism. MCTs were demonstrated to transport aliphatic monocarboxylates, including lactate, pyruvate and ketone bodies but also the branched-chain oxo acids derived from leucine, valine and isoleucine, and the ketone bodies acetoacetate, β-hydroxybutyrate and acetate (134,135); consequently, MCTs play a pivotal role in mammalian metabolism. We also observed that expression in normal adjacent cells is more marked for MCT1, what is in accordance to the broader distribution of this transporter and also because it transports butyrate, a substrate for colonic epithelial cells, and possess trophic effects in the colon (127,134,135,137).

Chaperones CD147 and CD44 immunohistochemical expression were also overexpressed in CRC when comparing with normal adjacent tissue and in plasma membrane expression pattern (p<0.001). Functional expression of MCTs is regulated by these accessory proteins (104,135,157–162), that are involved in trafficking, anchoring of plasma membrane proteins (135) and communication of cell-matrix interactions (181,182), respectively.

With regard to CD147, besides acting as MCT chaperone, CD147 expression seems to be dependent on MCT1 and MCT4 expressions (135,157,160) and in all tissues expressing MCT1 or MCT4, CD147 expression was consistently found co-localized in the same regions (158). In our study, we observed a higher expression and higher plasma membrane staining was in tumour cells than in normal adjacent cells. These results are consistent with those observed in the literature. Zheng et al. (177), Buergy et al. (178) and Jin et al. (179) documented that CD147 expression is stronger in C and metastatic carcinoma than normal adjacent cells.

The glycolytic metabolic marker GLUT1 has also a higher expression and higher plasma membrane staining in tumour cells than in normal adjacent cells. These results were expected because as a consequence of the high energetic demands observed in CRC, increased glucose metabolism and utilization is accomplished by upregulation of glucose transport across the plasma membrane (209,212), so increased GLUT1 expression reflects an increased glycolytic metabolism (209,210,212,213,215,367) in CRC.

Some studies suggest that GLUT1 expression may play an important role in the survival of tumour cells by promoting an adequate energy supply (210,213) and could be a useful biomarker for malignant transformation (210,214,216).

We studied the association between MCT isoforms and the remaining proteins and observed that in tumour samples, MCT1 positive cases were associated with CD147 plasma membrane expression and between MCT4 and both chaperones and GLUT1 plasma membrane expression. As stated before, functional expression of MCTs is regulated by these chaperones (104,135,157–162) and our results support these previously mentioned findings. Also the association found between MCT4 and the glycolytic metabolic marker GLUT1 can result from the fact that CRC cells upregulate GLUT1 to increase glucose uptake and, subsequent to "aerobic glycolysis", while the accumulated lactate is extruded by MCTs.

We studied the association between MCT chaperones, metabolic marker GLUT1 expression and clinical data, diagnosis/pre-operative staging data pathological and follow-up data and compared with other cancer literature data on CRC.

MCT1 positive cases were associated with the presence of "Pulmonary Metastasis" so more advanced CRC stage. In our previous study we documented a significant correlation between MCT1 plasma membrane staining and vascular invasion (109), that was not observed in this larger series, one possible explanation is that different methods may be used to evaluate vascular invasion.

We found that MCT4 positive cases were associated with "Personal History of CRC". Patients with a "Personal History of CRC" presented an increased risk to develop CRC, this higher expression of MCT4 in the patients may reflect an alteration of CRC metabolic profile conferred in the previous cancer.

There was also an association between MCT4 positivity and "Rectal Examination", namely with fixed rectal cancer. With digital rectal exam, the size, location, and degree of fixation of most low and some middle third rectal tumours can be detected and assessed. Assessment of the extent of local disease by digital rectal exam is imprecise (368,369), however, rectal fixed tumours are generally associated with an advanced rectal cancer stage (369).

There is some controversy in the literature when analyzing the correlation between CD147 expression and the clinicopathological characteristics in CRC. In our study, we only found association between CD147 positivity and "Tumour Size" and a tendency to associate with "Patient

Age" (p=0.056), also observed for MCT4 plasma membrane positive cases (p=0.052).

Zheng et al. (370) reported that CD147 expression was positively correlated with tumour size, depth of invasion, vascular or lymphatic invasion, grade of infiltration of CRC. On the other side, Jin et al. (167) documented a CD147 overexpression in CRC compared to normal mucosa, but no correlation was found with TNM stage. Also Jung et al. (149) and Stenzinger et al. (371) showed that the CD147 overexpression was not associated with clinicopathological parameters, although Stenzinger et al. (371) and Buergy et al. (372) observed that it was associated with a poor clinical prognosis.

Associations of CD147 expression with survival and prognosis have been suggested for other tumours, such as endometrial (373), ovarian carcinoma (173) and esophageal squamous cell carcinomas (374) although in esophageal squamous cell carcinomas Ishibashi et al. (176) it was reported that CD147 expression was not associated with the recurrence-free survival. In oral squamous cell carcinoma, increased expression of CD147 has been shown to correlate with lymphatic metastasis and tumour progression (375) and Yang et al. (376) found that CD147 expression in breast carcinoma cells rendered them resistant to anoikis, a form of apoptosis triggered by a lack of improper cell-matrix interactions, through an MAP kinase-dependent pathway. Marieb et al. (148) documented that upregulated CD147 expression stimulates hyaluronan production by elevating hyoluronan synthases, which is closely related to the anchorage-independent growth of cancer cells. Taken together, our result supported the opinion that CD147 might enhance tumour growth of CRC by disrupting the balance between apoptosis and proliferation.

In our study, we only documented a correlation of CD44 immunoexpression and "Vessel Invasion" in other words with metastatic spread also documented in the tendency to associate with "Hepatic Metastasis" (p=0.055). These results are in harmony with previous reports, which states that extracellular acidification induces invasion.

Several studies have suggested an important biological role for CD44 in tumour progression and metastasis, and the potential for the use of CD44 variant expression as a clinicopathological marker of disease progression in CRC (189–194) and other cancers (195–199). Some studies observe that protein expression of standard and variant isoforms of CD44 correlates with a poor prognosis in CRC (200–202) and that it can be a molecular marker for CRC and its micrometastasis to the regional normal lymph node (202), but divergent conclusions have been reached regarding a

potential relationship between variant CD44 expression and the prognosis of patients with CRC (181,203–205). More recent studies suggest either no role for CD44s or a worse clinical outcome (192,206–208), documented by correlation between CD44 expression and metastatic spread and survival (377–380).

Also studies performed in gastric cancer found no correlation of CD44 immunoexpression and clinicopathological characteristics such as tumour size, pathologic stage, histological grade, angioinvasion, perineural invasion and lymph node metastasis or prognosis in terms of survival (183,381). However, Ghaffarzadehgan et al. (377) reported significant correlation between CD44 expression and histological grade and patient survival.

GLUT1 positive cases in plasma membrane show a significant association with "Macroscopic Cancer type", namely with exofitic lesions, high CEA level (p=0.05) presence of "Hepatic Metastasis" (p=0.046), "Tumour Differentiation" (p=0.009), and a tendency for association with "Spread to nearby lymph nodes" (p=0.058) namely poorly-differentiated tumours, in other words, tumour characteristics associated with more aggressive tumours and poor prognosis, so tumours with high energetic demands to grow and metastize. Previous studies suggest that GLUT1 expression may play an important role in the survival of tumour cells by promoting an adequate energy supply (210,213) and could be a useful biomarker for malignant transformation (210,214,216). Many studies have reported a correlation between GLUT1 expression level and the grade of tumour aggressiveness (209,212,213,217,218), increased proliferative activity and energy requirements (212) which suggests that GLUT1 expression may be of prognostic significance (209,213,219).

In our study, we documented a significant correlation between GLUT1 and tumour differentiation, results which are in accordance with those of Sakashita et al. (382) that reported that GLUT1 expression was significantly different between well differentiated and less differentiated groups in CRC. Also, Ito et al. (383) in lung adenocarcinomas and Chen et al. (384) in breast cancer, demonstrated that GLUT1 immunostaining was stronger in tumours with lower differentiation. Others studies (214,217) reported that there was no correlation between GLUT1 expression and histological differentiation.

The relationship between GLUT1 expression the depth of invasion has been reported in CRC. Sakashita et al. (382) reported that GLUT1 expression was significantly different between T1

and T2 groups, however, Younes et al. (217) and Young Jin Jun et al. (213), demonstrated that there was no significant difference between GLUT1 expression and depth of invasion. Younes et al. (217), Young Jin Jun et al. (213) and Zhou et al. (218) documented that there was a close correlation between strong GLUT1 expression and the frequency of lymph node metastasis in CRC. Sakashita et al. (382) reported that the correlation of GLUT1 expression in CRC with nodal metastasis was higher than that in those without, but the difference was not significant. In our study, we did not observe that correlation but we documented a significantly correlation between "CEA level" and "Hepatic metastization" both associated with more advanced cancers. The greater degree of GLUT1 expression in these tumors indicates that GLUT1 may be important for maintaining the high-energy requirements of aggressive cancers.

Young Jin Jun et al. (213) documented that there was a close correlation between GLUT1 expression and tumour stage, and also showed that GLUT1 expression was significantly correlated with poor overall survival and disease-free survival. Also Shen et al. (219) found a worse prognosis in GLUT1 positive cancers; but Haber et al. (214) reported a association of GLUT1 staining status and stage; however, no statistical significance was revealed. In our study we did not observe any statistically significant relation with survival. Also Hong et al. (210) did not show these results, but suggest the possibility that tumours with absent GLUT1 staining might express another GLUT iso-form, which might be associated with poor prognosis (210,385). Also, for breast cancer, Avril et al. (386) find no association. On the other hand, other studies reported that GLUT1 correlates with poor prognosis and tumour aggressiveness in carcinomas of the lung (387,388) and bladder (389), and in squamous cell carcinoma of the head and neck (390,391) and in ovarian cancer (392,393).

Although the associations between MCTs, chaperones and GLUT1 and clinicopathological data associated with worse prognosis, when we observe colon and rectal cancer survival curves assessed by log-rank test, we only find a statistically significant association between MCT1 expression and stage IV for colon cancer; GLUT1 expression and stage I for rectal cancer and a tendency to association between MCT4 expression and stage III for colon cancer (p=0.060); thus suggesting that longer follow-up times may be necessary to document this relationship.

5.3 MCTs, Chaperones and Glycolytic Metabolic Markers Immunohistochemical expression in Colorectal Cancer Hepatic Metastasis and Normal Adjacent Tissues and correlation with epidemiological data

Our initial aim was to correlate the results of MCTs, chaperones and Glycolytic Metabolic Markers Immunohistochemical expression in Colorectal Cancer Hepatic Metastasis with the results obtained in CRC and ascertain if the metabolic profile observed in CRC was maintained in CRC Hepatic Metastasis, but due to the few number of patients that have been submitted to hepatic resection during this period this was not possible.

So we retrieved a new series with 45 patients that have been submitted to CRC hepatic metastasis resection in the period of 1 January 2003 to 1 de January 2011 and analyzed the expression of MCT4, CD147, CD44 and GLUT1 in CRC hepatic metastasis and normal adjacent tissue.

No data are available in literature about this issue, being this the first work performed with these proteins in CRC hepatic metastasis.

When analyzing CRC hepatic metastasis, the same expression patterns were observed in tumour positive cases, in immunoreaction and plasma membrane suggesting the same alterations in metabolic profile documented in CRC tissues. The lower significance observed in the metastases series may be justified by the lower number of cases.

Analyzing the associations between MCT4 and the other proteins we observed that MCT4 positive cases were associated with both chaperones and GLUT1 plasma membrane expression, as observed in CRC tissues, reinforcing the role of the chaperones in the function of MCT4 (104,135,157–162) and upregulation of GLUT1 to increase glucose uptake and, subsequent to "aerobic glycolysis".

Assessment with anatomopathological data from primary tumour and Hepatic metastasis, revealed CD147 positive cases were associated with "Venous Vessel Invasion" (p=0.042) and no other correlation was observed, perhaps because of the series size.

5.4 VEGF-A, VEGF-C, VEGFR-2 AND VEGFR-3 IMMUNOHISTOCHEMICAL EXPRESSION IN CRCS AND NORMAL ADJACENT TISSUES AND CORRELATION WITH EPIDEMIOLOGICAL DATA

Tumour angiogenesis is essential to allow neoplastic mass development favoring access to the blood components, and also strengthening the vascular routes in the metastatic process (4,241,242,244,247,248). Neovascularisation promotes tumour growth by supplying nutrients, oxygen and releasing growth factors that promote tumour cell proliferation (232,239,244,249,250).

Numerous studies have demonstrated that tumour overexpression of VEGF is associated with advanced tumour stage or tumour invasiveness in various common human cancers (232,240,394,395) and, its overexpression in colon cancer tissue indicates poor prognosis (395); although paradoxically, some data showed that VEGF has not a significant prognostic value in colon cancer tissue (396).

Our results corroborate the premises that angiogenesis plays a key role in tumourigenesis and metastatic processes (231,232,397), because all the markers involved with neovascularisation were consistently expressed in tumour cells. Additionally, VEGF-C, a lymphangiogenic maker, was more significantly expressed in cancer cells rather than in normal cells. This general view of our results clearly indicate that CRC are predominantly composed by cancer cell that are directly or indirectly associated to the high expression of molecular players related to the blood angiogenesis and that the major lymphangiogenic molecule is also more importantly expressed in cancer cells that primarily escape from primary site to metastatic route by lymphatic vessels.

Normally, VEGF family members are weakly expressed in a wide variety of human and animal tissues; however, high levels of VEGF expression can be detected at sites where physiologic angiogenesis is required, such as fetal tissue or placenta, or in the vast majority of human tumours and other diseases such as, chronic inflammatory disorders, diabetes mellitus, and ischemic heart disease (4). Furthermore, VEGF family and its receptors are expressed at high levels in metastatic human colon carcinomas and in tumour-associated endothelial cells, respectively (4,240). Consequently, VEGF is recognized as a prominent angiogenic factor in colon carcinoma and the assessment of VEGF expression may be useful for predicting metastasis from CRC (4,240).

In literature, the role of the VEGF family members in CRC has, to date, mainly concentrated

on VEGF-A, but the newer members of the family, VEGF-C and VEGF-D, may have important roles to play in both angiogenesis and lymphangiogenesis (398).

VEGF-A promotes angiogenesis through enhancement of permeability, activation, survival, migration, invasion, and proliferation of endothelial cells (4,399) and play a role in early tumour development at the stage of adenoma formation (4,12,400) and some studies document a overexpression of VEGF-A in CRC (4,401). In other studies, VEGF-A expression was also found to be higher in patients with metastatic tumours (4,240,243), and high levels of VEGF-A expression were associated with advanced cancer stage and related with unfavorable prognosis (4,395,396,402). VEGF-A was documented as a useful marker for prognosis by significantly correlating with angio-lymphatic invasion, lymph node status and depth of invasion, notwithstanding it was not an independent prognostic factor (4,244,401).

VEGF-C gene was also found to be poorly and at maximum moderately expressed in CRCs when compared to control tissue (398,403); however, the number of samples analysed in this study, particularly, was small (n=12). In a larger series, however, the immunohistochemical expression of VEGF-C was correlated with lymph node spread (398,404). In our study, in opposite to that observed in literature, we did not observe a statistically positive correlation between tumour and normal adjacent tissues of VEGF-A expression. The majority of the normal-looking tissues were strongly decorated by the VEGF-A reaction. On the other hand, we observed that VEGF-C was overexpressed in tumours when comparing tumour cell strongly decorated to the weak staining of the normal-adjacent tissue (p=0.004).

The effect of VEGF depends not only on tumour cell expression of VEGF, but also on the VEGF receptors in the endothelial cells (4,232) so we also analyzed the associations between VEGF-A, VEGF-C and the receptors VEGFR-2, VEGFR-3 expression in CRC tissues and we observed that in tumour samples, VEGF-C positive cases were associated with VEGFR-3 expression (p=0.047), this is consistent with the fact that lymphangiogenesis induced by VEGF-C is driven mainly by the activation of the tyrosine kinase-linked receptor VEGFR-3 (405) and supports the fact that CRC escapes through lymphatic vessels, although no correlation with pathological data of lymph node metastasis or lymphatic vessel invasion was observed.

The comparison of the correlation among VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 expressions and the clinical-pathological data, data from diagnosis/surgery and pathological data

revealed that VEGF-A positive cases were associated with “Patient Gender” (p=0.016) and “Tumour Differentiation” (p=0.001); VEGF-C expression with “Tumour Localization” (p=0.037), and Macroscopic Cancer type” (p=0.048), “Tumour Differentiation” (p=0.007) and “Tumour penetration” (p=0.010); VEGFR-2 shows association with Histological type” (p=0.007) and VEGFR-3 shows with “Hepatic Metastasis” (p=0.032). All this characteristics characterize a high expression of molecules that contribute for progression, invasion and metastasis and poorer survival and prognosis that we observe in overall-survival curves for rectal cancer in VEGF-C stage III (p=0.019) and VEGFR-3 expression stage IV (p=0.047).

6.1 EPIDEMIOLOGICAL DATA

As previously mentioned, the beginning of this thesis coincided with the creation of the Coloproctology Unit of Braga Hospital, responsible, among others diseases, by the treatment of patients with diagnosis of CRC. All the work was performed looking for the development of protocols as well estimulate multidisciplinary meetings with Surgery, Pathology and Oncology. Besides been necessary to the drawing of this thesis, this initiative also, allowed to standardize the diagnosis, staging, treatment and follow-up, leading to a significant improvement in the management of these patients.

As stated before, CRC epidemiological data are scarce in Portugal, and our results clearly demonstrated that CRC is a major problem of public health, impact due to the incidence and the degree of advanced stage of the tumors at the moment of diagnosis. This work not only allowed a better knowledge of our population, but with other parallel studies, improved patient treatment at Coloproctology Unit of Braga Hospital.

