UNIVERSITA' DEGLI STUDI DI MILANO

Facoltà di Medicina e Chirurgia

Dipartimento di Biotecnologie Mediche e Medicina Traslazionale

Scuola di dottorato in Scienze Biomediche Cliniche e Sperimentali

CORSO DI DOTTORATO DI RICERCA IN

PATOLOGIA E NEUROPATOLOGIA SPERIMENTALE

CICLO XXV, MED/03 - MED/04 - MED/05

TESI DI DOTTORATO DI RICERCA

"Adaptive and native immune cells as prognostic and predictive biomarkers along colorectal cancer progression"

Dr. Giuseppe DI CARO

Matricola N: R08876

Tutor: Dr. Raffaella Bonecchi

Coordinatore: Prof. Alberto Mantovani

Anno accademico 2011-2012

Abstract

Inflammation and cells of the innate immune system are known to contribute to tumour initiation and progression. Differently, the adaptive immune response controls growth and dissemination of established tumours . The double edge role of inflammatory and adaptive components of immune system in solid tumours are well represented in CRC. The progression and survival of patients with CRC is known to be modified by the interactions generated between the tumour and the host's response in a milieu named tumour microenvironment, composed by local immune responses. The quantification of the density and the type of immune cells in the tumour microenvironment has been a challenge since the early 60's of the last century. However their role and clinical significance in different human cancers has not been unequivocally addressed and still there is a strong interest in determining the dynamics of immunosurveillance and immunoevasion, and the role of immune cells infiltrating CRC. Recently, experimental support was provided that cancer infiltrating immune cells might be a crucial factor in chemotherapy mediated tumour cell death. Despite effort in this field there's still no clinical evidence in CRC regarding any effect modification by tumour infiltrating cells in enhancing the benefit of chemotherapy treatment, or whether this parameter might help to identify patients who would benefit from adjuvant therapy. In this context, tumour associated macrophages (TAM) represent the prevailing population in different cancers and are thought to enhance tumour cells proliferation and survival. Tissue macrophages are players of the innate immune response capable of phagocytosis and antigen presentation, that play a key role in directing immune responses through secretion of a plethora of factors. In CRC data regarding TAM and tumour progression are controversial. Of interest, in an experimental model of cancer TAM "re-educated" by CD40 ligand treatment, were found to be necessary to mediate antitumour activity, whereas tumour infiltrating lymphocytes (TILs) were irrelevant, supporting the hypothesis that TAM might mediate anti-tumour activity in certain conditions. The aim of this thesis was to study the prognostic significance of different populations ($CD3^+$ and $FOXP3^+$ TILs and $CD68^+$ TAMs) of immune cells in the tumour microenvironment, and their interactions with demographic and clinicopathological variables in a large dataset of stage II and III CRC patients. We first found that the cellular mediators of immunosurveillance seems to change along with the lymph-nodal involvement at diagnosis. Higher densities of TILs (both $CD3^+$ and $FOXP3^+$ cells) were associated with better prognosis among stage II CRC patients, but not in stage III. On the other hand, higher densities of TAM were associated with better prognosis only among stage III CRC patients,

but not in stage II. This data suggest that TILs mediate immunosurveillance in early stages of disease, while when the tumour has the ability to invade and spread to metastatic lymphnodes the mediators of surveillance seem to be macrophages. In detailed analysis, higher densities of TAM in stage III CRC were found to interact only with the variable 5-Fluoro-uracyl (5-FU) adjuvant chemotherapy treatment in predicting patients prognosis. We found that in stage III CRC patients, higher densities of TAM were associated with better survival only among those who received 5-FU chemotherapy. Moreover, the predictive effect of TAM in determining the efficacy of 5-FU chemotherapy showed significance only in microsatellite Stable (MSS) CRC patients. This is in accordance with the fact that microsatellite instability in CRC is a well-known negative predictor of response to 5-FU chemotherapy. The positive predictive effect of TAM in stage III CRC prompted us to confirm our findings in the non-colonic metastatic site of those patients. The densities of TAM in metastatic lymphnodes retained a positive predictive effect in identifying patients obtaining a prognostic advantage with 5-FU chemotherapy treatment. Therefore, the antitumour effect of TAM in 5-FU treated patients is likely to be exerted mainly on tumour micrometastasis which spread from the primary site and may cause recurrence of CRC. Ultimately, our data are in accordance with clinical guidelines supporting the use of 5-FU as adjuvant treatment only in stage III CRC. This study shed basis for the future identification of the molecular basis and the functional role of TAM in mediating 5-FU tumour cell death in reliable experimental models of CRC.

Table of Contents

Introduction

Chapter 1. Colorectal Cancer

1.1 Incidence and prevalence

In the most developed countries in 2008, according to IARC (International Agency for research on cancer), Colorectal cancer (CRC) ranked second for cancer prevalence and third for cancer mortality when considering only men. While it was the third most frequent type of tumour and the second cause of cancer death among women. Advances in population screening made possible an early detection of precancerous lesions in patients. On this regard, data obtained from the Surveillance, Epidemiology, and End Results (SEER) program of the National Cancer Institute of the United States population from 1998 to 2008 revealed a decrease in CRC incidence (-2.6%, -2.0%; for men and women respectively) and a decrease in CRC related mortality (-2.3%,-2.1%) [1]. However, regardless of improvement in preoperative imaging, surgical technique and adjuvant CHT, nearly a third of CRC patients experience disease recurrence. About 20%–25% of patients with CRC retain a metastatic disease at time of diagnosis, and about 20%–25% of patients will later develop metachronous metastases after surgery resulting in relatively high overall mortality rates of about 40%–45% [2]. On this regard, death from colorectal cancer parallels the incidence of metastatic disease and in 2008 CRC was still responsible for 9% of all cancer related deaths in the United States. Surgery still represent the backbone of CRC treatment, as it maintains the greatest influence on survival. However, while curative surgery in CRC patients without distant metastasis at diagnosis is in general macroscopically radical, occult metastases are thought to be the source of disease recurrence [3].

1.2 Pathological Staging

Cancer is not a single disease entity, cancer classification based on the tissue or organ where it arise was the first advance from considering all cancers as the same disease. Despite cancers have similar origins, clinical classification of cancers according to organ type and histopathological features helps clinicians in predicting cancer behaviour. Pathologists have a key role in the morphologic classification and final staging of surgically resected specimens and in the clinical diagnosis of patients with newly diagnosed CRC. The staging of resected CRC by pathologists remains the cornerstone for the prediction of future disease relapse and progression. The current edition of the CRC staging system named Nodal Metastasis Tumour System (TNM) predicts patients prognosis and is currently the only standard method available to address patients to postoperative adjuvant chemotherapy (CHT).[4] Despite many editions of TNM have been proposed by the years, those after the fifth edition didn't provide significant advantage. Accordingly, the fifth edition of TNM is still the most used in Western countries. [5] After many decades of utilization the current TNM staging system has now achieved near universal use. Developed by the cooperation between the International Union against Cancer (UICC) and the American Joint Committee for Cancer (AJCC), TNM is based on the extent of anatomic disease at the time of diagnosis, which is considered the main determinant of prognosis of colorectal cancer. This system was developed for CRC patients stratification according to the depth of tumour infiltration of the intestinal wall (T), the presence and the count of lymph nodes with metastases (N) and the presence and count of distant tumour distant metastasis(M).[6]

(a) clinical (cTNM)

(b) pathological (pTNM);

(c) post-surgical following neoadjuvant treatment (ypTNM)

The depth of tumour invasion is indicated with the acronym pT:
-pTis is a very early tumour lesion not invading underneath colorectal layers (*in situ*);
-pT1 is a tumour lesion invading colorectal mucosa tissue layer;
-pT2 is a tumour lesion invading colorectal muscular tissue layer;
-pT3 is a tumour lesion invading pericolorectal tissue layer;
-pT4 is a tumour lesion invading other structures or adjacent organs;
The number of metastatic lymph-nodes is indicated with the acronym pN:

-pN0 is a tumour without clinical evidence of metastatic regional lymph-nodes;
-pN1a is a tumour with clinical evidence of 1 metastatic regional lymph-node;
-pN1b is a tumour with clinical evidence of 3 or less metastatic regional lymph-node;
-pN2a is a tumour with clinical evidence of more than 3 metastatic regional lymph-node;
The presence of distant metastasis is indicated with the acronym pM:
-pM0 is a tumour without clinical evidence of distant metastasis;
-pM1 is a tumour with clinical evidence of distant metastasis;
(5th revision of the TNM staging system).

Stages of disease are identified by subgroups of CRC patients according to T, N and M status. Stages classification according to the fifth edition of the American Joint Committee on Cancer (AJCC) staging systems is shown in the table 1.2.

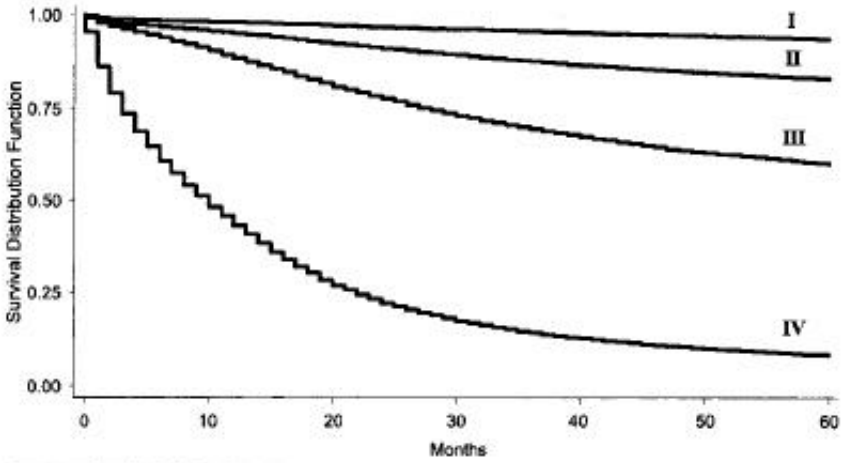
Table 1.2 CRC stages defined by the fifth edition of the American Joint Committee on Cancer (AJCC) staging systems.

