University of Trás-os-Montes and Alto Douro

Study of Inflammation in Colorectal Cancer

Master's Dissertation in Biotechnology for Health Sciences

Beatriz Alves Cardoso

Supervisors: Estela Maria Bastos Martins de Almeida, Ph.D.

Maria dos Anjos Clemente Pires, Ph.D.

Vila Real, 2019

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It is declared under an honour commitment that this work was expressly prepared by the author, as an original dissertation, for the purpose of obtaining a master's degree in Biotechnology for Health sciences, at University of Trás-os-Montes and Alto Douro. All non-original contributions were properly identified with indication of the source.

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Resumo

Globalmente, tanto em países economicamente desenvolvidos como em desenvolvimento, o cancro tornou-se um dos maiores problemas da sociedade hoje em dia. O Cancro colorretal é dos mais incidentes e frequentes na Europa.

A proteína inibitória de macrófagos (MIF) é uma citocina envolvida na imunidade célular, imunorregulação e inflamação. É capaz de desencadear respostas imunes significativas autócrina e/ou parácrinamente através da indução de citocinas pró-inflamatórias. Como mediador pró-inflamatório, o MIF tem sido demonstrado como estando envolvido no cancro. O gene *MIF* possui um SNP putativamente funcional, transversão de G>C na região flanqueadora do gene (rs755622), que tem sido reportado como associado ao cancro colorretal (CCR) e outras doenças.

Primeiramente, extraímos DNA de 172 amostras de sangue usando buffy coat. Posteriormente, procedeu-se à genotipagem das amostras extraídas utilizando-se a técnica de PCR em tempo real, com recurso a sondas Taqman, otimizadas para este SNP. Seguiu-se depois para sequenciação utilizando a técnica de Sanger para validação dos resultados. Obtivemos as seguintes frequências: GG 70%; GC 28%; CC 2%; consistente com frequências alélicas de 84% G e 16% C (similares às frequências alélicas presente na base de dados Ensembl: 81% -G; 19% -C). Após análise estatística entre o polimorfismo do MIF e os dados clínico-patológicos, não foi encontrada correlação significativa. Relativamente à técnica de imunohistoquímica também foi realizada em 32 amostras, não apenas para caracterizar a proteína MIF que foi o ponto focal, mas também na tentativa de estabelecer uma comparação entre MIF, macrófagos e a infiltração linfocitária em tumores utilizamos um anticorpo para a proteína MIF, outro para CD68 (receptor de macrófagos) e outro para CD3 (co-receptor de células T). Análises de genótipo-fenótipo foram realizadas entre TAMs, TILs e os genótipos MIF -173 G> C onde nenhuma significância foi encontrada. Em relação à intensidade e percentagem de células coradas com MIF, foram então correlacionados com os genótipos MIF -173 G> C,onde se encontrou significância na variável modelo aditivo quando referente à intensidade de coloração.

O principal objetivo foi correlacionar todos os dados clinicopatológicos, genéticos e imunohistoquímicos, a fim de obter dados / conclusões que possam vir a ser úteis para a prática clínica e melhorar o tratamento e a sobrevida do paciente no futuro.

Palavras-chave: MIF; colorretal; SNP; genotipagem; Imunohistoquímica

Abstract

Worldwide, in both economically developed and developing countries, cancer has become one of the biggest problems in society and colorectal cancer is the second most incident and most frequent cause of death in Europe.

MIF (macrophage inhibitory factor) protein is a cytokine involved in cell-mediated immunity, immunoregulation and inflammation. It is capable of triggering significant immune responses through autocrine/paracrine loops via the induction of pro-inflammatory cytokines. As a pro-inflammatory mediator, MIF has been implicated in cancer. *MIF* gene has a putatively functional SNP, G-to-C transversion at the 5'-flanking region (rs755622), which has been reported to be associated with Colorectal Cancer (CRC) and other diseases.

Firstly, DNA was extracted from 172 blood samples using its buffy coat. Thereafter we proceeded to the genotyping of the extracted samples using real time PCR technique using *Taqman* probes optimized for this SNP, followed by Sanger sequencing for result confirmation. We obtained the following frequencies: GG 70*%;* GC 28%; CC 2%; consistent with an 84% G and 16% C allelic frequencies (similar to Ensembl database allelic frequencies: 81%-G; 19%-C). After statistical analysis between clinicopathological parameters and *MIF* polymorphism, no significant correlation was found.

Immunohistochemistry technique using an antibody for MIF, CD68 (macrophage receptor) and for CD3 (T cell co-receptor) was also optimized in 32 case samples, not only to characterize the MIF protein, which was the focal point, but also in an attempt to establish a comparison between the MIF and the macrophage and lymphocyte infiltration in tumors. Genotype-phenotype analyses was performed between TAMs, TILs and *MIF* -173 G>C genotypes using Mann-Whitney U tests, and no significance was found. Concerning intensity and percentage of cells stained with *MIF* were then correlated with *MIF* -173 G>C genotypes using Fisher's exact and Pearson chi-square test. It was found significance in the additive and recessive model variables.

We aimed to establish correlations between all clinicopathological, genetic and immunohistochemistry data in order to draw data/conclusions that could possibly be useful for clinical practice and improve patient treatment and survival in the future.

Keywords: MIF; Colorectal cancer; SNP; Genotyping; Immunohistochemistry

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List of Abbreviations

APC - Adenomatous polyposis coli gene

CALD – Caldesmon

CAF – Cancer associated fibroblasts

CCL – C-C motif chemokine

CIMP – CpG island methylation phenotype

CIN – Chromosomal instability

CMS – Consensus molecular subtypes

COX-1 – Ciclooxygenase 1

CT – Centre of tumor

CTLA – Cytotoxic T-lymphocyte-associated protein

CRC – Colorectal cancer

CXCL – C-X-C motif ligand

DC – Dendritic cells

DCC – Deleted in Colorectal Cancer

DRE – Digital rectal examination

EGFR – Epidermal growth factor receptor

ERCC-1 – Excision repair cross-complementing 1

FAP - Familial adenomatous polyposis

FAP – Fibroblast activation protein

FOBT – Fecal occult blood test

HNPCC - Hereditary nonpolyposis colorectal cancer

IBD – Inflammatory bowel disease

IFN – Interferon

IGFBP – Insulin-like growth factor binding protein

IHC - Immunohistochemistry

IL – Interleukin

IM – Invasive margin

IMP3 – insulin-like growth factor II mRNA-binding protein 3

LGALS – Lectins, galactoside-binding

LOH – Loss of heterozygosity

LPS- Lipopolysaccharide

MAPK – Mitogen-activated protein kinase

MC – Mast cells

MCC – Mutated in colorectal cancer

MDSC - Myeloid-derived suppressor cells

MHC – Major histocompatibility complex

MIF – Macrophage inhibitory factor

MLH – MutL homolog 1

MMP - Matrix metalloproteinase

MMR – Mismatch repair genes

MSI-H – Microsatellite instability - high

MSI-L/EMAST - microsatellite instability-low/elevated microsatellite alterations at selected tetranucleotide

NK – Natural killer cells

OS – Overall survival

PCR – Polymerase chain reaction

PD-L – programmed death ligand

PDGF – Platelet-derived growth factor

PFS – Progression-free survival

PIK3 – Phosphoinositide-3-kinase

PKM2 – Pyruvate kinase M2

PLA – Phospholipase A

POSTN – Periostin gene

PTEN – Phosphate and tensin homolog

RAF – Rapidly accelerated fibrosarcoma kinase

ROR – RAR-related orphan receptor

SAA – Serum amyloid A

SNP – single nucleotide polymorphism

TAM – Tumor associated macrophage

TCR – T-cell receptor

TGF-β – Transforming growth factor β

TILs – Tumor-infiltrating lymphocytes

Tregs - Regulatory T-cells

VEGF - Vascular endothelial growth factor

1. Introduction

1.1. Colorectal cancer: Clinical and Pathological Landscape

Cancer refers to a group of diseases involving abnormal and uncontrolled cell growth infiltrating the surrounding tissue, with the potential to spread and colonize other organs (metastasis) (WHO, 2017). In order to establish an organizing principle for rationalizing the complexities of neoplastic diseases, Hanahan D & Weinberg R (2000) initially proposed six hallmarks of cancer, which included sustainable proliferative signalling, evasion of growth suppressors, cell death resistance, replicative immortality, induced angiogenesis, and activation of invasion and metastasis. Later, conceptual progress led to an updated paper on cancer hallmarks that included plus 4 emerging hallmarks: genome instability , inflammation that fosters multiple hallmark functions, reprogramming of energy metabolism and the capacity of evading immune destruction (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011). In addition to cancer cells' phenotype, tumors exhibit another dimension of complexity, once they contain a repertoire of recruited, seemingly normal cells that contribute to the acquisition of hallmark traits by creating the tumor microenvironment (Hanahan & Weinberg, 2011).

Colorectal cancer (CRC) is the second most commonly diagnosed cancer in Europe and a leading cause of death worldwide (Van Cutsem *et al*., 2016; Ferlay *et al*., 2018). CRC usually develops in the lining of the colon or rectum and many studies support that most tumors of the colon start as polyps (a benign or pre-malignant form), which might end up as cancer via multistep process involving an accumulation of successive genetic alterations (Das *et al*., 2017; Danese & Montagnana, 2017).

CRC can be generally classified as hereditary or sporadic (non-hereditary) forms. Hereditary CRC are stratified into two sub-groups: hereditary non-polyposis colorectal cancer (HNPCC), encompassing 1-6% of colorectal cancers, and multiple polyposis CRC, which includes familial adenomatous polyposis (FAP), hamartomatous polyposis syndrome and adenomatous polyposis. Each of the above mentioned CRC subtypes involve different genetic causes (Tsang *et al.*, 2014).

1.1.1. Epidemiology

Epidemiological studies proposed several risk factors and causes for developing colorectal cancer and as prognosis variables (Das *et al*., 2017; CDC, 2018), which are summarised in table 1.

Table 1: Risk factors and causes/ethiopathogenesis of colorectal cancer.

Risk factors	Description
1. Diet	Intake of some food items and nutrients is associated with the risk of colorectal neoplasia. The mechanisms seems to involve the direct effects on immune responsiveness and inflammation, and the indirect action of over-nutrition and obesity (Goldbohm *et al*., 1994). Feeding in high cholesterol food, saturated fats, and red and processed meat has been found to be associated with increased risk for CRC (Järvinen *et al*., 2001; Van Blarigan & Meyerhardt, 2015).
2. Age	Cancer incidence diverges significantly among different age groups. Apart from the hereditary colorectal cancer, the chance of developing this malignancy increases after the age of 40 and gradually rises after age of 50. It has been reported that a much higher prevalence rate is seen at age 60-79 years compared to younger ages (Amersi *et al*., 2005).
3. Smoking	Outcomes from the study of colorectal cancer survivors performed by Cosnes *et al.* (1996) and many others, suggested that smoking is associated with a much higher risk of death compared with non-smokers (Ordóñez-Mena *et al*., 2017).
4. Family history	Familial adenomatous polyposis (FAP) is one of the inherited genetic disorders that enhances the development of several polyps in the colon from a very young age. According to Heavy *et al.* (2004), hereditary nonpolyposis colorectal cancer (HNPCC) seems to accelerate the carcinogenic process through an increased mutation rate in microsatellite regions, which affects other genes involved in cell cycling and proliferation (Weigl *et al*., 2016).
5. Inflammatory bowel disease (IBD)	Ulcerative colitis drives inflammation of the mucosa of the colon and rectum. Crohn's disease enhances the inflammation of the full thickness of the bowel wall and may lead it towards any part of the digestive tract. Overall these conditions increase an individual's risk of developing colorectal cancer (Weitz *et al*., 2005).

Causes/Ethiopathogenesis	Description
1. Chromosomal instability	Colorectal cancers arise primarily through chromosomal instability which is characterized by imbalances in chromosome number, loss of heterozygosity and the promotion of the physical loss of a wild type copy of a tumor-suppressor gene like *APC*, *TP53* and *SMAD* family member 4 (Lengauer *et al.*, 1997; Orsetti *et al*., 2014).
2. Oncogenic mutations of RAS and BRAF genes	Oncogenic mutations are the most common causes to promote CRC carcinogenesis. *RAS* mutations can lead to the activation of Raf proteins (Hanahan & Weinberg, 2000). B-Raf is a component of MAPK signalling cascade that culminates with activation of several transcription factors important for cell survival, proliferation and metastasis (Andreyev *et al*., 2001; Lorentzen *et al*., 2017).
3. Adenomatous polyposis coli (APC) gene mutation	The adenomatous polyposis coli (*APC*) gene is a tumor suppressor that is mutated in most sporadic colon adenocarcinomas. These mutations promote the activity of other oncogenes such as Wnt and Ras mediated signalling pathways (Zhang & Shay, 2017).
4. Deleted in colorectal carcinoma* (DCC) gene mutation	*DCC* is a tumor suppressor gene, and one of the most frequent genetic abnormalities that occur in advanced colorectal cancers is loss of heterozygosity (LOH) of *DCC* (Shibata et al., 1996; Armaghany *et al*., 2012).

*HGNC approved name and symbol: DCC netrin 1 receptor

1.1.2. Staging

Staging of the disease provides a basis for stratification and analysis of treatment results in future studies and provides prognostic information (Fielding *et al*., 1991). The earliest stage of colorectal cancer is called I (a very early, localized cancer), and then progresses through stage IV (the most severe). Within each stage, an earlier letter means lower aggressiveness. Although each tumor is unique, cancers with similar stages tend to have a similar outlook and are often treated in a similar way (American cancer society, 2018; CDC, 2018; National cancer institute, 2018).

1.2. Genetic and Epigenetic factors

CRC is believed to be caused by a cascade of genetic mutations. According to Tsang *et al.* (2014), the molecular basis of CRC is genomic instability that facilitate the acquirement of tumor-associated mutations. Several forms of genomic instability have been identified, including chromosomal instability (CIN), microsatellite instability (MSI), and epigenetic gene silencing.

The classical model for the carcinogenesis of CRC, proposed by Fearon *et al*. (1990) can be described as the adenoma-carcinoma sequence. The adenoma-carcinoma sequence describes a gradual progression from normal epithelial mucosa to adenoma and subsequently to carcinoma as a result of a series of genetic changes such as mutation and gene amplification as shown in figure 1.

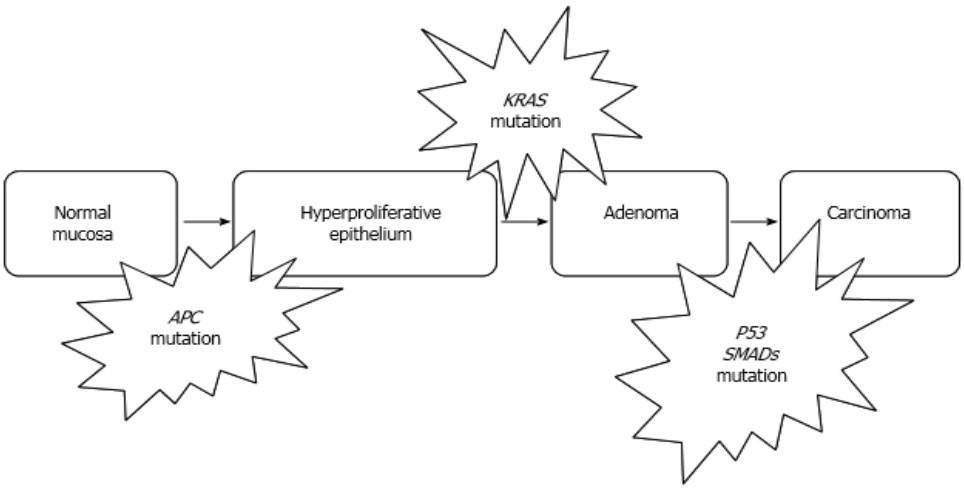


Figure 1: Adenoma-carcinoma sequence in colorectal cancer formation. This is a simplified presentation of colorectal cancer tumorigenesis. The true carcinogenesis progress of colorectal cancer is much more complex (Tsang *et al.*, 2014).

Tumorigenesis proceeds through a series of genetic alterations involving oncogenes, tumour suppressor genes and also system repair genes. In patients with familial adenomatous polyposis (FAP), a mutation on 5q region is inherited. This alteration may be responsible for the hyperproliferative epithelium present in these patients. In patients without polyposis the same region may also be lost and/or mutated at a relatively early stage of tumorigenesis. Hypomethylation of the 5q region is present in very small adenomas in patients with or without polyposis, and this alteration may lead to aneuploidy, resulting in the loss of suppressor gene alleles. RAS gene mutation appears to occur in one cell of a pre-existing small adenoma and through clonal expansion produces a larger and more dysplastic tumour (Fearon & Vogelstein, 1990).

The most frequently deletions occur in the chromosomes 5q; 17p (region where P53 suppressor gene resides), and 18q. Allelic deletions of 17p and 18q regions have been reported to be associated with an increased tendency of disease dissemination in CRC and usually occur at a later stage of tumorigenesis than do deletions of chromosome 5q or RAS gene mutations. However, the order of these changes is not invariant, and accumulation of these alterations, rather than their order seem to be most important. Tumors continue to progress once carcinomas are formed, and the accumulated loss of suppressor genes on additional chromosomes correlates with the ability of metastization and death (Fearon & Vogelstein, 1990; Cardoso *et al*., 2007).

More recently, an alternative mechanism has been proposed, named the serrated pathway (Snover, 2011), since different genetic alterations were detected, such as initial mutational and methylation burden B-Raf proto-oncogene, serine/threonine kinase (*BRAF*) gene mutation associated with high levels of methylation (Leggett and Whitehall, 2010). Alternatively, a third pathway has been reported to occur in gut-associated lymphoid tissue (GALT), a lymphoid tissue responsible for eliminating invasive agents in the gastrointestinal tract. This lymphoid tissue recognizes antigens, microorganisms and macro-molecules arising from the lumen, presenting them to immune cells, therefore having a rapid response (Rubio *et al*., 2018)

It is well established that both genetic and epigenetic events are key players in initiation and progression of CRC. Epigenetics are heritable alterations in gene expression that do not involve changes in the primary DNA sequence (Baretti & Azad, 2018). They occur widely across the genome and constitute an important cause of tumor heterogeneity. Nowadays, multiple ways for epigenetic modification are known to play pivotal role in CRC including, histone modifications, DNA methylation, chromatin remodelers and non-coding RNAs,

summarized in figure 2 (Pancione *et al*., 2012; Das *et al*., 2017; Danese & Montagnana, 2017).

Tsang *et al.* (2014) stated that the epigenetic silencing of genes is mostly caused by DNA methylation. Cancers with high degrees of methylation can be considered as CpG island methylation phenotype (CIMP) positive, and CIMP encompasses 35%-40% of sporadic CRC. DNA methylation is involved in normal cellular control of gene expression. A vast majority of methylated cytosines in the human genome are found in the CpG dinucleotide sequences. In normal cells, dense regions of CpG sequences (CpG Island) are usually found in the regions close to promoters. The methylation patterns of these CpG sequences are gene-specific. Aberrant CpG hypermethylation can lead to silencing of tumor-suppressor genes in carcinogenesis since the expression of the genes is repressed. In some cases, the presence of epigenetic silencing is overlapped with MSI. Some sporadic CRC with microsatellite instability is caused by DNA methylation. For example, DNA methylation of MLH1 gene promoter blocks its expression and destroys the ability of MMR system (Tsang *et al.*, 2014).

One of the epigenetic mechanisms are chromatin remodelers, multi-protein complexes that use the energy of ATP hydrolysis in order to slide, exchange or remove nucleosomes (Carlberg & Molnár, 2018) and can be grouped into four families:

- SWI/SNF (switching-defective/sucrose-non fermenting) this family functions in the sliding and eviction of nucleosomes.
- ISWI (Imitation-switch) operates in nucleosome assembly and spacing and is also proposed to have functions in higher levels of chromatin organization.
- CHD (chromodomain helicase DNA binding protein) remodelers are associated with nucleosome sliding, eviction, spacing and nucleosome assembly.
- INO80 (inositol requiring 80) family is specialized in restructuring the nucleosome by replacement of H2A-H2B dimers with dimers containing the histone variant H2A.Z.

Based in the current data, chromatin remodelers function via a mechanism called the hourglass model, where the four families of remodelers have a unifying remodelling mechanism but each family generate different outcomes (Clapier *et al*., 2017).

In short, chromatin remodelers may affect promoter and enhancer regions either more or less accessible to the transcriptional apparatus, thus allowing transcription factors to activate or suppress the transcription of their target genes (Clapier *et al*., 2017).

Another mechanism is histone modifications, which coordinate DNA accessibility through methylation, acetylation, phosphorylation, ubiquitination, deamination, sumoylation, ADP ribosylation, and proline isomerization of specific core histone residues (H2A, H2B, H3, and H4). Hundreds of histone modifications have already been identified and studied and the number keeps on growing (Zhao & Garcia, 2015). Post-translational modifications of histones are involved in balancing the states of chromatin between the active (euchromatin) and inactive (heterochromatin). Apart from their contribution in various physiological processes, histone modifications are responsible for the aberrant genetic and epigenetic profiles, which occur in numerous types of solid and hematological malignancies (Vaiopoulos *et al.*, 2014).

Non-coding RNAs are also part of a complementary mechanism of gene expression control in which they act by both regulating epigenetic alterations and being regulated by them. Non-coding RNAs can be divided into two subgroups, long or short noncoding RNAs. The subgroup of short endogenous noncoding RNAs (≈22 nucleotides) post transcriptionally downregulates gene expression through binding to a complementary site of target mRNAs. Upon binding, miRNAs incorporated into an RNA-induced silencing complex induce cleavage or translational repression of target-mRNAs. It also has been shown that miRNA behavioral pattern can sometimes be modulated through tumor-derived microenvironment stimuli. Aberrations of miRNA expression are often observed in CRC and seem to play a significant role in tumor development and progression (Vaiopoulos *et al.*, 2014).