The majority of our results are consistent to that observed in the literature. Most of our CRC patients were male and old patients, reinforcing the role of these data in CRC risk factors. Most of our cancers were located in colon more precisely left-sided colon. From these data, it would be expectable that flexible sigmoidoscopy would be a diagnostic procedure sufficient for most cases of CRC, but most cancers of our series were diagnosed by total colonoscopy, resulting in part from the fact that most of these patients have not done a screening exam but as investigation of some symptom, as documented by the higher percentage of symptomatic patients at diagnosis in our series. The low adhesion of our population to the CRC screening programs was also documented by the lower incidence of previous history of colorectal polyps, of previous personal and of a positive familiar story for CRC, than that observed in the literature.

As a measure of the re-structuring of Gastroenterology department of Braga Hospital and in part as a result of these observations, actually an annual screening programme is realized at the Braga Hospital.

From the reports collected from surgeons, we documented that most patients were submitted to a scheduled surgery, presenting similar results to that observed in the literature for emergent surgeries, what is associated to a worse prognosis as it influences staging besides the

patients being operated without a complete pre-operative staging.

Data from pathological reports reveals that although most of CRC in our series were small tumours, most of those tumours present macroscopic serosa involvement at diagnosis, what reflects a more advanced stage.

When analysing "Resection Margin involvement", we documented that this was more frequent in rectal than colon cancer. This data was expectable not only resulting from anatomical surgical reasons but also from technical reasons. This reflects the higher percentage of patients with local rectal cancer recurrence compared to colon cancer patients.

In what concerns "Vascular Invasion", venous vessel and lymph vessel are two routes of CRC metastization and actually considered as an independent risk factor. These data, and also the number of positive lymph nodes were not described in all specimens. For this reason we intend, with the Pathological department, and as it was already done for other cancers, to standardize the histological report of colon and rectal cancers.

Also, the results of "Staging at Diagnosis" were similar to that observed in the literature, with few patients diagnosed at stage I and almost 19% at stage IV, for rectal and colon cancer. "Metastization/Recurrence" during the follow-up were more frequent in rectal than colon cancer patients, but in both this was more frequent in the liver and most patients were asymptomatic, reinforcing the need of periodical follow-up. Despite expecting a worse prognosis in rectal cancer this fact was not documented in survival curves and longer follow-up may be necessary.

The results presented in this chapter were submitted for publication in international periodicals:

- Martins SF, Reis RM, Amorim R, Pinheiro C, Rodrigues AM, Baltazar F, Filho AL. An epidemiologic descriptive study of Colorectal Cancer patients treated at Braga Hospital, Northern Portugal.

Other results collected in CRC prospective database were used as material for Master thesis of medical students and some were posteriorly published:

. "Assessment of Quality of life (QoL) after rectal cancer surgery."

- Supervisor of Master thesis presented at School of Health Sciences in January 2009.

. "Sensibilidade da Ecografia Endorectal no estadiamento do Cancro do Recto: correlação com o estadiamento patológico."

- Supervisor of Master thesis presented at School of Health Sciences in January 2010.
- Carriço L, Martins SF. Sensibilidade da Ecografia Endorectal no estadiamento do Cancro do Recto: correlação com o estadiamento patológico. **Rev bras Coloproct**, 2011;30(4): 430-439. **(Appendix 9)**

. "Evaluation of quality parameters of rectal cancer surgery at the Coloproctology Unit of Hospital de Braga."

- Supervisor of Master thesis presented at School of Health Sciences in January 2011.
- Castro M, Martins SF. Evaluation of quality parameters of rectal cancer surgery at the Coloproctology Unit of Hospital de Braga. **J Coloproctol**, 2011;31(4): 362-371. **(Appendix 10)**

. "Assessment of surgical risk in CRC patients: possum vs. Acpgbi?"

- Presented as communication at "Congresso Nacional de Cirurgia 2012"
- Accepted for publication at Revista Portuguesa de Cirurgia. Goulart A, Martins SF. Assessment of surgical risk in colo-rectal cancer patients: possum vs. Acpgbi?

6.2 CRC AND HEPATIC METASTASIS METABOLIC MARKERS

One of cancer features is the ability to maintain a sustained proliferative signaling, that is responsible for the faster tumor growth comparing to normal cells. Thus, tumor cells present higher energy requirements, and this enhanced glucose consumption and glycolytic metabolism results in the production of high amounts of lactic acid. Therefore, in order to survive, cancer cells must reprogram their energy metabolism.

Recently, much attention has being given to the manipulation of tumour metabolism, in the context of therapeutic approaches and the expression of MCTs have already been documented by several authors (including our group), in CRC and other cancers.

The purpose of this work was not only to reinforce our previous results with a smaller series but also to expand the study to other metabolic markers, namely chaperones CD147, CD44 and the glycolytic metabolic marker GLUT1 to further understand the role of MCTs in CRC glycolytic metabolism, besides the advantage of the possibility of correlation with epidemiological patients' data.

Moreover, as well known, metastization is one of the main prognostic factors, so, apart from evaluating these metabolic markers in the primary cancer (CRC), we evaluated the same proteins in a seires of CRC Hepatic Metastasis, for which there is no data in the literature.

As stated before in the present study, it was demonstrated that MCT1, MCT4, CD147, CD44 and GLUT1 are overexpressed in human CRC samples, when compared with normal adjacent tissues. As expected, up-regulation of GLUT-1 is a result of the high energetic demands of CRC cells to promote an adequate energy supply. This, in turn, results in an increased lactic acid production, thus the up-regulation of MCTs is an expected result in order to maintain intracellular pH and prevent apoptosis.

Observing those results, we also documented that the expression of these metabolic markers in normal adjacent cells was more pronounced for MCT1 than the remaining proteins. This can reflect the influence of the tumor microenvironment, since the tissue evaluated is adjacent to the tumour, and may be under "tumour influence". However, it could also reflect the broader distribution of MCT1 as well the function of butyrate transport, a substrate for colonic epithelial cells, which possess trophic effects in the colon.

To overcome this limitation, evaluation of theses markers in normal colic epithelium may be necessary although it was not possible. This must be taken into account when we think of MCTs as potential therapeutic targets, making MCT4, chaperones and GLUT1 more attractive, since their lower expression in normal adjacent tissue will be associated to fewer side effects.

When analyzing CRC Hepatic Metastasis series, the same expression patterns were observed in tumour positive cases, suggesting that Hepatic Metastasis hold the same alterations in metabolic profile documented in CRC tissues. In CRC Hepatic Metastasis, the results observed in normal adjacent cells were still more promising, comparing to CRC, as no expression was observed for MCT4, CD44 and GLUT1 in normal adjacent tissue, but once again the evaluation in normal hepatic tissue and in a larger series will be important.

When we analyzed the association between MCT expression with chaperones, CD147 and CD44, and with GLUT1 in CRC and CRC Hepatic Metastasis as expected, by the reasons previously mentioned, we observed that in tumour samples MCT1 positive cases were associated with CD147 plasma membrane expression and MCT4 with both chaperones (plasma membrane expression) and GLUT1. Further, in this evaluation, CRC Hepatic Metastasis holds the same alterations in metabolic profile documented in CRC tissues for MCT4.

When analyzing the correlation between plasma membrane expression and epidemiological data, the association of these proteins with characteristics as: “Age”, “Personal History of CRC”, “Rectal examination”, Macroscopic cancer type”, “Tumour size”, “Vessel invasion” and presence of Hepatic metastasis” and “Pulmonary metastasis”, we documented that the association with these parameters that reflect a worse prognosis, reflects the metabolic advantage that these tumor cells have acquired. Analyzing these correlations in the Hepatic Metastasis series, no association was observed, being the small series and the retrospective access to the data possible limiting factors.

The results presented in this chapter were submitted for publication in international periodicals:

Martins SF, Amorim R, Pereira H, Pinheiro C, Pardal F, Rodrigues AM, Preto A, Filho AL, Baltazar F. Monocarboxylate Transporters (MCTs) as rational therapeutic targets in Colorectal Cancer.

Other results presented in this chapter were used as material for Master thesis of medical students:

. "Avaliação da expressão dos transportadores de monocarboxilatos nas metástases hepáticas do carcinoma Colorrectal"

- Supervisor of Master thesis presented at School of Health Sciences in January 2012.

Candidate to "Grande Prémio Fundação AstraZeneca 2008":

"Expression of monocarboxylase transporters in colorectal carcinomas". PI: Sandra Martins.

Candidate to "Concurso FCT 2012":

"Papel dos transportadores de monocarboxilatos (MCTs) na comunicação entre a sinalização oncogénica e a remodelação metabólica em Carcinoma Colorrectal". PI: Fátima Baltazar.

Candidate to "Concurso FCT/CAPES 2012":

"Avaliação da *crosstalk* entre o metabolismo tumoral e a sinalização oncogénica: papel dos transportadores de monocarboxilatos (MCTs)". PI: Fátima Baltazar.

6.3 CRC Angiogenic Markers

Angiogenesis is a key process for tumor growth and metastization. This study had as purpose to evaluate the expression of VEGF-A, -C and the receptors -2 and -3 in this large series of CRC and assess, if possible, correlations with clinicopathological data and impact on prognostic.

Assessing the expression of VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 in this series, we documented that all these markers were overexpressed in human CRC samples which suggest their role in tumour development and progression, by enabling new routes of oxygenation and nutrition of tumour cells, preventing tumour cell apoptosis.

When we compared CRC tissue and normal adjacent tissue we observed a statistically significant correlation for VEGF-C; a marker for lymphatic vessels, and its upregulation in the tumour tissue support the fact that lymphatic system is an escape route for metastization in CRC. We also observed a tendency for correlation with VEGFR-2, a receptor for the ligands VEGF-A and VEGF-C with action in terms of angiogenesis and lymphangenesis, contributing not only to tumour growth but also to tumour metastization. Observing the results of the expression of these markers in normal adjacent tissue, we observed that the staining was less pronounced for VEGFR-3 than the remaining, although present. This can reflect the biology of the tumor microenvironment, once the tissue evaluated is the normal-like adjacent tissue to the tumor, so it may be under the same "tumour influence".

To overcome this study limitation, evaluation of these markers in normal colonic epithelium may be necessary although it was not currently possible. When we analyzed the association between VEGF-A and -C and the receptor VEGFR-2, VEGFR-3 we observed that in tumour samples, VEGF-C positive cases were associated with VEGFR-3 expression. This is consistent with the fact that lymphangiogenesis induced by VEGF-C is driven mainly by the activation of the tyrosine kinase-linked receptor-3, VEGFR-3, and once again supports the fact that CRC escapes through lymphatic vessels.

When we evaluated the correlation of these markers with epidemiological data, we expected to find some particular associations namely with tumour size, vessel invasion and lymph node metastasis. Although these associations were not found, correlations were observed with data that demonstrate tumour progression, in specifically with the fact of VEGF-A correlates with "Tumour Differentiation", in particular well differentiated tumours takes into account that overexpression of

VEGF-A is an earlier event in tumour development as observed by its overexpression in CRC adenomas. On the other hand, the correlations observed with VEGF-C suggest that this marker was associated with more advanced stages and with histological characteristics that reveal a greater probability for metastization, as observed with the correlation with "Macroscopic Cancer type", namely exophytic tumours; "Tumour Differentiation", namely moderately differentiated tumours and "Tumour Penetration" and specifically more advanced tumour stages, T3/T4 lesions. Lastly, VEGFR-3 correlated with the presence of "Hepatic Metastasis". All these characteristics characterize a high expression of molecules that contribute for progression, invasion and metastasis and poorer survival and prognosis that we observed in overall-survival curves for rectal cancer in VEGF-C stage III and VEGFR-3 expression stage IV.

By documenting the overexpression of these markers in CRC, we can in the future improve CRC staging, by identifying at a early stage a group of patients that despite not present lymph node metastasis at diagnosis may present overexpression of these markers and so the potential for development of metastasis.

These findings also open a new door in CRC therapy. Most studies currently available are based on VEGF-A and VEGFR-2 expression on tumour cells and tumour vessels. With this study, also VEGF-C and VEGFR-3 are potential therapeutic targets, particularly if we associated the fact that the lymphatic pathway is a major route of escape in CRC and with the advantage of their expression in the tumour. Moreover, the fact that the drugs already approved and those that are under consideration are directed to VEGF-A and VEGFR-2 and resistance to these drugs are emerging, makes VEGF-C and VEGFR-3 promising new therapeutic options.

The results presented in this chapter were published or submitted to international peer review periodicals:

Martins SF, Reis RM, Rodrigues AM, Baltazar F, Filho AL. Role of endoglin and VEGF family expression in colorectal cancer prognosis and anti-angiogenic therapies World Journal of Clinical Oncology. **World Journal of Clinical Oncology.** 2011;2(6):272–80. **(Appendix 11)**

Submitted for publication:

Martins SF, Garcia EA, MA, Pardal F, Rodrigues AM, Filho AL. Clinicopathological correlation and prognostic significance of VEGF-A, VEGF-C, VEGFR-2, VEGFR-3 expression in Colorectal Cancer.

Candidate to "Grande Prémio Fundação AstraZeneca 2008":

"Evaluation of Angiogenesis and Lymphangiogenesis in Colorectal Cancer: Impact in Prognosis Assessment". PI: Sandra Martins.

Studies under development:

As Master thesis of "Mestrado Integrado em Medicina" and other studies:

- Assessment of D2-40 in CCR and correlation with clinicopathological data and prognostic significance.

- Assessment of Ki-67 in CCR and correlation with clinicopathological data and prognostic significance.

- Assessment of PROX-1 in CCR and correlation with clinicopathological data and prognostic significance.

- Assessment of correlations between *SPINT2* metilation, expression of the receptor MET, clinicopathological data and prognostic significance, in CRC.

- Relevance of HOXA9 Expression in Colorectal Cancer Patients.

- Assessment of Microsatellite Instability in Colorectal Cancer Patients.

- miR-28 targets in colorectal cancer

Candidate to RASPHAGY Project:

- The role of KRAS mutation signaling in autophagy regulation in colorectal carcinoma: towards identification of new therapeutic targets. PI: Ana Preto.

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Appendix 1:

"Protocolo de estudo de Cancro do Colon"

Hospital de São MarcoS

UNIDADE FUNCIONAL DE COLOPROCTOLOGIA

Protocolo de Estudo Cancro do Cólon

CÓDIGO: PRT.XXX.HSM.XXX

DATA:

CRITÉRIOS DE REFERÊNCIA: 19.22

EDIÇÃO N.º: 01

ÂMBITO: Aplica-se a todos os médicos do Hospital de São Marcos

REVISÃO N.º: 00

OBJECTIVO:

Uniformizar a avaliação pré-operatória dos doentes com cancro do cólon no Hospital de São Marcos.

RESPONSABILIDADES:

Compete aos Directores de Departamento de Cirurgia, dos Serviços de Cirurgia 1 e 2 e ao Coordenador da Unidade Funcional de Coloproctologia a implementação desta instrução de trabalho.

DEFINIÇÕES:

Cancro do cólon: Neoplasia, com confirmação histológica, do cólon.

DESCRIÇÃO:

Na avaliação pré-operatória do doente com cancro do cólon deve constar:

a. Exames analíticos, Rx tórax e ECG de acordo com protocolo existente
b. Estudo da função hepática (ALT, AST, LDH, FA, bilirrubina)
c. Avaliação do estado nutricional (proteínas totais, albumina e transferrina)
d. CEA e Ca 19.9
e. Colonoscopia total se não houver impedimento orgânico
f. Histologia da lesão
g. TAC abdominal
h. Relatório resultante da discussão do caso clínico em reunião multidisciplinar.

PROTOCOLO

ELABORADO POR
Director/Responsável
.....
( )
00-00-2005

APROVADO POR
Presidente do C.A.
.....
(Lino Mesquita Machado)
00-00-2005

HOMOLOGADO POR
Conselho de Administração
.....
(Lino Mesquita Machado)
00-00-2005

PRÓXIMA REVISÃO
00-00-2005

Appendix 2:

"Protocolo de estudo de Cancro do Recto"

Hospital de São MarcoS	UNIDADE FUNCIONAL DE COLOPROCTOLOGIA Protocolo de Estudo Cancro do Recto	CÓDIGO: PRT.XXX.HSM.XXX
		DATA:
CRITÉRIOS DE REFERÊNCIA: 19.22		EDIÇÃO N.º: 01
ÂMBITO: Aplica-se a todos os médicos do Hospital de São Marcos		REVISÃO N.º: 00

OBJECTIVO:

Uniformizar a avaliação pré-operatória dos doentes com cancro do recto.

RESPONSABILIDADES:

Compete aos Directores de Departamento de Cirurgia, dos Serviços de Cirurgia 1 e 2 e ao Coordenador da Unidade Funcional de Coloproctologia a implementação desta instrução de trabalho.

DEFINIÇÕES:

Cancro do recto: Neoplasia, com confirmação histológica, do recto.

Limites do recto: segmento do tubo digestivo, medido com rectosigmoidoscopio rígido, cujo limite superior se encontra aos 15 cm da margem anal.

Ressecção anterior recto: anastomose acima da reflexão peritoneal

Ressecção anterior do recto baixa: anastomose abaixo da reflexão peritoneal

Ressecção anterior do recto ultra-baixa: anastomose ao nível do pavimento pélvico

Anastomose colo-anal: anastomose à linha pectinea

Recidiva Local: recidiva pélvica excepto metástases ováricas

DESCRIÇÃO:

Na avaliação pré-operatória do doente com cancro do cólon deve constar:

a. Exames analíticos, Rx tórax e ECG de acordo com protocolo existente
b. Estudo da função hepática (ALT, AST, LDH, FA, bilirrubina)
c. Avaliação do estado nutricional (proteínas totais, albumina e transferrina)
d. CEA e Ca 19.9
e. Colonoscopia total se não houver impedimento orgânico
f. Histologia da lesão
g. TAC toraco-abdomino-pélvico
h. RMN pélvica
i. Ecoendoscopia rectal
j. Relatório resultante da discussão do caso clínico em reunião multidisciplinar.