Stage	Local invasion	Nodal Metastasis	Distant metastasis
I	T1 T2	N0 N0	M0 M0
II	T3 T4	N0 N0	M0 M0
III	Any T	N1	M0
IV	Any T	Any N	M1

TNM staging systems are designed to enable physicians to stratify patients in terms of expected predicted survival, to get information to select the most effective treatments, to determine prognoses, and to evaluate cancer control measures. To date TNM staging is the only method employed in clinical routine by physicians to predict CRC patients survival and to allocate patients to chemotherapy. Data from a study in 2004 by stratification of CRC patients in stages as defined by the AJCC fifth edition system from data obtained from a cohort study in 2004, 5-year colon cancer-specific survival was 93.2% for stage I, 82.5% for

stage II, 59.5% for stage III and 8.1% for stage IV [6]. The log-rank survival analysis for the cohort used in this study is shown in the figure below [6]. To date the most important prognostic marker of colorectal cancer survival is tumour cells detected in regional lymph nodes by histo-pathological examination[3].

Figure 1.2 Five year survival rate by stage I to IV according to American Joint Committee on Cancer fifth edition stages I-IV (Adapted from O'Connell [6]).



Stage	0 mo		30 mo			60 mo		
	Survival (%)	N	Survival (%)	N	P	Survival (%)	N	P
I	100	14500	96.1	8591	—	93.2	4515	—
II	100	34361	89.2	19492	<.0001	82.5	10105	<.0001
III	100	26949	72.7	12192	<.0001	59.5	5514	<.0001
IV	100	20802	17.3	1832	<.0001	8.1	432	<.0001

Despite the fact that TNM staging still remains the most important prognostic marker of survival and predictive marker of therapeutic response for CRC patients, clinicopathological staging lacks accuracy, and identification of all patients at greatest risk for disease recurrence or deriving optimum benefit from therapy for most CRC tumours is still not possible with this methodology. Accordingly, up to 30% of stage I and 50% of stage II patients develop recurrent disease. Stage III patients with radical cancer surgery including that metastasized to regional lymph nodes, exhibit recurrence rates of up to 70% [3]. Differences in recurrence rates among studies in patients with node-negative and node-positive disease is likely an effect of down-staging of CRC with stage III or IV respectively, also caused by histologically misidentification of cancer cells. Imprecision in predicting CRC patients at risk reflects in part limitations inherent to the method [3]. Microscopy has enhanced sensitivity, by improving the ratio cancer cells detection but pathologists typically reviews <0.01% of biopsied histological

tissue, producing sampling error, since more than 99.99% of available tissue is not examined and cancer cells do not distribute homogenously. Similarly, the outcome of CRC patients significantly differs between patients within the same histological tumour stage and the progression of advanced stage CRC can remain stable for years, while partial or full regression of large metastatic lesions can also occur spontaneously. An explanation for the limited accuracy in predicting outcome with traditional staging system lies in the estimation of tumour progression as an autonomous process without considering the evolution of the cancer as a balance of factors not histologically assessable. Therefore, the evidence that even histologically similar tumours arising from the same organ may have drastically different outcomes, prognosis and/or response to treatment gave rise to the hypothesis that CRC is a heterogeneous, multifactorial disease. In order to move to a more personalized cancer medicine there's the need for molecular classification that might identify patients with common molecular patterns and progression abilities. For this reason, the focus of CRC research moved from a clinical point of view to an understanding of its molecular basis.

1.3 Molecular carcinogenesis

The colorectal carcinogenesis develop through an ordered and partially defined series of event named as "the adenoma-carcinoma sequence" and "multistep tumourigenesis" which takes years to decades to progress and which has its onset with the transformation of normal colonic epithelium with the formation of a small adenomatous polyp followed by formation of a larger polyp with dysplasia and then ultimately to adenocarcinoma. Multistep carcinogenesis was describe by proposing a model wherein the triggering mechanisms were found to be multiple genetic events occurring in gatekeeper and caretaker gene pathways,. In this model the sequential evolution of specific genetic alterations were associated with the occurrence of neoplastic phenotype in the colon and were required for tumour progression [7] . In their seminal paper Vogelstein and Fearon proposed and described a four-step sequential pathway that was declared to be sufficient to the development of cancer, while further genetic events were required for tumour progression. The first step was identified to be the inactivation of the *adenomatous polyposis coli* (*APC*) tumour suppressor gene, which is a component of the Wnt signalling pathway and was found to be the earliest molecular defect to cause adenoma progression. In larger adenomas and invasive cancer APC mutation was accompanied by a second step consisting in the activating mutations of *KRAS* which promoted adenomatous growth. The third step consisted in biallelic loss of chromosome 18q which allowed progression and the last step was proposed to be *p53* inactivation which was able to triggers the transition to carcinoma. This sequence of molecular events was described 20 years ago, nowadays the multistep model has been implemented with the detection of mutations in additional target genes with a function in the oncogenic transformation, such as mutations in transforming growth factor–β (*TGFβ*) gene and *PIK3CA* pathways [8,9]. This model predicts that at least seven distinct mutations are required [7]. *APC* mutations are the necessary condition in adenoma formation in human and mouse models. In contrast, mutational activation of *KRAS* cannot initiate cancer *in vivo*, and only when combined with a mutation in *APC* mutant *KRAS* promote tumour progression. Genomic instability was recognized as an essential cellular feature that accompanies the acquisition of these mutations. Accordingly colorectal cancer is now classified in two main classes that represent genetic instability carcinogenesis pathways [10].

1.3.1 CIN pathway

The first class, which represents about 85% of sporadic cases, have chromosomal instability (CIN)[10]. This term refers to gains or losses of whole or large portions of chromosomes that results in karyotypic abnormalities, namely allelic imbalances at several chromosomal loci and chromosome amplification and translocation, which together contribute to imbalances in chromosome named aneuploidy, sub-chromosomal genomic amplifications, and a high frequency of loss of heterozygosity (LOH) [10]. Experimental evidence indicates that aneuploidy arises in CRC cancers because of CIN — when the rate of gains or losses of whole or large portions of chromosomes increases. CIN, is thought to enhance the acquisition of genetic changes that are required for tumorigenesis [11]. However, it is still unknown whether CIN is the first event in tumorigenesis, and therefore precedes mutation of *APC*. Data suggest that chromosomal instability can be observed in adenomas, therefore it is thought to be an early event in carcinogenesis progression. The most common karyotypic abnormalities in the CIN pathway are loss of 5q, 8q, 17q, 18q allele together with the accumulation of mutations in oncogenes and tumour suppressor genes such as *APC* and *K-ras* [12].

1.3.2 MSI Pathway

The second class of genomic instability, which comprise about 15% of sporadic CRC patients, have the phenotype of microsatellite instability (MSI). MSI represents a unique pathway for tumour development that does not involve LOH [10]. Accordingly, MSI tumour cells had a tendency to be diploid and are characterized by the accumulation of single nucleotide mutations, gene length alterations named frame-shift mutations and base-pair substitutions that occurs in repetitive microsatellite and in short tandemly repeated nucleotide that are common in the genome, named microsatellites nucleotide sequences[13]. The most frequent errors associated with microsatellites are base–base mismatches that escape the intrinsic proofreading activity of DNA polymerases, and insertion–deletion loops, which are extrahelical nucleotides that form DNA hairpins. Microsatellite are usually located in non-encoding regions, but they could also be included in regions of genes with functions of cell proliferation control or apoptosis. These nucleotides occur when the first nucleotide and template strand dissociate and incorrectly re-anneal in a microsatellite. DNA sequences of genes containing such microsatellites sequences were altered resulting in premature stop codons and frame-shift mutations that ultimately resulted in protein truncations and loss of function. The epiphenomenon of microsatellite instability is the loss of mismatch-repair (MMR) function which lead to the failure of repair activity of strand slippage within repetitive DNA sequence elements. The MMR system has the function to correct errors introduced in microsatellites and *MLH1, MSH2*, *MSH6* and *PMS2* are the principal proteins taking part in this task by their interaction as heterodimers. When the system works, *MSH2* interacts with either *MSH6* or *MSH3* and *MLH1* couples with *PMS2*, *PMS1* or *MLH3*[14-16]. Mutations and epigenetic silencing in the above genes lead to an accumulation of errors in DNA, which results in MSI. In sporadic colorectal cancer with microsatellite instability, somatic epi-genetic silencing blocks the expression of *MLH1* by hypermethylation of its promoter [11,13-16]. The MSI phenotype is strongly associated with mutations in specific oncogenes and tumour suppressor genes, especially *BRAF* and less to *K-Ras* in agreement with the fact that the latter mutation is mutually exclusive with the first [10]. MSI is more common among stage II (~20%) than stage III (~12%) CRC, and is less frequent also in stage IV CRC (~4%). MSI tumours are characterised by proximal location, mucinous histology, poor differentiation, and lymphocytic infiltration [17]. Different patterns of mutant genes can be seen in MSI vs. the CIN tumours suggesting that the underlying form of genomic instability in the cancer influences the susceptibility to and selection for specific mutations [10].