Consequently, epigenetic alterations represent an attractive target either for epidemiological and physiopathological studies or for therapeutic response evaluation and drug design (Pancione *et al*., 2012; Das *et al*., 2017; Danese & Montagnana, 2017).

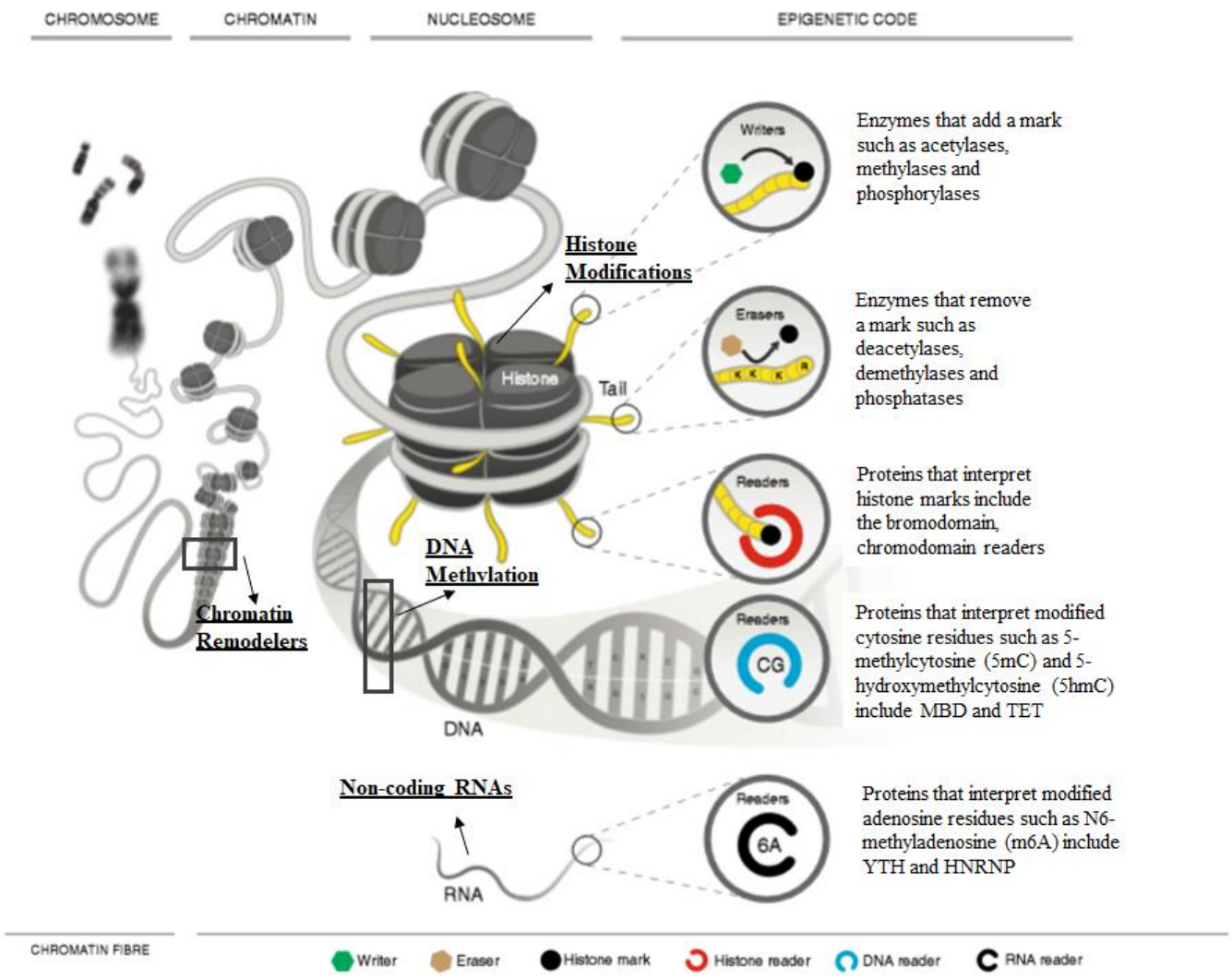


Figure 2: Summary scheme of all epigenetic mechanisms and respective action site, adapted from: (Keating *et al.*, 2018).

1.3. Screening and diagnosis

As an initial step, the patient is asked about their family history of bowel cancer and of symptoms in detail. Presence of local symptoms around the anus, for example may indicate rectal cancer. This is followed by a physical examination known as a digital rectal examination (DRE). In a DRE the physician gently places a gloved and lubricated tip of the finger into the anus and then up the rectum. This examination checks for lumps in the rectum or anus. The next step is a sigmoidoscopy which involves insertion of a thin flexible tube with a camera on its tip for viewing the inside walls of the lower part of the large intestine on the monitor. A sigmoidoscopy can also be used to remove small samples of suspected tissue known as a biopsy. A total colonoscopy is the most complete exam that allows the visualization of the whole rectum and colon. This examination also allows the physician to do a biopsy, if needed. Routine examination by a test of faecal occult blood (FOBT) is done when colorectal cancer is suspected. However, an effective and accurate diagnosis is only possible by the histopathology analysis of polyps or lesions collected during the colonoscopy or sigmoidoscopy (Institute CUF of oncology, 2018).

Once the diagnosis is confirmed, further testing is usually carried out either to check the possible spread of the cancer to other parts of the body or to stage the cancer to determine appropriate therapy (Peluso *et al*., 2017; News Medical, 2018).

Over the last few decades various molecular biomarkers for CRC have been studied regardless the fact that several screening strategies for CRC detection already exist. These screening strategies can be classified as non-invasive and/or invasive imaging techniques. Nevertheless, recent studies have highlighted that these screening strategies lack proper sensitivity and specificity. Subsequently, enormous research efforts have been made in order to identify molecular biomarkers based on DNA, RNA or protein, in order to develop novel, non-invasive detection methods and treatment of colorectal cancer (Das *et al*., 2017; Van Cutsem *et al*., 2016; Lin *et al*., 2016).

These markers can be divided into three different groups: diagnostic, predictive, and prognostic, summarized in table 2. Diagnostic markers permit an early diagnosis and risk stratification (Peluso *et al.*, 2017).

Table 2: Molecular biomarkers for CRC (Peluso *et al.*, 2017).

Diagnostic	Predictive	Prognostic
• MSI (microsatellite instability) • IGFBP2 (Insulin-Like Growth Factor Binding Protein 2) • Telomerase • PKM2 (pyrovate kinase M2)	• KRAS • BRAF • PIK3CA (Phosphoinositide-3-kinase) • PTEN (phosphatase and tensin homolog) • ERCC-1 (Excision repair cross-complementing-1) • Ezrin	• APC • p53 • VEGF (vascular endothelial growth factor) • EGFR (epidermal growth factor receptor) • 18q Loss of heterozygosity (LOH) • SMAD4 • MCC protein (mutated in colorectal cancer) • IMP3 (Insulin-Like Growth Factor II mRNA-Binding Protein 3)

Predictive biomarkers are useful for forecasting the patient's response to the therapy and so patients can be selected to undergo a particular treatment on the basis of an expected positive response. They can even be used to identify the right drug dose and to prevent its toxicity.

Prognostic biomarkers allow estimating the natural course of the disease and dividing tumors in two groups: the ones with a good outcome and the ones with a bad outcome (Peluso *et al.*, 2017). These can be molecules involved in different process, such as cellular proliferation, differentiation, angiogenesis, invasion, and metastasis (Peluso *et al.*, 2017; Tsang *et al.*, 2014).

1.4. Treatment

There are several ways to treat colorectal cancer, depending on its type and stage. Some treatments are called local therapies, meaning they treat the tumor without affecting the rest of the body. These types of therapies include: surgery; radiation therapy; ablation or embolization. Local therapies are more likely to be useful for earlier stage (less advanced) cancers, although they might also be used in other situations (American cancer society, 2018; National cancer institute, 2018).

Besides this, there are systemic treatments which use drugs to treat colorectal cancer. These drugs can be given by mouth or directly into the bloodstream. These systemic therapies can reach cancer cells anywhere in the body. Depending on the type of colorectal cancer, several different types of systemic therapies might be used, including: chemotherapy; targeted therapy or even immunotherapy (American cancer society, 2018; National cancer institute, 2018).

Depending on the stage of the cancer and other factors, different types of treatment may be combined at the same time or used after one another (American cancer society, 2018; National cancer institute, 2018).

Presently, treatments focused on altering the immune system have recently made their way broadly into clinical oncology practice, based upon the successes seen with immune checkpoint inhibitors. An important part of the immune system is its ability to keep itself from attacking normal cells in the body. To do this, it uses "checkpoint" proteins on immune cells, which act like switches needing to be turned on (or off) to start an immune response. Cancer cells sometimes use these checkpoints to avoid being attacked by the immune system. But drugs that target these checkpoints hold a lot of promise as cancer treatments (Bolan & Wen Wee Ma, 2017; American cancer society, 2018).

Pembrolizumab (Keytruda) and *Nivolumab (Opdivo)* are drugs that target PD-1, a protein on T cells that normally helps keep these cells from attacking other cells in the body. By blocking PD-1, these drugs boost the immune response against cancer cells and can diminished some tumors or slow their growth. These drugs can be used in people whose colorectal cancer cells have tested positive for specific gene changes, such as a high level of microsatellite instability (MSI-H), or changes in one of the mismatch repair (MMR) genes (Bolan & Wen Wee Ma, 2017; American cancer society, 2018; Smyth *et al*., 2015).

Emerging data demonstrates that subsets of patients, those with colorectal cancers with hypermutated genes, may benefit from immune checkpoint inhibitors. In addition, combinatorial approaches are evolving which may ultimately overcome this relative resistance across colorectal cancers (Bolan & Wen Wee Ma, 2017; Smyth *et al*., 2015).

1.5. Tumor microenvironment in CRC

Under normal conditions, the immune system is an effective "gate-keeper" against cancer. Antitumor activity of the immune system is initially mediated by innate immunity, mainly with effector cells such as Natural Killer (NK) cells, neutrophils, and macrophages. Subsequently, adaptive immunity mechanisms are activated. This type of response is specific and generates memory cells, mainly B and T-cells which encompass the humoral and cellular immunity (De La Cruz-Merino *et al.*, 2011).

The CRC–immune microenvironment consist of different types of cells which include malignant cells, innate immune cells (granulocytes, mast cells [MCs], dendritic cells [DC] and monocytes/macrophages), adaptive immune cells (T and B cells), fibroblasts and endothelial cells. These cells, either by themselves or associated with other cell types, contribute to the inflammatory and/or immunological status of tumor tissues via cell-to-cell contact and/or cytokine/chemokine production. Tumor infiltrating lymphocytes (TIL) are mixtures of T cells, B cells and NK cells, T cells being the most abundant. Macrophages and other innate cells are also present in variable proportions (Koi & Carethers, 2017).

The success of escape mechanisms displayed by the tumor against the host's immune response is responsible, at least in part, for cancer development. This is an area of great importance in the scientific research of tumor microenvironment, with evidence validating the hypothesis that an effective and potent immune reaction against certain tumor antigens may overcome escape mechanisms, leading to the elimination and control of the cancer (Swann & Smyth, 2007). Hereupon, cancer cells may escape the innate and immune host responses mostly by two mechanisms: selection of nonimmunogenic tumor cell variants (immunoselection) or by active suppression of the immune response (immunosubversion) (Swann & Smyth, 2007; Hanahan & Weinberg, 2011).

Tumor-associated macrophages (TAMs) are key elements of the tumor microenvironment and influence various aspects of cancer. TAMs do not become differentiated based on their location, but based on distinct signals derived from the microenvironment in which they reside (DeNardo *et al.*, 2009; Doedens *et al.*, 2010; Ruffell *et al.*, 2012). Therefore, combinations of different signals can result in production of a wide spectrum of TAM phenotypes with characteristic tumor-regulating properties (Qian & Pollard, 2010; Galdiero *et al.*, 2013).

Cytokines produced in the tumor microenvironment can originate macrophages with distinct phenotypes. Classical activated macrophages (M1) occur in response to interferon γ (IFN-γ). M1 macrophages stimulate tissue disruptive reactions by producing tumor necrosis factor α (TNF-α), interleukin 12 (IL-12), oxygen intermediates and reactive nitrogen. M1-activated macrophages are part of the Th1 response, as shown in figure 2. M2 (alternative activated) macrophages arise in response to various stimuli, including IL-4, IL-13, IL-10, and glucocorticoids and are part of the Th2 response, as presented in figure 3. Tumor-associated macrophages have properties of M2-activated cells and express many proangiogenic and angiogenic modulatory factors such as IL-1β, IL-6, IL-8, vascular endothelial growth factors (VEGFs), and matrix metalloproteinases (MMPs) (Varner & Schmid, 2010).

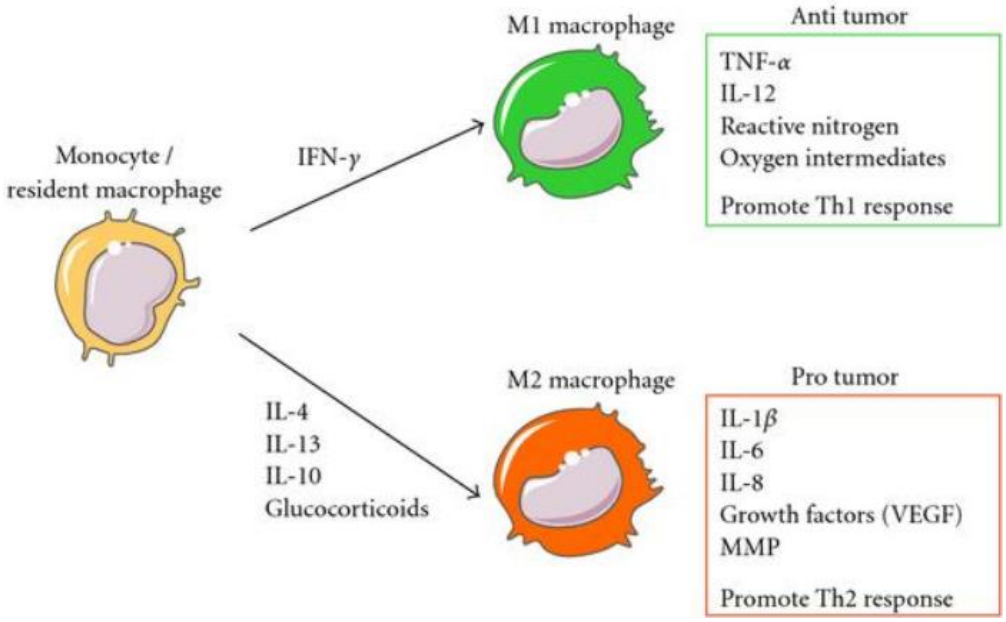


Figure 3: Macrophages subtypes and associated factors that influence the immune response (Varner & Schmid, 2010).

TAM products can influence many aspects of tumor growth and progression (Mantovani *et al*., 2008). In particular, they can regulate senescence; interact with and contribute to extracellular matrix remodeling; promote cancer cell proliferation, invasion and metastasis; sustain angiogenesis and lymphangiogenesis (Lin *et al*., 2006; Clear *et al*., 2010). TAMs express low levels of the major histocompatibility complex class II and reduced antimicrobial and tumoricidal activity. Finally, they suppress anti-tumoral adaptive immunity, presented in figure 4 (Galdiero *et al*., 2013).

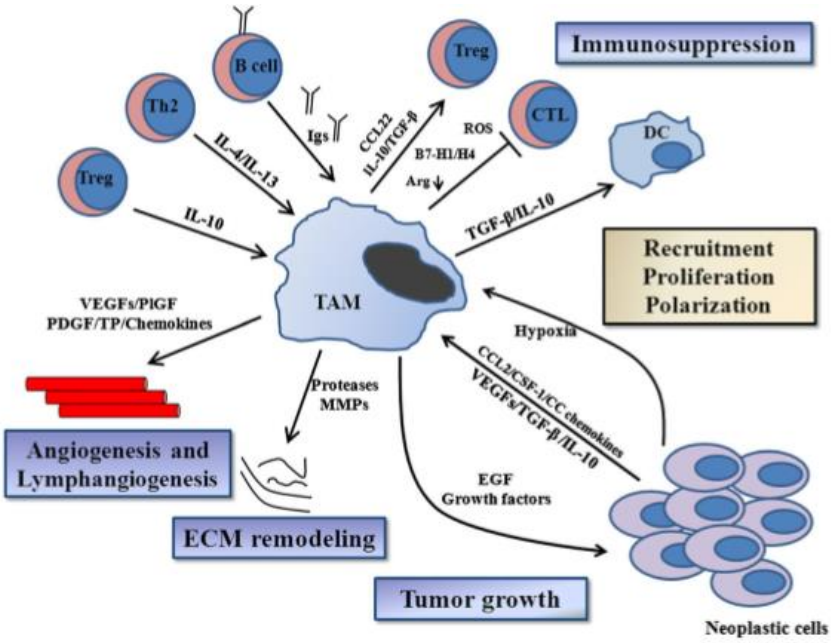


Figure 4: TAMs are key regulators of the tumor-related inflammation. Neoplastic cells recruit macrophages, supporting their proliferation and polarization toward a pro-tumor phenotype. Factors promoting TAMs polarization are also produced by immune cells. In contrast, TAMs influence many aspects of cancer progression: they induce tumor growth, contribute to the remodeling of the extracellular matrix, sustain angiogenesis and suppress anti-tumoral adaptive immunity (Galdiero *et al*., 2013).

In colorectal cancer (CRC), the role of TAM in tumor progression seems to be controversial. Indeed, even if some studies indicated a role of TAMs in CRC progression, evidences indicate a strong antitumoral activity of TAMs and their association with improved disease-free survival (Forssell *et al*., 2007). Interestingly and consistent with previous observations, this controversial role of TAMs in CRC could be due to distinct phenotypes depending on their localization within the tumor, with peritumoral TAMs exerting a better prognostic impact compared to intratumoral TAMs (Erreni *et al*., 2011; Galdiero *et al*., 2013).

Tumor-infiltrating lymphocytes (TILs) are located in the inflammatory infiltrates in islets and in the peritumoral stroma of solid tumors. TILs include cytotoxic T-lymphocytes (CD8), NK cells, and helper T-lymphocytes (CD4). Among the latter, there is a subpopulation of cells named regulatory T-cells (Tregs), formerly suppressor Tcells, mainly suppresses and controls the immune response (Nishikawa & Sakaguchi, 2010). Whilst Treg cells have a physiological role in the prevention of autoimmune events to avoid a disproportionate response to self-antigens, in case of malignant neoplasia, their presence seems more related to immunosuppressive mechanisms preventing tumor destruction (Vignali, Collison, & Workman, 2009).

The relationship between CD8/NK and Treg cells in the tumor-peritumor microenvironment offers an explanation to the final effect of a triggered immune response with an efficient or an immunosuppressive response resulting in tolerance or anergy (Galon et al., 2007; De La Cruz-Merino et al., 2011).

Over the last decade, the association between the patients' prognosis and the immunological landscape in primary CRC determined by high-throughput quantitative measurements of cellular and molecular characteristics has been examined through various studies (Galon *et al.*, 2006; Camus *et al.*, 2009; Pagès *et al*., 2009). The results shown in these studies suggest that the inflammatory/immunological response in CRC is heterogeneous among patients (Galon *et al.*, 2006; Camus *et al.*, 2009); an enhanced T-lymphocytic reaction in tumor tissues, especially in the generation of mature memory T cells, reflects an improved prognosis (Pagès *et al*., 2009); but cancer-associated fibroblasts (CAF) in tumor tissues antagonize T-cell antitumor activity and negatively contribute to patients' prognosis (Becht *et al.*, 2016). The balance between these two factors may determine disease outcome to a great extent. A classification of CRCs according to their immunological status in tumor microenvironment may accurately predict patient's outcome and identify patients with stage I/II/III CRCs for whom there is a high or low risk of recurrence after surgery (Koi & Carethers, 2017).

1.5.1. Immunologic mechanisms associated with tumor progression and prognosis

Cancer-related inflammation is an essential process in malignant diseases, with common and defined players at different stages of progression (Balkwill *et al*., 2005; Mantovani *et al*., 2008). Until recently, the scientific community has been driven by the hypothesis that extrinsic inflammatory pathways promote or, in some cases, initiate cancer—i.e., that chronic inflammation causes or promotes cancer (Balkwill & Mantovani, 2001), represented in figure 5. However, there is now evidence that there is an intrinsic inflammation pathway activated by the followed genetic events that cause neoplasia: activation of oncogenes such as MYC, RAS, and RET, or inactivation of tumor suppressors, leading to constitutive production of inflammatory cytokines by the initiated cell (Mantovani *et al.*, 2008). Oncogene and tumor suppressor pathways are proven intracellular targets for therapies. Recent data suggest that immune functions, inflammatory cytokines and their receptors are suitable targets for therapy in malignant diseases (Candido & Hagemann, 2012).

In line with early observations, the presence of a high level of lymphoid reactions in CRC tissues is associated with an improved prognosis (Jass, 1986; Ogino *et al.*, 2009) as shown in figure 5.