PROTOCOLO

ELABORADO POR	Director/Responsável ( ) 00-00-2005
APROVADO POR	Presidente do C.A. (Lino Mesquita Machado) 00-00-2005
HOMOLOGADO POR	Conselho de Administração (Lino Mesquita Machado) 00-00-2005
PRÓXIMA REVISÃO	00-00-2005
Página 1 de 1	

Appendix 3:

"Protocolo de Registo de Cancro Colorectal"

Hospital de São MarcoS	UNIDADE FUNCIONAL DE COLOPROCTOLOGIA Protocolo de Registo de Cancro Colorectal	CÓDIGO: PRT.XXX.HSM.XXX
		DATA:
CRITÉRIOS DE REFERÊNCIA: 19.22		EDIÇÃO N.º: 01
ÂMBITO: Aplica-se a todos os médicos do Hospital de São Marcos		REVISÃO N.º: 00

OBJECTIVO:

Uniformizar os registos dos doentes com cancro colorectal (CCR) no Departamento de Cirurgia.

RESPONSABILIDADES:

Compete aos Directores de Departamento de Cirurgia, dos Serviços de Cirurgia 1 e 2 e ao Coordenador da Unidade Funcional de Coloproctologia a implementação desta instrução de trabalho.

DEFINIÇÕES:

Cancro do cólon: Neoplasia, com confirmação histológica, do cólon.

Cancro do recto: Neoplasia, com confirmação histológica, do recto.

Limites do recto: segmento do tubo digestivo, medido com rectosigmoidoscopio rígido, cujo limite superior se encontra aos 15 cm da margem anal.

DESCRIÇÃO:

Dever ser registados todos os doentes tratados por CCR no Departamento de Cirurgia, onde conste:

i. Passado tumoral
ii. História familiar
iii. Modo de apresentação
iv. Avaliação pré-tratamento
v. Cirurgia e características patológicas do tumor

b. Sempre que um doente está proposto para cirurgia por CCR, o médico responsável pelo doente têm de preencher o formulário HSM.PC.CIRII. 194.1 – "Registo de Cancro do Cólon " ou HSM.PC.CIRII. 195.1 – "Registo de Cancro do Recto ", consoante a localização do cancro, que será entregue ao Coordenador da Unidade Funcional de Coloproctologia ou a quem ele delegue.

c. Eventuais medidas correctivas serão incluídas no Plano de Acção do Serviço de Cirurgia 2

DOCUMENTOS RELACIONADOS:

HSM.PC.CIRII. 194.1 – "Registo de Cancro do Cólon "

HSM.PC.CIRII. 195.1 – "Registo de Cancro do Recto"

PROTOCOLO

ELABORADO POR	Director/Responsável ( ) 00-00-2005
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Página 1 de 1	

Appendix 4:

"Protocolo Terapêutico de Cancro do Recto"

Hospital de São MarcoS

UNIDADE FUNCIONAL DE COLOPROCTOLOGIA

Protocolo Terapêutico de Cancro do Recto

CÓDIGO: PRT.XXX.HSM.XXX

DATA:

EDIÇÃO N.º: 01

REVISÃO N.º: 00

CRITÉRIOS DE REFERÊNCIA:

ÂMBITO: Aplica-se a todos os médicos do Departamento de Cirurgia e de Oncologia Médica do Hospital de São Marcos

OBJECTIVO: Uniformizar o tratamento oferecido a doentes com cancro do recto.

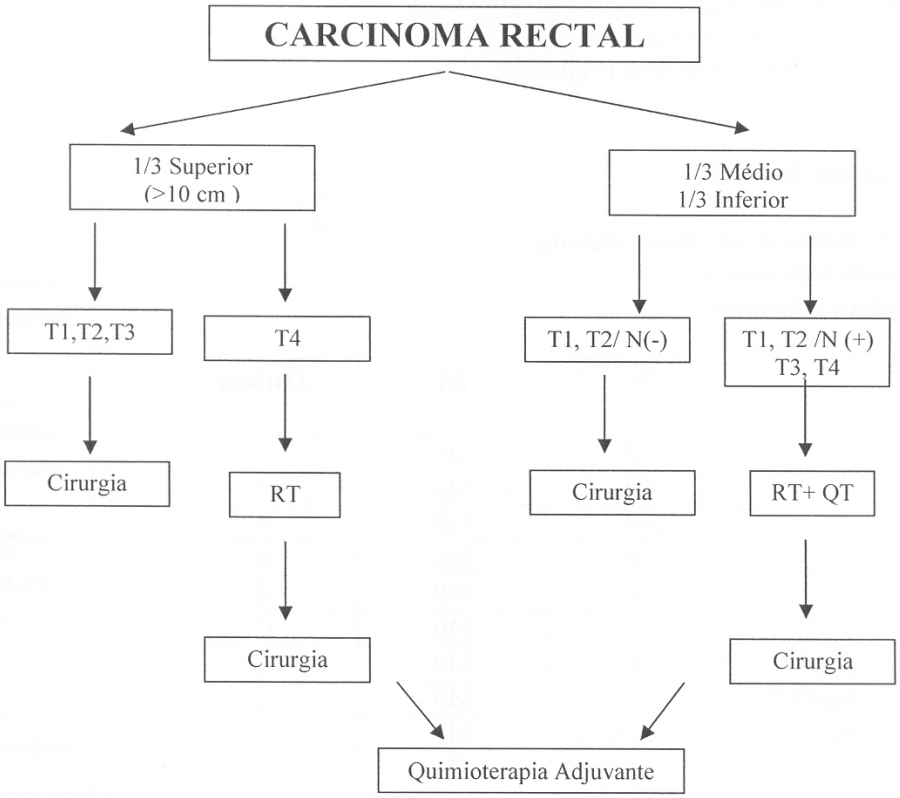
RESPONSABILIDADES:

Compete aos Directores de Departamento de Cirurgia, dos Serviços de Cirurgia 1 e 2, do Coordenador da Unidade Funcional de Coloproctologia e do Director de Serviço de Oncologia Médica a implementação desta instrução de trabalho.

DESCRIÇÃO

1. Após a avaliação e estadiamento pré-operatório do doente com cancro do recto, este é apresentado em reunião multidisciplinar para decisão do tratamento a seguir.

2. Para esta decisão é tomado em conta o fluxograma "Protocolo Terapêutico do Carcinoma Rectal"



PROTOCOLO

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( )
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(Lino Mesquita Machado)
00-00-2005

HOMOLOGADO POR
Conselho de Administração
(Lino Mesquita Machado)
00-00-2005

PRÓXIMA REVISÃO
00-00-2005

Hospital de São MarcoS	UNIDADE FUNCIONAL DE COLOPROCTOLOGIA Protocolo Terapêutico de Cancro do Recto	CÓDIGO: PRT.XXX.HSM.XXX
		DATA:
CRITÉRIOS DE REFERÊNCIA:		EDIÇÃO N.º: 01
ÂMBITO: Aplica-se a todos os médicos do Departamento de Cirurgia e de Oncologia Médica do Hospital de São Marcos		REVISÃO N.º: 00

3. O estadiamento TNM é efectuado respeitando a seguinte nomenclatura:

Tumor Primário (T)

Tx – Tumor primário não pode ser determinado
T0 – Sem evidência de tumor primário
Tis – Carcinoma em situ: intraepitelial ou invasão da lâmina própria
T1 – Tumor invade a submucosa
T2 – Tumor invade a muscularis própria
T3 – Tumor invade através da muscularis própria até à subserosa ou até aos tecidos peri-cólicos não peritonizados ou perirectal
T4 – Tumor invade a directamente outros órgãos ou estruturas e/ou perfura o peritoneu visceral

Gânglios linfáticos regionais (N)

Nx – Gânglios linfáticos regionais não podem ser determinados
N0 – Sem gânglios linfáticos regionais metastizados
N1 – Metástases em 1-3 gânglios regionais
N2 – Metástases em 4 ou mais gânglios regionais

Metástases à Distância (M)

Mx – Metástases à distância não determinadas
M0 – Sem metástases à distância
M1 – Com metástases à distância

Estadio	T	N	M	Dukes
0	Tis	N0	M0	
I	T1	N0	M0	A
	T2	N0	M0	A
IIA	T3	N0	M0	B
IIB	T4	N0	M0	B
IIIA	T1-T2	N1	M0	C
IIIB	T3-T4	N1	M0	C
IIIC	Qualquer T	N2	M0	C
IV	Qualquer T	Qualquer N	M1	-

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Appendix 5:

"Protocolo de Follow-up de Cancro Colorectal"

Hospital de São MarcoS	UNIDADE FUNCIONAL DE COLOPROCTOLOGIA Protocolo de Follow-up do Cancro Colorectal	CÓDIGO: PRT.XXX.HSM.XXX
		DATA:
CRITÉRIOS DE REFERÊNCIA:		EDIÇÃO N.º: 01
ÂMBITO: Aplica-se a todos os médicos do Departamento de Cirurgia e de Oncologia Médica do Hospital de São Marcos		REVISÃO N.º: 00

OBJECTIVO: Uniformizar o seguimento dos doentes com cancro colorectal.

RESPONSABILIDADES:

Compete aos Directores de Departamento de Cirurgia, dos Serviços de Cirurgia 1 e 2, do Coordenador da Unidade Funcional de Coloproctologia e do Serviço de Oncologia Médica a implementação desta instrução de trabalho.

FUNDAMENTAÇÃO:

. Não existe um protocolo universalmente aceite de follow-up de cancro colorectal.

. A maior parte dos estudos clínicos, mostram que cerca de 80% das recidivas ocorrem nos primeiros 3 anos após a ressecção cirúrgica e portanto a vigilância deve ser maior durante este período.

. Deste modo o protocolo que propomos para follow-up de cancro colorectal deve ser considerado um guia e ajustado ao estádio da doença, à idade e ao estado geral do doente.

. Após a realização de cirurgia com intenção curativa, a vigilância dos doentes com cancro colorectal é realizada com os seguintes objectivos:

1- **Avaliar possíveis complicações terapêuticas**
2- **Identificar a recorrência que é potencialmente ressecável para cura da doença**
3- **Identificar lesões metacrones num estadio pré-invasivo**

. Para follow-up recomendamos os seguintes meios:

1- **História clínica e Exame Objectivo**
2- **Colonoscopia**
3- **Rx tórax**
4- **TAC abdominal**
5- **CEA**

PROTOCOLO

ELABORADO POR	Director/Responsável ( ) 00-00-2005
APROVADO POR	Presidente do C.A. (Lino Mesquita Machado) 00-00-2005
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PRÓXIMA REVISÃO	00-00-2005

Hospital de São MarcoS	UNIDADE FUNCIONAL DE COLOPROCTOLOGIA Protocolo de Follow-up do Cancro Colorectal	CÓDIGO: PRT.XXX.HSM.XXX
		DATA:
CRITÉRIOS DE REFERÊNCIA:		EDIÇÃO N.º: 01
ÂMBITO: Aplica-se a todos os médicos do Departamento de Cirurgia e de Oncologia Médica do Hospital de São Marcos		REVISÃO N.º: 00

DESCRIÇÃO:

Nos doentes operados por carcinoma colo-rectal deve-se proceder do seguinte modo:

1. O seguimento é subdividido em três períodos: 1º e 2º ano, do 3º ao 5º e a partir do 6º ano de pós-operatório

1º e 2º ANO	
Estadio II/III	- História clínica e exame objectivo (3/3 meses) - CEA (3/3 meses) - Rx tórax - anual
Estadio I	- História clínica e exame objectivo (6/6meses) - CEA (6/6 meses) - Rx tórax - anual

3º ao 5º ANO
- História clínica e exame objectivo (6/6meses) - CEA (6/6 meses) - Rx tórax - anual

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Hospital de São MarcoS	UNIDADE FUNCIONAL DE COLOPROCTOLOGIA Protocolo de Follow-up do Cancro Colorectal	CÓDIGO: PRT.XXX.HSM.XXX
		DATA:
CRITÉRIOS DE REFERÊNCIA:		EDIÇÃO N.º: 01
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A partir do 6º ANO
- História clínica e exame objectivo anual - EDB segundo o plano de rastreio CCR para polupação em geral - Referenciar ao médico assistente

2. Relativamente à realização de colonoscopia:

- Deve ser realizada até 1 ano após a ressecção cirúrgica (ou 3-6 meses após a cirurgia se não foi realizada pré ou per-operatóriamente devido a lesão obstrutiva)

- Recomenda-se a repetição da colonoscopia aos 3 anos e após esta cada 5 anos, a não ser que a colonoscopia de follow-up evidencie adenoma avançado (pólipo viloso, pólipo> 1 cm ou com displasia de alto grau), neste caso deve ser repetida 1 ano após a polipectomia.

- Colonoscopias mais frequentes podem estar indicadas em doentes em que a idade de diagnóstico foi antes dos 50 anos de idade.

3. Relativamente à realização da TAC:

- Em doentes com alto risco de recorrência há autores que recomendam TAC toraco-abdomino-pélvica anual nos primeiros 3 anos de pós-operatório.

- TAC toraco-abdomino-pélvica: se sintomas positivos ou no estudo de elevação seriada do CEA

PROTOCOLO

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Hospital de São MarcoS	UNIDADE FUNCIONAL DE COLOPROCTOLOGIA Protocolo de Follow-up do Cancro Colorectal	CÓDIGO: PRT.XXX.HSM.XXX
		DATA:
CRITÉRIOS DE REFERÊNCIA:		EDIÇÃO N.º: 01
ÂMBITO: Aplica-se a todos os médicos do Departamento de Cirurgia e de Oncologia Médica do Hospital de São Marcos		REVISÃO N.º: 00

4. No caso particular do Cancro do Recto:

- Proctoscopia 6/6 meses nos 5 anos de follow-up de doentes submetidos a ressecção anterior do recto, para avaliar a recorrência local anastomotica

Os doentes operados por carcinoma rectal devem realizar, aos 6 meses pós-cirurgia, uma RMN pélvica que ficará como RMN de referência.

PROTOCOLO

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PRÓXIMA REVISÃO 00-00-2005	

Appendix 6:

"Protocolo de Registo de recidiva de Cancro Colorectal"

Hospital de São MarcoS	UNIDADE FUNCIONAL DE COLOPROCTOLOGIA Protocolo de Registo de Recidiva do CCR	**CÓDIGO:** PRT.XXX.HSM.XXX
		DATA:
CRITÉRIOS DE REFERÊNCIA: 19.22		**EDIÇÃO N.º:** 01
ÂMBITO: Aplica-se a todos os médicos do Departamento de Cirurgia do Hospital de São Marcos		**REVISÃO N.º:** 00

OBJECTIVO:

Registar os dados, em documento próprio e suporte informático, de doentes com cancro do cólon e recto aquando da recidiva tumoral.

RESPONSABILIDADES:

Compete aos Directores de Departamento de Cirurgia, Dos Serviços de Cirurgia 1 e 2 e ao Coordenador da Unidade Funcional de Coloproctologia a implementação desta instrução de trabalho.

DESCRIÇÃO:

O registo das recidivas dos doentes com cancro colorectal (CCR) processa-se do seguinte modo:

1. Serão registados todos os doentes com recidiva de cancro do cólon e recto tratados no Departamento de Cirurgia.
2. Sempre que um doente têm recidiva de cancro do cólon e recto, sendo proposto para cirurgia ou não, o médico responsável pelo doente têm de preencher o formulário HSM.PC.DCIR.196.1 – "Registo de Recidiva do doente com CCR", que será entregue ao Coordenador da Unidade Funcional de Coloproctologia.
3. Os formulários obtidos serão arquivados em pasta própria no Serviço de Cirurgia 2.
4. Eventuais medidas correctivas serão incluídas no Plano de Acção do Serviço de Cirurgia 2

DOCUMENTOS RELACIONADOS:

HSM.PC.DCIR.196.1 – "Registo de Recidiva do doente com cancro colorectal"

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PRÓXIMA REVISÃO	00-00-2005

Appendix 7:

"Protocolo de Antibioprofilaxia para Cirurgia Colorectal"

Hospital de São MarcoS	UNIDADE FUNCIONAL DE COLOPROCTOLOGIA Protocolo de Antibioprofilaxia para Cirurgia Colorectal	CÓDIGO: PRT.XXX.HSM.XXX
		DATA:
CRITÉRIOS DE REFERÊNCIA:		EDIÇÃO N.º: 01
ÂMBITO: Aplica-se a todos os médicos do Departamento de Cirurgia do Hospital de São Marcos		REVISÃO N.º: 00

OBJECTIVO: Definir as medidas a tomar para antibioprofilaxia na Cirurgia Colorectal

RESPONSABILIDADES: Compete aos Directores de Departamento de Cirurgia, dos Serviços de Cirurgia 1 e 2 e do Coordenador da Unidade Funcional de Coloproctologia a implementação desta instrução de trabalho.

DEFINIÇÕES:

Antibioprofilaxia: Consiste na administração de antibiótico aos doentes que vão ser submetidos a cirurgia, não havendo evidência de infecção no momento do acto cirúrgico.

FUNDAMENTAÇÃO:

. A Cirurgia colorectal, incluindo a realizada de forma electiva, é a cirurgia que apresenta maior incidência de ILC

. A ILC na cirurgia colorectal, ocorre frequentemente nos doentes que não realizam antibioprofilaxia, cerca de 40% dos casos. Por outro lado a ILC está associada a um aumento do número de admissões na UCIP assim como a um aumento de reinternamentos e da mortalidade.

. O risco de ILC depende ainda do ASA (3,4,ou 5), classificação da ferida (contaminada ou suja), tempo de duração de cirurgia (superior a 3 horas) entre outros factores (exemplo: transfusão per-operatória, realização concomitante de estoma etc.) (NNIS risk índex).

Este risco de infecção é superior para a cirurgia do recto relativamente à cirurgia cólica.

. A antibioprofilaxia reduz a incidência de ILC pós-operatórias. (de 40% para cerca de 7%, na maior parte dos estudos)

PROTOCOLO

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Hospital de São MarcoS

UNIDADE FUNCIONAL DE COLOPROCTOLOGIA

Protocolo de Antibioprofilaxia para Cirurgia Colorectal

CÓDIGO: PRT.XXX.HSM.XXX

DATA:

EDIÇÃO N.º: 01

REVISÃO N.º: 00

CRITÉRIOS DE REFERÊNCIA:

ÂMBITO: Aplica-se a todos os médicos do Departamento de Cirurgia do Hospital de São Marcos

PROTOCOLO:

- Cefoxitina, 2 g EV, idealmente durante a indução anestésica ou no máximo até 30min a 1 hora antes da cirurgia.