Chapter 2. Tumour associated inflammation

2.1 Inflammation in cancer

Rudolf Virchow 150 years ago was the first who described the presence of infiltrating leukocytes in tumours, and theorized that cancer arises at the chronic inflammation site. In support of this has been later reported that chronic infections are associated with 15–20% of malignant cancers [18,19]. Helicobacter pylori, hepatitis B and C viruses, and the human papilloma virus has been established to be risk factors associated with gastric cancer, hepatocellular carcinoma, and cervical cancer, respectively. Moreover, smoking and obesity have been associated with an increase of 20% and 30% of risk of cancer respectively [20], which both can in turn trigger inflammatory responses in the lungs and liver, respectively, which is the cause of tumorigenesis promotion [21,22]. According to such results and others, inflammation plays an important role in promoting cancer development , and recently inflammation has been included in the next generation of the criteria as a new ‘‘hallmarks of cancer” [23]. Solid cancers are organ-like structures and their growth and development has been shown to be modified by the behaviour of recruited cells in the tumour microenvironment, which typically are bone marrow derived cells [24]. Accordingly, along cancer progression the crosstalk between tumour cells and the microenvironment is crucial. Pro-inflammatory cytokines are released in the tumour microenvironment, and has been demonstrated that such molecules are very important for cancer development [25]. Inflammation may predispose to cancer through enhanced cellular proliferation and mutagenesis, inability to adapt to oxidative stresses, promotion of angiogenesis, inhibition of apoptosis, and secretion of mediators that may promote tumorigenesis[25]. Large amount of experimental, epidemiological, and clinical data suggest that chronic inflammation is linked causally to cancer occurrence [26].

2.2 Inflammation in Colorectal Cancer

Moving to intestine, chronic inflammation has been shown to be a risk factor of colorectal cancer (CRC) occurrence. Ulcerative colitis and Crohn's disease, which are thought to be the two major types of inflammatory bowel disease (IBD), have been both associated with an increased risk of developing colitis associated colorectal cancer (CAC). Ulcerative colitis patients were found to retain a risk to develop CRC of 2% after 10 years, 8% after 20 years and 18% after 30 years of active disease [27]. Interestingly, the relative risk of developing CRC was not changing when comparing patients who retain Crohn's colitis with patients who retain ulcerative colitis of similar severity [28]. The pathogenesis of IBD is thought to be related, in genetically susceptible individuals, to an excessive stimulation of the immune system directed to antigens of the gut microbiota and this series of events is thought to cause chronic inflammation [29]. On this regard, general consensus is reached on the fact that chronic inflammation of the colon such as that observed during either UC or CD increases the risk of developing CRC. However, it is worth considering that IBD-related CRC, named Colitis Associated CRC (CAC) is estimated to be responsible for less than 2% of all CRC appearing annually [30]. Moreover, further than inflammatory bowel disease the role of inflammation in sporadic colorectal cancer remains undefined from a clinical and an experimental point of view[31]. In colon the initial evidence for cytokine-regulated tumour promotion came from the studies in the mouse model of CAC [32]. However, according to Karin et al the same mechanisms might be applied to sporadic CRC [25]. Among the most important inflammatory mediators, tumour necrosis factor (TNF)-α and interleukin (IL)-6 has been shown to activate nuclear factor *NF-kB* and *Stat3*, in turn *NF-kB* induces the expression of COX-2, IL-6, and TNF- α [25,33] [34]. CRC tumours and cell lines were shown to retain activation of *NF-kB* and *Stat3* transcription factors which are thought to be essential components of inflammatory pathways [35,36]. However, no activating mutations in *NF-kB* or *STAT3* have been detected to date in colorectal or colitis associated tumours, this consideration is important to underline a likely activation of signalling pathways components upstream of such transcription factors or alternatively they might be activate in a paracrine or autocrine fashion[25]. Accordingly, these signalling pathways construct an inflammatory network in the tumour microenvironment, which plays an important role in tumour promotion [33,34]. The most convincing clinical association between inflammation and risk to develop sporadic CRC comes from an old drug. Many robust epidemiological studies, both observational and randomized controlled studies, have revealed that regular use of non-steroidal anti-inflammatory drugs (NSAIDs) as for

example aspirin is associated with a lower probability to experience gastrointestinal cancer [37-39]. This effect is supposed to be mediated through abrogation of chronic inflammation. NSAIDs are drugs that specifically target cyclooxygenase COX-1 and COX-2 molecules which are known to be involved in inflammatory pathways[40]. Accordingly, many studies indicated that COX-2 and its downstream product, prostaglandin E2 (PGE2), play an important role in cancer development to promote inflammation and cell proliferation [41]. On this regard, an epidemiological study from Chan which took advantage of more than 30 thousands women demonstrated that those with very high plasma levels of TNFR-2 had a higher risk of developing CRC and the chemo-protective effect of aspirin was shown to be retained only among women with high TNFR-2 levels [31]. This evidence is the most convincing to support the hypothesis that aspirin reduce risk of colorectal neoplasia through anti-inflammatory pathways. On the other hand, it is important to underline that the effect of aspirin on the survival after diagnosis of CRC is not clear, raising doubts on aspirin use as an agent for adjuvant therapy in CRC. On this issue, a recent paper from Ogino stated that regular aspirin use after CRC diagnosis had an impact on survival only among patients with mutated-*PIK3CA* tumour[42]. Mutations in *PIK3CA* (the gene encoding phosphatidylinositol-4,5-bisphosphonate 3-kinase) are present in only about 15 to 20% of colorectal cancers [42,43]. Therefore, NSAIDs protective effect in the progression of CRC seems to be retained only among subclasses of patients with peculiar molecular features, supporting a "tailored" or "personalized" chronic inflammation role to sporadic colorectal cancer pathogenesis from the onset to the recurrence and progression. Considering the heterogeneity of colorectal cancer evolution among patients, the complexity of the interactions between tumour cells and the various subtypes of innate and adaptive immunity make difficult to precisely define the role of different cell types, cytokines or growth factors in either promoting or containing cancer. Thus, the contribution of mediators of inflammation to cancer biology can't be generalised, since they might retain different roles along progression of tumours with the same histopathology and arising in the same organ.

2.3 Immunoediting and cancer

2.3.1.Immunosurveillance in experimental models

As mentioned in the previous paragraph, inflammation is a complex physiological process, in the early 1990 the possibility to develop new genetic models of immunodeficiency readdressed the role of immunity in cancer. The idea of cancer immunosurveillance started with the evidence that INFγ was implicated in rejection of tumour transplanted in mice [44]. Moreover subsequent studies revealed that by targeting INFγ receptor or *Stat1*, the transcription factor required for INFγ receptor signalling), or by inhibiting adaptive immunity with *RAG*-/- mice (without B, T and NK cells) were more susceptible to spontaneous tumours and to carcinogenesis induced tumourigenesis [45,46]. According to Schreiber's model [47], immunity has been shown to take part in cancer immune surveillance through different mechanisms. To eliminate viral infections which are potentially related to cancer and therefore suppresses virus-induced tumours; to eliminate pathogens which are thought to mediate an inflammatory microenvironment and thus might facilitates tumourigenesis if not killed; to eliminate tumour cells since new transformed epithelial cells often de-novo express ligands for receptors on innate immune cells and tumour antigens that are recognized by immune receptors on lymphocytes of the adaptive immune system [47]. The landmark principle of cancer immunosurveillance theory reside in the ability of cancer cells to express antigens that are not expressed by the normal tissue from which they arise. Seminal experimental models indirectly demonstrated the presence of tumour antigens which were named "transplantation rejection antigens" [48]. The first of such experiments showed that mice exposed to chemical-induced tumours were immunized and therefore protected by following challenges with the same tumour [48]. In 2001 a study from Shankaran revealed that only half of the tumours were growing when reimplanted in syngeneic immunocompetent mice if were arise and derived from carcinogen-treated immunodeficient mice, compared to tumours arising from immunocompetent mice [46]. Tumours arising in immunodeficient mice were named "unedited" while those arising in immunocompetent mice were "edited" [46]. Therefore immune system was able to shape tumour antigens content by selecting less immunogenic clones who might escape from immune system control and give rise to clinically relevant tumours. This evidence gave rise to the assumption that immune system has a dual role on cancer evolution, by both eliminating and promoting cancer. In 2002 Dunn and colleagues postulated the cancer immunoediting theory which proposed three phases of immunesurveillance: elimination, equilibrium and escape phase [49]. In elimination phase

adaptive and innate immune systems interacts to detect tumour antigens and to eliminate it. The release of ligands that are expressed by dying tumour cells might bind to innate immune cells which in turn release cytokines that facilitate tumour cell killing by adaptive immune cells in a coordinated activation of both cellular types. Different studies which took advantage of immunodeficient hosts for specific immunity subsets or cytokines and pathway effectors deficiency has proved that immunity requirements for cancer elimination are dependent on the specific tumour characteristics, its anatomical site of onset and its origin [50]. 129/*Rag2*−/− mice developed more spontaneous epithelial tumours (of which 35% were gastrointestinal tumour and 15% were lung tumour) than mouse wild-type [46]. In agreement with such statements, 129/Sv *RAG2*−/− mice that also lack *STAT1*, which is required for INFγ signalling, retained an earlier onset of disease and more aggressive, with the development of colon and mammary adenocarcinomas [46]. Consistent with a role for the innate immune cells in cancer immunosurveillance, mice chronically depleted of NK cells displayed increased tumour incidence [51]. Therefore both adaptive and innate immune responses were involved into elimination process. However, C57BL/6 β2microglobuline-deficient mice that lack NKT cells and many CD8+ T cells was shown not to increase tumour formation upon aging [52], suggesting that distinct lymphocyte populations may play distinct roles, if any, during cancer immunosurveillance of spontaneous tumours. Tumour cells which survive elimination phase may enter into equilibrium phase. In this phase immune system might shapes tumour cells immunogenicity by keeping them in a low number. The host immune system and tumour cells enter a dynamic balance, wherein powerful antitumor immunity contains, but does not fully eradicate, a heterogeneous population of tumour cells. Experimental evidence of this tumour latency comes from studies which shown that mice injected with low dose carcinogens had no macroscopic evidence of cancer [53]. When T-cells (CD4 and CD8) or INFγ signalling were experimentally depleted by mAbs, tumours became visible at the site of injection. In contrast mAbs that deplete NKcells, block NK cell recognition (anti-NKG2D), or inhibit NK cell effector function (anti-TRAIL) failed to cause the appearance of growing tumours at the site of carcinogen injection [53]. Consistently cells isolated from arising tumours were highly immunogenic and further characterization revealed that adaptive but not innate immunity was responsible for keeping tumour cells in a dormant state [53]. This evidence separate equilibrium phase by elimination phase as the latter required the cooperation of both immune systems to work. The natural selection process occurring in the equilibrium phase results in the selection of immuno-edited tumour clones which acquired mutations that gave tumour cells immune evasion abilities. Edited tumour cells are those who gained the ability to evade immunity