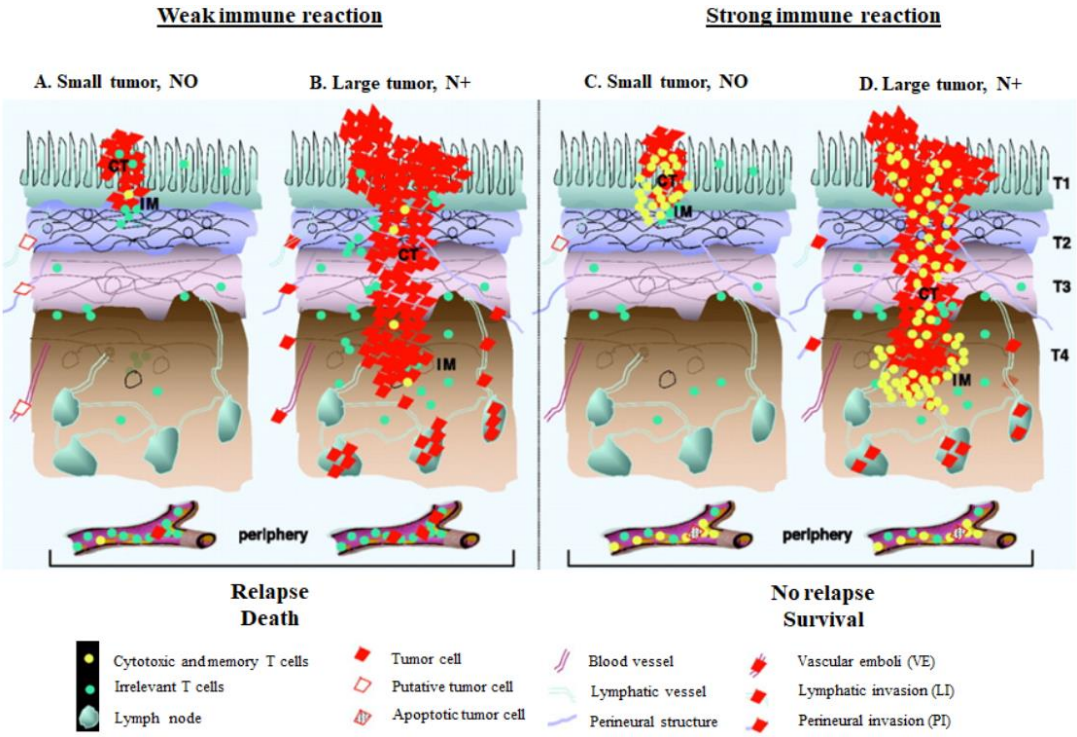


Figure 5: A proposed model of the mechanisms by which the immune system may control tumor dissemination and influence clinical outcome. The main observations supporting this model can be summarized as follows. (*1*) Correlation between a high density of $CD45RO^+$ memory T cells and $CD8^+$ T cells infiltrating colorectal cancer and the absence of lymphovascular and perineural invasion (*C* compared to *A* and *D* compared to *B*). (*2*) A weak adaptive immune reaction is associated with a very poor prognosis even in patients with minimal tumor invasion (*A* and *B*). (*3*) A high density of lymphocytes correlates with a favourable prognosis whatever the local extent of the tumor and the invasion of regional lymph nodes (*C* and *D*). (*4*) The combined analysis of infiltrating T cells within tumor regions (centre of the tumor plus invasive margin) improves the accuracy of prediction of survival for the different patient groups compared with single-region analysis. (*5*) Effector and memory T cells *in situ* limit tumor dissemination. Effector and memory T cells could reflect a quality of systemic effectors for recognition and killing of circulating cancer cells. (*6*) The histopathologic variables (T (tumor) stage, N (node) stage, and differentiation) are no longer informative for the evaluation of the clinical outcome when analysed together with adaptive immune variables. Adaptive immune reaction within the tumor may be an ultimate variable influencing clinical outcome following surgical treatment with curative intent. *Adapted from*: (Galon *et al.*, 2007).

In general, the transformation of naive CD4+ T cells into Th1 cells is triggered by specific binding of their T-cell receptors (TCR) to tumor-derived antigens with class II MHC presented by antigen-presenting dendritic cells (DC). IL-2 and IFN-γ produced by antigen-activated Th1 T cells promote priming and expansion of CD8+ effector T cells. Naive CD8+ T cells are primed to effector T cells expressing high levels of perforin and granzymes when their TCR specifically binds to the antigen presented with class I MHC on the DC (Schoenberger *et al*., 1998). Naito *et al*. (1998) first described the infiltration of cytotoxic CD8+ T cells within CRC cell nests as an independent prognostic factor. Later on, Pagès *et al*. (2005) showed that CRC tumors without signs of early metastasis (vascular-lymphatic invasion and perineural invasion) exhibit higher densities of Th1 cells, early memory and effector memory CD45RO+ CD8+ T cells compared with the CRCs with vascular-lymphatic invasion and perineural invasion. High levels of CD45RO+ cells in the tumor microenvironment correlated with increased disease-free survival (DFS) and overall survival (OS) of CRC, represented in figure 5 and table 3 (Koi & Carethers, 2017).

Galon *et al*. (2006) showed that tumor recurrence in CRC inversely correlates with high expression level of genes including TBX21, IRF1, IFNG, CD3-ζ, CD8A, GZMB and GLNY that are components of Th1 T/effector CD8+ T-cell functions; a high density of T lymphocyte subpopulations including CD45RO (memory), GZMB (cytotoxic effector), CD8 (effector) and CD3 (total T cells) is associated with prolonged OS (table 3). Galon's group (2012) also reported that high-risk stage I/II CRCs are identifiable as CRCs containing low levels of CD8+ and CD45RO+ T lymphocytes at CT and IM sites (Pagès *et al.*, 2009). Moreover, Mlecnik *et al*. (2016) demonstrated that a low immunoscore - immunological classification based on type, density, and location of immune cells - determined by density of CD3+ and CD8+ at CT and IM sites of primary CRC is associated with the metastatic potential of tumors.

All together, these retrospective studies suggest that the activation of Th1 and cytotoxic memory T cells play a key role in preventing recurrence and/or metastasis in CRC.

Although improved prognosis of CRC seem to be associated with a high level of antitumor T-cell activity, a large number of CRCs exhibited a decreased level of antitumor activity and associate with a shorter patient survival time (table 3). For instance, approximately 60% of stage I/ II/III CRC showed a low level of antitumor immune reaction and approximately 80% of stage IV CRC exhibited a high mortality rate in a large cohort studied by Rozek *et al*. (2016).

Table 3: Factors associated with improved and poor prognosis (Koi & Carethers, 2017).

Improved survival	Poor survival
High level of lymphocytes	Low level of lymphocytes
High level of CD8+ T lymphocytes	Low levels of CD8+ and CD45RO+ T lymphocytes
High level and density of CD45RO+ T lymphocytes	High levels of myeloid-derived suppressor cell
Th1 and **T/effector CD8+ T-cell expression**	High levels of mast cells
Granzyme B expression	CAFs (cancer associated fibroblasts) that produce immunosuppressive factors

Furthermore, whereas the level of antitumor T-cell activity decreases with stage, the density of B cells and of innate immune cells, such as neutrophils, mast cells (MCs), macrophages and immature DCs, increases (Bindea *et al.*, 2013).

Angelova *et al*. (2015) showed that the presence of myeloid-derived suppressor cells (MDSCs), MCs and Th17 in TILs are significantly associated with stage progression and poor prognosis of CRC as shown in table 3. MDSCs are immature myeloid cells (macrophage, DCs or neutrophils), generated in the bone marrow (i.e. polymorphonuclear-MDSC) and/or spleen (i.e. monocytic-MDSC) in response to tumor-derived factors including cytokines, chemokine and metabolites. Migrated MDSCs in various tissues, including tumors, are suppressive to T-cell antitumor immunity through the increased production of arginine, reactive oxygen species and nitric oxide and the induction of Treg cells and TGF-β secretion. MCs influence tumor angiogenesis and invasion and immune suppression, contribute to an immune suppressive tumor microenvironment and they promote angiogenesis and tumor invasion into surrounding tissues (Dalton & Noelle, 2012). Thus, CRC patients with high microvessel and MC densities had significantly poorer prognoses than patients with low microvessel and MC densities. The Th17 cells are differentiated from naive CD4+ T cells upon IL6 and TGF-β stimulation. The IL-6 induces IL-21 production, which promotes the expression of the transcription factor RORγt together with IL-23, resulting in IL-17 production. There is contradictory evidence on whether the presence of IL-17-producing cells are either associated with improved or poor prognosis of CRC patients. Thus, further study is necessary to determine the role of Th17 cells in immune-suppression of CRC (Bindea *et al.*, 2013; Amicarella *et al.*, 2017).

Treg cells expressing FOXP3 are derived from naive CD4+ T cells upon TGF-β and IL-2 stimulation. Treg cells in CRC not only produce TGF-β and IL-10 with immune-suppressive activity but also express several immune checkpoints including PD-L1, PD-L2 and CTLA-4 to inhibit effector T-cell functions. Expression of genes characteristic to TIL-Treg cells in CRC is negatively associated with patient survival (De Simone *et al*., 2016). The molecular signature of cancer-associated fibroblasts (CAFs) were found to produce immunosuppressive factors such as LGALS, CXCL12 and COX-1 and proangiogenic factors such as VEGFB, VEGFC and PDGFC and inflammatory factors such as CCL11, CCL8, CCL2, SAA3 and CXCL5. CAFs are over-represented for a subset of CRC, which shows the worst prognosis (table 3) (Torres *et al*., 2013).

Taken together, the above studies, as shown in figure 5, suggest that an inflammatory environment with depressed or loss of antitumor reactions is associated with a poor prognosis in CRC.

1.5.2. Classification and Immune-based predictive markers

Classifying CRCs based on the immunological, genetic and transcriptional landscapes identified through the studies described so far may contribute to lowering the incidence of recurrence and associated death. On average, approximately 10% of stage I/II and approximately 30% of stage III CRC patients experience recurrence with distant metastasis after curative surgery. To reduce the recurrence rate, progress could be made in two critical areas. First, a predictive marker(s) that identifies patients at high risk for recurrence should be discovered and developed. Second, new adjuvant therapies, alone or in combination with current 5-Fluorouracil-based chemotherapy, that are specifically effective to high-risk patients identified by new markers should be developed (Koi & Carethers, 2017).

The apparently promising immunoscore (immunological classification based on type, density, and location of immune cells) system developed by Galon's group showed that high-risk stage I/II CRCs exhibited a low immunoscore (low levels of CD8+ and CD45RO+ T lymphocytes densities in the centre and invasive margin of the tumor sites) (Galon *et al*., 2012). However, immunoscores alone may not accurately predict patient outcomes, as they cannot distinguish between CRCs with high levels of Th1/CD8+ T cells that exhibit poor prognoses (CMS4 subtype CRC) and those that exhibit improved prognoses (Koi & Carethers, 2017). On the other hand, expression of cancer associated fibroblasts (CAF)

markers including CALD1, POSTN, FAP and IGFBP7 can be a predictive factor for poor prognosis in CRC (Galon *et al*., 2007; Pagès *et al*., 2009; Calon *et al*., 2015).

Developing a new classification system of primary CRC based on the current lymphnode metastasis system combined with Th1/CD8+ T-cell markers, CAF markers, microsatellite markers, and other new biomarkers that are arising in the scientific community, would more accurately and cost-effectively identify high-risk CRCs that might relapse after surgery and could also facilitate the discovery of critical targets for adjuvant therapy (Koi & Carethers, 2017).

1.6. Macrophage migration inhibitory factor (MIF)

Macrophage Migration Inhibitory Factor (MIF) was first described in the 1960s, making it one of the first cytokines to be identified (David, 1966). The *MIF* gene is located on the chromosome 22q11.2 and has a total of 1223 base pairs and has three possible transcripts, MIF-201 transcript has 3 exons and encodes a protein constituted by 115 residues of 12,5 kDa total represented in figure 6. This cytokine is involved in cell-mediated immunity, immunoregulation and inflammation (Ensembl, 2018; NCBI, 2018; Renner, Roger, & Calandra, 2005).

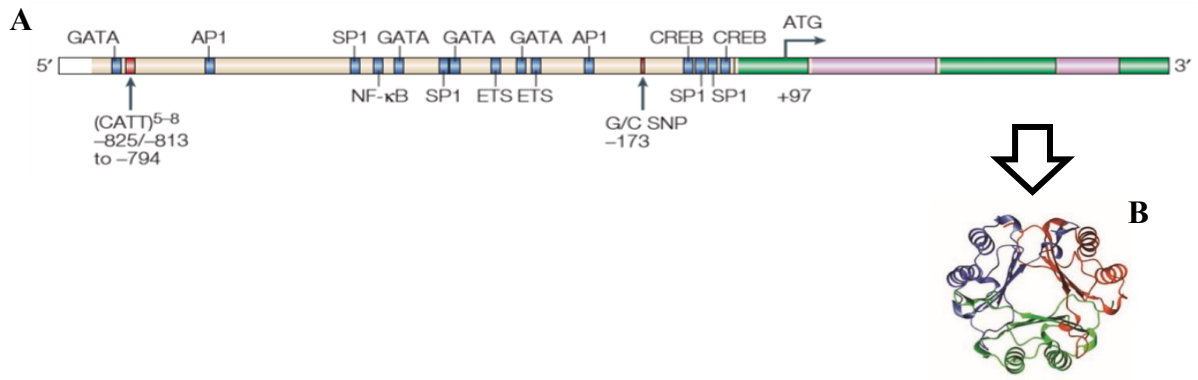


Figure 6: A. Structure of the human *MIF* gene. Composed of three short exons (green boxes) of 107, 172 and 66 base pairs, and two introns (pink boxes) of 188 and 94 base pairs. Its 5′regulatory region contains several consensus DNA-binding sequences for transcription factors, notably activator protein 1 (AP1), nuclear factor-κB (NF-κB), ETS, GATA, SP1 and cAMP response element binding protein (CREB). It's also represented two polymorphisms of the human *MIF* gene (arrows) — a CATT-tetranucleotide sequence repeated five to eight times at position –794 and a G-to-C single nucleotide polymorphism (SNP) at position –173. **B. MIF protein structure:** three-dimensional ribbon diagram of human *MIF*, revealing its homotrimeric subunit structure. Each colour denotes one monomer (Calandra & Roger, 2003; Leng & Bucala, 2006).

The three dimensional crystal structure of MIF was solved by three laboratories in 1996, revealing a new protein fold and structural superfamily with MIF as its defining member. These studies also provided insights into the probable native form of the protein - a homotrimer represented in figure 6 (Sugimoto *et al*., 1996; Leng & Bucala, 2006).

The unique biological functions associated with MIF have led to it being described as a cytokine, enzyme, hormone, and chemokine. It is capable of triggering significant immune responses through autocrine and paracrine loops via the induction of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, nitric oxide, COX2, and IFN-γ (O'Reilly *et al*., 2016). As a pro-inflammatory mediator, MIF has been shown to be implicated in the pathogenesis of severe sepsis and septic shock, acute respiratory distress syndrome, and several other inflammatory and autoimmune diseases, including rheumatoid arthritis, glomerulonephritis, and inflammatory bowel diseases (De Jong *et al.*, 2001; Gregersen & Bucala, 2003; Renner, Roger & Calandra, 2005).

1.6.1. Signalling pathway

The effect of MIF on cellular signalling is initiated through receptor-mediated pathways and intracellular interactions, represented in figure 7. MIF forms a complex with CD74/CD44, leading to phosphorylation of ERK 1/2 and therefore MAPK ERK 1/2 activation; this triggers downstream processes such as the release of pro-inflammatory cytokines and cell proliferation. During this process, the activation of phospholipase A2 (PLA2) and cyclooxygenase 2 (COX2) leads to the downregulation of tumor suppressor p53, hence inhibiting apoptosis. The Akt pathway is also activated via CD74, leading to phosphorylation of pro-apoptotic proteins like BAD which further contributes to cell survival and the inhibition of apoptosis (O'Reilly *et al*., 2016). HIF-1α is a key transcription factor for angiogenic proteins such as VEGF (vascular and endothelial growth factor) and MIF can also play a role in its stabilization through a p53-dependent mechanism and in chemotactic recruitment of cells to the inflammation sites via its interaction with CXCR 2/4 (figure 7) (Renner, Roger & Calandra, 2005; O'Reilly *et al*., 2016).

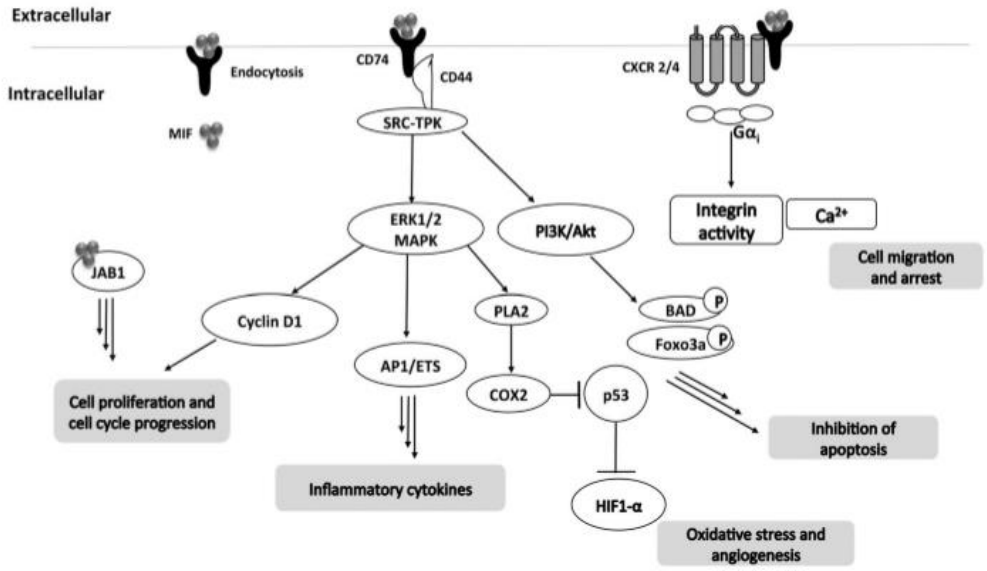


Figure 7: MIF's signalling pathways. MIF interacts with CD74/CD44 receptors which leads to the activation of a series of downstream processes (Renner, Roger & Calandra, 2005; O'Reilly *et al.*, 2016).

Figure 8 summarizes the hypothesis proposed by Leng and Bucala (2006) regarding the potential mechanisms for MIF signal transduction via CD74 based on the known molecular biology of these proteins. CD74 RIP is an interesting and well understood mechanism for signalling in B lymphocytes.

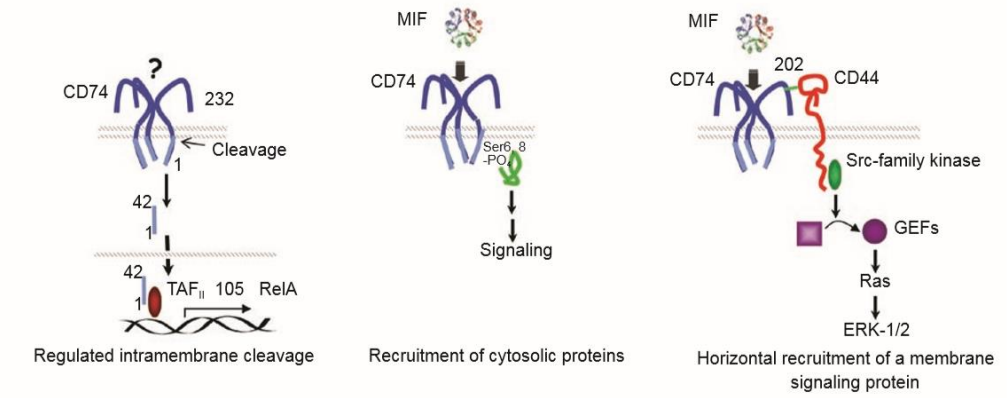


Figure 8: Proposed pathways for MIF signal transduction through its cell surface binding receptor (CD74) (Leng & Bucala, 2006).

MIF action in B lymphocytes has not yet been studied and the activation of the RelA/NF-kB family of transcription factors has yet to be reported to be a feature of MIF action. The phosphorylation of the CD74 intracytoplasmic domain is strongly suggestive of a signal transduction function requiring contact with second messengers (Leng & Bucala, 2006).

It is believed that the recruitment of a second transmembrane protein, such as CD44, is a possible mechanism for MIF signalling, especially since CD44 is known to activate Src-family kinases leading to downstream ERK phosphorylation. Receptor activation by such "horizontal" recruitment is typical of proteins that span the membrane only once, and it is the structural basis for signal transduction by several known cytokine receptors. The highly polymorphic nature of the CD44 ectodomain, which additionally has a role in cell adhesion, also lends itself to cell-specific pleiotropism. Such a pathway is within the expectation for MIF's broad regulatory role in cell survival and apoptosis and suggests that MIF's actions, which have long been considered to be extremely broad for a classical "pro-inflammatory" cytokine, may be productively regulated by the interaction between a cell surface binding protein (i.e. CD74) with a polymorphic, signal transduction molecule (CD44) (Fingerle-Rowson *et al*., 2003; Leng & Bucala, 2006).

1.6.2. Single nucleotide polymorphisms

A single nucleotide polymorphism (SNP) is a variation at a single position in a DNA sequence among individuals. The primary DNA sequence is formed from a chain of four nucleotide bases: A, C, G, and T. If more than 1% of a population does not carry the same nucleotide at a specific position in the DNA sequence, then this variation can be classified as a SNP. If a SNP occurs within a gene, then the gene is described as having more than one allele. In these cases, SNPs may lead to variations in the amino acid sequence.

SNPs, however, are not just associated with coding genes; they can also occur in noncoding regions of DNA. Although a particular SNP may not cause a disorder, some SNPs are associated with certain diseases. These associations allow scientists to look for SNPs in order to evaluate an individual's genetic predisposition to develop a disease (Nature, 2018).

As regards to CRC, few SNPs of *MIF* gene have been studied and associated with this type of cancer. In general, there is 9538 SNPs of *MIF* gene registered in the Ensembl database. Of these, 222 have already been cited in scientific papers. The SNP chosen for this work is included in the category of 5' untranslated region variants and has already been cited in various scientific papers (Ensembl, 2018).