- Repicagem com 1 g às 2 h da cirurgia

- Prolongar até as 24 h de pós-operatório: 1 g EV 8/8h

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Appendix 8:

"Protocolo de Processamento da peça operatória"

Hospital de São MarcoS

UNIDADE FUNCIONAL DE COLOPROCTOLOGIA

Protocolo de Processamento da Peça Operatória

CÓDIGO: PRT.XXX.HSM.XXX

DATA:

EDIÇÃO N.º: 01

REVISÃO N.º: 00

CRITÉRIOS DE REFERÊNCIA: 19.22

ÂMBITO: Aplica-se a todos os médicos do Departamento de Cirurgia e de Anatomia Patológica do Hospital de São Marcos

OBJECTIVO:

Uniformizar as medidas de processamento da peça cirúrgica.

RESPONSABILIDADES:

Compete aos Directores de Departamento de Cirurgia, dos Serviços de Cirurgia 1 e 2, do Coordenador da Unidade Funcional de Coloproctologia e do Serviço de Anatomia Patológica a implementação desta instrução de trabalho.

DESCRIÇÃO:

O processamento da peça operatória prévio ao envio para o Serviço de Anatomia Patológica é efectuado do seguinte modo:

1. Proceder à limpeza adequada da peça cirúrgica,

2. Abrir pelo bordo antimesentérico tentando não interceptar a neoplasia,

3. Referenciar os topos proximal e distal,

4. Enviar a peça cirúrgica, a fresco, no caso de neoplasia,

5. Enviar os anéis de sutura em recipiente separado,

6. Caso haja outras biópsias, enviar em frasco separado e referenciado.

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Appendix 9:

"Sensibilidade da Ecografia Endorectal no estadiamento do Cancro do Recto: correlação com o estadiamento patológico."

Sensibilidade da ecografia endorectal no estadiamento do cancro do recto: correlação com o estadiamento patológico

Sensitivity of endorectal ecography in the staging of rectal chancre: correlation with pathological staging

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CARRIÇO LFC; MARTINS SFF. Sensibilidade da ecografia endorectal no estadiamento do cancro do recto: correlação com o estadiamento patológico. **Rev bras Coloproct**, 2011;30(4): 430-439.

RESUMO: Objectivo: Avaliar a sensibilidade da ecografia endorectal, em nossa experiência, no estadiamento do cancro do recto comparando com o resultado anatomopatológico. Material e métodos: Estudo retrospectivo, realizado entre Janeiro de 2005 e Agosto de 2009. Calculou-se a sensibilidade, a especificidade, o valor preditivo positivo e negativo para cada estadio T e N. Por meio da elaboração de curvas ROC avaliou-se a precisão do estadiamento ecoendoscópico e por meio do teste de McNemar comparou-se com o resultado anatomopatológico. Resultados: Dos 112 doentes, 76 cumpriram os critérios de inclusão. Obtivemos uma eficácia de 75 a 97% para uT e de 75% para uN. Verificou-se sensibilidade, especificidade, valor preditivo positivo e negativo, respectivamente, de 63;98;92 e 89% para uT1; 71;76;54 e 88% para uT2; 67;81;73 e 76% para uT3; 100;97;60 e 100% para uT4; e 39;91;62 e 78% para uN. As curvas ROC indicaram que a ecografia endorectal é um bom teste para o estadiamento do T e razoável para o N. O teste de McNemar revelou que não há diferenças significativas entre o estadiamento ecoendoscópico e anatomopatológico (p>0,05). Conclusões: Conclui-se que a ecografia endorectal é uma importante ferramenta no estadiamento do cancro do recto, apresentando boa correlação com o resultado anatomopatológico.

Descritores: Ecografia; Valor preditivo dos testes; Patologia.

INTRODUÇÃO

O cancro colorectal (CCR) é a doença oncológica gastrointestinal mais comum e a segunda maior causa de mortes oncológicas nos países Ocidentais[1]. Em Portugal, segundo o Instituto Nacional de Estatística, é a principal causa de morte por doença oncológica[2]. A sobrevida do CCR está relacionada com o estadio da doença, apresentando no geral uma sobrevida de 78% no primeiro ano de seguimento e de 54% aos 5 anos[3]. Cerca de 15 a 20% dos doentes morrem da doença em fases iniciais e 40 a 80% em fases mais avançadas[4].

O cancro do recto apresenta particularidades em termos de diagnóstico, estadiamento e tratamento. Constitui cerca de 5% dos tumores malignos, sendo diagnosticados cerca de 140 mil novos casos por ano, na Europa[5].

Tradicionalmente, o estadiamento era obtido pelo exame anatomopatológico da peça cirúrgica. Hoje em

Escola de Ciências da Saúde da Universidade do Minho em colaboração com o Hospital de Braga.

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Sensibilidade da ecografia endorectal no estadiamento do cancro do recto: correlação com o estadiamento patológico
Luís Filipe Carvalho Carriço e Cols.

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dia, o estadiamento pré-operatório é de grande importância para gerir adequadamente as decisões terapêuticas bem como para determinar o prognóstico do doente[6], uma vez que vai permitir a selecção dos doentes candidatos a terapêutica primária com o principal objectivo de reduzir a recidiva local e que paralelamente beneficiam com a redução local do tumor, facilitando a ressecção e potencialmente podendo resultar em recessões que preservem o esfíncter[7]. Também em termos de terapêutica observou-se nos últimos 1970 anos uma evolução de um tratamento meramente cirúrgico para uma terapêutica multimodal[8].

A utilização da terapêutica primária é actualmente recomendada em doentes com cancro do recto localmente avançado, ou seja, em que se verifique extensão do tumor na gordura perirectal e/ou envolvimento ganglionar ou do mesorecto (T3/T4 N0 ou Tx N1/N2)[9]; pois doentes com estádios II e III têm elevada taxa de recorrência local depois da cirurgia[10,11] e tem-se obtido uma redução significativa da recorrência local e da ocorrência de metástases à distância, com consequente aumento da sobrevida, por meio da combinação da ressecção cirúrgica do cancro com a quimioradioterapia primária[11,12]. Nos doentes com doença no estádio IV, a mesma atitude permite aumentar a taxa da ressecção cirúrgica e a sobrevida dos doentes[11,13]. Assim, hoje em dia, devido à utilização da terapêutica primária, a "cirurgia poupadora de esfíncteres" pode ser oferecida também a doentes com cancro do recto localmente avançados sem compromisso do resultado oncológico[14].

Nesses doentes, a terapia primária seguida de cirurgia resulta num melhor controlo local e numa redução da toxicidade quando comparada com a terapia adjuvante pós-operatória estandardizada[15,16]. Verificando-se ainda uma redução de 13% da recidiva tumoral[17].

O controlo locoregional do tumor também melhorou significativamente nos últimos 15 anos com melhoria da técnica cirúrgica, nomeadamente com a introdução da excisão total do mesorecto (ETM)[18]. Esta permitiu diminuir a taxa de recorrência local de 16 para 9%, sendo ainda um predictor independente da sobrevida geral[19].

O estadiamento pelo sistema tumor-node-metastasis (TNM) para o cancro do recto é baseado na profundidade da invasão da lesão (T), a extensão da invasão local a gânglios linfáticos (N) e a presença de metástases à distância (M)[18].

Actualmente dispomos de várias opções para o estadiamento pré-operatório, tais como, tomografia computorizada (TC), ecografia endorectal, ressonância magnética (RM) e tomografia de emissão de positrões (PET)[20,21]. Apesar desses avanços tecnológicos, o exame objectivo, nomeadamente o toque rectal, dá-nos informações relevantes relativamente à localização, distância da margem anal e tonicidade dos esfíncteres, aspectos importantes para planear a cirurgia. No entanto, trata-se de um exame subjectivo ao avaliar a invasão tumoral[6].

Em 1984, Hildebrandt e Fielfe preconizaram o estadiamento ecoendoscópico dos tumores rectais com base na classificação TNM[6].

A ecografia endorectal (2 dimensões) é realizada com um aparelho provido com sonda que proporciona uma imagem de 360°, possibilitando, portanto, a avaliação circunferencial das camadas do recto. Ecograficamente, o recto está dividido em camadas circulares e concêntricas, alternadas entre imagens hiperecoicas e hipoecoicas. São visualizadas cinco camadas, sendo a mais interna a mucosa, seguida da muscular da mucosa, submucosa, muscular própria e gordura perirectal. Actualmente, existem ecoendoscópios com imagem a três dimensões com melhor resolução e precisão em visualizar a infiltração e tamanho tumoral[22].

Segundo alguns autores, a sensibilidade e especificidade da ecografia endorectal (2 dimensões) para o estadiamento do T ronda os 80 a 95% comparando com a RM (75 a 85%) e com a TC (65 a 75%)[17,23,24]. Enquanto para determinar o N é aproximadamente de 70 a 75% comparado com a RM (60 a 70%) e com a TC (55 a 65%)[23,25,26]. Assim, a ecografia endorectal tem emergido como modalidade de diagnóstico de escolha para o estadiamento clínico dos tumores rectais[27,28].

MATERIAIS E MÉTODOS

População

A população em estudo é constituída por todos os doentes com cancro do recto estadiados e tratados no Hospital de Braga, desde 1° de Janeiro de 2005 a 31 de Agosto de 2009.

Definiram-se para esse estudo, como critérios de inclusão: doentes com diagnóstico histológico de adenocarcinoma do recto; estadiamento pré-operatório completo, incluindo ecografia endorectal conclusiva; e resultado histológico da peça cirúrgica.

Definiram-se como critérios de exclusão: diagnóstico histológico distinto de adenocarcinoma, como por exemplo, carcinomas epidermoides; doentes com diagnóstico de cancro do recto que não realizaram ecografia endorectal ou em que esta não foi conclusiva, por exemplo: impossibilidade de visualização da totalidade da lesão; doentes submetidos a radioterapia pélvica e doentes sem o resultado do estadiamento histológico.

Amostra

Utilizou-se uma amostra de conveniência, de 76 doentes com diagnóstico de adenocarcinoma do recto que respeitam os critérios de inclusão/exclusão previamente definidos.

Métodos e recolha de dados

Entre 1º de Janeiro de 2005 e 31 de Agosto de 2009 foram realizadas, no Hospital de Braga, um total de 112 ecografias endorectais para estadiamento do cancro do recto. Destas, 76 preenchiam os critérios previamente determinados.

De maneira a poder avaliar a sensibilidade da ecografia endorectal no estadiamento do cancro do recto, elaborou-se uma base de dados a partir dos relatórios da ecografia endorectal e do resultado anatomopatológico da peça cirúrgica.

Os parâmetros estudados foram: sexo e idade do doente; localização da lesão (1/3 inferior, médio ou superior, isto é, 0 a 5cm, 6 a 10cm e 11 a 15cm da margem anal respectivamente) e estadiamento ecoendoscópico do tumor e histológico da peça cirúrgica.

Análise estatística

Após a recolha dos dados, estes foram armazenados na forma de base de dados no programa Statistical Package for the Social Sciences, (SPSS Inc. R, Chicago, Illinois, Estados Unidos), versão 17.0, de onde, posteriormente, se procedeu à análise.

Numa primeira fase do estudo, foi realizada a análise descritiva dos dados para se obter as frequências, médias, desvios-padrão e variância. Foi utilizado o Microsoft® Excel 2007 para a elaboração de gráficos e tabelas.

Posteriormente, procedeu-se ao cálculo da sensibilidade, especificidade, valor preditivo positivo e negativo do estadiamento pela ecografia endorectal relativamente ao T e N comparativamente com os resultados da anatomia patológica (Tabela 1).

Realizou-se ainda um estudo comparativo entre o estadiamento ecoendoscópico e o histológico por meio de curvas ROC com o cálculo da área abaixo das curvas (AUC). A curva ROC com o cálculo da AUC é um bom preditor da precisão de um teste, em que quanto mais perto tiver a área da AUC de 1 melhor será o exame. Valores abaixo de 0,50 representam um teste ruim ou ineficaz; entre 0,50 a 0,70 significa um teste de precisão média ou razoável, de 0,70 a 0,90 prediz um bom ou excelente teste.

Tabela 1 – *Fórmulas estatísticas utilizadas para cálculo da sensibilidade, especificidade, valor preditivo positivo (VP positivo) e valor preditivo negativo (VP negativo).*

		Teste		Total
		T^+	T^-	
Gold Standard	D	VP	FN	n_D
	$\overline{D}$	FP	VN	$n_{\overline{D}}$
Total		n_{T^+}	n_{T^-}	n

Sensibilidade $S = \frac{VP}{n_D} = \frac{VP}{VP + FN} = P(T^+ \mid D)$

Especificidade $VP^+ = \frac{VP}{n_{T^+}} = \frac{VP}{VP + FP} = P(D \mid T^+)$

VP Negativo $VP^- = \frac{VN}{n_{T^-}} = \frac{VN}{VN + FN} = P(\overline{D} \mid T^-)$

VP Positivo $E = \frac{VN}{n_{\overline{D}}} = \frac{VN}{VN + FP} = P(T^- \mid \overline{D})$

Por último, utilizou-se o teste de McNemar para avaliar se existem diferenças significativas entre o estadiamento ecoendoscópico e o histológico. Admitiu-se que existem diferenças significativas quando $p<0,05$.

RESULTADOS

Dos 76 exames realizados, 68,4% (52 doentes) eram do sexo masculino e 31,6% (24 doentes) eram do sexo feminino. A média de idades dos doentes é de 68,9±10,7, com idade mínima de 49 anos e máxima de 93.

No que respeita à localização tumoral, 69,7% (53 doentes) localizavam-se no 1/3 médio do recto, 26,3% (20 doentes) no 1/3 inferior do recto e 3,9% (3 doentes) no 1/3 superior do recto. A localização mais comum, em ambos os sexos, foi no 1/3 médio do recto, nomeadamente 76,9% (40 doentes) no sexo masculino e 54,2% (13 doentes) no sexo feminino. Relativamente ao estadiamento tumoral obtido pela ecografia endorectal, dos 76 exames realizados, 17,1% (13 tumores) foram classificados como T1, 36,8% (28 tumores) foram estadiados como T2, 39,5% (30 tumores) como T3 e 6,6% (5 tumores) foram classificados como T4 (Tabela 2). Em relação ao envolvimento ganglionar, 82,9% (63 tumores) foram classificados como N0 e 17,1% (13) com envolvimento ganglionar (N1) (Tabela 3). Relativamente ao estadiamento anatomopatológico das peças cirúrgicas, 25% (19 tumores) foram classificados como T1, 27,6% (21 tumores) estadiados como T2, 43,4% (33 tumores) classificados como T3 e 3,9% (3 tumores) foram estadiados como T4 (Tabela 4). Respeitante ao envolvimento ganglionar, em 71,1% (54 tumores) não foi observado envolvimento ganglionar e em 28,9% (22 tumores) verificou-se envolvimento ganglionar regional (N1) (Tabela 5).

Procedendo-se à comparação do estadiamento efectuado pela ecografia endorectal com o resultado histológico da peça cirúrgica (Tabela 6), verificou-se: sub-estadiamento em 1 doente (1,3% dos casos) estadiado como uT1; sobre-estadiamento em 5 doentes (6,6% casos) estadiados como uT2; sub-estadiamento em 8 doentes (10,5% casos) estadiados como uT2; sobre-estadiamento em 8 doentes (10,5% casos) estadiados como uT3 e sobre-estadiamento em 2 doentes (2,6% casos) estadiados como uT4.

Em relação à comparação do estadiamento referente ao envolvimento ganglionar, notou-se um sub-estadiamento de 18,4% (14 doentes) e um sobre-estadiamento de 6,6% (5 doentes) (Tabela 7).

Quanto aos resultados obtidos para a sensibilidade da ecografia endorectal no estadiamento pré-operatório do cancro do recto, observou-se uma sensibilidade de 63% para T1, de 71% para T2, 67% para T3 e de 100% para T4. Em relação à especificidade, verificou-se uma especificidade de 98% para T1, de 76% para T2, de 81% para T3 e de 97% para T4. No que diz respeito ao valor preditivo positivo, constatou-se um valor preditivo de 92% para T1, de 54% para T2, de

Tabela 2 – *Estadiamento obtido pela ecografia endorectal em relação ao T*

	Frequência	%
T1	13	17,10
T2	28	36,80
T3	30	39,50
T4	5	6,60
Total	76	100

Tabela 3 – *Estadiamento obtido pela ecografia endorectal em relação ao N.*

	Frequência	%
N0	63	82,90
N1	13	17,10
Total	76	100

Tabela 4 – *Estadiamento anatomopatológico respeitante ao T.*

	Frequência	%
T1	19	25,00
T2	21	27,60
T3	33	43,40
T4	3	3,90
Total	76	100

Tabela 5 – *Estadiamento anatomopatológico respeitante ao N.*

	Frequência	%
N0	54	71,10
N1	22	28,90
Total	76	100

Tabela 6 – *Comparação entre o estadiamento histológico e ecográfico respeitante ao T.*

		Estadiamento ecoendoscópico				
		T1	**T2**	**T3**	**T4**	**Total**
Estadiamento histológico	T1	12(15,8)	5(6,6%)	2(2,6%)	0	19(25%)
	T2	0	15(19,7%)	6(7,9%)	0	21(27,6%)
	T3	1(1,3%)	8(10,5%)	22(28,9%)	2(2,6%)	33(43,4%)
	T4	0	0	0	3(3,9%)	3(3,9%)
	Total	13(17,1%)	28(36,8%)	30(39,5%)	5(6,6%)	76(100%)

Tabela 7 – *Comparação entre o estadiamento histológico e ecográfico em relação ao N.*

		Estadiamento ecoendoscópico		
		N+	N-	Total
Estadiamento histológico	N+	8(10,5%)	14(18,4%)	22(28,9%)
	N-	5(6,6%)	49(64,5%)	54(71,1%)
	Total	13(17,1%)	63(2,9%)	76(100%)

Tabela 8 – *Resultados da sensibilidade, especificidade, valor preditivo positivo e negativo e eficácia do estadiamento ecoendoscópico em relação ao T e ao N.*

	Sensibilidade (%)	**Especificidade (%)**	**VP positivo (%)**	**VP negativo (%)**	**Eficácia (%)**
T1	63	98	92	89	89
T2	71	76	54	88	75
T3	67	81	73	76	75
T4	100	97	60	100	97
N	39	91	62	78	75

73% para T3 e de 60% para T4. Quanto ao valor preditivo negativo, observou-se um valor de 89% para T1, de 88% para T2, de 76% para T3 e de 100% para T4. Quanto à eficácia da Ecoendoscopia, esta foi de 89% para T1, de 75% para T2 e T3 e de 97% para T4 (Tabela 8). Em relação ao N, observou-se uma sensibilidade de 39%, especificidade de 91%, um valor preditivo positivo e negativo de 62 e 78%, respectivamente, e ainda uma eficácia de 75% (Tabela 8).