control after a long selection process during equilibrium. In a process that might last many years cancer cells undergo stochastic genetic and epigenetic changes in order to generate the critical modifications necessary to evade both innate and adaptive immunological protection. Immune evasion occurs because a heterogeneous population of tumour cells changes in response to immune system selective pressure, wherein immune system contributes to tumour progression by a Darwinian selection of more aggressive tumour variants more prone to survive and to suppress the antitumor immune response[50]. In another perspective, immune evasion might occur as a result of host immune system impairment by external factors or by cancer ability to suppress immunity response. In the first option, loss of tumour antigens is the main driver of immune evasion and it occurs through different mechanisms, tumour cells can acquire defects in antigen processing and presentation pathways that facilitate evasion from adaptive immune recognition. MHC class 1 is the molecule that present antigens to T cells, interference in INFγ pathway such as mutations and epigenetic silencing in INFγ receptor leads to insensitivity to its ligand and therefore the inability to increase MHC1 protein expression required for antigens presentation. On this regard loss of proteins involved in the MHC1 pathway machinery presentation such as TAP1 and 2 and B2microglobuline might affect tumour cells recognition by immune cells [50,54]. In a different escape strategy, tumour cells might start expression of immune inhibitory ligands that avoid immune recognition or cytotoxic ability in order to generate an immunosuppressive environment. The occurrence of immunosuppression is concomitantly with tumour development by release from tumour cells immunosuppressive cytokines as VEGF, TGFβ and by recruiting T regulatory (T-regs) cells , macrophages and myeloid derived suppressor cells (MSDC)[50]. T-regs are $CD4^+$ cells expressing *FOXP3* transcription factor and produce TGFβ and IL10 and expressing CTLA4 [50]. Tumours may also attract MSDC, which have the ability to recruit T-regs ,and tumour-associated macrophages (TAMs) by producing IL-4 and IL-13. M2 macrophages can inhibit antitumor immunity through the production of TGF-β and IL-10 and can promote stromal development and angiogenesis through secretion of platelet-derived growth factor (PDGF) [34]. Chronic inflammation has been clearly demonstrated to contribute to cancer initiation by generating genotoxic stress, cancer promotion by inducing cellular proliferation, and cancer progression by enhancing angiogenesis and tissue invasion. On this regard, Vesely and others suggested that there is overwhelming evidence that cancer immunity might exert also immunosurveillance that shouldn't be seen as a mutually exclusive process with cancer inflammation but rather as potentially overlapping immune algorithms. For example inflammation participates in the cancer immunoediting process during the tumour escape

phase, when inflammatory cells and regulatory immune cells are recruited and activated by cancer-derived products to dampen antitumor immunity and subvert immune cells to promote cancer progression[50].

2.3.5 Clinical evidences of immunoediting

Many evidences suggest that cancer immunoediting is an alternative concept that integrates immune system roles in shaping cancer cells immunogenicity. Human immunodeficiency (AIDS) patients has been shown to have an higher risk of colon, lung, pancreas, kidney, head and neck and melanoma cancers [50]. Moreover reports exist of patients receiving organ transplantation from the same donor and later developing the same tumour from which donor was diagnosed, treated and recovered [55]. A plausible explanation for this evidence is that the tumour cells were present in the donor, even though not clinically detectable, and were kept in a dormant state from donor's immune system equilibrium phase. The transplant of such cells in an immunodepressed and naive host gave the ability to cancer cells to grow and kill the patients. Cancer immunoediting theory fits to progression of early stages of CRC not associated with CAC. In this case surgery can remove macroscopically detectable colon cancer burden by physical excision while adjuvant chemotherapy is administered by assuming that it will kill circulating tumour cells and micrometastasis which spread out the whole body. Such cells are not detectable by conventional diagnostic methods and are kept in a dormant state, while after many years they may give rise to metachronous metastasis which are the main cause of death in colorectal cancer. Immune system might play a role in this process by keeping not detectable micrometastasis in an equilibrium phase for many years, while the evolution of such tumour cells might give them the chance to escape immune system recognition and cause recurrence events. Adjuvant chemotherapy gives a survival advantage CRC patients, though its role on immune system micrometastasis recognition is still unknown.

2.4.Immune cells in colorectal cancer microenvironment

2.4.1.Contribution to tumour microenvironment

2.4.1.1 Tumour infiltrating Lymphocytes (TILs)

In CRC immune cells infiltrates tumour stroma and thus take part in tumour microenvironment. The adaptive cells of immune system are represented by $CD8^+$ CTLs and the $CD4^+$ T helper lymphocytes. $CD4^+$ T cells main function is to release cytokines, such as IL-2, TNFα and INFγ, by doing this T helper cells influence and promote stimulation of CTLs cells, on the other hand $CD8^+$ T cells are able to produce perforin and granzyme B, which mediate the cytotoxic activity of CTLs on target cells [56]. Therefore, CTLs have the ability to mediate identification of tumours and their specific elimination [57,58] . T-lymphocytes are also kept under control by a subpopulation of T- lymphocytes named T-regs which have the role to control immune responses [59]. The transcription factor forkhead box protein P3 (*FOXP3*) is recognised to be a sensitive T-reg cell marker [59,60]. T-reg cells are represented by different subpopulations but most studies detected T-reg cells as a population expressing $CD4^+$ $CD25^+$ T cells markers of which the latter is a subunit of the receptor for the T cell-stimulating cytokine IL-2, and *FOXP3* [59]. However, none of these markers is fully restricted to T-reg cells because CD25 and *FOXP3* are also expressed by activated effector T cells[61]. According to Fridman, the antigen specificity of tumour-infiltrating T-reg cells has yet to be established in humans and for this reason T-reg cells may have different functions according to the type of tumour contexture, as they might block anti-tumour immunity or decrease chronic pro-tumour inflammation [61]. T-lymphocytes recognition of antigens after immune response is kept at a higher activation level compared to the baseline. Activated T-lymphocytes have long life and are characterized by the expression of specific surface molecules and are more sensitive to stimulation than naïve T-lymphocytes. Such CTL cells phenotypically switch CD45 isoform from CD45RA to CD45RO [61]. In a paper from Koch was functionally demonstrated for the first time activation and cytotoxic activity of $CD8^+$ TIL and migration of $CD4^+$ T helper cells that was tumour specific in CRC tissues [62]. Accordingly, in this study authors found a higher proportion of activated and cyto-toxically active $CD8^+$ TIL in colorectal cancer compared with normal mucosa and the increased activation, the cytotoxic activity, and the functional reactivity of TILs were correlating with the presence of functional tumour antigen-reactive T cells in the blood and bone marrow. Moreover, they found that the proportion of activated TILs decreased significantly in higher tumour stage (from stage II through stage III to stage

IV), giving functional assessment of increasing immune evasion along with more advanced clinical histopathologically staging [62]. In a study from Atreya authors proposed the proportion of activated $CD8^+$ TILs is not the only relevant feature in mediating CTL antitumor activity, as their cytolytic abilities is determinant to mediate an effective antitumor activity [63]. Authors demonstrated that *eomesodermin*, a T-box transcription factor involved in controlling the cytotoxic activity of $CD8^+$ CTLs, is inversely correlated with the presence of lymph node metastasis at diagnosis in CRC patients [63]. In accordance with these data a paper from Laghi group showed clinical evidence of cancer immunoescape along with the progression of CRC from stage II to III and thus along lymph-node metastasis. Accordingly $CD3^+$ densities lost their prognostic abilities in advanced stage of colorectal cancer patients while $CD3^+$ densities were a strong prognostic factor only in patients without local metastatic disease at diagnosis.

2.4.1.2 Tumour associated macrophages (TAM)

It is generally accepted that in the majority of cancers TAM have a protumoural effect. TAM recruitment at the tumour site favours angiogenesis, and their secretion of chemokines stimulate proliferation and invasion of tumour cells [64-69]. Macrophages play a key role in directing immune responses through secretion of a plethora of immune mediators such as cytokines, tumour necrosis factors alpha and beta, interleukins (IL-1, IL-8 and IL-10) and prostaglandins. Accordingly one of the early roles of macrophages is to release pro-inflammatory cytokines . Moreover tissue macrophages are cells of the innate immune response capable of phagocytosis and antigen presentation [70]. Macrophages are very versatile and plastic molecules which show different functional activities even opposite to each other depending on the local environment. Accordingly, diversity has emerged as a hallmark of mononuclear phagocytes and the same applies to the various forms of macrophage activation [64-69]. During bacterial infections macrophages are the first defence of the host which arrange an acute inflammation to eliminate pathogens, afterward they become scavengers and in such configuration they have the function to heal the damaged tissue and to create new vessels and to recruit fibroblasts. In the attempt to oversimplify macrophages phenotypes Mantovani and coll. proposed two different polarization status named M1 and M2. M1 macrophages are the classical activated ones and are stimulated by bacterial products and T helper type 1 cytokines as for example INFγ. When macrophages are switched to M1 phenotype they start releasing immunostimulatory and inflammatory cytokines which enhance adaptive responses, reactive oxygen radicals (ROS) and nitrogen derivates (iNOS) which retain cytotoxic activity on bacteria and transformed cells [64-69]. Macrophages exert cytotoxic activity by different mechanisms such as release of reactive nitrogen intermediates and members of the TNF receptor family. Antitumour activity of M1 macrophages is exerted by negatively affecting vascular cells and activating coagulation which in turn cause tissue- and tumour-destructive reactions named hemorrhagic necrosis. M1 macrophages activates adaptive immunity to exert cytotoxic activity by releasing IL12 which support the formation of T-helper 1 (Th1) response. Differently, M2 type of macrophages differentiation is supported by tumour microenvironments rich in T helper type 2 cytokines as for example IL4 and IL13. It is important to underline that M2 macrophages has been shown to inhibit adaptive immune response while they acquire scavenging activity and release different growth factor necessary for tissue repair. Tumours might release the chemokine CCL2, which is a powerful activator of chemotaxis and thus attract monocytes to the site of tumour. Once in the tumour monocytes are differentiated to macrophages by M-CSF which is produced as well by CRC cells [64-69].