A G-to-C substitution in the 5'-flanking region of *MIF* gene, at position -173 G>C (rs755622), represented in figure 9, which has been reported to be associated with susceptibility to adult inflammation and cancer (Morris *et al*., 2014).

Several studies have shown this single nucleotide polymorphism (SNP) of *MIF* gene has been linked to the risk of CRC (Dessein *et al*., 2010; Morris *et al*., 2014). Although the rs chosen for this project is situated in the 5'UTR region and does not directly affect the structure of the final protein, it might influence the transcriptional process.

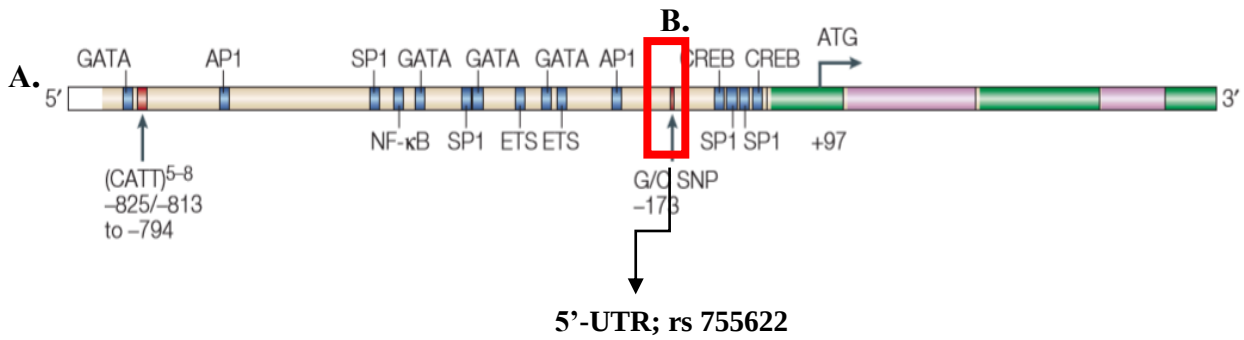


Figure 9: A. Representation of the *MIF* gene structure. B. representation of the position of rs755622, situated in the 5'-UTR (Calandra & Roger, 2003; Ensembl, 2018).

The variant rs755622 has a worldwide allelic frequency of G: 73%; C: 27%, and European allelic frequencies of G: 81%; C: 19%. When compared to the other variants lodged in the Ensembl database it seems to exhibit a superior probability in obtaining an accurate and precise genotyping of the SNP in order to associate it with CRC (Ensembl, 2018). In fact, the meta-analysis conducted by Zhang *et al.* (2015) revealed that *MIF* -173G/C polymorphism might increase the risk of cancer although more studies are needed to confirm the association between *MIF* polymorphism and the risk of cancer.

Although other variants have been studied, the variant rs755622 (G/C) seems to be the one that provides the best possible data to draw conclusions on whether the SNP of *MIF* gene has or not an influence on an individual's susceptibility of CRC (Ensembl, 2018).

1.6.3. Association with Colorectal Cancer

Chronic inflammatory states such as *Helicobacter pylori* infection for gastric cancer and inflammatory bowel disease for colorectal cancer are key risk factors associated with the development of these malignancies (Gillen *et al*., 1994).

Chronic inflammation is thought to have a role in carcinogenesis by increasing cell proliferation and cell resistance to apoptosis, and yet, a complete understanding of the underlying mechanisms by which inflammation and carcinogenesis are linked remains elusive. It has been previously shown that MIF is highly produced by gastrointestinal tract

during inflammatory diseases and acts in an autocrine manner when exposed to *H. pylori* inducing transactivation of the epidermal growth factor receptor (EGFR), which suggests an important link to pro-carcinogenic mechanisms (Beswick *et al*., 2006; Morris *et al*., 2014).

Strong evidence has been presented linking chronic inflammation to the onset and pathogenesis of cancer, in several studies MIF, as multifunctional pro-inflammatory protein occupies a central role in the inflammatory pathway, can promote tumor growth and viability by modulating immune responses and has been implicated and supporting the angiogenesis and metastasis of many cancer phenotypes *(*Ramireddy *et al*., 2014) (O'Reilly *et al.*, 2016). A few experiments suggested that *MIF* mRNA and MIF protein are overexpressed in a number of cancers (Zhang *et al.*, 2015).

Morris *et al*., (2014) and her research team reported that MIF and its receptor, CD74, were found to be increased in most tumors from patients with lymph node metastasis. MIF was also found to be highly produced by cancer associated fibroblasts isolated from human tumors compared to fibroblasts from matched normal tissues from uninvolved areas. Fibroblast-produced MIF highly increased cancer cell proliferation, which was decreased upon neutralizing MIF or CD74. Chronic MIF treatment led to sustained proliferation and signalling events in non-transformed fibroblast cells, which was maintained upon removing MIF treatment for eight weeks. Additionally, chronic treatment of normal cells expressing fibroblast markers for up to 16 weeks with MIF led to a drastic decrease of fibroblast markers with concurrent increase of epithelial markers. These results suggest that MIF promotes mesenchymal epithelial transition, cell transformation and tumorigenesis in cancers, and may be an important link between chronic inflammation and tumorigenesis (Morris *et al.*, 2014).

2. Objectives

The main goal of this research was to contribute to the study of the clinical genomics of colorectal cancer. Grounded on the possibility of this disease being influenced by genetic factors, several interesting candidate genes arise, possibly involved in patient's survival and disease progression. In detail, the proposed objectives were to:

- Characterize the genotype of the *MIF* functional polymorphism rs755622 in a CRC population;
- Assess whether any genotype of this SNP influences clinicopathological parameters, particularly overall survival and progression-free survival in colorectal cancer;
- Analyse MIF, macrophages and T lymphocytes in CRC tissues, by immunohistochemistry, and its association with genotype data and clinicopathological parameters.

3. Materials and Methods

3.1. Sample Harvesting, Processing and Storage

Sample collection started in January 2017 in the Department of Medical Oncology in the Centro Hospitalar de Trás-os-Montes & Alto Douro (CTMAD), Vila Real. Two blood samples were collected (6ml in an EDTA coated tube, each) from 172 CRC patients by health professionals at CTMAD. The patients were chosen based on the fact that they had a colorectal cancer (adenocarcinoma) with more than one-year follow-up after diagnosis. Those samples were stored immediately at 4ºC. Only CRC patients who agreed to sign the informed consent (in agreement with the declaration of Helsinki) were included in this study.

The samples were transported to the Laboratory of Applied Molecular Genetics of the Department of Genetics and Biotechnology (DGB), in UTAD in order to proceed with sample processing. This consisted in centrifuging the blood samples, in an *Eppendorf centrifuge 5804R A-4-44*, for ten minutes at 2500 rpm and 10ºC. With this, it was possible to obtain a separation between the plasma, buffy coat and the haematocrit, represented in figure 10, in order to proceed with serum, plasma and buffy coat isolation from blood. Approximately 700µL of buffy coat and plasma were transferred to separate Eppendorf tubes of 1,5ml each (1 Eppendorf tube of buffy coat per sample and 4 Eppendorf tubes of plasma per sample). Samples were then stored at -20ºC for further processing.

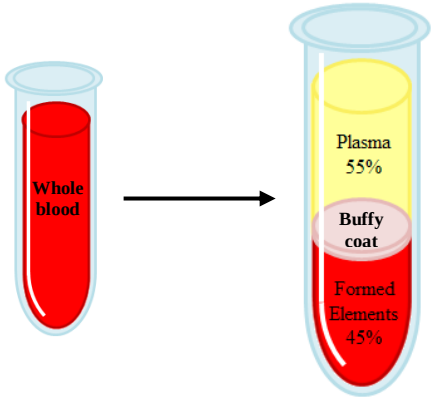


Figure 10: Representation of the separation blood after processing.

3.2. DNA Extraction and Quantification

DNA extraction aims to separate the DNA from proteins, membranes and other cell components. This process requires careful handling of the biological material to prevent sample contamination and crossover. Tubes and recipients should be carefully labelled, especially when transfers are required (Elkins, 2013).

In this study, we started by the technique optimization of different DNA extraction kits: *Isolate II Genomic DNA kit* (Ref.: BIO-52065); *Invisorb® Spin blood Mini kit* (Ref.:1031100200, STRATEC Molecular) and *Extract Me® DNA blood kit* (Ref.: EM05-050, BLIRT). *Extract Me DNA blood kit* was the selected one as it presented the best quality/price ratio. Buffy coat from all blood samples was utilized for DNA extraction with columns using the selected kit. The DNA extraction protocol, performed conform the manufacturer instructions, was the following:

1. Transfer **350µL** of sample to a sterile 1.5 Eppendorf tube and add the same volume of the RBC Lysis Buffer.

Note: When isolating from less than 200 µL of sample, add Elution Buffer or PBS buffer to 200 µL and then add 200 µL of RBC Lysis Buffer.

2. Mix well by inverting the tube until a clear red solution is obtained.
3. Centrifuge* for **4 min** at 8.6k x g **(~9000rpm, in the Eppendorf centrifuge 5430R FA-45-30-11 aerosol-tight)**.

Note: Higher speeds are not recommended as they may hinder the subsequent suspension of the white blood cell pellet in the lysis buffer.

4. Carefully discard the supernatant from over the white blood cell pellet.
5. Add **375 µL BL Lysis Buffer** and ressuspend the cell pellet completely.
6. Add **6 µl Proteinase K** and mix by vortexing.
7. Incubate at **55°C for 10 min** vortexing every 2 minutes extending the incubation time if necessary, until the cells are completely lysed.
8. Add **400 µl BB Buffer** and mix thoroughly.
9. Vortex vigorously for **15-20 sec.**
10. Transfer the lysate onto a purification minicolumn placed in a collection tube. Centrifuge for **1 min** at 11-15k x g **(11481rpm)**.
11. Transfer the purification minicolumn to a new collection tube (2 ml).
12. Add 600 µl BW1 Buffer and centrifuge for **30 s** at 11-15k x g **(11481rpm)**.
13. Discard the filtrate and reuse the collection tube.

14. Add 400 µl BW2 Buffer and centrifuge for **30 s** at 11-15k x g **(11481rpm)**.
15. Discard the flow-through and reuse the collection tube.
16. Centrifuge for **1.5min** at 15-21k x g **(13722rpm)**.

Note: The wash buffer contains alcohol, which may interfere with some enzymatic reactions and also decrease the elution efficiency. It is therefore vital to remove the alcohol completely from the column before elution.

17. Discard the collection tube and the flow-through and carefully transfer the purification minicolumn to a sterile 1.5 ml Eppendorf microcentrifuge tube.
18. **Add 100 µL Elution Buffer**, **pre-heated to 70°C**, directly onto the purification minicolumn membrane.
19. Incubate the minicolumn at room temperature for **2 min**.
20. Centrifuge at 11-15k x g **(11.481rpm)** for **1 min.**
21. Remove the minicolumn. The isolated DNA is ready for use in downstream applications or for either short-term storage at +4°C or long-term storage at -20°C.

*The centrifuge used in the extraction protocol was *Eppendorf centrifuge 5430R FA-45-30-11 aerosol-tight.*

After DNA extraction we proceeded with the quantification of all samples using *Nanodrop ND-1000 spectrophotometer*, in order to assess the quality, concentration and purity of the extracted DNA. Only 1µL per sample was used for quantification.

3.3. Real-time PCR based on *Taqman* probes

Real time PCR relies on the same components as a standard PCR: target DNA, free nucleosides triphosphates, primers and the Taq polymerase; the only difference is that this technique uses a fluorescent oligonucleotide probe (*Taqman* probe), which allows the supervision of the process as it occurs in real time. The probe contains two fluorescent dyes a reporter dye on the 5' end and a quencher dye on the 3' end. When the reporter is excited by light, it transfers its energy to the quencher. This process of energy transfer is called FRET (Fluorescence Resonance Energy Transfer) and prevents the reporter dye from emitting light. The PCR cycles begin with the heated denaturation of the DNA, the temperature is then lowered and the probe and primers anneal to the specific sequences on the DNA strands. Taq DNA polymerase synthesises complementary DNA using the primers as starting points. The polymerase has an exonuclease activity which is used when the enzyme encounters the probe, digesting it thus allowing the polymerase to continue to elongate the DNA strands. The cleavage of the probe is an essential part of the reaction, it releases the fluorophore of the reporter dye and breaks the close proximity to the quencher, thus relieving the quenching effect and allowing fluorescence of the fluorophore. Hence, fluorescence detected in the quantitative PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR. RT-PCR based on *Taqman* probes mechanism is represented in figure 11 (A. Heid *et al*., 1996).

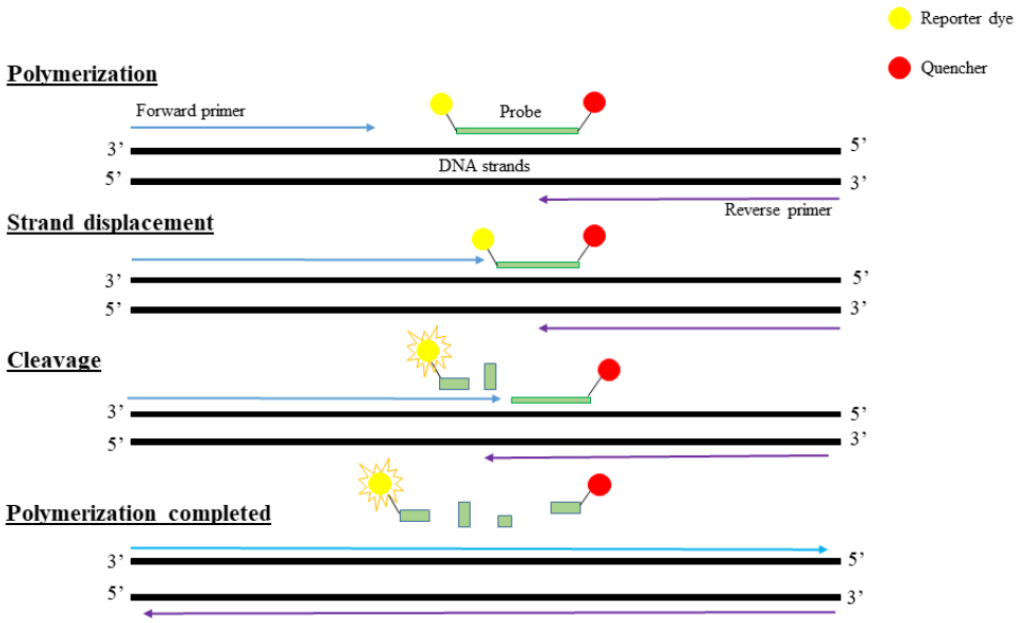


Figure 11: Schematic representation of the real time PCR based on *Taqman* probes mechanism.

In the present work the real time PCR based on *Taqman* probes protocol used was optimized for the SNP rs755622 of *MIF* gene. The *STRATAGENE Mx3005P Multiplex Quantitative PCR System* was used for the genotyping of all samples.

The *MIF* genotyping assay probe used in this protocol had the ancestral allele (G) marked with FAM fluorescent dye and the mutated allele (C) marked with VIC fluorescent dye. The optimized amplification conditions were the following: 95ºC for 3 minutes; 95ºC for 10 seconds; 60ºC for 45seconds; during 40 cycles. The genotyping buffer used was the *SensiFAST Genotyping Hi-ROX Mix* 2x and the optimized conditions used for the reaction were: 7,5 µL of *SensiFast Genotyping Hi-ROX Mix;* 5,8 µL of H_2O; 0,70 µL of Assay ; 1 µL of DNA for a final volume of 15 µL.

3.4. Standard PCR

Polymerase chain reaction (PCR) is a powerful amplification technique that can generate an abundant supply of amplicons (specific segments of DNA) from only a small amount of starting material (i.e., DNA template or target sequence) and uses an enzyme named *Taq* DNA polymerase (Lorenz, 2012).

In order to confirm and assess the quality of the results obtained and the integrity of the extracted DNA, a standard PCR was optimized, using primers designed for the targeted sequence, followed by an electrophoresis in agarose gel.

The optimized PCR conditions used were the following: 10µl Master mix; 0,8µl DMSO; 6µl Water; 1µl Primer forward; 1µl Primer reverse; 1µl DNA. The temperatures used were: 95ºC for 1 min.; 95ºC for 15 sec.; 59ºC for 15 sec.; 72ºC for 10 sec., for 40 cycles.

3.5. Sample purification and Sanger Sequencing

To proceed with further confirmations, about 5% of the samples were purified and subjected to Sanger sequencing.

Illustra ExoProstar-1-step was used for sample purification. The protocol was the following:

1. Put 10µl of PCR product in an Eppendorf tube
2. Add 3µl of *Illustra ExoProstar-1-step* to the Eppendorf tube
3. Incubate for 15 min. at 37ºC
4. Incubate for 15 min at 80ºC

After this process the samples are ready to be sequenced.

Sanger sequencing is a method of DNA sequencing, developed by Frederick Sanger and colleagues in 1977 and is based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication, see figure 13.

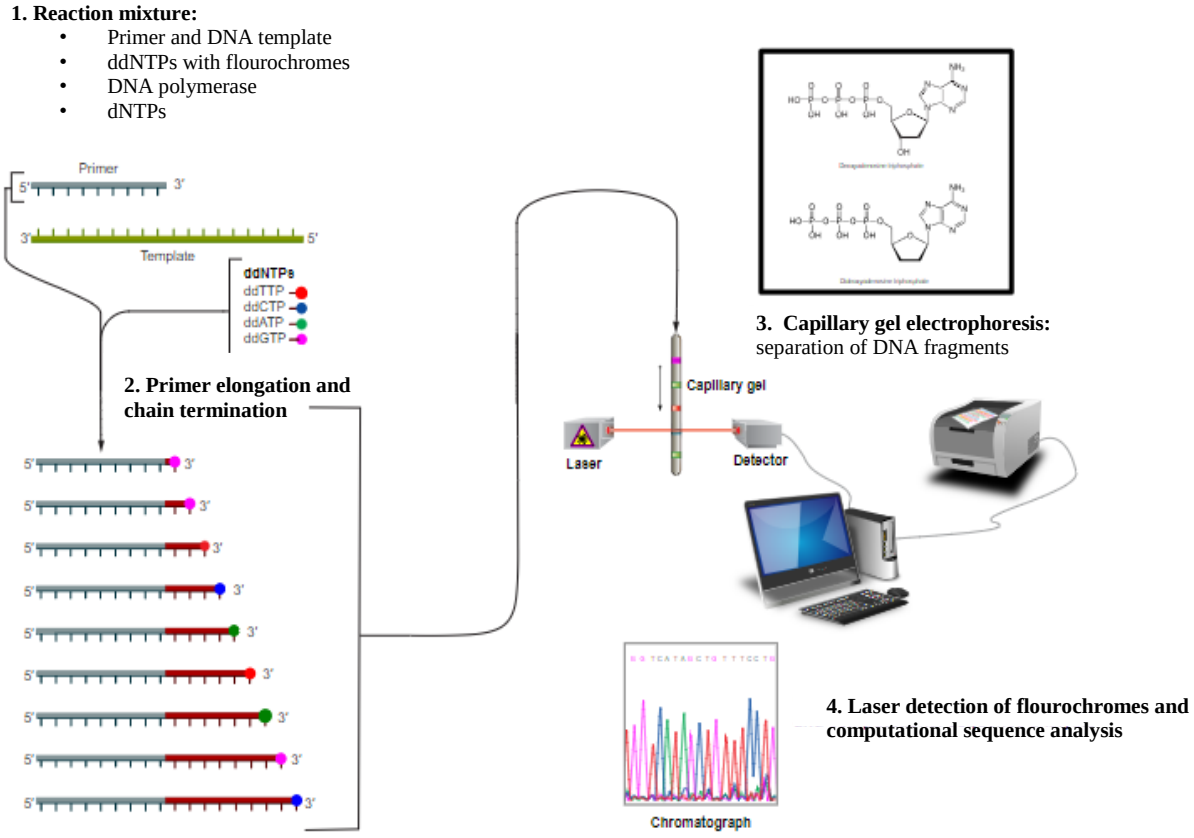


Figure 12: Schematic representation of the Sanger sequencing method. *Adapted from:* https://en.wikipedia.org/wiki/Sanger_sequencing.

3.6. Immunohistochemistry

Immunohistochemistry (IHC) is a technique largely used for diagnosis and investigation of a broad range of disease processes with several applications such as research on disease pathogenesis, prognosis and therapeutic actions to tailor a personalized treatment. IHC is based on an immune complex formation on cell or tissue target molecules and its identification and visualization. Essentially, IHC builds a bridge between classical histopathology and molecular pathology (Ramos-Vara & Miller, 2014).

IHC detection methods can be direct or indirect. Direct detection methods are a one step process which uses a labelled primary antibody conjugated with molecules that could be identified, as for instance fluorochromes, enzymes, colloidal gold, or biotin (figure 14). Indirect detection consist in using two or more layers of antibodies in which the first layer is not labelled but the others are, thus increasing the sensibility and specificity, as shown in figure 14 (Ramos-Vara & Miller, 2014).