Na avaliação da precisão estadiamento ecoendoscópico, por meio da elaboração de curvas ROC e cálculo das AUC, obteve-se um valor de AUC de 0,807 para T1, de 0,739 para T2, de 0,740 para T3, de 0,986 para T4 e um AUC de 0,636 para o estadiamento N (Figura 1).

No que diz respeito ao teste de McNemar, não se verificou diferença significativa entre o estadiamento ecoendoscópico e o estadiamento anatomopatológico (Tabela 9).

DISCUSSÃO

O cancro do recto é uma doença oncológica de elevada incidência[1] e o seu prognóstico depende não só de um diagnóstico precoce, mas também de um estadiamento pré-operatório preciso, o que vai permitir a selecção da terapêutica mais apropriada com o objectivo de diminuir a recidiva local e assim aumentar a sobrevida do doente[10,11,13,15-17]. Dessa forma, torna-se de extrema importância auditar a eficácia dos métodos disponíveis na gestão dessa patologia, no caso do nosso estudo, os resultados da ecografia endorectal, uma vez que o erro no estadiamento pré-operatório poderá levar a sub ou sobretratamento do doente. Dado a precisão da ecografia endorectal ser muito variada na literatura, pretende-se, com este estudo, avaliar a sensibilidade e especificidade desta no estadiamento do cancro do recto, em nossa série, por meio da comparação com os resultados histológicos da peça cirúrgica.

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Sensibilidade da ecografia endorectal no estadiamento do cancro do recto: correlação com o estadiamento patológico
Luís Filipe Carvalho Carriço e Cols.

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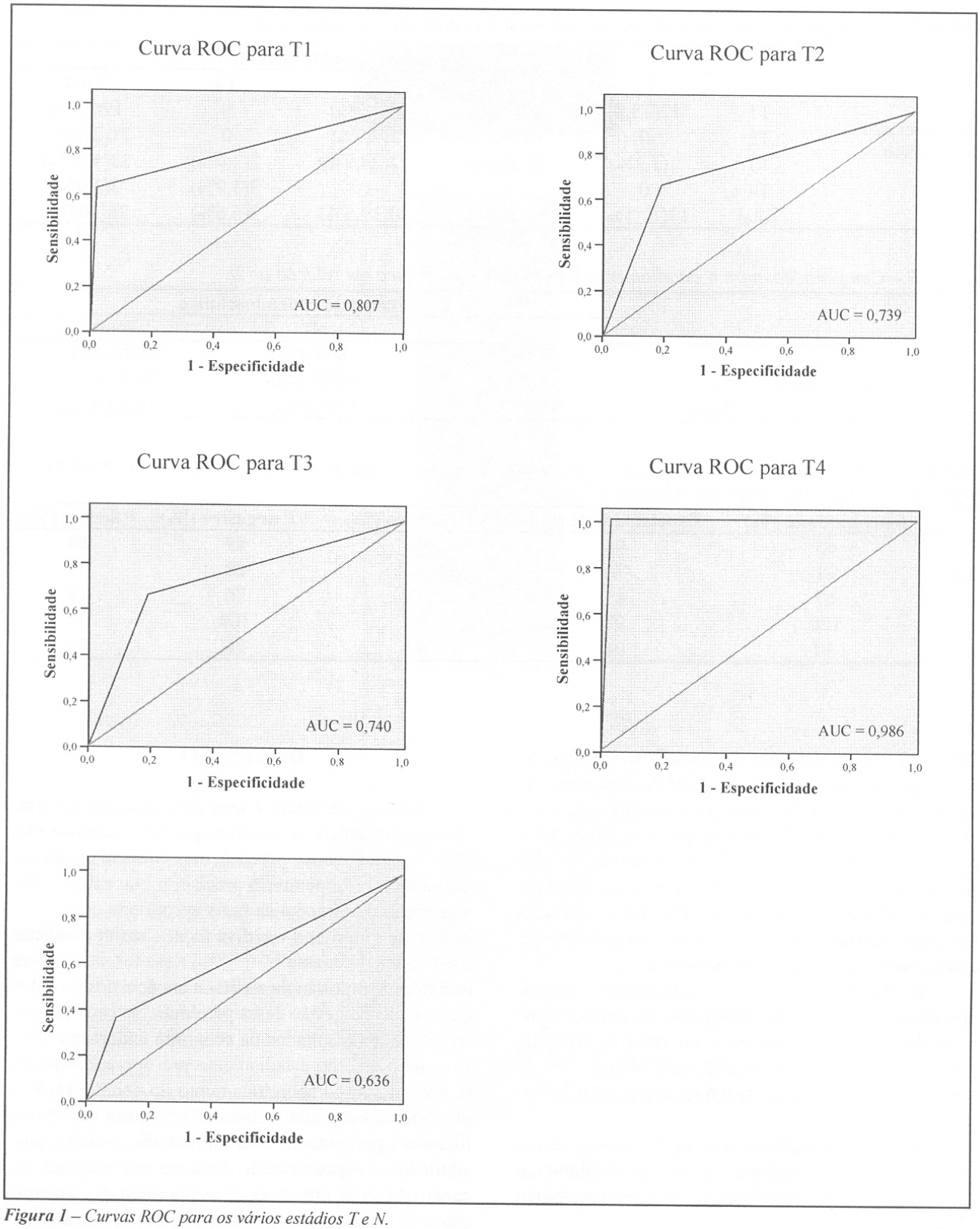

Figura 1 *– Curvas ROC para os vários estádios T e N.*

Actualmente, as técnicas de estadiamento do cancro do recto incluem o exame objectivo, TAC, ecografia endorectal e a RM com bobina endorretal; destes, os dois últimos são considerados os melhores exames para determinar o T[29].

A ecografia endorectal é uma das técnicas mais precisas no estadiamento do cancro do recto, tendo emergido nos últimos tempos como modalidade de escolha nesse processo[22,27]. Entre as vantagens apontadas, consta a realização fácil, baixo custo e uma precisão muito elevada segundo alguns autores[17,23,26]. Contudo, tem as suas limitações, sendo o facto de ser operador-dependente uma das mais significativas[29-31]. Por outro lado, é um exame com sensibilidade limitada para detecção de metástases ganglionares regionais, assim como, para o re-estadiamento de doentes que realizaram radioterapia pré-operatória[29]. Por último, essa técnica pode também ser influenciada por inúmeros factores, nomeadamente, a incapacidade de a sonda ultrapassar a lesão tumoral, uma exploração incompleta devido à angulação do recto, um contacto irregular com o recto devido a fezes ou gases, defeitos anatómicos provocados por intervenções cirúrgicas no recto, inflamação tumoral que poderão levar a interpretações erradas[32].

A RM com bobina endorretal, fornece informação em relação ao T sobreponível à ecografia endorectal, mas o elevado custo é uma das principais limitações[29]. Permite uma avaliação precisa do mesorecto e possibilita a determinação correcta da margem de ressecção radial tumoral, sendo esse último um preditor muito forte da recorrência local do tumor[33-34].

Quer a ecografia endorectal quer a RM com bobina endorretal apresentam sensibilidade limitada na avaliação do envolvimento ganglionar[29].

Neste estudo, quando se procedeu à comparação do estadiamento efectuado pela ecografia endorectal com o resultado histológico da peça cirúrgica (Tabela 5), verificou-se: sub-estadiamento em 1 doente (1,3% dos casos) estadiado como uT1; sobre-estadiamento em 5 doentes (6,6% casos) estadiados como uT2; sub-estadiamento em 8 doentes (10,5% casos) estadiados como uT2; sobre-estadiamento em 8 doentes (10,5% casos) estadiados como uT3 e sobre-estadiamento em 2 doentes (2,6% casos) estadiados como uT4. Tendo em conta que doentes com o estadiamento pré-operatório T1-2N0 realizam somente terapêutica cirúrgica e que doentes com estadio T3,4Nx e TxN1 realizam terapêutica primária[9] verificou-se um subtratamento em 8 doentes (10,5% casos), uma vez que foram estadiados com T2 e o resultado histológico demonstrou que na realidade se tratavam de T3, não tendo portanto realizado terapêutica primária. Relativamente aos oito doentes que aparentemente foram sobre-estadiados como uT3, tendo portanto realizado terapêutica primária, não podemos afirmar com certeza este sobre-estadiamento, pois o resultado histológico da peça cirúrgica pode tratar-se de um sobre-estadiamento ou então de um subestadiamento resultante da terapêutica primária.

O efeito downstaging dessa modalidade terapêutica tem sido confirmado em vários estudos. Após a radioterapia pré-operatória em esquema longo (45Gy, 5 semanas) verificou-se existir downstaging histológico, com sinais de regressão tumoral, em 94,4% dos doentes e tem sido constatada regressão tumoral completa inferior a 10% dos casos submetidos a terapia radica isolada, subindo essa taxa para valores até 30% após radioquimioterapia[35].

A respeito do cálculo da sensibilidade da ecografia endorectal no estadiamento T, neste estudo observou-se uma sensibilidade de 63% para T1, de 71% para T2, 67% para T3 e de 100% para T4. Esses valores são ligeiramente inferiores aos referidos na literatura, exceptuando a nível de T4, em que referem valores de sensibilidade mais altos, a rondar os 80 e 95%[17,23,24]. Esta diferença poderá ser explicada pelo facto de muitos estudos não incluírem muitos doentes com cancros do recto localmente avançados[36,37]. Por outro lado, alguns autores concluíram que existe um enviesamento de publicações, no que respeita a edição dos estudos com melhores resultados[38].

No que diz respeito à especificidade, verificou-se uma especificidade de 98% para T1, de 76% para T2, de 81% para T3 e de 97% para T que demonstram valores entre 80 e 98%[17,23,24].

Em relação ao valor preditivo positivo, constatou-se um valor de 92% para T1, de 54% para T2, de 73% para T3 e de 60% para T4. Quanto ao valor preditivo negativo, observou-se um valor de 89% para T1, de 88% para T2, de 76% para T3 e de 100% para T4. Esses valores obtidos estão de acordo com estudos anteriores que apontam para valores semelhantes de valor preditivo positivo e negativo[39].

Quanto à eficácia, esta foi de 89% para T1, de 75% para T2 e T3 e de 97% para T4, conforme resultados demonstrados por estudos anteriores que apontam para níveis de eficácia muito altos da ecografia endorectal na avaliação da invasão tumoral na parede do recto[17,23,26,39].

Ao analisar o envolvimento ganglionar, observou-se uma sensibilidade de 39%, o que difere de alguns estudos publicados que apontam para valores mais altos de sensibilidade, mas que vai ao encontro de outro estudo, que demonstra uma sensibilidade de 33% na avaliação do N[23,25,26,40]. Isso poderá ser explicado pelo enviesamento de publicação referido anteriormente, mas também pelo facto da inclusão nesses estudos de doentes submetidos à terapia neoadjuvante, que poderá resultar numa subestimação da sensibilidade da ecografia endorectal[39,40].

Verificou-se uma especificidade de 91%. Esse valor é ligeiramente superior ao encontrado na literatura que aponta valores de especificidade entre 76 e 86%[23,19,25,26,39]. Em relação ao valor preditivo positivo e negativo, observou-se um valor preditivo positivo e negativo de 62 e 78%, respectivamente. Esse resultado é concordante com o publicado em estudos anteriores, que demonstra que a ecografia endorectal é melhor na exclusão de envolvimento ganglionar do que propriamente a confirmar a invasão ganglionar[26].

Foi observada uma eficácia de 75% da ecografia endorectal na avaliação da invasão ganglionar, conforme o já descrito na literatura que aponta para uma eficácia entre 64 e 75%[26,39,40].

De modo a comprovar melhor a precisão da ecografia endorectal, elaborou-se curvas ROC e calculou-se a AUC destas. Esse teste estatístico é um bom preditor da precisão de um teste, sendo que uma área de 1 representa um teste perfeito. Na avaliação da precisão, obteve-se um valor de AUC de 0,807 para T1, de 0,739 para T2, de 0,740 para T3, de 0,986 para T4 e um AUC de 0,636 para o estadiamento N (Figura 1). Neste estudo, as curvas ROC mostraram valores de AUC muito perto de 1, indicando que a ecografia endorectal é um bom teste para estadiar a invasão tumoral no recto (T) e que é um teste razoável no estadiamento da invasão ganglionar. Esses resultados são ligeiramente inferiores a estudos previamente efectuados, que apontam para valores de AUC mais altos, indicando, assim, que a ecografia endorectal é um excelente teste no estadiamento global do Cancro do Recto[17,26]. No entanto, essa diferença pode ser explicada pelo maior número de doentes incluídos neste estudo relativamente aos estudos já efectuados, o que por sí poderá levar a uma melhor estimação da precisão da ecografia endorectal.

Por meio do teste de McNemar verificamos se existiam ou não diferenças significativas entre a ecografia endorectal e o estadiamento anatomopatológico. Neste estudo, verificou-se que há concordância significativa entre ambos pois não se obteve valores de $p<0,05$ (Tabela 8). Esse resultado vem reforçar que a ecografia endorectal é um exame essencial no estadiamento pré-operatório do cancro do recto.

Em jeito de conclusão, os resultados deste estudo permitem confirmar que a ecografia endorectal é uma importante ferramenta, de alta precisão para o estadiamento pré-operatório do cancro do recto. Os dados são melhores no estadiamento do T do que do N, sobretudo a nível da sensibilidade, com valores entre 63 e 100% comparativamente a 39%. O mesmo acontece relativamente à eficácia, com valores compreendidos entre 75 e 97% contra 75% na avaliação da invasão ganglionar. Apesar disso, ecografia endorectal é um teste moderado para averiguar o envolvimento ganglionar, sendo mais preciso na exclusão do que na confirmação de invasão ganglionar.

No futuro próximo, com os avanços tecnológicos que a ecoendoscopia 3D poderá acrescentar a esta modalidade de estadiamento, será possível atingir maior precisão no estadiamento TN do cancro do recto pré-operatoriamente e assim obter uma gestão mais adequada da doença.

ABSTRACT: Objective: This study aimed to evaluate endorectal ultrasound sensibility, in our experience, in rectal cancer staging comparing with pathologic result. Methods: A retrospective study between January 2005 and August 2009. We calculated sensibility, specificity, positive and negative predictive value for T and N. Through ROC curves we evaluated endoscopic ultrasound accuracy and through McNemar test we compared it with the anatomopathological result. Results: Of 112 patients, 76 met the inclusion criteria. We obtained an efficiency of 75 to 97% for uT and 75% in uN. There was a sensibility, specificity, positive and negative predictive value, respectively of 63, 98, 92 and 89% for uT1, 71% and 76, 54 and 88 for uT2, 67, 81; 73 and 76% for uT3, 100, 97, 60 and 100% uT4,

and 39, 91, 62 and 78% for uN. The ROC curves indicated that endorectal ultrasound is a good test for T staging and reasonable for N staging. The McNemar test revealed no significant differences between endoscopic ultrasound and histological staging (p>0.05). Conclusions: We concluded that endorectal ultrasound is an important tool in rectal cancer staging, showing a good correlation with histopathological results.

Key words: Ultrasonography endorectal; Sensibility; Specificity; Positive predictive value; Negative predictive value; Pathological outcome.

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Appendix 10:

"Evaluation of quality parameters of rectal cancer surgery at the Coloproctology Unit of Hospital de Braga."

Original Article

Evaluation of quality parameters of rectal cancer surgery at the Coloproctology Unit of Hospital de Braga

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ABSTRACT: Introduction: Rectal cancer (RC) represents 1/3 of all diagnosed colorectal cancers. After the creation of specialized units to treat RC, it became fundamental to establish criteria to assess the quality of the service. Objective: To evaluate the surgical treatment provided to RC patients at the Coloproctology Unit of *Hospital de Braga* (BH-CU) by means of quality parameters. Methods: We conducted an observational cross-sectional descriptive study with a convenience sample of 149 patients undergoing surgical treatment in this unit, from January 1st, 2007 to June 30, 2010. Results: We observed that the postoperative mortality rate (4%) and the global dehiscence rate (14.8%) were in accordance with recommended values. Sphincter sparing surgery rate (65.8%) was higher than the recommended minimum; however, more than 12 resected ganglia (36.6%) is inferior than what is recommended. The oncological results were analyzed by the local recurrence rate (6.7%) and the two-year survival rate (91.1%); both values are in accordance with literature. Conclusion: We conclude that the BH-CU surgical treatment has a quality level similar to that observed in literature.

Keywords: rectal cancer; functional coloproctology unit; quality parameters of surgical treatment.

INTRODUCTION

Colorectal cancer (CCR) is the third most common cancer and ranks the fourth position as a cause of death by cancer worldwide[1-3]. Its incidence is geographically varied, and there are higher rates in North America, Australia and Western Europe. The lower rates are in developing countries[4], but the incidence in these countries[5] has been increasing in the past few years.