Analysis of different type of tumours revealed that TAM at the tumour site are mainly polarized to M2. It has been demonstrated that M2 macrophages in the tumour microenvironment facilitate tumour progression. In human cancers M2 macrophages revealed a typical gene expression profile, with over-expression of osteopontin, fibronectin, scavenger $CD163^+$ and mannose receptor. Moreover factors release by the tumour microenvironment such as M-CSF, PGE2, TGFβ, IL6 and IL10 retain the ability to differentiate macrophages to an M2 phenotype [64-69]. In turn macrophages when polarized to M2 release epidermal growth factor (EGF), TGFβ, VEGF, metalloproteases (MMPs), cathepsins that promote tumour progression and increase expression of MHC class II. In CRC higher release of IL6 by macrophages induce IL10 production in tumour cells, which has been correlated with worst prognosis. Via secretion of immuno suppressive mediators, such as IL10, TGFβ and IDO, TAM retain the ability to suppress T-cell activation. As a source of TGFβ TAM in intestinal inflammation can directly induce T-regs differentiation which in turn can suppress $CD8^+$ mediated cytotoxicity likely contributing to immune evasion. On this regard TAM are thought to retain suppressive activity of adaptive responses by directly releasing T-cells inhibitory factors or indirectly by stimulating T-regs activity. According to Mantovani and colleagues TAM can also be associated with anti-tumour activities [64-69]. However, the mechanisms behind the antitumor effects of TAM in different studies were not fully elucidated and could potentially be ascribed to the presence of classically activated M1 macrophages as it has long been known that M1 macrophages mediate extracellular killing of tumour cells. In this perspective, a paper from Forssell group interestingly pointed out that the degree to which macrophages exert their antitumorigenic abilities may partly depend on the possibility to get direct contact with tumour cells and a high macrophage to cancer cell ratio [71]. In this study in vitro co-culture experiments revealed that a high ratio of macrophages to colon cancer cells inhibited cancer cell growth [71]. Importantly, this effect was partially dependent on cell to-cell contact, on the other hand Boyden chamber cocultivation without macrophage-tumour cell contact promoted cancer cell spread. Accordingly, Forssell proposed that protumorigenic properties could be exerted by macrophages only when tumour cell were not in direct contact with macrophages. In accordance with this data was previously shown that glioma cells were killed by murine macrophages in a phagocytosis process only when transfected with the membrane but not the secreted isoform of macrophage colony-stimulating factor in [72]. More significantly, it was recently reported that macrophage depletion in rats bearing colon cancer xenografts promoted enhanced cancer cell growth and impaired survival [73]. Taken together, these results might suggest a role for macrophages in antitumor defense in colon cancer.

2.4.2 Prognostic value

2.4.2.1 Undefined Lymphocytic infiltration, not characterized by CD antigen.

A bunch of studies since late 60s have been reporting that an inflammatory cell response in CRC was recognised to confer an improved clinical outcome [74-78]. The first report giving evidence of an association between a better CRC related survival and tumour infiltrating lymphocytes at the tumour periphery and at the tumour centre dated 1967 [74]. Lymphocytic infiltration was assessed by pathologists on haematoxylin and Eosin staining and was not informative on specific subpopulation of immune cells. A seminal paper from Jass in 1987 proposed new criteria to classify immune lymphocytic infiltration determined by a semi-quantitative quantification score [77]. The selected variables were given weighted scores and the score range was divided to provide four prognostic groups. The prognostic classification of Jass was simple to use and was superior to staging by the method of Dukes because it placed twice as many patients into groups that provided a better prediction of clinical outcome [77]. Accordingly, later studies confirmed lymphocytic infiltration as an predictor of survival independent of other histopathological characteristics [79-84]. With the same methodology, in 1990 a paper from Graham first described the presence of a "Chron's like reaction" at the tumour site composed of discrete lymphoid aggregates with germinal centres [85]. Such structures were later associated with better prognosis and higher lymphocytic infiltration. Later studies revealed that lymphocytic infiltration and Chron's like reaction was positively associated with MSI in CRC tumours [84]. More recently, by taking advantage of immunohistochemistry it was possible to assess tumour infiltration of specific subsets of immune cells.

Table 2.4.2.1 Summary table of studies reporting the associations between the in situ local inflammatory response and survival in colorectal cancer (Adapted from Roxburgh[86])

Immune cell infiltration	Number of studies	Studies reporting significant survival association	Studies reporting no survival association
Undefined Lymphocytic infiltration	39	36	3
CD3+ expression	12	10	2
CD4+ expression	5	1	4
FOXP3+ expression	7	3	4
CD45RO+ expression	8	8	0
CD8+ expression	25	20	5
CD68+	13	9	4

2.4.2.2 Tumour infiltrating lymphocytes (TILs), classified by specific CD antigen.

CD3 marker is employed for specific recognition of all subsets of T-lymphocytes, namely CTLs, T-helpers and T-regs. As shown in above $CD3^+$ and $CD8^+$ antibodies has been employed as tumour markers and associated to CRC patients prognosis in many studies to date, of which 5 were contradictory while trends toward better survival was detected in two of those studies (reviewed in Roxburgh [86] and Table 2.4.2.1) . The first paper employing CD8 marker was reported by Naito et al. which demonstrated that semiquantitative scoring of $CD8^+$ cells was independently associated with better survival [87]. The first paper which analyzed CD3 and survival came out in 2001 and took in consideration only rectal cancers, at multivariate analysis $CD3^+$ density was not predicting outcome independently of TNM staging system [88]. A later paper by Guidoboni et al. which took advantage of right sided colon cancers revealed that high infiltration of $CD3^+$ was associated with MSI status and positively correlated with better survival independently of MSI status [89]. In the study from galon et al. authors stated that $CD3^+$ density assessed by quantitative analysis was independently associated with better survival, but despite previous evidence, $CD3^+$ density correlation with survival was found to be better than TNM staging, therefore suggesting to include $CD3^+$ density in clinical routine instead of TNM staging [90]. On the contrary, in a recent and robust study by Nosho and co-workers in a large cohort of stage I-IV CRC, no survival relationships were reported for $CD3^+$ T-lymphocytes at univariate analysis when measured at the epithelial neoplastic area, while this marker was significantly positively associated with prognosis when measured in the whole tumour tissue core or in tumour stromal areas [91]. However, on multivariate analysis nor $CD3^+$ or $CD8^+$ cell densities were associated with better survival. The author explained this discrepancy compared to other studies by asserting that $CD8^+$ cell density lost prognostic significance when adjusting statistical multivariate models for TNM tumour staging and $CD45^+$ cell densities [91]. Therefore the independent prognostic effect of $CD8^+$ cells in other studies might be explained by the confounding effect of TNM tumour staging and $CD45^+$ cells. Together with this evidence is important to underline that in a paper from Laghi et al. $CD3^+$ cells were associated with better survival only in stage II CRC but not in stage III CRC [92]. Here $CD3^+$ TIL density was statistically interacting with TNM tumour staging in predicting prognosis and for this reason was not a stage independent predictor of survival. This paper raised the notion that $CD3^+$ TILs subsets are relevant to the fact that it can help in clinical decision making in the post-surgical management of stage II CRC. In this respect, the density of TIL subpopulations should be particularly evaluated in stage II CRC in which it might be of help in stratifying high- and low-risk patients in decision making for

allocation to chemotherapy. Importantly, this was the first clinical evidence of immunoescape in CRC, as the prognostic value of T-lymphocytes was lost in patients which retained a metastatic disease at diagnosis. Moving to $CD4^+$ marker few studies reported relationships between tumour $CD4^+$ T lymphocyte and cancer survival (reviewed in Roxburgh [86] and Table 2.4.2.1). A report from Canna described a positive correlation with survival in patients with increasing general intratumoural $CD4^+$ T-lymphocyte infiltration [93]. $CD45RO^+$ staining for memory T lymphocytes were assessed in different studies and were related to survival in colorectal cancer (Reviewed in Roxburgh [86] and Table 2.4.2.1). Higher densities of $CD45RO^+$ cells which were assessed at both the tumour centre and invasive margin were associated with better survival o patients prognosis. Pages et al in their study took advantage only of stage I and II (node negative) colorectal cancers and $CD45RO^+$ which were measured at the tumour margin and centre was associated with better patients prognosis in both cases [94]. Moreover authors combined $CD45RO^+$ and $CD8^+$ T-lymphocytes and produced an ‘‘immune score’’ that was showed to be an independent prognostic factor [94]. The paper from Nosho above described showed that CD3 densities were not predicting survival while T-lymphocyte subsets $CD8^+$ $CD45RO^+$ and *FOXP3*$^+$ cells were all significantly related to better cancer survival at univariate analysis [91]. When multivariate analysis was performed among all those immune markers included in the model $CD8^+$ cells were not predicting prognosis independently of CD45 RO^+ cells [91]. Some studies took advantage of $FoxP3^+$ cells to examine the prognostic value of T-reg cells in CRC patients (Reviewed in Roxburgh [86] and Table 2.4.2.1). Among them only two studies reported that *FOXP3*$^+$ expression was an independent prognostic factor [95,96]. The first study from salama group examined densities of *FOXP3* in stage II and III CRC in intra-tumoral randomly chosen sections[95], while the second study from Frey group found prognostic independence of *FOXP3* only in MSS CRC[96]. In contrast, the study from Nosho stated that intraepithelial $Foxp3^+$ cells were not independent from $CD45RO^+$ cells when predicting outcome [91].