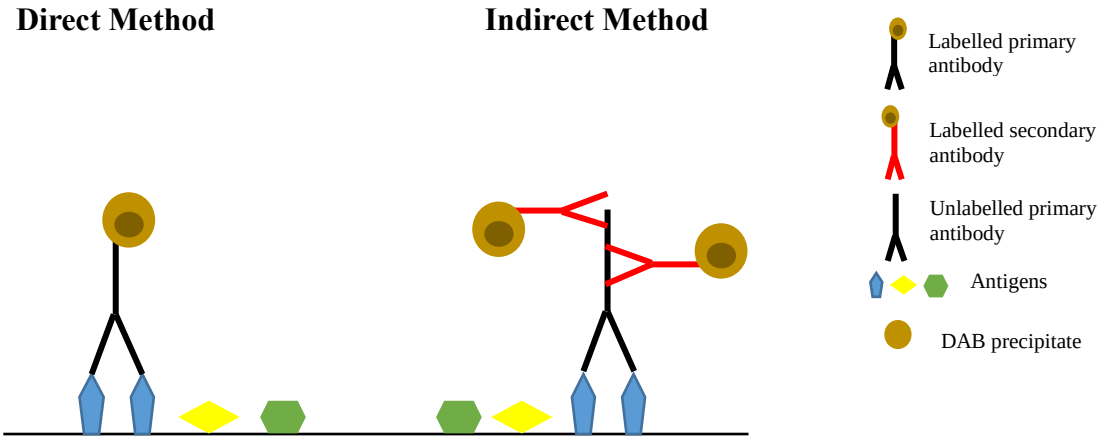


Figure 13: Schematic representation of the direct and indirect methods of detection in IHC.

In the present work, we performed the IHC technique optimized in the Laboratory of Histology and Anatomical Pathology (LHAP) of UTAD.

In an attempt to establish a comparison between tumor infiltrated lymphocytes (TILs), tumor associated macrophages (TAMs) and MIF, we used the monoclonal antibody anti-MIF (D-2: clone sc-271631 from Santa Cruz Biotechnology, Inc.); the monoclonal antibody anti-CD68 (to identify macrophages, Ab955, AbCam) and the polyclonal antibody anti-CD3 (to recognize T cell, A0452, DAKO) all at 1:50 dilution. Incubation time and antigen retrieval method, for each antibody is depicted in table 4. The positive control was a lymphoid tissue (tonsil).

Table 4: Reference, incubation time and antigen retrieval method, for each antibody.

Primary antibody	Reference	Antigen retrieval method	dilution	Incubation time*
MIF	D-2: clone sc-271631 from Santa Cruz Biotechnology, Inc.	3 min. pressure cooker	1:50	2 hours
CD68 (macrophages)	Ab955, AbCam	3 min. pressure cooker	1:50	2 hours
CD3 (T lymphocytes)	A0452, DAKO	3 cycles, 5 min. each, microwave	1:50	3 hours

*At room temperature in a horizontal humid chamber.

After all optimizations, 32 CRC tissue samples were analysed by IHC. The final optimized protocol used was the following:

1. **Deparaffinization**: Put the slides in xylene for 15 minutes;
2. **Hydrate the slides with decrescent alcohol degrees** (100º; 95º; 80º; 70º each during 5 minutes);
3. Pass the slides through distilled water;
4. **Thermic treatment for antigenic retrieval:** in a pressure cooker for 3 minutes in citrate buffer or 3 cycles, 5min. each on the microwave when using the antibody CD3 (pH= 6,0± 0,2);
5. Deep the slides in cold PBS and emerge them in hydrogen peroxide (3%) for 30 minutes;
6. Delimit the tissue in the slides with an hydrophobic pen;
7. Wash the slides 3 times with PBS;
8. **Incubate with universal serum** (Ultra V Block® Thermo Fisher Scientific, Labvison Corporation, Freemont, CA, USA**)** for 5 minutes at room temperature;
9. **Drain off the excess of serum**;
10. **Incubate the slides with the primary antibody:** for 2 hours at room temperature in a horizontal humid chamber;
11. Wash the slides 3 times with PBS;
12. **Incubate the slides with universal biotinylated serum (**biotinylated goat polyvalent plus® antibody; Thermo Fisher Scientific, Labvison Corporation, Freemont, CA, USA**):** for 10 minutes at room temperature;
13. Wash 3 times with PBS;

14. **Incubate the slides with universal streptavidin serum (**Streptavidin-peroxidase Plus® antibody; Thermo Fisher Scientific, Labvison Corporation, Freemont, CA, USA**):** 10 minutes at room temperature;
15. Wash 3 times with PBS;
16. Incubate for 10 minutes with DAB (previously activated with 3% hydrogen peroxide);
17. Rinse slides in running tap water for 10 minutes;
18. **Counterstain** slides with Gill's haematoxylin for 2 minutes;
19. Rinse slides in warm tap water for 10 minutes;
20. **Dehydrate the slides with crescent alcohol degrees** (100º; 95º; 80º; 70º each during 5 minutes);
21. **Clarification:** put the slides in xylene for 10 minutes;
22. **Mount the slides with Entellan® solution.**

The slides are then ready to be analysed at the microscope.

Interpretation of results are the following: 32 case samples available for immunohistochemistry were analysed by two independent individuals first with a 4x objective to overall labelling visualization. The labelling evaluation was made in a 40x objective.

For MIF, a grading scale of intensity and percentage were used. For intensity ranging from 0 to 3 was used for sample assessment, where 0 represented a negative staining, 1 weak, 2 moderate and 3 a strong staining (figure 17). In a percentage grading, 0 represented 0% of the stained cells, 1 where 1-75% of cells were stained and 2 where >75% of the cells were labelled.

For CD68 and CD3 analysis, in each slide, 10 hot-spots areas in the tumor or surrounded stroma were selected, where the labelled lymphocytes and macrophages were in greater number. The CD3 and CD68 cells were counted manually with the help of the ImajeJ Software (version 1.52a). The results were given in total number per 73 cm^2.

3.7. Statistical analysis

Descriptive analyses included absolute count and frequencies, median with respective inter-quartile range (IQR) and mean ± standard deviation (SD) or standard error of mean (SEM). Departure from normality was tested using the Shapiro-Wilk test. Comparison of tumor macrophage and lymphocyte counts, and TILs/TAMs ratio between genotypes were conducted using Kruskall-Wallis or Mann-Whitney tests.

The primary end-points were overall survival (OS) and progression-free survival (PFS). Survival analyses were the primary end-points included initial empirical time-to-event and comparison of Kaplan-Meier estimates, in order to test robustness of *MIF* SNP and clinicopathological variables to explain the outcomes. Then, only variables with $P<0.10$ on univariate analysis were included in multivariate Cox regression proportional hazards to define whether the resulting model would include *MIF* genetic information. Another Cox regression analysis was conducted to estimate the best fitting Cox regression models for PFS and OS end-points in a subgroup analysis including patients stratified as lymphopenic (decrease of lymphocytes in peripheral circulation) and normal lymphocyte count (1.0–3.0×10^9 amount of lymphocytes in peripheral circulation).

Genotype-to-phenotype analysis tested the association between *MIF* genotypes and the immunohistochemical staining intensity and percentage of cells stained, using the Pearson chi-square test, followed by *Eta* analysis as a measure of association.

Statistical analyses were conducted in SPSS17.0 and STATA 12.0.

4. Results

With this study, the main goal was to contribute to the molecular epidemiology/genetics of colorectal cancer. In order to achieve the goals proposed (remote to page 36 in the objectives section), after sample processing and DNA extraction, first we performed a standard PCR so as to evaluate the integrity and quality of the results obtained from sample processing and DNA extraction. We later genotyped all 172 sample via real-time PCR using *Taqman* probes, and 5% (9 samples) of these were sequenced using Sanger sequencing technique.

As to enhance the study's quality and robustness, and to possess more information not only on the genetic part related to the *MIF* gene and the -173 G>C polymorphism, but also on the pathology stand point, we performed the immunohistochemistry technique on 32 tissue samples using MIF antibody, CD68 (macrophages) and CD3 (T-lymphocytes). The number of samples decreased from 172 to 166 because 6 patients had other concomitant cancers and couldn't be included in the statistical analysis. The median follow up time, since CRC diagnosis was 43.8 months (interquartile range, 25.5-67.0).

4.1. Standard PCR

A standard PCR was optimized, using primers designed for the targeted sequence (~762bp amplicon), followed by an electrophoresis in agarose gel, in order to confirm and assess the quality of the results obtained. The primer forward utilized had a melting temperature of 58ºC, and the primer reverse had a melting temperature of 62ºC thus meaning that the recommended annealing temperature was, approximately, of 53ºC. Many conditions were tested and the gel shown in figure 12 contains all the optimizations performed.

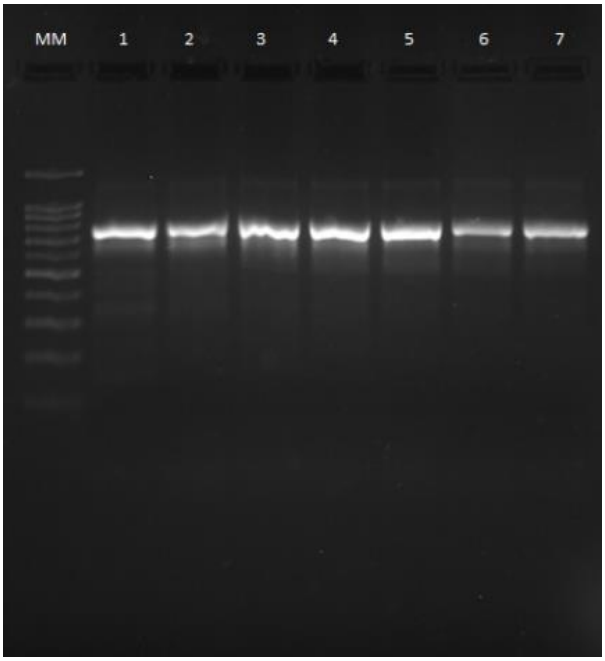
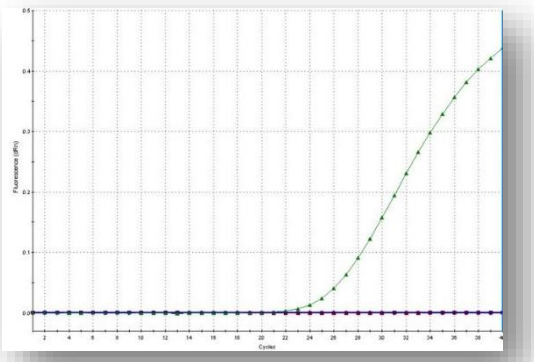


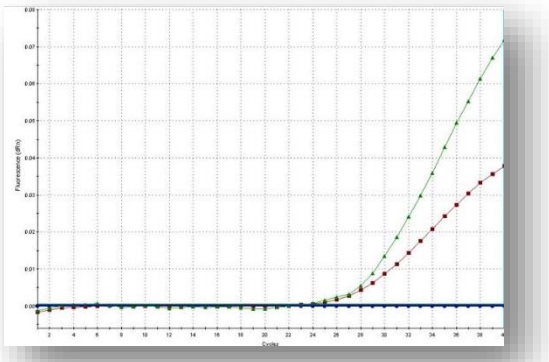
Figure 14: Standard PCR optimizations. MM- Molecular marker (100bp ladder); 1- Ta=55ºC; 35 cycles; 2- Ta= 57ºC; 35 cycles; 3- Ta= 59ºC; 40 cycles; 4- Ta= 60ºC; 40 cycles; 5- Ta= 59ºC; 35 cycles; 6- Ta= 59ºC; 35 cycles and 1µl of DMSO was added to the prepared solution; **7- Ta= 59ºC; 40 cycles and 0,8µl of DMSO was added to the prepared solution (selected conditions for amplification).**

4.2. Real-time PCR and Sanger sequencing

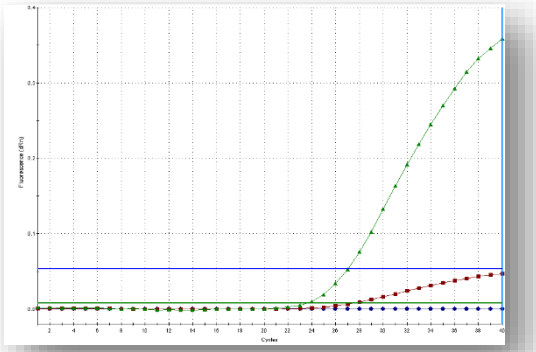
During real-time PCR genotyping analysis of all 172 samples, 120 seem to have GG genotype, 49 the GC genotype and 3 the CC genotype. All three genotypes found during this analysis are represented in figure 15.



A. Representation of GG genotype



B. Representation of CC genotype
**clarification in the text below*



C. Representation of GC genotype

Figure 15: Representation of all 3 genotypes found during real-time PCR. Green curve represents the G allele; red curve represents the C allele, the blue line represents the control dye rox.

Of all analysed samples, 5% of them were sequenced using Sanger technique in order to confirm the results obtained in real-time PCR. Of all 120 samples with GG genotype found in real time PCR, 3 of them were sequenced and confirmed GG genotype found; of all 49 samples with questionable GC genotype, 2 of them were sequenced and confirmed the heterozygous GC genotype; of all 3 samples with GC genotype, 2 of them were sequenced and the results show that these samples are in fact homozygous CC and not GC (figure 16).

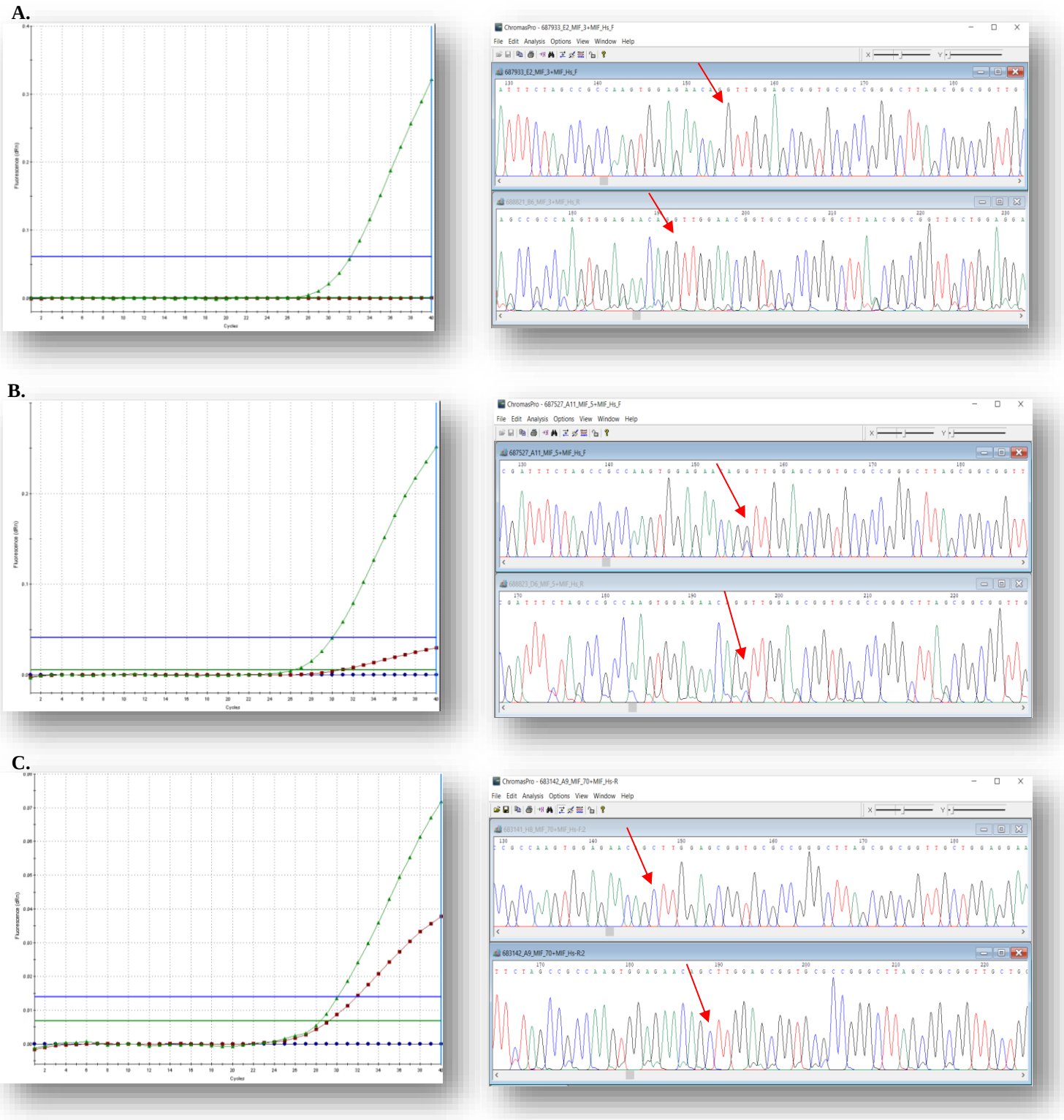


Figure 16: Real-time PCR results vs Sanger sequencing confirmation of the results. A). GG homozygous genotype; B). GC heterozygous genotype; C). CC homozygous genotype.

The probe used in this study seems to have a lack of specificity. It appears that the probe correspondent of the G allele (green curve) is permanently binding to the DNA template non-specifically, thus leading to the constant presence of the green curve.

Via Sanger sequencing we could confirm that samples that presented doubtful real time-PCR results (GC/GG) are in fact heterozygous GC, samples that presented a GC genotype are in fact homozygous CC and GG samples found during real-time PCR were confirmed to be homozygous GG (figure 16).

We obtained the following genotypic frequencies: GG: 70*%;* GC: 28%; CC: 2%; and allelic frequencies: 84% for the G allele and 16% for the C allele.

4.3. Morphological classification and Immunohistochemistry results

In this study all cases were morphologically classified as adenocarcinoma (some examples in the figure 17). In some cases, the neoplastic cells have a strong mucous production. In other cases, ulcer was noted near the apparently normal mucosa. In this cases, necrotic tissues and sometimes bacteria are present. The inflammatory cells were present in higher numbers in the neoplastic stroma, and in the *lamina propria* and submucosa of normal adjacent mucosa. Small amounts of inflammatory cells were observed inside the tumor. In rare cases eosinophils are present too. Surrounded tissues (muscular layer, serosa and peritoneum) have proliferation of neoplastic cells. Lymphoid aggregates are seen in some cases.

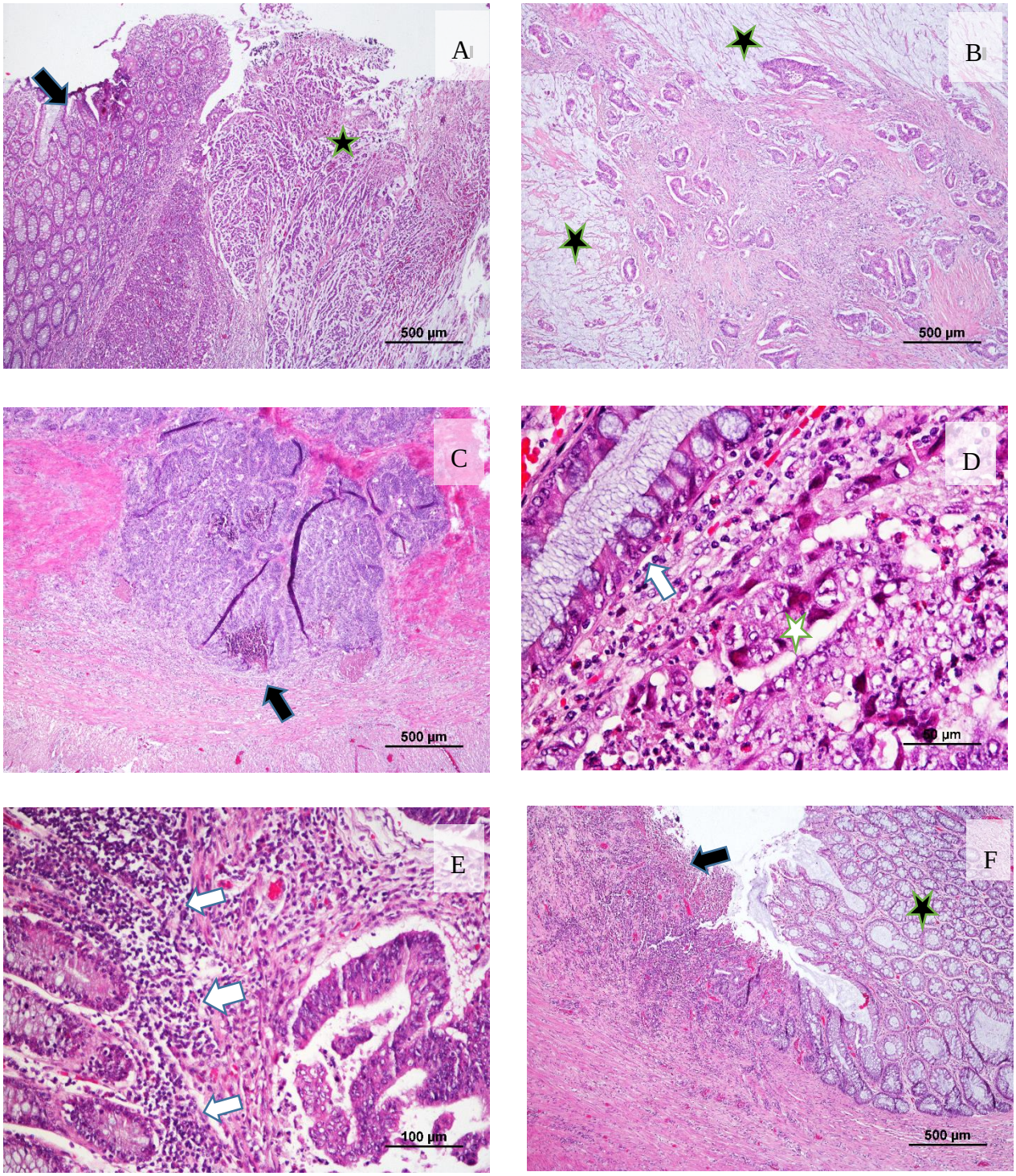


Figure 17: Morphological aspects of colon adenocarcinomas. A) Adenocarcinoma (*) adjacent to normal mucosa (arrow). B) Adenocarcinoma invading the adjacent tissues. Some mucous produced by tumour cells (*). C) Muscular layer invaded by adenocarcinoma (arrow). D) Inflammatory cells in the neoplastic stoma. Some eosinophils are seen in this tumour, adjacent to normal mucosa (arrow) and tumour cells (*). E) Intense inflammatory cells infiltrate (arrow) surrounding the normal mucosa F) An ulcer is present in this adenocarcinoma (arrow), adjacent to normal mucosa (*).