According to the World Health Organization (WHO), CCR is the second most common cancer in Europe, followed by lung cancer among males and breast cancer among females[6]. Despite the high incidence, data from WHO from 1997 to 2007 show that mortality caused by CCR decreased[7]. The reduction in mortality rates was mainly due to the advances in knowledge concerning the molecular mechanisms that are responsible for the development and progression of CCR[8] and for the introduction of tracking programs with the population aged more than 50 years[9]. In Portugal, according to the National Institute of Statistics, CCR is the second most common cancer and the main cause of death due to neoplastic disease[10]. It is more common in urban centers, such as Lisbon, Porto and Setubal[11]. To the north of Portugal, data from *Registro Oncológico Regional do Norte* (RORENO) show that CCR was the most prevalent cancer in 2005 for both genders, and the second cause of death due to cancer, after lung cancer[12,13].

Rectal cancer (RC) makes up to 1/3 of the total number of diagnosed cases of CCR[14]. Even though the north of Portugal presents an incidence rate of 24.8/100,000 inhabitants, which is higher to the incidence in Europe (21.2/100,000 inhabitants), the five-year survival rate (53%) has a much closer value to the European mean (53.2%)[12]. The therapeutic approach to RC has been through significant changes in the past decades, going

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from a merely surgical treatment to a multidisciplinary approach[15]; however, despite the aforementioned advances, surgical exeresis is still essential[16], since it is the only potentially curative treatment nowadays. There are currently many therapy options related to the location of the cancer; thus, the performance of an anterior rectal resection (ARR) for superior rectal tumors is indicated; a low anterior rectal resection with coloanal anastomosis is indicated for inferior rectal tumors[17]. As to the latter, since this procedure has risk of dehiscence, it is established that is should be complemented with protective ileostomy[18]. The abdominoperineal resection (APR) is currently limited; it is recommended for tumors that present with sphincter infiltration, for cases of fecal incontinence prior to tumoral symptomatology and elderly patients with associated comorbidity that does not allow an anastomosis. The same happens with the Hartmann's operation (HO), which is performed when there are factors that contraindicate anastomosis that would enable the preservation of the sphincters with a safe distal margin[17]. Also, the local transanal resection is only indicated for tumors that are limited to the mucosa and the submucosa (T1N0M0), with no lymphovascular invasion, well or moderately differentiated, with less than 3 cm in diameter and located up to 8 cm from the anal margin (AM)[17]. One of the great advances in the past decades, in terms of surgical treatment for CR, was the introduction of the concept of total mesorectal excision (TME). Heald et al. showed the importance of TME in the two lower thirds of the rectum, with dissection under direct visualization and preservation of the nervous plexus. The introduction of TME enabled the reduction of local recurrence rates for values of 6%, with a five-year survival rate of 80%, and ten-year survival rate of 78%[15]. The decrease in local recurrence rates was due to the fact that TME enabled the resection of RC with a negative circumferential margin[19]. This technique has also led to the improvement in pathological staging of cancer, as well as in the quality of life of the operated patient because of the reduction in the incidence of vesical dysfunction and sexual impotence[14].

The concept of margin is important to be considered in resection with a curative intent. Regarding RC, we should consider the distal, proximal and radial margins, in which the currently accepted values are 1 cm, 5 cm and 1 mm, respectively. The involvement of these margins is associated with increased locoregional recurrence; more specifically, the involvement of the radial margin is associated with a recurrence risk of 56 – 80%, with a five-year survival rate, decreasing from 79 to 40%[20]. Another margin to be considered is the distal margin of the mesorectal dissection, which has an important prognostic value and should be 5 cm distal to the tumor, once it showed the presence of tumor niche 2 to 3 cm below the tumor[17].

As to the surgical treatment of RC, together with negative resection margins, a proper lymphadenectomy is the most important aspect[21]. In this context, it is important to perform a proper lymphadenectomy, with resection of at least 12 ganglia; besides reducing the risk of lymphatic invasion, it also prevents the substaging of the tumor[22].

Despite the improvements observed in the recurrence rate of the resectable RC, the local recurrence is still an issue in cases of locally advanced fixed rectal cancer. The current strategy to treat such cases is multidisciplinary[23]. The primary therapy enables to increase respectability, decrease the locoregional recurrence rate and improve the survival of the patient[19,23]. Thus, the initial treatment for locally advanced RC (T3-4 or N+) consists of the administration of chemotherapy and primary radiotherapy[16,19].

The creation of units that are specialized in treating RC contributed with better results, since it improved the preoperative staging by using: the pelvic magnetic resonance and endoluminal ultrasound; the primary therapy after establishing the dose and proper times of application[24] in cases of locally advanced RC; the implementation of TME as a qualified technique to assess the obtained results[22]; and the establishment of standards concerning anatomopathological tecniques[24]. According to a study conducted in the United States, these changes are reflected in the decreased local recurrence rate, from 9.6 to 6.9%[25]. In a study group from Norway, the implementation of TME showed a decrease in the local recurrence rate, from 12% to 6%, and the survival rate after four years increased from 60% to 73%. The same happened in a randomized study conducted in the Netherlands, in which the local recurrence rate after two years was significantly lower in patients submitted to surgery and radiotherapy (2.4%) than in the group treated only with surgery (8.2%)[16]. Due to this evolution, many European countries, such as Germany, Sweden and Spain, showed the need to define new quality

standards, with the minimum required values, which are essential for the evolution of the diagnosis, staging and treatment of RC, for beyond the ones that are usually used, such as morbidity and mortality[22,24,26].

By studying large samples, some indicators have been established to enable the evaluation of surgical quality concerning the RC treatment; these can be divided into general and specific criteria, and criteria that study oncologic results[27]. General criteria are: postoperative mortality rate, which should be inferior to 5%, ideally between 2 and 3%[24,27], and the global dehiscence rate, whose required value lies between 10 and 15%[22,24,27]. Regarding the sphincter sparing surgery rate, it is recommended to be higher than 65%[24,27], and the number of cases with more than 12 resected ganglia should be higher than 75%[22]; both are considered to be specific criteria. Finally, the criteria that study the oncologic results are assessed by the local recurrence rate, that should be lower than 10%[22,24,27], and the ideal value for the survival rate after two years is between 70 and 80%[24,25]. Besides the aforementioned, these indicators enable a proper evaluation of quality in assistance, because it accounts for the final health status of the patient and its functional capacity[24].

OBJECTIVE

To assess the surgical treatment given to patients with rectal cancer in the Coloproctology Unit of *Hospital de Braga,* from January 1st, 2007, and June 30, 2010, according to quality standards.

METHODS

Population

The study population was comprised of patients treated for RC from January 1st, 2007, to June 30, 2010, at the Coloproctology Unit of *Hospital de Braga*. This study considered as "rectal cancer" all the cases of histopathological diagnosis of adenocarcinoma, located up to 15 cm from the anal margin, measured with the rigid sigmoidoscopy. Inclusion criteria were: patients with histological diagnosis of rectal adenocarcinoma submitted to surgery (local resection, anterior rectal resection, low anterior rectal resection or abdominoperineal resection). Exclusion criteria were: patients with histological diagnosis of rectal adenocarcinoma that did not undergo surgery, or those in which the derivative stoma was performed.

Sample

A convenience sample of 149 patients diagnosed with RC was used, respecting the inclusion/exclusion criteria previously established.

Methods and data collection

Before data collection, the work was submitted to and approved by the Ethics Committee of *Hospital de Braga*. A prospective database of patients diagnosed with RC was consulted; it consisted of sociodemographic data, location of the tumor, treatment of choice, number of resected ganglia, resection margins, presence of postoperative morbidity and data related to the follow-up period as the date of local recurrence and death.

Statistical analysis

All statistical analyses were performed with the 18.0 version of the software *Package for the Social Sciences*, (SPSS Inc. R, Chicago, Illinois, USA). A simple descriptive analysis of all the variables was conducted by defining the total number of cases and the absolute and relative frequencies for valid cases. As for the treatment of continuous quantitative variables (age, distance to anal margin and number of resected ganglia) central tendency (mode and mean) and dispersion (standard deviation [SD]) were measured.

RESULTS

Sample caracterization

From January 1st, 2007, and June 30, 2010, 164 patients with RC were treated at the Coloproctology Unit of *Hospital de Braga*. At first, 15 patients were not eligible for the study, once they were in no conditions to be submitted to surgery (n=7) or in case they had been submitted to the isolated performance of a derivative stoma (n=9). Thus, after the establishment of exclusion criteria, 149 patients were included in the study, that is, 91% of the patients that had been initially identified. As to gender distribution, we observed that 57% of the patients (n=85) were males, and 43% (n=64) were females. Mean age was 68±12 years; among females, it was 66±13 years, and for males it was 70±11

years. Mode was equal to 80 years. After observing the age group analysis, we noticed that most cases, 35.6%, occurs between the ages of 70 and 80 years (n=53) (Figure 1). The most common location of RC was the medium rectum, in 53% of the cases, followed by the lower and upper rectum, in 27.5 and 19.5% of the cases, respectively (Table 1). The mean distance to the anal margin was 8.5±4.3 cm. After staging, 27.5% (n=41) of the patients underwent primary therapy followed by surgery; out of these, chemoradiotherapy was used in 25.5% of the patients (Table 2).

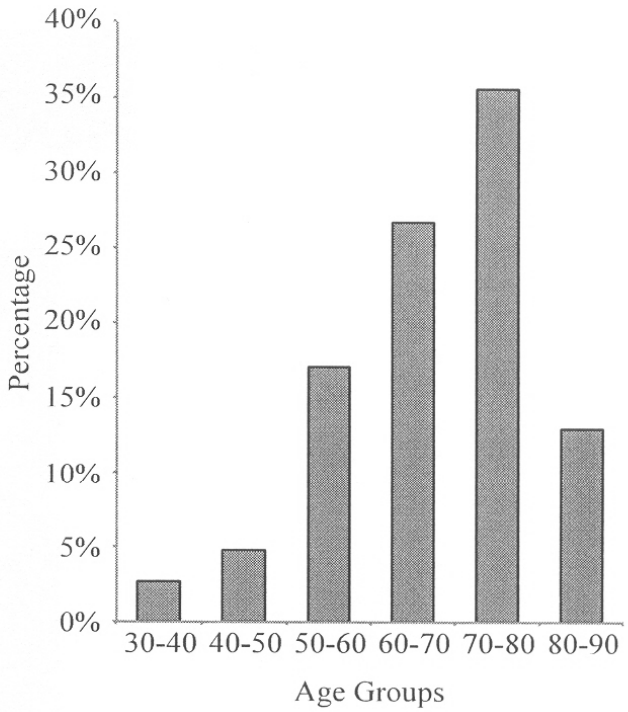

Figure 1. *Distribution of the "Age" variable by age groups.*

Evaluation of surgery quality parameters

Type of surgery

Concerning the performed surgeries, 98.7% (n=147) were elective, and 93.3% (n=139) of the cases, it had a curative intent. The most common surgery was the low anterior rectal resection, 30.2% (n=45), followed by the abdominoperineal resection (22.1%) (n=33). As demonstrated in Table 3, 65.8% of the surgeries were classified as "Sphincter Sparing Surgery".

Anastomotic dehiscence

Out of the 149 studied cases, 22 presented with postoperative morbidity classified as "anastomotic dehiscence". In this group, 9 patients needed surgical reintervention. After crossing the variables "type of surgery" and "anastomotic dehiscence", it was possible to show that the low anterior rectal resection is the surgical procedure that presents the highest global anastomotic dehiscence rate, with 6.8% of the cases; out of these, 3.4% were conservatively treated, and the other 3.4% needed surgical reintervention (Table 4). After analyzing the global dehiscence rate along the years of the study, we observed that 2007 and 2009 presented the highest percentage, with 4.7% of the cases; in 2010, this value decreased (Figure 2). Out of the 22 patients who presented with anastomotic dehiscence, only 1 (0.7%) was submitted to primary radiotherapy.

Postoperative mortality

The postoperative mortality rate was 4.0% (n=6). From these patients, 3 presented with postoperative morbidity characterized as anastomotic dehiscence; two were submitted to conservative treatment, and one underwent surgery.

Table 1. *Characterizing the variable "Anatomical Location".*

Anatomical Location		
	Absolute N° (n)	**Frequency (%)**
Superior rectum	29	19.5
Medium rectum	79	53.0
Inferior rectum	41	27.5
Total	149	100.0

Table 2. *Characterizing the variable "Primary Treatment".*

Primary Treatment		
	Absolute N° (n)	**Frequency (%)**
No primary treatment	108	72.5
CT + RT	38	25.5
CT	1	0.7
RT	2	1.3
Total	149	100

CT: chemotherapy; RT: radiotherapy.

Table 3. *Characterizing the variable "Type of surgery".*

Type of Surgery	Absolute N° (n)	Frequency (%)	Sphincter Sparing Surgery
Low anterior rectal resection	45	30.2	65.8 %
Anterior rectal resection	28	18.8	
Low anterior rectal resection + ileostomy	21	14.1	
Local Resection	4	2.7	
Hartmann's operation	18	12.1	34.2 %
Abdominoperineal resection	33	22.1	
Total	149	100.0	

CT: chemotherapy; RT: radiotherapy

Table 4. *Crossing variables "Type of surgery" and "Anastomotic dehiscence".*

	Absolute N°(n)	Frequency (%)
Dehiscence – Conservative treatment	13	8.8
Low anterior rectal resection	5	3.4
Abdominoperineal resection	5	3.4
Low anterior rectal resection + ileostomy	3	2
Dehiscence – Surgical treatment	9	6.0
Low anterior rectal resection	5	3.4
Abdominoperineal resection	4	2.6
Low anterior rectal resection + ileostomy	0	0
Total	22	14.8

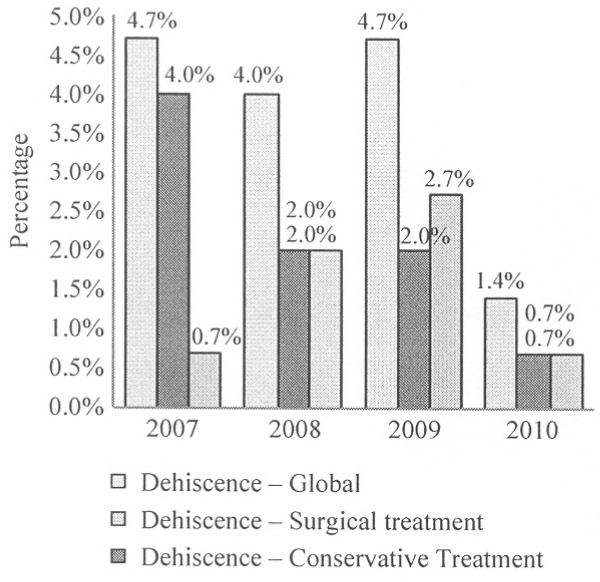

Figure 2. *Evolution of the variable "Anastomotic dehiscence".*

Number of analyzed ganglia

The mean of analyzed ganglia (gg) was 11±7 ganglia, the median was 9.5 and the mode was 6 ganglia.

The analysis of 12 or more ganglia was only observed in 36.6% of the cases (n=49); in the other 63.4% (n=85), an inferior number of ganglia were analyzed. Out of the 41 cases submitted to primary therapy, 70.7% (n=29) presented a number of analyzed ganglia inferior to 12. From the 85 cases with less than 12 analyzed ganglia, 29 cases (34.1%) had primary therapy.

Locoregional recurrence

The global recurrence rate was 6.7% (n=10). The patients submitted to primary therapy presented an inferior recurrence rate, 1.3%, in relation to those who underwent isolated surgery (5.4%).

Survival after 2 years

The survival rate after 2 years was 91.9% in the studied sample.

DISCUSSION

The treatment of RC has progressed for the past few decades[15], and this progress is mostly due to the creation of functional units that are specifically directed to this pathology[24]. Many European countries, such as Norway, the Netherlands, Germany, Sweden, France, Denmark and Spain, have been working to define new quality standards to establish minimum required values for the surgical treatment of RC[22,24,26,28-32].

The requirement for the creation of coloproctology functional units is based on many studies that demonstrate that the treatment of patients with specific diagnoses, such as RC, is better in hospitals that receive a lot of cases of this pathology; and although it might sound true, this may be more related to specific characteristics of the surgeon than to the number of cases in the hospital[33,34]. In Europe, it is acknowledged that the surgeon factor, especially the technique, the ability and the practice, are essential and influence the results of the treatment for RC[35]. Thus, the sub specialization of colorectal surgeons who are especially trained and have performed a high number of surgeries, is one of the most important predictors of quality concerning colorectal surgery[33,34]. In 2006, Rogers et al. suggested at least 13 rectal resections per surgeon as the minimum value required for maintaining the certificate in colorectal surgery for a period of 4 years, and in hospitals that have at least 84 surgeries of this type during this period[34]. In Sweden, as in this study, Martling et al. observed that the high number of surgeries is associated with better results, and defined that a group reaches such classification when each surgeon performs at least 12 rectal resections in a year[36].

In Portugal, there are many coloproctology functional units; however, there are few studies that evaluate quality standards. So, this study aims to audit the quality of the health care service that is present at the functional units of *Hospital de Braga* in order to provide a work base that allows its improvement.

After analyzing the data concerning the functional units of *Hospital de Braga*, from January 1st, 2007, to June 30, 2010, 164 patients with RC were treated, and since the unit had three surgeons, these values are clearly above the suggested by the two aforementioned studies[34,36] for the performance of RC surgery, so to offer quality standards to these patients.

Concerning the treated patients, it was observed that males are more affected, in 57% of the cases, and that 92.4% of the cases are comprised in age groups older than 50 years, which is in accordance with literature[1,3,37]. As to the location of the RC, our studied showed that 53% of the cases were in the medium rectum, which is similar to findings from studies conducted in Germany, Spain and the United States of America, in which 40 to 55% of the cancers had this anatomical location[22,25,38].

The administration of primary therapy is currently essential to approach locally advanced RC or with ganglion invasion, since it increases the possibility of resection, decreases the locoregional recurrence rate and increases survival rates[23]. In this study, after staging, 27.5% (n=41) of the patients underwent primary therapy followed by surgery.