2.4.2.3 Tumour associated macrophages (TAM), classified by specific CD antigen.

When considering TAM in CRC many studies have examined their predictive ability in colorectal cancer (Reviewed in Roxburgh [86] and Table 2.4.2.1). Nine studies reported significant positive correlations between macrophages either at the tumour margin or tumour centre and survival. In five studies the better associations with survival were observed when macrophages were assessed at the tumour invasive front. While four studies of modest patients cohort extent reported no relationships between $CD68^+$ macrophages and survival (Reviewed in Roxburgh [86] and Table 2.4.2.1). Forssell and Zhou proposed two different studies which took advantage of 446 and 160 patients, respectively and reported that higher counts of macrophages at the CRC tumour margin was an independent prognostic marker associated with better patient outcome and survival [71,97]. However both studies took advantage of a semiquantitatively methodology for $CD68^+$ cells infiltration assessment, which were evaluated by taking advantage of a manual count performed by pathologists which is subjected to operator's estimation. Moreover the authors proposed a $CD68^+$Hotspot value which was defined as the infiltration grade of the two highest view fields. Forssell suggested that a "vigorous macrophage response" that authors proposed as "hotspot" at sites of ongoing invasion might be of help in interpreting the protective action of macrophages with respect to patient prognosis. In this study were included patients from stage I to IV and patients which also may have received adjuvant radiotherapy (before surgical treatment). According to the fact that only rectal cancer patients included in this study may had received neo-adjuvant radio and chemotherapy it is worth to underline that in this study $CD68^+$ cells values were predicting better prognosis only among colon cancer patients [71]. In the report from Zhou only patients with stage III or IV CRC patients which didn't receive chemo or radiotherapy as neo-adjuvant treatment were taken into consideration and moreover stage IV patients were correctly excluded from survival analysis[97]. Accordingly, clinical follow-up was only provided to stage IIIB patients, as patients with stage IV are a group with high heterogeneity, including solitary or multiple liver metastases, liver only or other sites involved with metastases; these variables affects the treatment protocols and eventually the response rate and prognosis. Interestingly, in this study $CD68^+$ densities were lower in tumour stage IV and also in patients with synchronous compared to metachronous liver metastasis [97]. This suggests that $CD68^+$ densities were lower in stage IV regardless of disease progression and patients survival, therefore CD68 score in this study doesn't seem appropriate to predict prognosis in patients with metastasis at diagnosis. Interestingly, in the study from Kang authors stated that intratumoral TAMs cause CRC cells to have a more aggressive behaviour, as $CD68^+$ densities

were increasing along lymphnodal tumour metastasis in CRC patients [98]. Differently from other studies on TAM, in this report $CD68^+$ cell densities were quantitatively assessed, which is a more reproducible and objective method of measurement. However the authors didn't provide any survival and prognostic associative data to support their assumptions and a mere association of higher recruitment of CD68 densities in CRC patients with lymphnodal metastasis compared to patients that were not metastatic is not indicative of TAM pro tumour activities. Examples of $CD68^+$ cells and $CD3^+$ TILs and at the CRC invasive front and among tumour glands are shown in figure 2.4.2.3A. Examples of $FOXP3^+$cells in CRC Chron's like reactions, the CRC invasive front and intratumoural space is shown in figure 2.4.2.3B

Figure 2.4.2.3A Innate and adaptive infiltrating immune cells are detectable irregularly distributed in the tumour-stromal interface of the colorectal invasive front or among the tumoral glands. (Objective magnification, 10x).

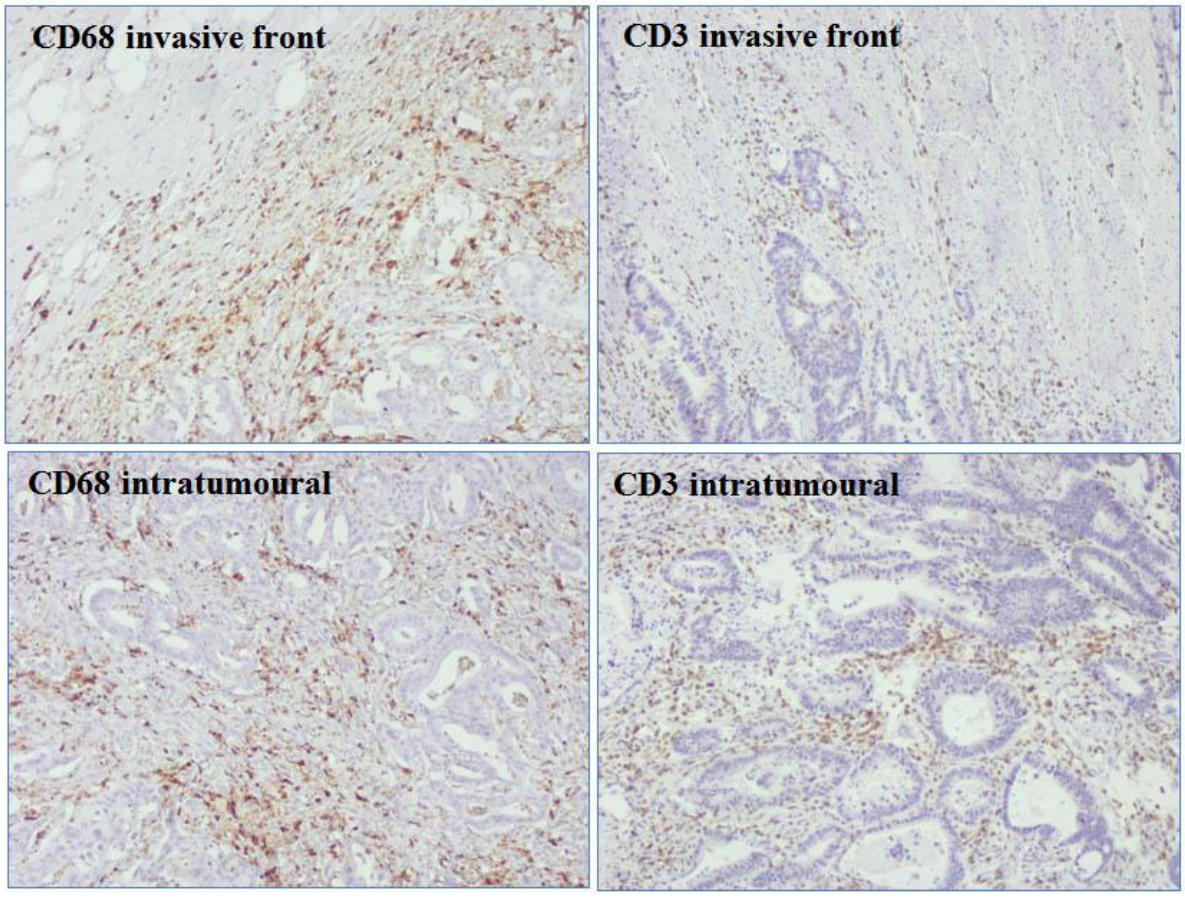
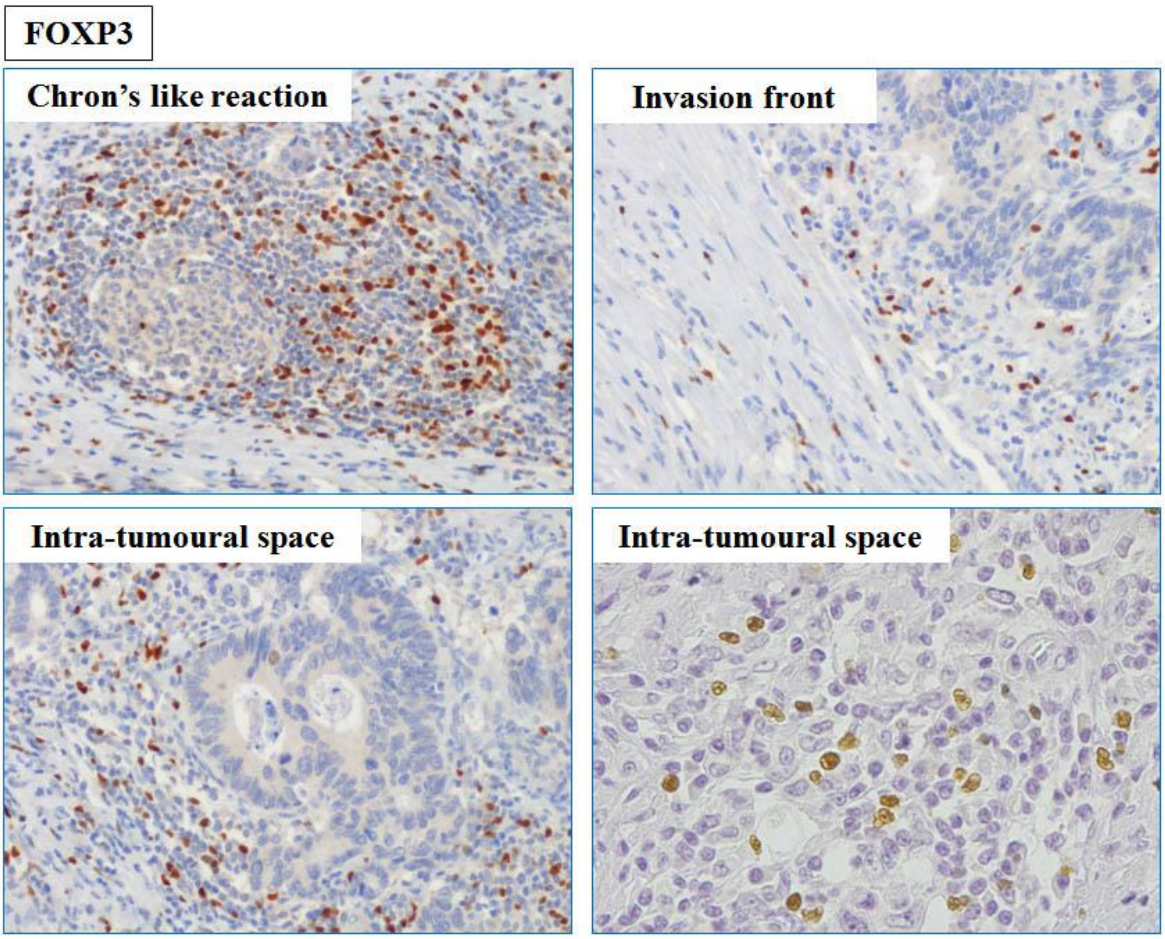


Figure 2.4.2.3B. Forkhead box P3 (FoxP3)+ lymphocytes have been observed into Chron's like reaction, at the tumor-stromal interface or among the tumoral glands. A clear nuclear immunoreactivity has been always found. (Objective magnification 20x and 40x).