Immunohistochemistry (IHC) was performed using anti-*MIF* antibody. In agreement of the used grading scale (see page 45 of material and methods sections) of the 32 samples analysed, intensity wise, 4 of the cases were negative, 1 case shown weak labelling (grade 1 in the intensity), 11 cases were moderated positive (grade 2) and 16 cases were strongly labelled (grade 3 in the grading scale). From the positivity cases, 6 were labelled in 75% of neoplastic cells and the other 22 cases have positivity in more than 75% of cells (figure 18).

Both intensity and percentage graphs were elaborated, (figure 19).

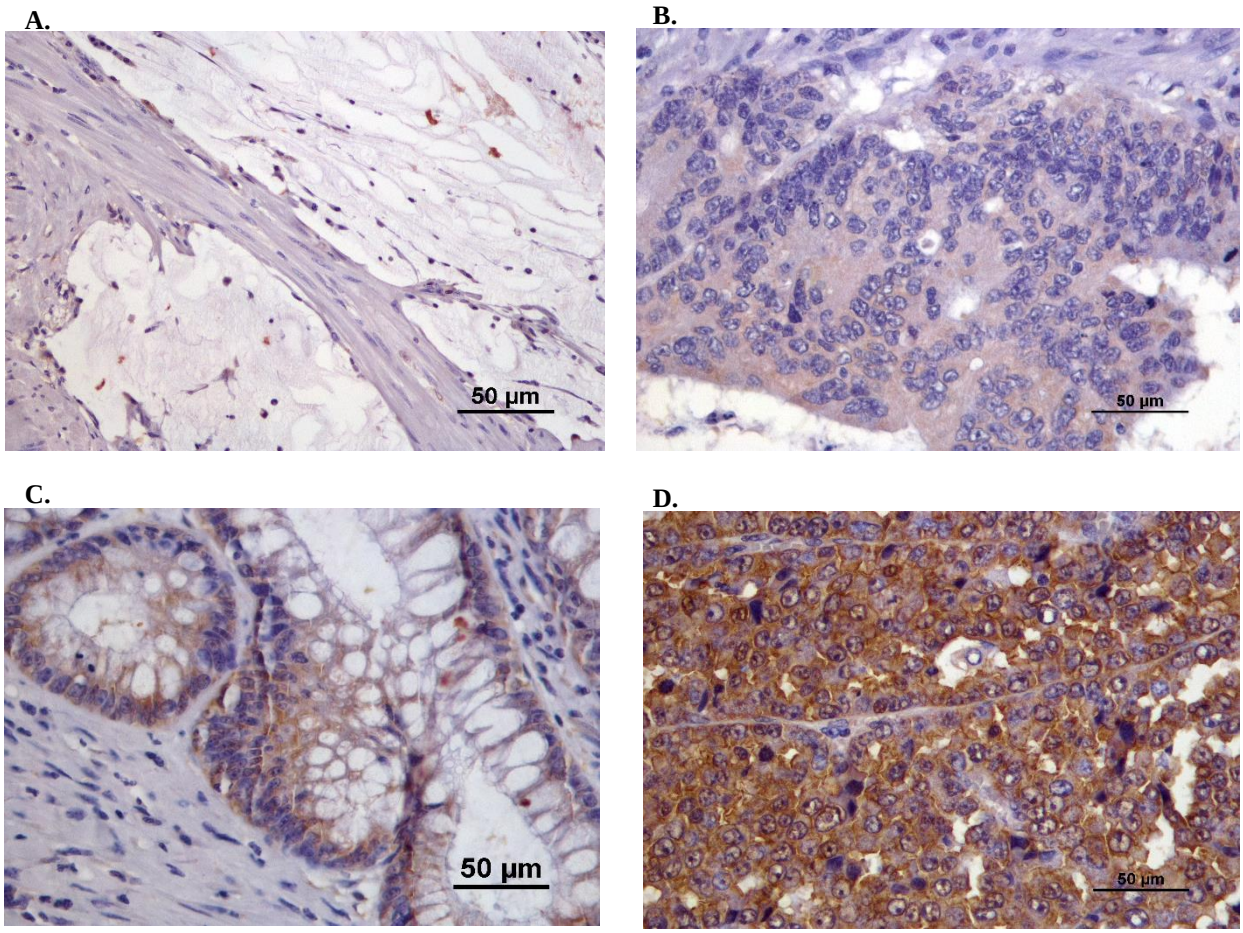


Figure 18: Immunolabelling for *MIF* antibody. A). Negative case; B). Weak positivity; C). Moderate intensity labelling; D). Case with strong intensity. Counterstain with Gill's Hematoxilyn.

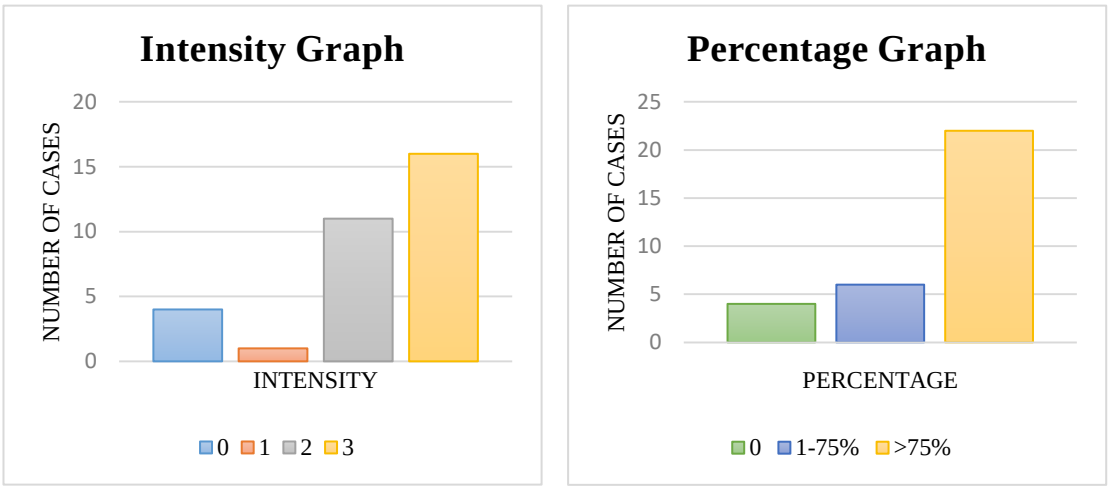


Figure 19: Intensity and percentage graphs referent to immunohistochemistry results.

In an attempt to establish a comparison between the MIF and the macrophage and lymphocyte infiltration in tumors, an antibody for CD68 (macrophage receptor) and for CD3 (T cell co-receptor) was also used, see figure 20 and in sector 3.3 figure 21 and table 9 for the statistical analysis made.

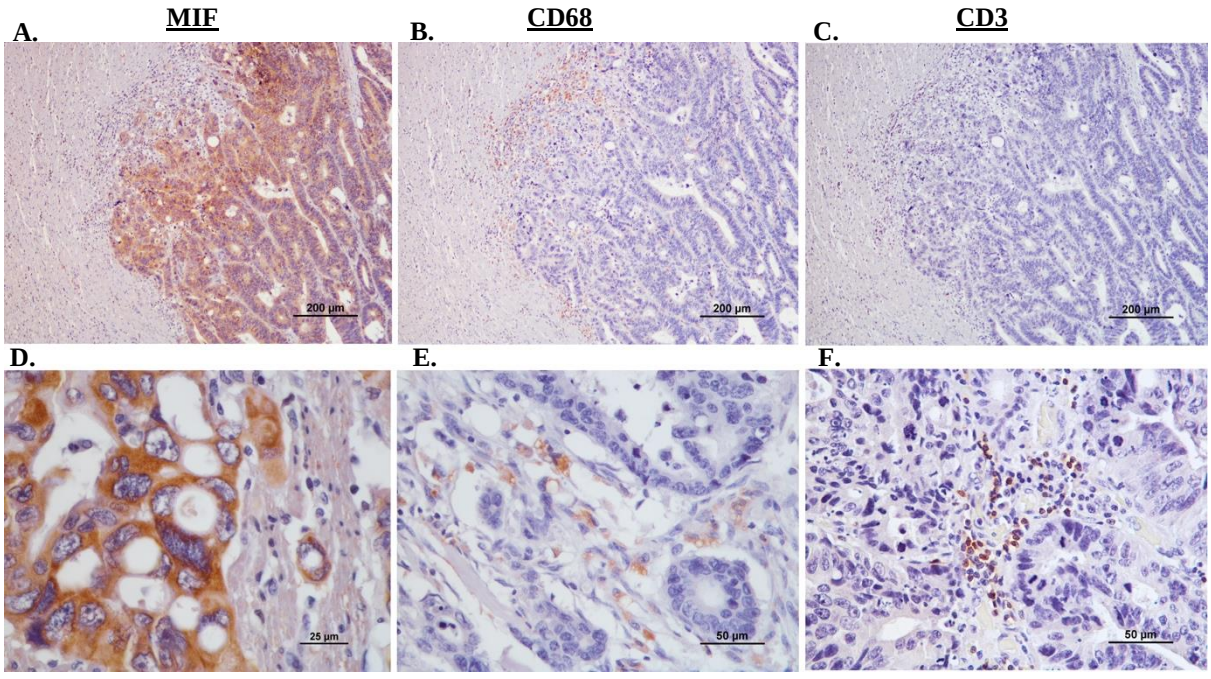


Figure 20: Comparison between *MIF*, CD68 and CD3 staining, using the same sample. Counterstain with Gill's Hematoxilyn. The brown color identifies the positivity. A) MIF in a strong positive tumor; B) Macrophages identified by CD68 antibody, in the stroma, near the tumor; C) A few T lymphocytes were identified in the stroma near the tumor cells; D) MIF as positive labelling inside the cytoplasm of tumor cells. E) Macrophages surrounded the tumor; F) T lymphocytes in the tumoral stroma.

4.4. Statistical Results

As a primary approach, the descriptive clinicopathological data of participants in this study is presented in Table 5.

Table 5: Descriptive data of colorectal cancer patients included in the study (n=166).

	N (freq.)	Median (IQR)	Mean ± SD
Age, yrs	163	65.9 (57.5-74.3)	65.2 ± 11.6
BMI, kg.m^{-2}	104	26.2 (22.9-29.4)	26.3 ± 4.5
Lymphocyte count	160	1395.0 (835.0-1905.0)	1434.1 ± 696.6
Neutrophils/Lymphocytes ratio	160	2.9 (1.9-5.5)	5.8 ± 9.2
Gender			
Female	62 (0.37)		
Male	104 (0.63)		
Localization			
Colon	96 (0.58)		
Rectum	70 (0.42)		
Tumor side			
Right	40 (0.24)		
Left	123 (0.75)		
Synchronous	1 (0.01)		
Clinical stage			
Localized	54 (0.33)		
Locally advanced	77 (0.47)		
Metastatic	32 (0.20)		
Surgery/Radiotherapy			
No	9 (0.06)		
Yes	156 (0.94)		
Surgical margins			
Negative	130 (0.87)		
Positive	19 (0.13)		
Adjuvant chemotherapy			
No	54 (0.33)		
Yes	112 (0.67)		
Palliative chemotherapy			
No	75 (0.45)		
Yes	90 (0.55)		

N (freq.), number of cases and relative frequency; BMI, body mass index; IQR, inter-quartile range; SD, standard deviation; yrs, years.

An empirical analysis using Kaplan-Meier (univariate) plots on the cohort of patients, using as endpoint all-cause of death was elaborated and also a multivariate analysis on the variables that had shown significance on the univariate analysis, all of which is represented in table 6. The variables that showed most significance were lymphocyte % (p= 0,029), tumor side (p=0,035), stage (p=0,008), surgical margins (p=0,013) and adjuvant chemotherapy (p=0,010), as it was expected and no significance was found for the genetic variables.

Furthermore, using disease progression as endpoint the approach was also done, (table 7). The results with more significance were once again lymphocyte % (p=0,005), tumor side (p=0,002), stage (p=<0,001), surgical margins (p=0,001) and adjuvant chemotherapy (p=0,001), with no significance found for the genetic variables.

Table 6: Empirical univariate (Kaplan-Meier) and multivariate (Cox regression) analyses of the endpoint survival for clinicopathological and genetic variables.

	Univariate analysis (Kaplan-Meier)		Multivariate analysis (Cox regression)	
	Mean (95%CI)	**P (Log-rank)**	**HR (95%CI)**	**P**
Age, years				
< median	120.2 (91.8-148.6)			
> median	96.1 (83.2-109.0)	0.560		
Gender				
Female	138.1 (111.2-165.0)			
Male	105.3 (83.6-127.1)	0.296		
Lymphocyte %				
< median	113.0 (85.9-140.0)		Reference	
> median	97.3 (89.1-105.4)	0.029	0.4 (0.2-0.8)	0.017
Localization				
Colon	91.1 (82.0-100.2)			
Rectum	134.9 (114.4-155.3)	0.854		
Tumor side				
Right	74.4 (63.4-85.5)		Reference	
Left	118.2 (97.0-139.3)	0.035 *	0.7 (0.3-1.8)	0.502
Stage				
Localized	142.2 (122.3-162.1)		Reference	
Locally advanced	92.9 (81.1-104.7)		1.6 (0.6-4.3)	0.340
Metastasis	60.2 (47.2-73.3)	0.008	0.7 (0.1-6.5)	0.792
Surgical margins				
Negative	116.5 (87.7-135.6)		Reference	
Positive	59.4 (41.2-77.6)	0.013	3.1 (0.3-28.5)	0.317
Adjuvant chemotherapy				
No	116.0 (91.5-140.6)		Reference	
Yes	119.8 (95.6-144.0)	0.010	0.4 (0.1-1.2)	0.106
***MIF* SNP, additive model**				
GG	--- [a]			
GC	--- [a]			
CC	--- [a]	0.599		
***MIF* SNP, recessive model**				
GG/GC	--- [a]			
CC	--- [a]	0.642		
***MIF* SNP, dominant model**				
GG	110.1 (87.5-132.8)			
GC/CC	143.5 (122.2-164.8)	0.343		

* Breslow test. [a] all CC cases were censored, no statistics were computed. SNP, single nucleotide polymorphism. HR, hazard ratio. 95%CI, 95% confidence interval.

Table 7: Empirical univariate (Kaplan-Meier) and multivariate (Cox regression) analyses of the endpoint progression of disease for clinicopathological and genetic variables.

	Univariate analysis (Kaplan-Meier)		Multivariate analysis (Cox regression)	
	Mean (95%CI)	**P (Log-rank)**	**HR (95%CI)**	**P**
Age, years				
< median	71.6 (51.0-92.2)			
> median	64.8 (52.6-76.9)	0.811		
Gender				
Female	80.9 (60.1-101.7)			
Male	62.6 (52.4-72.8)	0.591		
Lymphocyte %				
< median	67.7 (48.4-87.1)		Referent	
> median	71.6 (60.8-82.5)	0.005	0.5 (0.3-0.9)	0.014
Localization				
Colon	58.0 (47.7-68.4)			
Rectum	77.1 (54.0-100.2)	0.149		
Tumor side				
Right	35.3 (26.0-44.6)		Referent	
Left	82.4 (63.2-101.5)	0.002	0.4 (0.3-0.8)	0.003
Stage				
Localized	71.9 (58.7-85.1)		Referent	
Locally advanced	61.6 (50.5-72.6)		1.4 (0.8-2.5)	0.278
Metastasis	29.6 (21.2-38.0)	<0.001	2.0 (0.7-5.9)	0.215
Surgical margins				
Negative	86.7 (67.9-105.4)		Referent	
Positive	30.3 (19.1-41.6)	0.001	1.3 (0.4-3.8)	0.278
Adjuvant chemotherapy				
No	45.6 (33.2-58.1)		Referent	
Yes	93.4 (74.1-112.7)	0.001	0.7 (0.3-1.4)	0.278
***MIF* SNP, additive model**				
GG	--- [a]			
GC	--- [a]			
CC	--- [a]	0.492		
***MIF* SNP, recessive model**				
GG/GC	--- [a]			
CC	--- [a]	0.262		

[a] all CC cases were censored, no statistics were computed. [b] removed due to collinearity. SNP, single nucleotide polymorphism. HR, hazard ratio. 95%CI, 95% confidence interval.

In order to amplify the strength of the statistical analysis, we stratified the analysis by lymphopenia cut-off as well, for both survival and progression of disease endpoint (tables 8 and 9). Referent to the survival endpoint, the only variable that showed significance was the localization of the tumor (p=0,058). On the other hand, for the progression of disease endpoint, localization of the tumor (p=0,002), tumor side (p=0,002) and stage (p=0,055) all showed significance. Again, no significance was found for the genetic variables.

Table 8: Empirical univariate (Kaplan-Meier) and multivariate (Cox regression) analyses of the endpoint survival for clinicopathological and genetic variables stratified by lymphopenia cut-off.

	Lymphopenia (lymphocyte count < 1000) (n=47)				**Normal lymphocytes (lymphocyte count > 1000) (n=110)**			
	Univariate		**Multivariate**		**Univariate**		**Multivariate**	
	Mean (95%CI)	**P**	**HR (95%CI)**	**P**	**Mean (95%CI)**	**P**	**HR (95%CI)**	**P**
Age, years								
< median	137.0 (99.3-174.7)				90.8 (79.7-102.0)			
> median	61.1 (53.1-69.1)	0.438			91.6 (80.1-103.2)	0.770		
Gender								
Female	122.9 (47.9-197.9)				94.9 (81.7-108.0)			
Male	75.1 (65.2-85.0)	0.898			87.8 (76.7-98.9)	0.306		
Localization								
Colon	64.9 (51.6-78.2)		Referent		96.3 (87.2-105.5)		Referent	
Rectum	156.5 (125.6-187.3)	0.058	0.2 (0.05-0.9)	0.038	67.5 (57.1-78.0)	0.044	2.2 (0.9-5.4)	0.084
Tumor side								
Right	75.0 (58.3-91.7)				75.8 (63.8-87.8)			
Left	134.8 (102.9-166.6)	0.842			92.5 (83.5-101.5)	0.218		
Stage								
Localized	68.9 (50.8-87.0)				85.5 (78.3-92.7)		Referent	
Locally advanced	79.1 (68.8-82.3)				86.9 (72.9-100.9)		2.2 (0.7-7.2)	0.203
Metastasis	50.6 (42.0-59.2)	0.245			60.7 (45.5-75.9)	0.019	3.0 (0.8-11.0)	0.101
Adjuvant chemotherapy								
No	71.0 (54.6-87.4)				62.4 (51.3-73.5)		Referent	
Yes	127.5 (84.3-170.6)	0.643			96.7 (88.0-105.4)	0.003	0.3 (0.1-1.2)	0.092
***MIF* SNP, additive model**								
GG	--- [a]				--- [a]			
GC	--- [a]				--- [a]			
CC	--- [a]				--- [a]	0.549		
***MIF* SNP, recessive model**								
GG/GC	--- [a]							
CC	--- [a]							
***MIF* SNP, dominant model**								
GG	120.5 (79.6-161.4)				87.8 (77.6-98.0)			
GC/CC	76.3 (58.3-94.3)	0.857			83.3 (74.5-92.1)	0.300		

* Breslow test. [a] all CC cases were censored, no statistics were computed. [b] removed due to collinearity. SNP, single nucleotide polymorphism. HR, hazard ratio. 95%CI, 95% confidence interval.

Table 9: Empirical univariate (Kaplan-Meier) and multivariate (Cox regression) analyses of the endpoint progression of disease for clinicopathological and genetic variables stratified by lymphopenia cut-off.

	Lymphopenia (lymphocyte count < 1000) (n=47)				Normal lymphocytes (lymphocyte count > 1000) (n=110)			
	Univariate		Multivariate		Univariate		Multivariate	
	Mean (95%CI)	P	HR (95%CI)	P	Mean (95%CI)	P	HR (95%CI)	P
Age, years								
< median	84.8 (50.5-119.1)				51.0 (42.2-59.9)			
> median	45.1 (32.7-57.5)	0.950			64.9 (50.7-79.0)	0.979		
Gender								
Female	94.7 (45.4-144.0)				58.4 (44.8-72.1)			
Male	52.7 (39.8-65.5)	0.936			59.9 (47.9-72.0)	0.624		
Localization								
Colon	28.2 (18.3-38.0)		Referent		62.7 (51.2-74.2)			
Rectum	111.7 (79.1-144.3)	0.002	0.3 (0.1-1.1)	0.063	48.9 (37.2-60.5)	0.633		
Tumor side								
Right	22.2 (12.4-32.0)		Referent		39.5 (28.5-50.6)		Referent	
Left	103.5 (74.9-132.0)	0.002	0.4 (0.1-1.3)	0.128	64.6 (53.6-75.5)	0.092	0.5 (0.3-0.9)	0.029
Stage								
Localized	44.6 (29.8-59.5)		Referent		59.5 (50.1-69.0)		Referent	
Locally advanced	60.5 (46.0-75.1)		0.6 (0.2-1.6)	0.273	56.0 (41.9-70.0)		1.9 (1.0-3.7)	0.068
Metastasis	25.4 (13.6-37.3)	0.055	3.2 (1.0-10.7)	0.060	31.3 (20.8-41.8)	0.002	1.7 (0.7-4.0)	0.204
Adjuvant chemotherapy								
No	46.6 (31.6-61.7)				33.1 (24.5-41.7)		Referent	
Yes	96.1 (66.7-25.6)	0.651			71.5 (60.0-83.1)	<0.001	0.4 (0.2-0.9)	0.023
***MIF* SNP, additive model**								
GG	--- [a]				--- [a]			
GC	--- [a]				--- [a]	0.582		
CC	--- [a]	0.552			--- [a]			
***MIF* SNP, recessive model**								
GG/GC	--- [a]							
CC	--- [a]	0.621						
***MIF* SNP, dominant model**								
GG	98.1 (68.9-127.3)				61.2 (49.3-73.1)			
GC/CC	43.8 (24.5-63.2)	0.384			51.3 (41.3-61.3)	0.681		

* Breslow test. [a] all CC cases were censored, no statistics were computed. [b] removed due to collinearity. SNP, single nucleotide polymorphism. HR, hazard ratio. 95%CI, 95% confidence interval.