Concerning the performed surgeries, 93.3% (n=139) of the cases had curative intent, which is higher than the values found in literature, that shows values such as 91.5% in Norway[28], 83% in Sweden[39] and 64% in the Netherlands[40]. This result can be due to the fact that we are located in a region with high incidence of colorectal cancer; this is why patients have been tracked for the past few years, which enabled the early diagnosis, as well as the relation between the functional unit and the health centers; this way, patients were rapidly referred.

The most common surgery in the coloproctology functional unit was the low anterior rectal resection (30.2%), which is in accordance with rates found in literature, of 39.5%[38] and 47.3%[23].

As to the parameter "sphincter sparing surgery", in Sweden and Spain the recommended values are higher than 70%[24,39] of the performed surgery; in Norway and the Netherlands, the ideal value is between 65 and 70%[28,40]. The result was 65.8%, which is close to the minimum value required in these studies. This value can be explained because the ultralow anterior rectal resection is not performed with coloanal anastomosis, and also because of the high percentage of cases in comparison to other series of performed Hartmann's operation, 12.1% (n=18). Out of these patients, only one was submitted to urgent surgery; the others underwent elective surgery, in which the "sphincter sparing" resection could be performed, but due to the old age of the patients (mode of 80 years), with comorbidities associated with sphincter malfunctions, it

was chosen to perform a definitive stoma in order to avoid the high risk of fecal incontinence.

The rate of abominoperineal resections performed was 22.1% (n=33), which is within the limits described in literature, from 22 and 27%[41], strongly influenced by the number of patients in the center. For tumors that are under the 8 cm from the anal margin, the described values range from 44.6 to 44.8%[41].

This rate has been considered as one of the reliability criteria of the functional units[41-43]; however, such criteria are being discussed[41,42], since they depend on the percentage of RC located in the inferior 1/3 of the rectum that each unit presents; in this study, it was 27.5% of the cases.

Concerning the postoperative morbidity analysis, we chose to only characterize the anastomotic dehiscence since it is the main cause of morbimortality of rectal resection[35]. Values of 15%[24] are described in Spanish studies, but other countries presented inferior numbers: 9% in Sweden[39], 10% in Germany, 10% in Norway[28], and 12% in the Netherlands[40]. The first issue we face to compare values concerning the coloproctology functional units at *Hospital de Braga* with data presented in literature is the definition of this concept. Except for the German study, none of the others define "anastomotic dehiscence". This problem is registered in literature, since there are many studies related to dehiscence values; a review conducted by Bruce et al. on the incidence of anastomotic dehiscence post colorectal surgery analyzed 97 studies, in which 57 different definitions of anastomotic dehiscence were defined by the need of surgical reintervention, clinical findings or radiological criteria, thus making the comparison between studies more difficult[44].

In this study, the anastomotic dehiscence was defined as colorectal anastomotic failure, diagnosed by clinical or radiological criteria, with or without the need for the surgical treatment, which represents a total dehiscence rate of 14.8% (n=22); this value would decrease to 6% (n=9) in case only the patients who needed surgical reintervention were considered. When we analyze which "Type of surgery" presents the higher total dehiscence rate, we observe that the low anterior rectal resection is the highest, in 6.8% of the cases, which is in accordance with literature, since the risk of dehiscence depends on the level of anastomosis, among other factors, that is, bigger in the medium and inferior rectum[45].

Another important aspect in the data analysis is that the low anterior rectal resection with ileostomy presents the lowest total dehiscence value, 2%, and also that all the other cases (n=3) were treated without the new surgical intervention.

Even though the primary therapy increases the risk of dehiscence, this study did not have enough data to establish such a relation[45].

Data obtained after the analysis of the evolution of the variable "anastomotic dehiscence" throughout the studied years are inconclusive. Annual values are very similar, however, a gradual increase in dehiscence cases that needed surgical reintervention was observed. This can be a result of lower anastomoses that are performed with the years, due to the accumulated experience, thus causing a higher risk of dehiscence. The lowest dehiscence value was observed in 2010, concerning the first six months of the year; although, there is a tendency to reduce such number.

As to the postoperative mortality rate, according to countries like Sweden, Norway, the Netherlands and Spain, it should be around 2 and 3%[24,28,39,40]; however, this interval is not a consensus, and in Germany the recommendation is that it should be inferior to 5%[22]. In our study, the postoperative mortality rate was 4.0% (n=6) and, as described in literature, this rate is directly related to the anastomotic dehiscence rate, once it is the main cause of death at the postoperative for the colorectal patient[24]. Out of these six patients, three had anastomotic dehiscence, and one underwent a new surgery. Besides, other aspects are also important, especially the old age of most patients in the sample, which leads to low resistance to the intercurrences that occur during admission, as well as associated comorbidities[25]; thus, it was the cause of death for other 3 patients (respiratory failure, myocardial infarction and pulmonary edema).

The evaluation of the ganglia involvement is essential for the staging of the RC, and significant correlations have been established between the number of analyzed ganglia and the survival of patients[46]. In order to study the number of analyzed ganglia, the cohort value was established based on criteria of different surgeon associations, which recommend the analysis of at least 12 negative ganglia[41,46,47]. This way, it is possible to confirm with 90% accuracy that the patient is free of lymphatic invasion[38,48]. In one of the studies conduct-

ed in Germany, it was defined that more than 75% of the surgeries should have more than 12 analyzed ganglia; in Spain, the value presented for such indicator is around 71%[22,38]. In this context, the percentage of cases in which 12 or more ganglia were analyzed (36.6%) is lower than the minimum required value. Three types of factors can contribute with this value: the ones that depend on anatomy and on the biological conditions of the patient; the ones that depend on surgical technique; and the ones that depend anatomopathological technique[48].

Concerning the factors that depend on the patient, the anatomical factors stand out, with individual variations related to the number of lymphatic ganglia, the age of the patient, with the tendency to perform surgeries that are less aggressive in oncological terms, with the old age of the patients48 and the administration of the primary treatment, which causes the ganglia to decrease in size, thus making resection harder[46].

Concerning this last aspect in the analyzed study, 70% of the cases that were submitted to primary therapy presented a number of analyzed ganglia inferior to 12; however, they represent only 34.1% of the cases with less than 12 analyzed ganglia, thus, the low percentage cannot be only related to that fact.

As to the surgical technique, the analysis of resection margins that led to the observation that out of the 164 operated patients, only one presented with radial margin invasion; with this, we concluded that a proper total mesorectal excision was performed, and that the lymphatic ganglia that were present in the mesorectum were completely removed; they might or might not have been accounted for. In literature, abominoperineal resection is described as the surgery with the lowest number of ganglia[48]. Since this surgery ranks in second place in our series as to the most performed surgeries, this might have contributed with the obtained results.

Finally, these results can be justified by the anatomopathological technique, since this unit is still based on the classical model of visual identification and ganglion palpation, which is a slow and delicate process, and also, in 70% of the cases, ganglia have less than 5 mm in diameter and could easily go unnoticed during the resection process[48].

The locoregional recurrence of RC is one of the most feared situations, since it is usually inoperable and the patient could die slowly and painfully[43]. As 55 to 80% of the recurrence cases happen in the first two years after surgery[49], the local recurrence rate in this period is one of the main indicators of the oncological results. The maximum value established for that rate is 10%, and it is presented by the Spanish series[24]; however, in decreasing order, we found the following values: 9% in the Netherlands[40], 6% in Sweden[28,39], and 4% in Norway[28]. In these three countries, this limit is lower for patients submitted to the primary treatment, and the minimum required value is between 1.5% and 2.4%[28,39,40]. In this area, the studied unit presents good numbers, with a local recurrence rate of 6.8%, subdivided into 6.1% of recurrence without primary treatment and 0.7% with primary treatment.

CONCLUSION

The periodic evaluation of quality standards concerning the surgery of RC is important in any coloproctology functional unit, since it enables internal monitoring, identifies the key points as to how to intervene for better results, and yet, at the same time, it enables to inform the patients in the unit about the expected results at the institution, instead of those in literature.

In this study, quality standards were classified as: general, specific and those that study oncological results. Concerning general criteria, the postoperative mortality rate, 4%, and the global dehiscence rate, 14.8%, are within the values recommended in literature. In the category of specific criteria, the rate of sphincter sparing surgeries, 65.8%, was higher than the recommended limit; however, the rate concerning more than 12 resected ganglia, 36.6%, is lower than recommended. Finally, the analysis of oncological results was conducted by a local recurrence rate, 6.7%, and survival rate after two years, 91.1%, both within recommended values.

With this study, we can observe that the values in this unit are within the values recommended in literature for most of the quality criteria. The exception, and one of the items that should receive short term investments, is the improvement of the anatomopathological characterization of the number of assessed ganglia. However, it is important to emphasize that with the rapid therapeutic advances, it is necessary to discuss and regularly rethink the minimum required values, as well as to define a limit of standards that are easy to calculate, so that the evaluation of the results by each of the surgeons in the unit can be a simple and periodic process.

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RESUMO: Introdução: O câncer do reto (CR) constitui cerca de 1/3 da totalidade dos casos de câncer colorretal diagnosticados. Após a criação de unidades especializadas no tratamento do CR, tornou-se fundamental estabelecer critérios que permitam avaliar a qualidade do tratamento prestado. Objetivo: Avaliar, segundo parâmetros de qualidade, o tratamento cirúrgico prestado aos doentes com CR, na Unidade Funcional de Coloproctologia (UFC) do Hospital de Braga (HB). Métodos: Realizou-se um estudo observacional, transversal e descritivo com uma amostra de conveniência constituída por 149 doentes operados de CR entre 1º de Janeiro de 2007 e 30 de Junho de 2010. Resultados: Observou-se que a taxa de mortalidade pós-operatória (4%) e a taxa global de deiscência (14,8%) se encontram dentro dos valores recomendados. A taxa de realização de cirurgia poupadora de esfíncteres (65,8%) foi superior ao limite mínimo aconselhado; no entanto, a taxa de número de gânglios ressecados superior a 12 (36,6%), encontra-se aquém do exigível. Os resultados oncológicos foram analisados através da taxa de recidiva local, 6,7%, e da taxa de sobrevida aos 2 anos, 91,1%, ambos dentro dos valores propostos na literatura. Conclusão: Concluímos que o tratamento cirúrgico prestado na UFC do HB apresenta um nível de qualidade semelhante ao globalmente recomendado.

Palavras-chave: câncer do reto; unidade funcional coloproctologia; parâmetros de qualidade do tratamento cirúrgico.

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Appendix 11:

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REVIEW

Role of endoglin and VEGF family expression in colorectal cancer prognosis and anti-angiogenic therapies

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Abstract

Colorectal cancer (CRC) is one of the cancer models and most of the carcinogenic steps are presently well understood. Therefore, successful preventive measures are currently used in medical practice. However, CRC is still an important public health problem as it is the third most common cancer and the fourth most frequent cause of cancer death worldwide. Nowadays, pathologic stage is a unique and well-recognized prognostic indicator, however, more accurate indicators of the biologic behavior of CRC are expected to improve the specificity of medical treatment. Angiogenesis plays an important role in the growth and progression of cancer but its role as a prognostic factor is still controversial. Probably the most important clinical implication of tumor angiogenesis is the development of anti-angiogenic therapy. The goal of this review is to critically evaluate the role of angiogenic markers, assessed by either endoglin-related microvessel density or expression of vascular endothelial growth factor family members in the CRC setting and discuss the role of these angiogenic markers in anti-angiogenic therapies.

Key words: Angiogenesis; Colorectal cancer; Colorectal cancer treatment; Endoglin; Prognosis; Vascular endothelial growth factor

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COLORECTAL CANCER EPIDEMIOLOGY

Colorectal cancer (CRC) is the third most common cancer and the fourth most frequent cause of cancer death worldwide[1-3]. Globally, CRC incidence varies widely, with higher rates in North America, Australia and Western Europe and lower rates in developing countries [4], although, in recent years, high CRC rates have also been reported in these countries[5]. In terms of mortality, geographic disparities have also been observed[6]. In Western countries, CRC is

the second most common cause of death from malignant disease, and despite improvements in treatment mortality remains high with metastatic spread to the liver occurring in about 50% of patients[7].

European countries rank highest in the global statistics, both in terms of CRC incidence and mortality. From 1998 to 2002, the incidence of CRC in Europe for men and women was 38.5 and 24.6 (world age standardization (ASR-W)) per 100000 inhabitants and mortality over the same period was 18.5 and 10.7 (ASR-W) per 100000 inhabitants, respectively[8]. However, over the past twenty-five years, mortality rates among Caucasians have steadily declined[9]. Data from the World Health Organization (WHO), between 1997 and 2007 have revealed that mortality from CRC declined by around 2% per year from 19.7 to 17.4/100000 for men (world standardized rates), and from 12.5 to 10.5/100000 for women, and these recent decreases in CRC mortality rates in several European countries are likely due to improvement in earlier diagnosis and treatment, with a consequent higher survival[10].

CRC incidence is generally higher in men, and the risk increases with age, as the majority of cases are diagnosed in patients older than 50 years[1, 3, 8], with only 5% of cases recorded in patients younger than 40 years[1]. A large nationwide study identified CRC as one of the 10 most commonly diagnosed cancers among men and women aged 20-49 years[11]. The prevalence of advanced CRC also increases with age and is higher among men than women[12].

COLORECTAL CANCER PROGNOSIS AND DISEASE PROGRESSION

The main prognostic factors in CRC are tumor size (T), lymph node involvement (N), grade of differentiation (G) and distant disease spread (M)[1-3,9,13,14]. Other important factors include invasion of blood and/or lymphatic vessels and penetration or perforation of the bowel wall[14].

Long-term survival correlates with stage of the disease[9, 15-17], and this is the most important predictor of mortality. The five-year survival rate for localized disease is 90.4%, but only 39% of CRC is diagnosed at this early stage[9, 16]. Approximately 15-20% of patients die as a consequence of CRC in early stages compared with 40-80% in advanced stages[15]. The overall 5-year survival rate varies among studies but is approximately 60%[9, 15, 16]. Stage-specific survival rates are 96%, 87%, 55%, and 5% for TNM stage Ⅰ, Ⅱ, Ⅲ, and Ⅳ, respectively[9,17,18].

One third of the patients submitted to curative intent surgery die of local and/or distant tumoral recurrence [15]. Among the sites of metastasis, liver is the organ most frequently involved (38%-60% of cases), followed by abdominal lymph nodes (38%), lung (38%) and peritoneum (28%)[14]. Of those diagnosed with metastatic disease, less than 10% are still alive after 5 years[16]. The 5-year overall survival rates for patients in whom hepatic resection was technically feasible and who had metastasis confined to the liver was only 25%-40%[7,19,20]. Better results were reported by Abdalla *et al* and Choti *et al*, with a 5-year overall survival rate of 58% following resection[21] and a rate of 67% described by de Haas *et al*[22]. These higher survival rates likely reflect improvements in patient selection, perioperative and postoperative care, multidisciplinary treatment, and an appropriately aggressive approach to safe hepatic resection[21]. Therefore, early diagnosis is critical to improve survival rates in CRC[23] and owing to its typically slow growth, there is a large potential for reducing the burden of the disease by early detection and removal of precancerous lesions or early cancer stages[24].

On the other hand, the pathologic clinical stage is currently the single most well-established prognostic indicator, but it does not fully predict individual clinical outcome[7, 25, 26]; also, the response of clinically-identical tumors to the same treatment may be vastly different[1]. This is particularly contentious for those tumors with intermediate stage disease (Stage Ⅱ, T3-T4N0M0)[7], where one third of patients with tumor-free lymph nodes have recurrences, and therefore, adjuvant chemotherapy may be beneficial[27]. In this group, carcinoma cells are not detected in lymph nodes by conventional staging methods in 24% of patients. Surgical technique and specific pathological staining may improve staging accuracy and the appropriate selection of patients for chemotherapy[27]. Furthermore, the identification of cancer penetration or perforation is particularly important in defining CRC aggressiveness[14]. Accordingly, identification of prognostic molecular markers capable of categorizing those patients at high-risk, would be very helpful for improving treatment strategies mainly in lymph node negative patients, determining the characteristics of patients' outcome, predicting cancer dissemination and recognizing which patients might benefit most from adjuvant chemotherapy and those unlikely to benefit thus sparing them the toxicities of treatment[14, 27-29].

Molecular markers may improve clinicopathologic staging and provide a basis to guide novel therapeutic strategies which target specific tumor-associated molecules according to individual tumor biology[1, 2, 7, 14], however, so far, no ideal molecular marker has been found to predict disease progression[29].

HIGHLIGHTS OF THE ANGIOGENESIS PHENOMENON

Angiogenesis plays a key role in tumorigenesis and metastatic processes[1, 28, 30]. It consists of the formation of new blood vessels from the endothelium of pre-existing vasculature[2, 30]. Sprouting from existing blood vessels is the principal process of angiogenesis and involves proliferation of activated endothelial cells, migration of endothelial cells to reach remote targets, assembly of endothelial cells into new capillary tubes, followed by synthesis of a new basement membrane and maturation of vessels with formation of a vascular lumen[30]. However, recruitment and *in situ* differentiation of bone marrow-derived endothelial

progenitor cells are also involved[30].

Tumor angiogenesis is essential to allow neoplastic mass development favoring access to the blood components, and also strengthening the vascular routes in the metastatic process[25, 31-33]. Neovascularization as a whole promotes tumor growth by supplying nutrients, oxygen and releasing growth factors that promote tumor cell proliferation[25, 30, 34-36]. Hypoxia in solid tumors occurs at a distance of ≥ 70 μm from functional blood vessels and it is generally accepted that tumors do not exceed a volume of 1-2 mm^3 without induction of angiogenesis[36]. Intratumoral vasculature density is believed to be associated directly with cancer cell entrance into the systemic blood circulation, with the ability of cancer cells to invade locally normal anatomic structures, and the establishment of blood-borne metastases in distant organs[32, 37]. Regulation of tumor angiogenesis is the result of a complex balance between many stimulatory and inhibitory factors, which are secreted by both tumor cells and host-infiltrating cells as well as by tumoral stroma-cells activity[2, 30, 34]. Malignant neoplastic cells promote angiogenesis by secreting growth factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF), among others that stimulate endothelial migration and proliferation[2,25,31,33,37,38].