2.4.2.4 Interplay with the type of genetic instability.

Different studies have shown that MSI tumours are characterized by a stronger lymphoid reaction compared to patients with MSS molecular pattern. A Crohn's like reaction consisting of lymphoid aggregates with germinal centres has been found to be predominant in microsatellite unstable tumours [84]. Moreover, despite the fact that MSI is emerging as a biomarker of better prognosis, many studies showed that tumour infiltrating lymphocytes densities are independent of MSI when predicting better survival (Reviewed in Roxburgh [86] and Table 2.4.2.1). Detailed analyses have shown that MSI-H CRC are characterized by tumour infiltrating immune cells predominantly cytotoxic and activated and moreover such tumours release mediators of target cell death which were found to be located in proximity of activated lymphocytes [99], suggesting that an higher lymphocytic reaction in MSI CRC might be one of the molecular pathways of this type of cancer involved in the longer survival compared to MSS patients . In a large study from Salama which employed tissue microarrays, MSI CRC was associated with higher densities of $CD8^+$ and $CD45^+$ cells, while $FOXP3^+$ cells were not correlated with MS-status [95]. In the same study, only $FOXP3^+$ cells were independently associated with better survival. A large study from Frey et al. revealed that higher density of $FOXP3^+$ cells was predicting prognosis only in MSS patients but not in MSI patients [96]. In another large study from Nosho, which took advantage of intraepithelial measurements of immune markers, was revealed that in an adjusted analysis MSI patients were associated with a marked lower degree of $FOXP3^+$ and higher densities of $CD45RO^+$ cells, while in the same model MSI was not correlated with the densities of $CD3^+$ cells and $CD8^+$ [91]. This result suggests that lymphocytes infiltration in MSI CRC in this study was greatly composed of $CD45RO^+$ and therefore of activated CTLs, while poor of Tregs. Moreover, in this study only $CD45RO^+$ cells were independently predicting better survival. Moving to innate immunity, in a paper from Bauer was shown that infiltration of $CD163^+$ macrophages was significantly elevated in MSI-H compared to MSS CRC [100]. The authors suggested that the high numbers of antigen-presenting cells such as macrophages in MSI tumours might be induced by MSI-H CRC-specific antigens and by the induction of frameshift antigen-specific immune responses commonly observed in such type of cancers. However the authors didn't measure CD68 densities which is a pan-macrophages marker and didn't took in account whether the burden of $CD163^+$ cells was representing a subpopulation of macrophages infiltration in the tumour. It has been speculated and afterward suggested that frameshifts truncated peptides (FSP) that are aberrantly produced in MSI tumours might be immunogenic and for this reason might be presented to CTL[101-103]. This theory was proposed

as a biological explanation for the higher lymphocytic infiltration in such class of tumours since such peptides accumulate in the tumour cells and might be antigenic by eliciting immune response in the host [101-103]. In vitro experiments with frameshift peptides demonstrated that such molecules are immunogenic and have the ability recruit T cells [104,105]. However, later studies demonstrated that frameshift mutations have the ability to initiate nonsense mediated RNA decay system which cause RNA degradation of such microsatellite sequences when truncated and thus avoiding translation of FSP mutant proteins, for this reason it is still a matter of debate whether such FSP are generated in vivo [106]. Moreover, MSI tumour microenvironment retain a pro-inflammatory cytokine milieu which is known to contribute toTh1 response [107], in line with an antitumoral immune response. Many studies demonstrated specific T cell responses directed against different MMR deficiency-specific antigens in individuals with MSI-H CRC [101]. This was a convincing evidence that DNA sequences with frameshift mutations might elicit antigenic structures able to trigger antigen-specific antitumor T cell responses. Genes with important functions in immunity regulation have been found to retain microsatellites sequences. In this regard TGFB receptor 2 gene, which is a major regulator of adaptive and innate immunity, retain an A10 repeat mutated in >90% of MSI-H CRCs [8]. Frameshifts mutations of this repeat lead to an abrogation of TGFBR2-mediated signalling that lead to an increased growth rate of MSI-H tumours [108]. At the same time was proposed that TGFB pathway perturbation in different cancers might be important for regulation of immunity in the tumour microenvironment [109]. Accordingly, loss of tgfbr2 in MSI CRC might account for the increased lymphocytic reaction detected in the tumour site. Interestingly, it is worth to notice a paradox in the evolution of MSI tumourigenesis pathway, because frameshift mutations in MSI CRC while promoting tumour development by inactivating gatekeepers genes, at the same time they intrinsically make the tumour susceptible to recognition and elimination by the host's immune system.

Chapter 3 Colorectal cancer response to 5-FU based treatment

3.1 Chemotherapy in immunity driven tumour cell death

The National Cancer Institute strategies to select conventional antineoplastic agents with the best ability to kill cancer cells of most solid tumours have been developed to date on murine immunodeficient host based on drugs ability to directly interact with cancer cells and thus to inhibit their growth or induce cell death [102, 103]. However, this strategy completely neglect that the host immune system might have any effect or interaction on tumour eradication in the context of chemotherapy. Most anticancer treatments target rapidly proliferating cells without distinction whether they are immune system cells or tumour cells, since several cell types continuously proliferate in physiological conditions. For this reason conventional chemotherapy treatments are often associated with severe side effects which include myelosuppression, mucositis (linked to gastrointestinal toxicity) and alopecia. The immunosuppression caused by such treatments has not prevented oncologists to perform studies to assess whether immune system might have any role in the activity of conventional anticancer therapies. By taking advantage of different syngeneic solid tumours as in vivo models Apetoh and collaborators compared the ability of conventional anticancer treatments such as anthracyclines or oxaliplatin to promote tumour regression in immunodeficient versus competent mice. By taking advantage of this methodology, authors found that $CD8^+$ T cells mice depleted or carrying the knockout of either INFγ or the INFγ receptor reduced the efficacy of chemotherapy treatments compared to immunocompetent mice [110,111]. On this issue the group of zitvogel performed experimental studies to assess whether anticancer compounds might retain any ability to induce immunogenic cancer cell death. In the absence of any adjuvant therapy, inoculation of syngeneic CT26 tumour cells conditioned with sub-lethal chemotherapy drugs could prevent tumour growth when live CT26 cell were rechallenged in immunocompetent Balb/c mice, while this therapeutic response was lost when treated CT26 cells were implanted in immunodeficient nude mice [112]. Despite such evidences nowadays chemotherapy is still thought to kill cancer cells by apoptosis and thus in a non-immunogenic fashion. Apoptotic cells express ligands that are different by those expressed on living cells and that are recognized by phagocytes. Phagocytosis signals expressed by apoptotic cells include calreticulin, oxidized low-density lipoprotein particles, thrombospondin-1-binding sites, C1q- or C3b/bi-binding sites, and mannose-binding lectins [113,114]. These molecules are detected by phagocytic cells by recognition of scavenger receptors such as CD68 and CD36. Moreover, it has been reported that apoptotic cells can release

chemotactic signals which are known to recruit phagocytes, such as phospholipid lysophosphatidylcholine [113,114]. Therefore it is reasonable to assume that massive apoptosis generated by chemotherapy agents might activate phagocitosis that might cross present tumour antigens to T-cells. Macrophages are the most prevalent antigen-presenting cells in tumours and in certain cases may account for about 50% of the tumour mass [70]. Both DCs and macrophages have the ability to pick up tumour antigens for cross-presentation on MHC class I molecules [115]**.** Accordingly, splenic marginal metallophilic macrophages (MMM) have been found to efficiently capture and transfer antigens exclusively to dendritic cells for cross-priming cytotoxic T lymphocytes [116] . In this context it is important to consider that a recent paper from De Visser challenged the idea that adaptive system may increase chemotherapy-mediated tumour cell death proposed by Zittvogel and colleagues[117]. In this study authors took advantage of two different genetic models of spontaneous murine breast cancer MMTV-*NeuT or K14cre*; *Cdh1flox/flox*; *Trp53flox/flox* (FVB/N) crossbreeded with *Rag2–/–* mice. Cisplatin, oxaliplatin or doxorubicin ability to restrain the growth of mammary tumours was not changing in T and B cell deficient and immunocompetent mice [117]. Therefore the absence of the adaptive immune system did not affect mammary tumorigenesis in both chemotherapy treated and untreated mice. This study employed spontaneous murine tumour instead of syngeneic ones and moreover they employed specifically adaptive immunity deficient models as controls instead of nude mice. Therefore understanding why the adaptive immune system does not contribute to chemoresponsiveness may yield to new strategies or new cellular mediators able to enhance chemotherapy-driven antitumor activity.