In an IHC point of view, genotype-phenotype analyses was performed between TAMs, TILs and *MIF* -173 G>C genotypes using Mann-Whitney U tests (figure 22) where no significance was found.

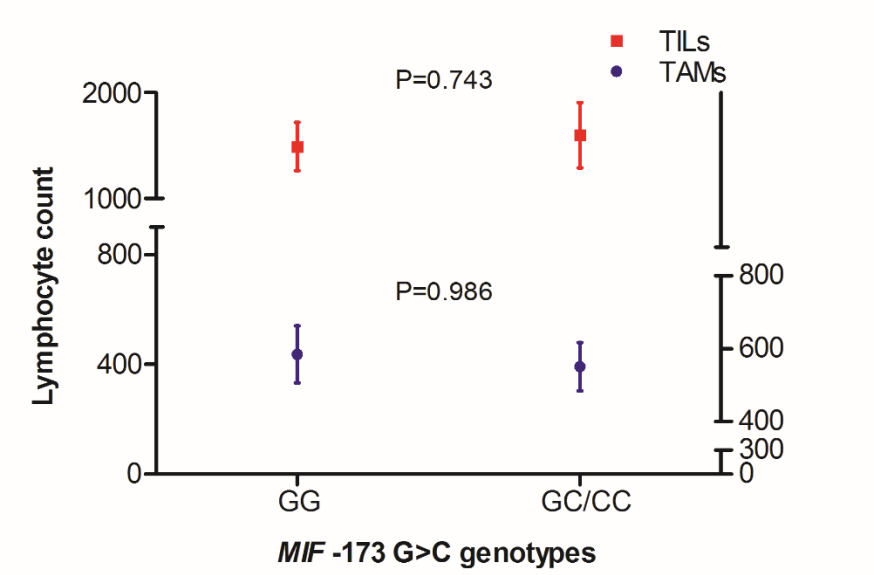


Figure 21: Genotype-phenotype analyses. Comparison of tumor macrophages and lymphocytes between *MIF* -173 G>C genotypes. Data is presented as mean±SEM; TILs, tumor infiltrating lymphocytes; TAMs, tumor associated macrophages. Mann-Whitney U tests were used to compare the differences between genotypes.

On the other hand, immunohistochemical intensity and percentage of cells stained with *MIF* were then correlated with *MIF* -173 G>C genotypes using Fisher's exact and Pearson chi-square test (table 10). It was found significance in the additive model variable (p=0,044) when referent to the intensity of staining. When referent to the percentage of cells stained, both additive (p=0,001) and recessive model (p=0,071) showed significance.

Table 10. Association between *MIF* genotypes and immunohistochemical intensity and percentage of cells stained with *MIF*.

	Intensity of staining					**Percentage of cells stained**			
	Negative/weak	**Moderate/strong**	**P ***	***Eta***		**Negative (0%)**	**Positive (>1%)**	**P ***	***Eta***
Additive model									
GG	2 (0.50)	15 (0.63)				1 (0.50)	16 (0.62)		
GC	1 (0.25)	9 (0.37)				0 (0.0)	10 (0.38)		
CC	1 (0.25)	0 (0.0)	0.044**	0.472		1 (0.50)	0 (0.0)	0.001**	0.702
Recessive model									
GG/GC	3 (0.75)	24 (1.00)				1 (0.50)	26 (1.00)		
CC	1 (0.25)	0 (0.0)	0.143	0.471		1 (0.50)	0 (0.0)	0.071	0.694
Dominant model									
GG	2 (0.50)	15 (0.63)				1 (0.50)	16 (0.62)		
GC/CC	2 (0.50)	9 (0.37)	1.000	0.090		1 (0.50)	10 (0.38)	1.000	0.061

* Fisher's exact test; ** Pearson chi-square test. The strength of association was tested using the *Eta* test.

5. Discussion

All over the world, in both economically developed and developing countries, cancer has become a major burden on society. Due to aging of the worldwide population as well as the increase in established risk factors such as smoking, obesity, sedentary lifestyles, the overall incidence and prevalence of cancer is increasing at a fast passed rate (Douaiher *et al*., 2017).

Colorectal cancer is the second most incident and most frequent cause of death in Europe (Ferlay *et al.*, 2018). CRC usually develops in the lining of the colon (large intestine) or rectum, commonly beginning by forming polyps (a benign or non-cancerous form) and turning rapidly into "full-blown" cancer via a multistep process involving a succession of genetic alterations (Das *et al*., 2017; Danese & Montagnana, 2017).

The CRC–immune microenvironment consists of different types of cells which include malignant cells, innate immune cells (granulocytes, mast cells [MCs], dendritic cells [DC] and macrophages), adaptive immune cells (T and B cells), fibroblasts and endothelial cells. These cells, either by themselves or associated with other cell types, contribute to the inflammatory and/or immunological status of tumor tissues via cell-to-cell contact and/or cytokine/chemokine production (Koi & Carethers, 2017). Direct or indirect interaction by autocrine and/or paracrine signalling of cytokines and chemokines via intercellular communication, controls and regulates tumor growth and progression. Infiltration of inflammatory and immune cells such as lymphocytes and macrophages that participate in defence against injury and infection can also persuade tumor progression and metastasis (Kitano *et al.*, 2017).

Macrophage migration inhibitory factor, also known as *MIF* or glycosylation-inhibiting factor, is a secreted, homotrimeric, multifunctional pro-inflammatory cytokine that modulates macrophage and T cell function and is an important regulator of host response to infection (Ensembl, 2018; Renner, Roger, & Calandra, 2005). MIF is expressed at sites of inflammation, which suggests that it plays a role in regulating macrophage function in host defence. MIF is produced by the pituitary gland and is found in monocytes, macrophages, differentiating immunological cells in the eye lens and brain, and fibroblasts. MIF promotes the systemic inflammatory response by counter-regulating glucocorticoid-mediated inhibition of immune-cell activation and pro-inflammatory cytokine production (Ietta *et al.*, 2018; Lechien *et al*., 2017).

MIF, for example, is released by both macrophages and DC after stimulation with LPS, Gram-positive exotoxins, glucocorticoid or pro-inflammatory cytokines. MIF then acts in both autocrine and paracrine fashions, activating cells to produce pro-inflammatory cytokines, and counteracting glucocorticoid activity at the site of infection. MIF directly or indirectly promotes production of a large panel of inflammatory cytokines (such as TNF-α, IFN-γ, IL-1β, IL-2, IL-6, IL-8), nitric oxide, prostaglandins and several matrix metalloproteases (Popa *et al*., 2006).As a pro-inflammatory mediator, MIF has been shown to be implicated in the pathogenesis of severe sepsis and septic shock, acute respiratory distress syndrome, and several other inflammatory and autoimmune diseases, including rheumatoid arthritis, glomerulonephritis, and inflammatory bowel diseases (De Jong *et al.*, 2001; Gregersen & Bucala, 2003; Renner, Roger & Calandra, 2005).

Strong evidence has been presented linking chronic inflammation to the onset and pathogenesis of cancer. In several studies, MIF is described as occupying a central role in the inflammatory pathway, can promote tumor growth and viability by modulating immune responses and has been implicated and supporting the angiogenesis and metastasis of many cancer phenotypes *(*Ramireddy *et al*., 2014; O'Reilly *et al*., 2016). A few experiments suggested that *MIF* mRNA and MIF protein are overexpressed in a number of cancers (Zhang *et al.*, 2017).

MIF gene, has a SNP (G to C substitution) in the 5'-flanking region at position -173G/C (rs755622) which has been reported to be associated with susceptibility to adult inflammation and risk of colorectal cancer (Dessein *et al*., 2010; Morris *et al*., 2014).

Here, 172 DNA samples were isolated from peripheral blood of CRC patients and genotyped via real time PCR using *Taqman* probes. We obtained the following genotypic frequencies: GG: 70*%;* GC: 28%; CC: 2%; and allelic frequencies of 84% for the G allele and 16% for the C allele. These results are in agreement with the literature that shows a worldwide allelic frequency of G: 73% and C: 27% for the variant rs755622, and a European allelic frequencies of G: 81% and C: 19% (Ensembl, 2018). Of the 166 patients eligible for data recovery, 62 were female and 104 were male, and for most (96/166), the adenocarcinoma was located in colon and 70/166 in rectum.

Other studies, such as those performed by Renner *et al*. (2005) and Ramireddy *et al.* (2014) submitted their samples to genotyping of the functional *MIF* polymorphism -173 G/C and obtained similar allele and genotypic frequencies.

We later proceeded to elaborate a robust statistical analysis, in order to consolidate all the information obtained during research.

An empirical analysis using Kaplan-Meier plots on the cohort of patients, using as endpoint all-cause of death, revealed that lymphocyte percentage (P=0.029), tumor side (P=0.035), stage of disease (P=0.008), surgical margins (P=0.013) and adjuvant chemotherapy (P=0.010) are each associated with all-cause mortality. The Kaplan-Meier curve analysis of *MIF* SNP showed that the dominant model was not associated with all-cause mortality. Additive and recessive models were not analysed since only 3 patients were C homozygous and none of them died during follow up. On multivariate analysis, increased lymphocyte % protected for all-cause death, whereas all other variables with significance on univariate analysis were not relevant for prognosis. Furthermore, using disease progression as endpoint it showed no association of *MIF* dominant model with progression (P=0.842). On multivariate, increased lymphocyte % and tumor localization on the left side were associated with protection for disease progression. Then, we stratified the analysis by lymphopenia cutoff (1000 lymphocytes), and no association for *MIF* SNP was observed for both mortality (P>0.05) or disease progression (P>0.05).

After immunohistochemistry analysis it is shown on figure 21 that tumor infiltrated lymphocytes (TILs) and tumor associated macrophages (TAMs) did not varied according to genotypes of *MIF* dominant model. We observed a significantly higher macrophage count in tumors located at the colon (633.9±55.8) compared to rectum (450.9±100.1) (P=0.012), whereas no differences between anatomical location were found for infiltrating lymphocyte count (P=0.911). Concerning to the MIF IHC, most cases had a high percentage of positive tumor cells (68,75% of the cases had more than 75% labelled cells), whereas for intensity 84,4% of the cases presented a strong staining intensity (level 2/3), which seems to indicate a high level of MIF in these tumors cells. The analysis of phenotype-by-genotype, showed a positive association between *MIF* additive and recessive models with the percentage of MIF-stained cells in the tumor microenvironment. De Benedetti *et al.* (2003) studied the functional and prognostic relevance of the –173 G-to-C polymorphism in *MIF* gene of patients with systemic-onset juvenile idiopathic arthritis, and showed that the C allele is somewhat related to the increase of MIF in circulation and therefore responsible for a poorer prognosis. Renner *et al*. (2005) in an attempt to associate *MIF* polymorphisms and susceptibility to inflammatory diseases were able to correlate the presence of -173*C allele with an increased risk of

developing disease. The authors hypothesised that the C allele may provide a binding site for transcription factors such as AP4.

Mittelbronn *et al*. (2011), Ramireddy *et al.* (2014), Kuai *et al.* (2015) and O'Reilly *et al*. (2016), using different techniques and research methods, agreed that higher levels of MIF appear to be related to higher risk for disease, higher inflammatory response and reduced patient survival, and that the presence of the -173*C allele in patients with many diseases as well as CRC seem to have a negative impact such as increased risk for diagnosis, for disease progression, reduced response to treatment, poorer prognosis and reduced overall survival. Conversely, Choi *et al*. (2012) and Lechien *et al*. (2017), suggest that MIF promotes tumor growth by increasing Treg generation and upregulating IL-2 production. They also indicated that the inhibition of MIF pathways in patients with various types of diseases will lead to better treatment response, better prognosis. Nevertheless, some concerns remain about the heterogeneity of MIF binding patterns and the lack of knowledge about its biological role.

In comparison with the above mentioned studies, our findings demonstrated lack of association between the *MIF* -173G>C polymorphism with each endpoint, overall survival or disease progression, in patients with CRC. The reduced number of participants might have influenced and diminished the statistical strength, preventing the acquisition of statistical significance. The number of subjects with the -173*C allele was very low, influencing the analysis; with increasing sample size more cases with the C allele could be found, and increased proportion of C-allele carriers would be found and add power to endpoint analysis.

Concerning the immunohistochemistry findings, they should be considered as a result of preliminary analysis. Here, as well, the low sample power may have restrained from finding significant genotype-to-phenotype associations using a robust statistical analysis. Several situations could influence our IHC data which we cannot control or correlate. For instance, treatment performed prior to surgery or type of treatment may inhibit the presence of inflammatory cells within the tumor. The individual's immune system might be correlated with the psychological state and this is also an uncontrollable factor. The fact that only one slide was chosen for IHC and we did not know if those slides best represented the tumor for this evaluation once again couldn't be controlled.

Considering that the levels of MIF were high in tumors of most patients which further corroborates with findings from other studies such as Mittelbronn *et al.* (2011), Ietta *et al.* (2018), Ranganathan *et al.* (2017), the number of samples should be increased in order to

correlate significantly not only between MIF, TILs and TAMs but also with polymorphism genotypes found in CRC patients who participated in this study.

Briefly, the overall results obtained in this study do not allow us to draw of any significant conclusions about the association between the *MIF* polymorphism studied and CRC patient's overall death and progression of disease. And neither do the results obtained in IHC leaving us with only assumptions and hypothesis on the relation of MIF with TILs, TAMs and the polymorphism and on *MIF*'s biological function and what it can or can't influence during disease.

As a starting point, in this project we were able to create a platform with all clinicopathological, genetic and immunohistochemistry data, providing a wide dynamic in research that might benefit from it in the future.

6. Conclusion

All over the world, in both economically developed and developing countries, cancer has become a major burden on society. The fact that colorectal cancer has an elevated incidence in Portugal was the driving force for the present study that aimed to contribute with clinicopathological and genetic knowledge to the scientific community and ultimately to the clinical practice.

We chose to study the *MIF* gene because of its biological importance in inflammatory pathways, tumor growth and viability by modulation the immune responses and due to its potential implication in angiogenesis and metastasis of many cancer phenotypes. We chose to study the macrophage inhibitory factor functional polymorphism rs755622 because of its previous associations with CRC and other diseases, in other studies.

In the literature, there are few studies that involve three different areas and search for an association between clinicopathological, genetic and immunohistochemistry data, especially in colorectal cancer.

Genotyping techniques used in this research were optimized and allowed to attain the allelic and genotypic frequencies of our studied population (G allele: 84%, C allele: 16%; GG: 70%; GC: 28%; CC: 2%).

Immunohistochemistry technique was also optimized which will be of great importance for the continuation of this project.

Concerning the cause of death and disease progression endpoints, relating to clinicopathological data, the variables that showed most significance were lymphocyte percentage, tumor side, stage, surgical margins and adjuvant chemotherapy. As for a genetic stand point, no significance was found relating either of the endpoints studied.

Relating to Immunohistochemistry, genotype-phenotype analyses was performed between TAMs, TILs and *MIF* -173 G>C genotypes, and no significance was found. However, concerning intensity and percentage of cells stained with MIF were then correlated with *MIF* -173 G>C genotypes and it was found significance in the additive and recessive model variables when referent to the intensity of staining and percentage of cells stained, respectively.

In the future, it would be important to increase the number of samples as to improve the robustness of the study. It also would be interesting to include a healthy control group of

individuals with coincidental factors such as age, sex, living area, etc., as to possibly do case/control studies. Likewise, other genes could be of interest to further study, such as genes related to inflammation, tumor microenvironment and tumor progression.

This is an innovative study that intends to be expanded, hoping to produce more robust results and to present data / conclusions that may be useful for clinical practice in the future.

7. References

A. Heid, Ch., Stevens, J., J. Livak, K., & Williams, P. M. (1996). Real Time Quantitative PCR. *Genome Research*, ***6,*** 986–993.

Amersi, F., Agustin, M., & Ko, C. Y. (2005). Colorectal cancer: Epidemiology, risk factors, and health services. *Clinics in Colon and Rectal Surgery*, ***18(***3), 133–140. http://doi.org/10.1055/s-2005-916274

American Cancer Societyhttps://www.cancer.org/cancer/colon-rectal-cancer/treating.html. Consulted in 2018

Amicarella, F., Muraro, M. G., Hirt, C., Cremonesi, E., Padovan, E., Mele, V., Iezzi, G. (2017). Dual role of tumour-infiltrating T helper 17 cells in human colorectal cancer. *Gut*, ***66***(4), 692–704. http://doi.org/10.1136/gutjnl-2015-310016

Andreyev, H. J. N., Norman, A. R., Cunningham, D., Oates, J., Dix, B. R., Iacopetta, B. J., Urosevic, N. (2001). Kirsten ras mutations in patients with colorectal cancer: The "RASCAL II" study. *British Journal of Cancer*, ***85***(5), 692–696. http://doi.org/10.1054/bjoc.2001.1964

Armaghany, T., Wilson, J. D., Chu, Q., & Mills, G. (2012). Genetic alterations in colorectal cancer. *Gastrointestinal Cancer Research : GCR*, ***5***(1), 19–27.

Balkwill, F., Charles, K. A., & Mantovani, A. (2005). Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell*, ***7***(3), 211–217. http://doi.org/10.1016/j.ccr.2005.02.013

Balkwill, F., & Mantovani, A. (2001). Inflammation and cancer: Back to Virchow? *Lancet*, ***357***(9255), 539–545. http://doi.org/10.1016/S0140-6736(00)04046-0

Baretti, M., & Azad, N. S. (2018). The role of epigenetic therapies in colorectal cancer. *Current Problems in Cancer*, 1–19. http://doi.org/10.1016/j.currproblcancer.2018.03.001

Becht, E., De Reyniès, A., Giraldo, N. A., Pilati, C., Buttard, B., Lacroix, L., Fridman, W. H. (2016). Immune and stromal classification of Colorectal cancer is associated with molecular subtypes and relevant for precision immunotherapy. *Clinical Cancer Research*, ***22***(16), 4057–4066. http://doi.org/10.1158/1078-0432.CCR-15-2879

Beswick, E. J., Pinchuk, I. V., Suarez, G., Sierra, J. C., & Reyes, V. E. (2006). Helicobacter pylori CagA-Dependent Macrophage Migration Inhibitory Factor Produced by Gastric Epithelial Cells Binds to CD74 and Stimulates Procarcinogenic Events. *The Journal of Immunology*, ***176***(11), 6794–6801. http://doi.org/10.4049/jimmunol.176.11.6794

Bindea, G., Mlecnik, B., Tosolini, M., Kirilovsky, A., Waldner, M., Obenauf, A. C., Galon, J. Ô. (2013). Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity*, ***39***(4), 782–795. http://doi.org/10.1016/j.immuni.2013.10.003

Bolan, P. M., & Wen Wee Ma. (2017). Immunotherapy for colorectal cancer. *Cancers*, ***9***(50), 247–255. http://doi.org/10.3390/cancers9050050

Calandra, T., & Roger, T. (2003). Macrophage migration inhibitory factor: A regulator of innate immunity. *Nature Reviews Immunology*, ***3***(10), 791–800. http://doi.org/10.1038/nri1200

Calon, A., Lonardo, E., Berenguer-Llergo, A., Espinet, E., Hernando-Momblona, X., Iglesias, M., Batlle, E. (2015). Stromal gene expression defines poor-prognosis subtypes in colorectal cancer. *Nature Genetics*, ***47***(4), 320–329. http://doi.org/10.1038/ng.3225

Camus, M., Tosolini, M., Mlecnik, B., Pages, F., Kirilovsky, A., Berger, A., Galon, J. (2009). Coordination of intratumoral immune reaction and human colorectal cancer recurrence. *Cancer Research*, ***69***(6), 2685–2693. http://doi.org/10.1158/0008-5472.CAN-08-2654

Candido, J., & Hagemann, T. (2012). Cancer-related inflammation. *J Clin Immunol*, ***454***(7203), 436–444. http://doi.org/10.1007/s10875-012-9847-0

Cardoso, J., Boer, J., Morreau, H., & Fodde, R. (2007). Expression and genomic profiling of colorectal cancer. *Biochimica et Biophysica Acta - Reviews on Cancer*, ***1775***(1), 103–137. http://doi.org/10.1016/j.bbcan.2006.08.004

Carlberg, C., & Molnár, F. (2018). Chromatin Remodelers and Organizers. In *Human epigenomics*, 103–120. Springer Nature Singapore. http://doi.org/10.1007/978-981-10-7614-5_7

CDChttps://www.cdc.gov/cancer/colorectal/basic_info/risk_factors.htm. Consulted in 2018

Choi Susanna, Hang-Rae Kim, Lin Leng, Insoo Kang, William L. Jorgensen, C.-S., & Cho, Richard Bucala, and W.-U. K. (2012). Role of macrophage migration inhibitory factor in the regulatory T cell response of tumor-bearing mice. *J Clin Immunol*, ***189***(8), 3905–3913. http://doi.org/10.4049/jimmunol.1102152