The role of angiogenesis as a prognostic factor, however, is still controversial[13, 39]. Weidner *et al* first reported a direct correlation between the incidence of metastasis and the number and density of blood vessels in invasive breast cancers. Similar studies have endorsed this correlation in gastrointestinal cancers[33] and in a variety of malignancies[2, 7, 13, 25, 35, 37]. An association between increased angiogenesis and an increased incidence of metastases and a subsequent decrease in survival curve rates was observed for the vast majority of solid tumors[2, 7, 3, 25, 35, 37].

Several studies revealed high angiogenic activity in CRC, which was more likely correlated with aggressive histopathological features that included parietal invasion, tumor stage, grade of tumor differentiation, metastatic potential and poor patient survival[1, 13, 32]. Tanigawa[35] *et al* confirmed this premise, although a significant variation in patient populations and techniques was used, which can explain, in part, the inverse relationship between tumor vascularity and patient survival observed by these authors. Gurzu[13] *et al* added that augmented angiogenesis in CRC was higher in early-stages of tumoral proliferation but was not a progressively increasing process, having rather an oscillating character.

However, other studies revealed that angiogenesis does not provide any significant information[13, 28, 30]. These controversial statements may be credited to the lack of standardization of the different methods of counting tumoral blood vessels and to the different cut-offs used to define relevant parameters to consolidate the results and, lastly, to the different antibodies used to highlight the blood vasculature[13, 28, 30].

Despite the debates, assessment of tumor angiogenesis may be particularly useful in prognostic classification of patients with apparent early cancer by conventional tumor staging, some of which may still develop early recurrence or metastasis (despite being staged as having early cancers by conventional parameters such as tumor size)[30].

De Vita[37] *et al* observed that highly angiogenic tumors were associated with the presence of lymph node invasion . Nevertheless, a higher percentage of patients with node-positive colon cancer than those without will experience recurrence and might benefit from anti-angiogenic adjuvant therapy. Thus, angiogenesis can be used to identify a subset of patients at high risk for recurrence regardless of their lymph node involvement[35].

There is evidence that blood vessel density is also important in predicting cancer response to chemotherapy or radiotherapy[20]. Angiogenic tumors have a more aggressive phenotype and the degree of intra-tumoral microvessels is significantly predictive of poor response to platinum-based chemotherapy in terms of complete response, as seen in two studies, one in squamous cell carcinoma patients[40] and the other in patients with epithelial ovarian cancers[41]. In addition, Takagi[42] *et al* observed that blood vessel density was a valid predictor of the effects of intra-arterial targeted carboplatin chemotherapy and concurrent radiotherapy for treating human oral and oropharyngeal squamous cell carcinomas. Zhang[43] *et al*, trying to identify reliable predictive factors for local control of hypopharyngeal cancer (HPC) treated by radiotherapy, observed that microvessel density (MVD) in biopsy specimens was closely correlated with local control of HPC treated by radiotherapy. In one study of 28 patients with advanced gastric cancer treated by paclitaxel and carboplatin, tumors with medium MVD showed a significantly higher response rate compared with those with either a high or low MVD[44]. Long course of radiotherapy significantly decreased angiogenesis in rectal cancer tissue. MVD have been found to be a favorable marker for tumor behavior during radiotherapy and a predictor of overall survival after a long course of radiotherapy. Further investigations are now needed to determine the changes in angiogenesis during a shorter course of radiotherapy[1]. However, the most important clinical implication of tumor angiogenesis is probably the development of anti-angiogenic therapy, targeting tumor vessels instead of cancer cells[30].

ENDOGLIN AND ASSESSMENT OF MICROVESSEL DENSITY AS ANGIOGENIC MARKERS

Microvessel density (MVD) assessment is the most common technique used to quantify intratumoral and peritumoral angiogenesis in cancer[2, 7, 28, 30, 39]. It was first developed by Weidner *et al* in 1991 who used pan-endothelial immunohistochemical staining of blood microvessels, mainly with Factor VIII related antigen (F. VIII Ag or von Willebrand's factor), CD31 or CD34, and rarely CD105[2].

Measurement of angiogenesis is complicated by the

fact that it is a dynamic process. Intra-tumoral microvessels can be identified by immunostaining of endothelial cells by two categories of human endothelial cell-specific antibodies: the pan-endothelial cell markers and specific antibodies that bind selectively to proliferating endothelium[44, 45]. CD31 is utilized as the pan-endothelial marker of choice; it is characterized by equal intensity of staining for small and large vessels. The disadvantages associated with staining for CD31 antigen include co-staining of inflammatory cells. The selective antibodies, such as endoglin, distinguish quantitatively between tumor neovascularization and pre-existing vessels with no or poor staining of lymphatics and normal quiescent blood vessels[46]. Most studies revealed that high MVD predicts occurrence of metastatic disease[2, 7, 13, 25, 32, 35, 37], and although tumor angiogenesis is unlikely to be the only factor responsible, it provides large numbers of leaking blood vessels for vascular invasion[25].

Endoglin (CD105) is a receptor for the TGF-β1 molecule that is up-regulated in tumor angiogenesis [13, 25, 29]. Its secretion is induced by hypoxia[29] and, as it is present mainly in new vessels, it is very useful in the assessment of newly formed vessels in malignant neoplasms[13, 25, 29]. It is also currently accepted as a potential target for anti-angiogenic therapy, especially in cancer patients at risk of developing metastases[29]. The endoglin antibody binds preferentially to the activated endothelial cells that participate in tumor angiogenesis, however, endoglin expression is weak/or negative in vascular endothelium of normal tissues; accordingly, it is a more specific and sensitive marker of tumor angiogenesis than the others commonly used such as pan-endothelial markers[25, 29]. Intra-tumoral MVD determined by immunohistochemical staining for endoglin has been reported to be an indicator of poor prognosis in many types of solid neoplasia such as breast carcinoma, cervical cancer, endometrial carcinoma, gastric carcinoma, melanoma, some testicular tumors, non-small cell lung cancer, prostate cancer, renal cell carcinoma and squamous cell carcinoma[29].

In CRC, many reports indicate that endoglin assessed immunohistochemically correlates not only with MVD, but also with survival curves, and it has also been identified as a valuable parameter for predicting increased risk of developing metastatic disease[25, 29,42]. Yan[47] *et al* reported that MVD was higher in CRC patients with metastases than in those without and observed that the specificity and sensitivity of MVD in predicting metastatization in CRC was 66.22% and 51.72%, respectively. In other studies, the presence of endoglin also had a prognostic meaning, showing a positive correlation with the presence of angio-lymphatic invasion, lymph node metastases, tumor stage and hepatic metastases, reinforcing the premise that endoglin might be considered for further therapeutic trials as anti-angiogenic therapy[25, 29].

Endoglin is not only expressed on the cell surface but its soluble form can also be detected in the blood[29, 48]. Myśliwiec[29] *et al* demonstrated an apparent continuous endoglin rise in plasma from patients with metastatic colorectal cancer, and Li[48] *et al* reported that circulating endoglin levels positively correlated with CRC Dukes' stage and survival; patients with a high MVD, above the median 3.10 × 250, showed the worst prognosis. Takahashi[49] *et al* observed that increased serum endoglin was associated with metastasis in patients with solid tumors including colorectal and breast carcinomas; and, in CRC patients, the difference in endoglin levels between the metastasis-negative patients and the metastasis-positive patients was statistically significant. Conversely, it was recently demonstrated that assessment of endoglin in plasma is not a useful maker of CRC, but might be helpful in selecting patients with metastatic diseas[29].

VASCULAR ENDOTHELIAL GROWTH FACTOR FAMILY AND CRC

Quantification of angiogenic factors in solid malignant tumors provides an alternative to MVD evaluation in assessing tumor angiogenic activity[28, 30]. Numerous studies have demonstrated that tumor overexpression of vascular endothelial growth factor (VEGF) correlates with high tumor MVD and is associated with advanced tumor stage or tumor invasiveness in various common human cancers[30, 37, 50, 51] and, its overexpression in colon cancer tissue indicates poor prognosis[51]; although paradoxically, some data showed that MVD might have a significant prognostic value in colon cancer tissue, whilst VEGF has not[52].

VEGF is the most widely studied angiogenic factor; it increases vascular permeability and is the most potent, direct acting, angiogenic protein known[28, 29, 36, 37, 52]. Normally, VEGF is weakly expressed in a wide variety of human and animal tissues; however, high levels of VEGF expression can be detected at sites where physiologic angiogenesis is required, such as fetal tissue or placenta, or in the vast majority of human tumors and other diseases i.e., chronic inflammatory disorders, diabetes mellitus, and ischemic heart disease[37]. Furthermore, both VEGF and its receptors are expressed at high levels in metastatic human colon carcinomas and in tumor-associated endothelial cells, respectively[37]. Consequently, VEGF is recognized as a prominent angiogenic factor in colon carcinoma and the assessment of VEGF expression may be useful for predicting metastasis from CRC[37]. In fact, VEGF expression was found to be higher in patients with metastatic tumors than in those with non-metastatic tumors[37,38], and high levels of VEGF expression were associated with advanced cancer stage and related with unfavorable prognosis[51-53].

De Vita *et al* [37] reported that preoperative serum VEGF levels might be useful for predicting the outcome of colon cancer patients following surgery. After surgery, VEGF levels tend to decrease compared with preoperative concentrations[30, 37]. Conversely, elevated VEGF levels after surgery may indicate significant residual disease, even

if it is not evident macroscopically[37].

Other studies have shown that VEGF is also a useful marker for prognosis by significantly correlating with angio-lymphatic invasion, lymph node status and depth of invasion, notwithstanding it was not an independent prognostic factor[25, 29].

Although numerous publications dealing with the measurement of circulating VEGF for diagnostic and therapeutic monitoring have been published, the relationship between the production of tissue VEGF and its concentration in blood is still unclear[31]. Some of the controversies regarding the clinical value of VEGF serum level measurement are related to the well-known fact that circulating VEGF is largely found in platelets, and as a consequence an open debate is ongoing to clarify if VEGF serum levels truly reflect tumor expression of VEGF or whether there are other potential sources of circulating VEGF, such as blood cells[30]. Cressey[31] *et al* noted that the cell-associated isoform (VEGF189), but not the soluble isoforms (VEGF121 and VEGF165) appear to play an important role in tumor progression. In addition, Serum VEGF protein levels are a prognostic parameter for progression-free and overall survival in CRC. Patients with high soluble VEGF levels might have a more aggressive disease, and the improved outcome observed in their series might be a reflection of the disease biology[54,55].

The effect of VEGF depends not only on tumor cell expression of VEGF, but also on the VEGF receptors in the endothelial cells[30]. The ligands of the VEGF family include VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-E; and the receptors are VEGFR-1, R-2 and R-3[56].

VEGF-A is commonly overexpressed by a wide variety of human tumors, and this overexpression has been correlated with progression, invasion and metastasis, MVD, and poorer survival and prognosis[56]. In CRC, VEGF-A is the ligand of the VEGF family most abundantly expressed[29]. VEGF-A promotes angiogenesis through enhancement of permeability, activation, survival, migration, invasion, and proliferation of endothelial cells[57]. VEGF-A and VEGF-B play a role in early tumor development at the stage of adenoma formation[7, 58].

Myśliwiec[29] *et al* found a strong positive association with VEGF-A plasma concentrations assessed postoperatively and the presence of distant metastases. Zlobec[59] *et al* also correlated high VEGF expression with response to preoperative radiotherapy in patients with rectal tumors.

VEGF-C and -D are glycoproteins structurally similar and sharing areas of sequence homology with VEGF-A. In CRC, augmented VEGF-C expression has been found to correlate with lymphatic invasion and lymph node metastasis[60]. Elevated levels of serum VEGF-C have been found in patients with breast cancer, lung cancer and cervical cancer and it appears to be an independent marker for early diagnosis of cancer metastasis. Moreover, increased VEGF-C mRNA expression in tumor tissues correlates positively with lymphatic metastasis and poor prognosis[61]. A correlation between VEGF-D expression levels in the primary tumor and lymph node metastasis is still disputable, with controversial data reported[62].

Another important fact is that through the development of anti-angiogenic therapy, CRC prognosis is improving[30, 63-65]. Median survival of patients with metastatic CRC (mCRC) treated with best supportive care is approximately 6 mo. Palliative chemotherapy considerably improves treatment outcome, with fluorouracil (FU) plus irinotecan and/or oxaliplatin extending median overall survival to approximately 20 mo[66]. Thus, in the past decade, the median overall survival of patients with mCRC has increased from 12 mo to approximately 20 mo, mainly due to the development of new combinations with standard chemotherapy[67]. Currently, anti-angiogenic treatment can prolong the survival time by some months, however, the results are not reproducible for all cases[13]. There have been clinical trials which show as many as 94% of invasive carcinomas and 88% of *in situ* carcinomas having a complete response[68]. Unfortunately, there are no tumor characteristics or molecular markers at present that help to identify patients who are likely to benefit from anti-angiogenic treatment[69].

Bevacizumab (BV) is a monoclonal antibody against VEGF with anti-angiogenic properties, and several clinical trials supported the use of BV in the first-line treatment of mCRC[70]. BV is typically used in combination with other chemotherapeutic agents such as oxaliplatin, irinotecan, leucovorin, and 5-fluorouracil (5-FU) for treatment of patients with mCRC[70, 71]. In addition to its direct anti-angiogenic effects, BV may also improve the delivery of chemotherapy by changing tumor vasculature and decreasing the elevated interstitial pressure in tumors[69]. When combined with standard chemotherapy regimens, it has been associated with significant improvements, compared with chemotherapy alone, in the efficacy end points of overall survival, progression-free survival, and response rates in patients with mCRC and for some facilitates secondary resections[72]. Jubb[73] *et al* demonstrated that in patients with mCRC, the addition of BV to irinotecan, 5-FU/leucovorin (IFL) improves survival regardless of the level of VEGF expression, or MVD. In a review by Tappenden[74] *et al*, the addition of BV to IFL resulted in a statistically significant increase in median overall survival (OS) of 4.7 mo, and in a median progression-free survival (PFS) of 4.4 mo. An overall tumor response rate of 44.8% was reported for BV plus IFL compared with 34.8% for IFL plus placebo within one study. In a pivotal, placebo-controlled, phase III trial in patients with mCRC (Genentech Study 2107), the addition of BV to IFL resulted in a significantly longer survival time (20.3 *vs* 15.6 mo) and progression-free survival time (10.6 *vs* 6.2 mo) than with IFL plus placebo[73, 75-78]. In a placebo-controlled, phase II trial (Genentech Study 2192), adding BV to 5-FU plus LV resulted in a significantly longer progression-free survival time than with 5-FU and LV plus placebo in

Table 1 The main results of CD105 and VEGF studies

Study	*n*	High levels of CD105 were associated with	High levels of VEGF were associated with
Barozzi *et al*[7]	101	M1	M1
Saad *et al*[25]	150	M1, N1 and angiolymphatic invasion	N1, angiolymphatic and depth of invasion
De Vita *et al*[37]	81	NE	NCs (serum levels)
Cascinu *et al*[38]	121	NE	RR
Myśliwiec *et al*[29]	48	M1	Colorectal cancer patients (plasma levels)
Li *et al*[48]	111	Dukes' stages and survival	NE
Takahashi *et al*[49]	34	M1	NE
Liang *et al*[51]	114	NE	N1, TNM staging and poor prognosis
Zheng *et al*[52]	97	NE	Poorly differentiated adenocarcinoma
Cressey *et al*[31]	76	NE	TNM
Cao *et al*[53]	71	NE	N1, M1, TNM, and OS
Miyazaki *et al*[58]	127	NE	RR, DF, OS (plasma levels)

DF: Disease-free; M1: Positive distant metastasis; N1: Positive lymph node metastasis; NCs: Non-curative surgery; NE: Not evaluated; OS: Overall survival; RR: Recurrence rate

patients with mCRC who were unsuitable candidates for first-line therapy with irinotecan (9.2 *vs* 5.5 mo). There was also a trend towards a longer survival time in patients receiving 5-FU, LV, and BV (16.6 *vs* 12.9 mo)[77]. BV was also tested in mCRC combined with an oxaliplatin-based regimen in the second-line setting. In this randomized phase III trial (E3200), patients with previously treated CRC were randomized into 3 arms: FOLFOX4 plus BV, FOLFOX4 and BV only. Results showed superior survival and progression-free survival in the FOLFOX4 plus BV arm. In this study, BV was equally effective with the oxaliplatin-based regimen[78].

BV ultimately achieved FDA approval in 2004 as a first-line treatment for mCRC in combination with chemotherapy, based on its statistically and clinically meaningful benefits on progression-free survival and OS and has since garnered additional approval[79]. BV is the most used VEGF inhibitor with clear proof of efficacy in CRC, however, optimal use of this agent at various stages of the disease is still under investigation. Additionally, there are numerous other angiogenic agents targeting VEGF and other pro-angiogenic systems in clinical development[80]. These novel targeted agents inhibit the VEGF pathway by targeting the VEGF ligand, its receptors or by blocking downstream signaling pathway components. Anti-angiogenic agents include antibodies, small molecule tyrosine kinase (TK) inhibitors, antisense oligonucleotides and aptamers[81].

Table 1 summarized the main results of CD105 and VEGF studies.

CONCLUSION

Despite major advances, in terms of knowledge and treatment of CRC in recent years, the single most well-documented prognostic marker of pathologic stage remains the gold standard for disease stage at diagnosis. Angiogenesis plays an important role in the growth and progression of cancer but its role as a prognostic factor is still controversial. Most studies report that endoglin and vascular endothelial growth factor family expression are indicators of poor prognosis in CRC patients. Beyond these controversies, the ultimate clinical implication of tumor angiogenesis is the development of anti-angiogenic therapy, targeting tumor vasculature.

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