Clapier, C. R., Iwasa, J., Cairns, B. R., & Peterson, C. L. (2017). Mechanisms of action and regulation of ATP-dependent chromatin-remodelling complexes. *Nature Reviews Molecular Cell Biology*, ***18***(7), 407–422. http://doi.org/10.1038/nrm.2017.26

Dalton, D. K., & Noelle, R. J. (2012). The roles of mast cells in anticancer immunity. *Cancer Immunol Immunother*, ***61***(9). http://doi.org/10.1007/s00262-012-1246-0

Danese, E., & Montagnana, M. (2017). Epigenetics of colorectal cancer: emerging circulating diagnostic and prognostic biomarkers. *Annals of Translational Medicine*, ***5***(13), 279–279. http://doi.org/10.21037/atm.2017.04.45

Das, V., Kalita, J., & Pal, M. (2017). Predictive and prognostic biomarkers in colorectal cancer: A systematic review of recent advances and challenges. *Biomedicine and Pharmacotherapy*, ***87***, 8–19. http://doi.org/10.1016/j.biopha.2016.12.064

David, J. R. (1966). Delayed hypersensitivity in vitro: its mediation by cell-free substances formed by lymphoid cell-antigen interaction. *Proceedings of the National Academy of Sciences*, ***56***(1), 72–77. http://doi.org/10.1073/pnas.56.1.72

De Benedetti, F., Meazza, C., Vivarelli, M., Rossi, F., Pistorio, A., Lamb, R., Martini, A. (2003). Functional and prognostic relevance of the -173 polymorphism of the macrophage migration inhibitory factor gene in systemic-onset juvenile idiopathic arthritis. *Arthritis and Rheumatism*, ***48***(5), 1398–1407. http://doi.org/10.1002/art.10882

De Jong, Y. P., Abadia-Molina, A. C., Satoskar, A. R., Clarke, K., Rietdijk, S. T., Faubion, W. A., Terhorst, C. (2001). Development of chronic colitis is dependent on the cytokine MIF. *Nature Immunology*, ***2***(11), 1061–1066. http://doi.org/10.1038/ni720

De La Cruz-Merino, L., Henao Carrasco, F., Vicente Baz, D., Nogales Fernández, E., Reina Zoilo, J. J., Codes Manuel De Villena, M., & Pulido, E. G. (2011). Immune microenvironment in colorectal cancer: A new hallmark to change old paradigms. *Clinical and Developmental Immunology*. http://doi.org/10.1155/2011/174149

De Simone, M., Arrigoni, A., Rossetti, G., Gruarin, P., Ranzani, V., Politano, C., Pagani, M. (2016). Transcriptional Landscape of Human Tissue Lymphocytes Unveils Uniqueness of Tumor-Infiltrating T Regulatory Cells. *Immunity*, ***45***(5), 1135–1147. http://doi.org/10.1016/j.immuni.2016.10.021

Dessein, A. F., Stechly, L., Jonckheere, N., Dumont, P., Monté, D., Leteurtre, E., Huet, G. (2010). Autocrine induction of invasive and metastatic phenotypes by the MIF-CXCR4 axis in drug-resistant human colon cancer cells. *Cancer Research*, ***70***(11), 4644–4654. http://doi.org/10.1158/0008-5472.CAN-09-3828

Douaiher, J., Ravipati, A., Grams, B., Chowdhury, S., Alatise, O., & Are, C. (2017). Colorectal cancer—global burden, trends, and geographical variations. *Journal of Surgical Oncology*, ***115***(5), 619–630. http://doi.org/10.1002/jso.24578

Elkins, K. M. (2013). DNA Extraction. *Forensic DNA Biology*, 39–52. http://doi.org/10.1016/B978-0-12-394585-3.00004-3

Esemblhttps://www.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000240972;r=22:23894004-23895227. Consulted in 2018

Esemblhttp://www.ensembl.org/Homo_sapiens/Variation/Population?db=core;v=rs755622;vdb=variation. Consulted in 2018

Fearon, E. F., & Vogelstein, B. (1990). A genetic model for Colorectal Tumorigenesis. *Cell*, ***61***(1), 759–767.

Ferlay, J., Colombet, M., Soerjomataram, I., Dyba, T., Randi, G., Bettio, M., Bray, F. (2018). Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018. *European Journal of Cancer*. http://doi.org/10.1016/j.ejca.2018.07.005

Fingerle-Rowson, G., Petrenko, O., Metz, C. N., Forsthuber, T. G., Mitchell, R., Huss, R., Bucala, R. (2003). The p53-dependent effects of macrophage migration inhibitory factor revealed by gene targeting. *Proceedings of the National Academy of Sciences*, ***100***(16), 9354–9359. http://doi.org/10.1073/pnas.1533295100

Galdiero, M. R., Garlanda, C., Jaillon, S., Marone, G., & Mantovani, A. (2013). Tumor associated macrophages and neutrophils in tumor progression. *Journal of Cellular Physiology*, ***228***(7), 1404–1412. http://doi.org/10.1002/jcp.24260

Galon, J., Costes, A., Sanchez-Cabo, F., Kirilovsky, A., Mlecnik, B., Lagorce-Pagès, C., Pagès, F. (2006). Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science*, ***313***(5795), 1960–1964. http://doi.org/10.1126/science.1129139

Galon, J., Fridman, W. H., & Pages, F. (2007). The adaptive immunologic microenvironment in colorectal cancer: A novel perspective. *Cancer Research*, ***67***(5), 1883–1886. http://doi.org/10.1158/0008-5472.CAN-06-4806

Galon, J., Pagès, F., Marincola, F. M., Angell, H. K., Thurin, M., Lugli, A., Fox, B. A. (2012). Cancer classification using the Immunoscore: A worldwide task force. *Journal of Translational Medicine*, ***10***(1). http://doi.org/10.1186/1479-5876-10-205

Gillen, C. D., Walmsley, R. S., Prior, P., Andrews, H. A., & Allan, R. N. (1994). Ulcerative colitis and Crohn's disease: A comparison of the colorectal cancer risk in extensive colitis. *Gut*, ***35***(11), 1590–1592. http://doi.org/10.1136/gut.35.11.1590

Goldbohm, R. A., van den Brandt, P. A., van 't Veer, P., Brants, H. A. M., Dorant, E., Sturmans, F., & Hermus, R. J. J. (1994). A Prospective Cohort Study on the Relation between Meat Consumption and the Risk of Colon Cancer. *Cancer Research*, ***54***(3), 718–723. http://doi.org/10.1155/2015/812961

Gregersen, P. K., & Bucala, R. (2003). Macrophage migration inhibitory factor, MIF alleles, and the genetics of inflammatory disorders: Incorporating disease outcome into the definition of phenotype. *Arthritis and Rheumatism*, ***48***(5), 1171–1176. http://doi.org/10.1002/art.10880

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell*, ***100***(1), 57–70. http://doi.org/10.1007/s00262-010-0968-0

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell*, ***144***(5), 646–674. http://doi.org/10.1016/j.cell.2011.02.013

Ietta, F., Ferro, E. A. V., Bevilacqua, E., Benincasa, L., Maioli, E., & Paulesu, L. (2018). Role of the Macrophage Migration Inhibitory Factor (MIF) in the survival of first trimester human placenta under induced stress conditions. *Scientific Reports*, ***8***(1), 12150. http://doi.org/10.1038/s41598-018-29797-6

Järvinen, R., Knekt, P., Hakulinen, T., Rissanen, H., & Heliövaara, M. (2001). Dietary fat, cholesterol and colorectal cancer in a prospective study. *British Journal of Cancer*, ***85***(3), 357–361. http://doi.org/10.1054/bjoc.2001.1906

Jass, J. R. (1986). Lymphocytic infiltration and survival in rectal cancer. *Journal of Clinical Pathology*, ***39***(6), 585–589. http://doi.org/10.1136/jcp.39.6.585

Keating, S. T., van Diepen, J. A., Riksen, N. P., El-Osta, A., & Keating SamKeating, S. T. (2018). Epigenetics in diabetic nephropathy, immunity and metabolism, 6–20. http://doi.org/10.1007/s00125-017-4490-1

Kitano, Y., Okabe, H., Yamashita, Y. I., Nakagawa, S., Saito, Y., Umezaki, N., Baba, H. (2017). Tumour-infiltrating inflammatory and immune cells in patients with extrahepatic cholangiocarcinoma. *British Journal of Cancer*, ***118***(2), 171–180. http://doi.org/10.1038/bjc.2017.401

Koi, M., & Carethers, J. M. (2017). The colorectal cancer immune microenvironment and approach to immunotherapies. *Future Oncology*, ***13***(18), 1633–1647. http://doi.org/10.2217/fon-2017-0145

Koi, M., Garcia, M., Choi, C., Kim, H.-R., Koike, J., Hemmi, H., & Al., E. (2016). Microsatellite Alterations with Allelic Loss at 9p24.2 Signify Less-aggressive Colorectal Cancer Metastasis. *Gastroenterology*, ***150***(4), 944–955. http://doi.org/10.1053/j.gastro.2015.12.032

Kuai, S. G., Ou, Q. F., You, D. H., Shang, Z. B., Wang, J., Liu, J., Huang, L. H. (2015). Functional polymorphisms in the gene encoding macrophage migration inhibitory factor (MIF) are associated with active pulmonary tuberculosis. *Infectious Diseases*, ***48***(3), 222–228. http://doi.org/10.3109/23744235.2015.1107188

Lechien, J. R., Nassri, A., Kindt, N., Brown, D. N., Journe, F., & Saussez, S. (2017). Role of macrophage migration inhibitory factor in head and neck cancer and novel therapeutic targets: A systematic review. *Head and Neck*, ***39***(12), 2573–2584. http://doi.org/10.1002/hed.24939

Leggett B, Whitehall V (2010). "Role of the serrated pathway in colorectal cancer pathogenesis". *Gastroenterology*. **138**(6): 2088-2100.

Leng, L., & Bucala, R. (2006). Insight into the biology of Macrophage Migration Inhibitory Factor (MIF) revealed by the cloning of its cell surface receptor. *Cell Research*, ***16***(2), 162–168. http://doi.org/10.1038/sj.cr.7310022

Lengauer, C., Kinzler, K. W., & Vogelstein, B. (1997). Genetic instability in colorectal cancers. *Nature*. http://doi.org/10.1038/386623a0

Lin, J. S., Piper, M. A., Perdue, L. A., Rutter, C. M., Webber, E. M., O'Connor, E., Whitlock, E. P. (2016). Screening for Colorectal Cancer. *Jama*, ***315***(23), 2576. http://doi.org/10.1001/jama.2016.3332

Lorentzen, J. A., Grzyb, K., De Angelis, P. M., Hoff, G., Eide, T. J., & Andresen, P. A. (2016). Oncogene mutations in colorectal polyps identified in the Norwegian Colorectal Cancer Prevention (NORCCAP) screening study. *Clinical Medicine Insights: Pathology*, ***9***(1), 19–28. http://doi.org/10.4137/CPath.s40143

Lorenz, T. C. (2012). Polymerase Chain Reaction: Basic Protocol Plus Troubleshooting and Optimization Strategies. *Journal of Visualized Experiments*, **63**, 1–15. http://doi.org/10.3791/3998

Mantovani, A., Allavena, P., Sica, A., & Balkwill, F. (2008). Cancer-related inflammation. *Nature*, ***454***(7203), 436–444. http://doi.org/10.1038/nature07205

Mittelbronn, M., Platten, M., Zeiner, P., Dombrowski, Y., Frank, B., Zachskorn, C., Wischhusen, J. (2011). Macrophage migration inhibitory factor (MIF) expression in human malignant gliomas contributes to immune escape and tumour progression. *Acta Neuropathologica*, ***122***(3), 353–365. http://doi.org/10.1007/s00401-011-0858-3

Morris, K. T., Khan, H., Ahmad, A., Weston, L. L., Nofchissey, R. A., Pinchuk, I. V., & Beswick, E. J. (2014). G-CSF and G-CSFR are highly expressed in human gastric and colon cancers and promote carcinoma cell proliferation and migration. *British Journal of Cancer*, ***110***(5), 1211–1220. http://doi.org/10.1038/bjc.2013.822

Morris, K. T., Nofchissey, R. A., Pinchuk, I. V., & Beswick, E. J. (2014). Chronic macrophage migration inhibitory factor exposure induces mesenchymal epithelial transition and promotes gastric and colon cancers. *PLoS ONE*, ***9***(6). http://doi.org/10.1371/journal.pone.0098656

National cancer institutehttps://www.cancer.gov/types/colorectal. Consulted in 2018

Naturehttps://www.nature.com/scitable/definition/single-nucleotide-polymorphism-snp-295. Consulted in 2018

NCBIhttps://www.ncbi.nlm.nih.gov/gene/4282. Consulted in 2018

Nishikawa, H., & Sakaguchi, S. (2010). Regulatory T cells in tumor immunity. *International Journal of Cancer*, *127*(4), 759–767. http://doi.org/10.1002/ijc.25429

News medical https://www.news-medical.net/health/Colorectal-Cancer-Diagnosis.aspx. Consulted in 2018

O'Reilly, C., Doroudian, M., Mawhinney, L., & Donnelly, S. C. (2016). Targeting MIF in Cancer: Therapeutic Strategies, Current Developments, and Future Opportunities. *Wiley Online Library (Wileyonlinelibrary.Com)*, ***29***(6), 1292–1327. http://doi.org/10.1002/med.21385

Ogino, S., Nosho, K., Irahara, N., Meyerhardt, J. A., Baba, Y., Shima, K., Fuchs, C. S. (2009). Lymphocytic Reaction to Colorectal Cancer is Associated with Longer Survival, Independent of Lymph Node Count, MSI and CpG Island Methylator Phenotype. *Clin Cancer Res.*, ***15***(20), 6412–6420. http://doi.org/10.1158/1078-0432.CCR-09-1438.Lymphocytic

Ordóñez-Mena, J. M., Walter, V., Schöttker, B., Jenab, M., O'Doherty, M. G., Kee, F., Brenner, H., *et al.* (2018). Impact of prediagnostic smoking and smoking cessation on colorectal cancer prognosis: A meta-analysis of individual patient data from cohorts within the CHANCES consortium. *Annals of Oncology*, ***29***(2), 472–483. http://doi.org/10.1093/annonc/mdx761

Orsetti, B., Selves, J., Bascoul-Mollevi, C., Lasorsa, L., Gordien, K., Bibeau, F., Theillet, C. (2014). Impact of chromosomal instability on colorectal cancer progression and outcome. *BMC Cancer*, ***14***(1), 1–13. http://doi.org/10.1186/1471-2407-14-121

Pagès, F., Kirilovsky, A., Mlecnik, B., Asslaber, M., Tosolini, M., Bindea, G., Galon, J. (2009). In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. *Journal of Clinical Oncology*, ***27***(35), 5944–5951. http://doi.org/10.1200/JCO.2008.19.6147

Peluso, G., Incollingo, P., Calogero, A., Tammaro, V., Rupealta, N., Chiacchio, G., Carlomagno, N. (2017). Current tissue molecular markers in colorectal cancer: A literature review. *BioMed Research International*. http://doi.org/10.1155/2017/2605628

Popa, C., van Lieshout, A. W. T., Roelofs, M. F., Geurts-Moespot, A., van Riel, P. L. C. M., Calandra, T., Radstake, T. R. D. J. (2006). MIF production by dendritic cells is differentially regulated by Toll-like receptors and increased during rheumatoid arthritis. *Cytokine*, ***36***(1–2), 51–56. http://doi.org/10.1016/j.cyto.2006.10.011

Ramireddy, L., Chen, W. T.-L., Peng, C.-T., Hu, R.-M., Ke, T.-W., Chiang, H.-C., Lo, W.-Y. (2014). Association Between Genetic Polymorphism of the MIF Gene and Colorectal Cancer in Taiwan. *Journal of Clinical Laboratory Analysis*, ***7***, 1–7. http://doi.org/10.1002/jcla.21763

Ramos-Vara, J. A., & Miller, M. A. (2014). When Tissue Antigens and Antibodies Get Along: Revisiting the Technical Aspects of Immunohistochemistry-The Red, Brown, and Blue Technique. *Veterinary Pathology*, ***51***(1), 42–87. http://doi.org/10.1177/0300985813505879

Ranganathan, V. ; F. C. F., & Haroon, G. G. J. M. E. G. N. I. (2017). Macrophage Migration Inhibitory Factor induces inflammation and predicts spinal progression in Ankylosing Spondylitis. *Arthritis & Rheumatology*. http://doi.org/10.1002/art.40175

Renner, P., Roger, T., & Calandra, T. (2005). Macrophage Migration Inhibitory Factor: Gene Polymorphisms and Susceptibility to Inflammatory Diseases. *Clinical Infectious Diseases*, ***41***(7), 513–519. http://doi.org/10.1086/432009

Rubio CA, Puppa G, de Petris G, Kis L, Schmidt PT (2018). "The third pathway of colorectal carcinogenesis". *Journal of clinical pathology.* **71**(1): 7-11.

Schoenberger, S. P., Toes, R. E. M., Van der Voort, E. I. H., Offringa, R., & Melief, C. J. M. (1998). T-cell help for cytotoxic T-lymphocytes is mediated by CD40-CD40L interactions. *Nature*, ***393***(6684), 480–483. http://doi.org/10.1038/31002

Shibata, D., Reale, M. A., Lavin, P., Silverman, M., Steele, G. J., Jessup, J. M., Summerhayes, I. C. (1996). T H E D C C P ROT E I N A N D P RO G N O S I S I N C O LO R E C TA L CA N C E R THE DCC PROTEIN AND PROGNOSIS IN COLORECTAL CANCER. *The New England Journal of Medicine*, ***335***(23), 1727–1732.

Smyth, M. J., Ngiow, S. F., Ribas, A., & Teng, M. W. L. (2016). Combination cancer immunotherapies tailored to the tumour microenvironment. *Nature Reviews Clinical Oncology*, ***13***(3), 143–158. http://doi.org/10.1038/nrclinonc.2015.209

Snover DC (2011). "Update on the serrated pathway to colorectal carcinoma". *Human Pathology.* **42**(1): 1-10.

Sugimoto, H., Suzuki, M., Nakagawa, A., Tanaka, I., & Nishihira, J. (1996). Crystal structure of macrophage migration inhibitory factor from human lymphocyte at 2.1 A resolution. *FEBS Letters*, ***389***(2), 145–8.

Swann, J. B., & Smyth, M. J. (2007). Review series Immune surveillance of tumors. *The Journal of Clinical Investigation*, ***117***(5), 1137–1146. http://doi.org/10.1172/JCI31405.antigens

Torres, S., Bartolomé, R. A., Mendes, M., Barderas, R., Fernandez-Aceñero, M. J., Peláez-García, A., Casal, J. I. (2013). Proteome profiling of cancer-associated fibroblasts identifies novel proinflammatory signatures and prognostic markers for colorectal cancer. *Clinical Cancer Research*, ***19***(21), 6006–6019. http://doi.org/10.1158/1078-0432.CCR-13-1130

Tsang, A. H.-F. (2014). Current and future molecular diagnostics in colorectal cancer and colorectal adenoma. *World Journal of Gastroenterology*, ***20***(14), 3847. http://doi.org/10.3748/wjg.v20.i14.3847

Vaiopoulos, A. G., Athanasoula, K. C., & Papavassiliou, A. G. (2014). Epigenetic modifications in colorectal cancer: Molecular insights and therapeutic challenges. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, ***1842***(7), 971–980. http://doi.org/10.1016/j.bbadis.2014.02.006

Van Blarigan, E. L., & Meyerhardt, J. A. (2015). Role of Physical Activity and Diet After Colorectal Cancer Diagnosis. *Journal of Clinical Oncology*, ***33***(16), 1825–1834. http://doi.org/10.1200/JCO.2014.59.7799

Van Cutsem, E., Cervantes, A., Adam, R., Sobrero, A., Van Krieken, J. H., Aderka, D., Arnold, D. (2016). ESMO consensus guidelines for the management of patients with metastatic colorectal cancer. *Annals of Oncology*, ***27***(8), 1386–1422. http://doi.org/10.1093/annonc/mdw235

Varner, J. A., & Schmid, M. C. (2010). Myeloid cells in the tumor microenvironment: Modulation of tumor angiogenesis and tumor inflammation. *Journal of Oncology*, *2010*. http://doi.org/10.1155/2010/201026

Vignali, D. a, Collison, L. W., & Workman, C. J. (2009). How regulatory T cells work. *Nature Reviews: Immunology*, ***8***(7), 523–532. http://doi.org/10.1038/nri2343.How

Weigl, K., Jansen, L., Chang-Claude, J., Knebel, P., Hoffmeister, M., & Brenner, H. (2016). Family history and the risk of colorectal cancer: The importance of patients' history of colonoscopy. *International Journal of Cancer*, ***139***(10), 2213–2220. http://doi.org/10.1002/ijc.30284

Weitz, J., Koch, M., Debus, J., Hölher, T., Galle, P. R., & Büchler, M. W. (2005). Colorectal cancer. *Lancet (London, England)*, ***365***, 153–165. http://doi.org/10.1016/S0140-6736(05)17706-X

WHOhttp://www.who.int/cancer/en/. Consulted in 2017

Xu, W., & Pan, Q. (2015). The association between the migration inhibitory factor - 173G / C polymorphism and cancer risk : a meta-analysis. *Oncotargets and Therapy*, 601–613.

Zhang, L., & Shay, J. W. (2017). Multiple Roles of APC and its Therapeutic Implications in Colorectal Cancer. *Journal of the National Cancer Institute*, ***109***(8), 1–10. http://doi.org/10.1093/jnci/djw332

Zhao, Y., & Garcia, B. A. (2015). Comprehensive catalog of currently documented histone modifications. *Cold Spring Harbor Perspectives in Biology*, ***7***(9), 1–20. http://doi.org/10.1101/cshperspect.a025064