3.2 Predictive factors of 5-FU based adjuvant treatment responsiveness in stage I-III Colorectal cancers

3.2.1 TNM staging and 5-FU based chemotherapy

When excluding patients with distant metastatic spread of the disease, surgery has always been the primary treatment for patients from stage I-III CRC. In parallel, since the early 1990s, 5-fluorouracil (5-FU) has been the mainstay of postsurgical chemotherapeutic treatment for patients with CRC in the adjuvant setting. Nowadays, almost all adjuvant chemotherapy regimens involve the use of 5-FU, typically in combination with leucovorin and more recently with oxaliplatin which retains better prognosis in CRC patients compared to 5-FU only based treatments [118]. Since stage IV CRC retain a dismal prognosis and mostly doesn't receive surgical treatment, chemotherapy is considered as a palliative medication among such patients which doesn't retain a substantial benefit in terms of survival [119]. In clinical practise administration of adjuvant chemotherapy in CRC patients is intended only for stage III CRC and stage II with poor prognostic features [120]. The clinical guidelines suggest that only certain poor prognostic features in stage II CRC might recommend clinicians to consider adjuvant therapy [121,122]. Poor prognostic features in CRC are: levels of preoperative carcinoembryonic antigen more than 5 ng/mL, diagnosis of bowel obstruction or perforation, need for emergent operation, T4 local invasion, improper nodal resection (<12 lymph-nodes examined), or peritumoral lymphatic/venous invasion [121,122]. However, there is no consensus that such clinicopathological tumour characteristics, are predictive of a good response to adjuvant chemotherapy [121]. The QUASAR prospective trial, which is commonly cited, reported a an improved survival for patients with stage I to III colon and rectal cancer receiving adjuvant chemotherapy compared with surgery alone. However, when considering only stage II colon cancer subgroup of patients this study couldn't prove any significant survival benefit of 5-FU based chemotherapy [123]. A large meta-analysis including many clinical trials concluded that chemotherapy in patients with stage II disease provided an survival improvement that was statistically non-significant [121]. Despite controversial and uncertain data, adjuvant chemotherapy is commonly administered to stage II patients with poor prognostic features. A recent paper on the issue from o'connor took advantage of about 43'000 stage II and III colon cancer patients which were obtained retrospectively from the SEER registry and diagnosed from 1992 to 2005, stage II colon cancer were stratified in poor prognostic features [124]. This epidemiological statistically robust paper revealed that patients with stage II colon cancer, even those with any of six identified poor prognostic features, do

not have a survival benefit from chemotherapy. The authors pointed out that the dataset included patients recruited for long period of time, and since adjuvant treatment regimen recommendations have changed during the period of the analysis many patients in this study population did not receive oxaliplatin, thus creating an heterogeneous distribution of chemotherapy regimens in the population studied, although all patients received chemotherapy 5-FU based [124]. Accordingly, when considering only stage I-III CRC it is tempting to speculate that chemotherapy have a beneficial effect only at later stage of disease when tumour clones are more likely to have spread in to the body although not yet clinical detectable and thus immunoescape mechanisms may have selected tumour clones not detectable by immune system for their elimination. In this view I hypothesis that chemotherapy might cause alteration in tumour cells or *de novo* expression of molecules that might restore tumour cell immunogenicity and recognition by immune system. It is important to notice that despite clinical evidences, the biological basis of discrepancies in terms of chemotherapy benefit along CRC progression are still unknown. The current need of valid experimental models which correctly reproduce CRC patients progression might explains at least in part this lack of knowledge.

3.2.2 Microsatellite-status and 5-FU based chemotherapy

Different studies performed in vitro suggested a strong association between MSI-H and resistance to 5-FU chemotherapy. Koi and colleagues by taking advantage of *hMLH1*-deficient HCT116 MSI CRC cell line, which is known to be resistant to 5-FU treatment, showed that chromosome 3 transfer in the same cell line which restored *hMLH1* gene function also reinstated HCT116 cell line sensitivity to 5-FU chemotherapy [125]. In another study the treatment of HCT116 MSI CRC cell line with the de-methylating drug 5-azacytadine, which restored transcription of *hMLH1*, also reinstated HCT116 cell line sensitivity to 5-FU chemotherapy [126]. Moving to in vivo studies, the employment of ortothopic colon cancer xenografts confirmed that MSI tumours are not sensitive to 5-FU chemotherapy treatment [127]. Moving to clinical studies, few reports gave no evidence of any predictive ability of MSI to 5-FU treatment [128,129]. However, a bunch of studies showed that MSI is a negative predictive marker of response to 5-FU. Such reports included randomized clinical trials[130,131], retrospective case series [132,133], and a meta-analysis [134] and together provided evidence that CRC patients with MSI tumours had no survival increase from 5-FU adjuvant treatment and in the study from Ribic authors even suggested that 5-FU chemotherapy might worsen overall survival of MSI CRC patients [130]. A pooled analysis of stage II and III CRC patients which included also patients from Ribic [130] study showed that MSI retained better prognosis compared with MSS only in the subgroup of chemotherapy untreated patients, while in the subgroup of CRC patients that received 5-FU adjuvant treatment MSI didn't retain any prognostic advantage compared to MSS and this evidence suggest that only MSS tumours had survival benefit by chemotherapy treatment [131]. It is important to consider that in this study authors also pointed out that among MSS CRC patients 5-FU adjuvant chemotherapy was associated with better prognosis only in stage III subgroup of patients but not in stage II CRC. However, according to the low prevalence of MSI patients in the overall CRC population (about 10-15%) authors acknowledged that data produced in this study were based on a low prevalence of MSI that didn't give proper statistical power to assess significant interaction effect [131]. Ideally, predictive marker analyses should be conducted in studies that incorporate untreated control arms, however most studies do not meet this criteria. Nowadays it is clinically recommended that patients with stage II colon cancer which retain MSI should not receive 5-FU as adjuvant therapy [131,135].

9.3 Immune cells and 5-FU based chemotherapy

As mentioned previously, many clinical studies measured immune densities in CRC and reported immune cells as positive prognostic factors. However, very few study assessed with efficacy the predictive impact of such infiltrates on the efficacy of conventional cancer therapies in CRC. In the context of immune infiltration chemotherapy treatment has important clinical effects as it might lead to myelosuppression, although speculation raised whether this effect might also give rise to suppression of inhibitory immune cell function and thus stimulating the peritumoral T cells to infiltrate and attack cancer cells [136]. Alternatively, as explained previously, chemotherapy might induce an immunogenic cell death, which involves the de novo expression of antigens on tumour cells that might be immunogenic and thus providing immune cells antitumour abilities [110-112,137]. Despite this strong experimental evidence most of the clinical studies which related immune cells presence and CRC prognosis did not reveal any predictive value of immune effectors. In the paper from Laghi, authors stated that the extent of $CD3^+$ cells immunostaining did not affect the survival of the subset of stage III patients treated with fluorouracil adjuvant therapy [92]. However, a study by Morris and colleagues reported that in an adjusted analysis, stage III colon cancer patients with higher densities of tumour infiltrating lymphocytes were gaining a survival advantage from adjuvant chemotherapy [138]. It is important to notice that in this study detection of lymphocytes was obtained merely by pathological assessment. Moreover, was counterintuitive that in patients with low or high lymphocytes 5-FU chemotherapy was significantly associated with better survival in both subgroup of patients. To explain their results authors suggested that conventional chemotherapy might be an enhancer of antitumor immune responses and that the effects of chemotherapy might include the formation of a large amount of apoptotic tumour cells that enters the antigen presentation pathway. Authors also suggested that chemotherapy could also create lymphopenia as a side effect of such treatment, and in this phase immune system might be more receptive to the breaking of tolerance and thus stimulating their recognition and elimination of tumour cells. On this issue another study from Halama analysed immune infiltration and prognosis in stage IV CRC patients that received palliative chemotherapy treatment. In this study immune infiltration at the border with hepatic metastases was predicting better prognosis in CRC patients that received palliative chemotherapy and also included "technically nonresectable" liver metastasis [139,140]. It has to be considered that the study employed a low number of patients with a dismal prognosis and very importantly lacked of a control “arm” of untreated patients. In this regard it is important

to notice that immune infiltrate in CRC of stage IV primary tumour has been found to be scarce [141], since this subgroup of CRC retain colorectal metastases in the liver and a dismal prognosis, the low immune infiltration might be an effect of immunoescape [142]. Moreover, it is worth to consider that surgery in such patients is unlikely to be radical in the metastatic site. However, according to Fridman and colleagues at this late stage of disease an immune control of the neoplastic disease may still persist, although in very rare cases retaining a relatively favourable prognosis [142].In a study from Prall authors reported that the prognostic positive association with high CD8+ infiltration was more significantly marked in stage III CRC patients who received adjuvant chemotherapy treatment compared to the whole cohort of patients that included also those who were only treated by surgery [143]. However, also in the whole cohort of patients a significant association with better prognosis and higher densities of $CD8^+$ cells was reached [143]. Thus, there's some clinical suggestion that the amount of immune infiltrate before chemotherapy may have an impact on the efficacy of the treatment of colorectal cancer.

Material and methods

Patients cohort

Tissue specimens were taken from consecutive patients who underwent radical surgical resection for pT3 or pT4 colorectal cancer (CRC) at the Humanitas Clinical and Research Center, Rozzano, Milan, Italy, from January 1997 to November 2005. Patients' demographics and pathological data were available from the Institutional Intranet. To investigate the occurrence of patient relapse, tissues from patients with pT1 or pT2 colorectal cancer, who have a very low risk of progression, and tissues from patients with perioperatively detected metastases were excluded. Patients who underwent neoadjuvant radiotherapy for rectal cancer were excluded from the study, because of the possibility of interference with the assessment of the local immune response. Investigators who were blinded to the results of the morphological analysis performed a clinical database. The absence of metastasis at diagnosis was assessed in all patients by combining histopathological findings, surgical records and perioperative imaging. The observation period started immediately after surgical procedure. To exclude postsurgical tumor recurrences, thoraco-abdominal computed-tomography (CT) abdominal ultrasonography, and chest radiography, were done according to common protocols for surveillance. Microsatellite status was screened preliminarily for all cancers included in the study by testing instability at mononucleotide repeats, as previously described [17]. Ethics Committee of the Humanitas Clinical and Research Center approved the study, and written informed consent was obtained by the referring physician, at the time of surgery by each patient.

Immunohistochemistry

Formalin-fixed, paraffin-embedded, 2-μm thin sections of tumor were deparaffined and exposed to an antigen-retrieval system before being incubated with specific monoclonal antibodies raised against CD3 (dilution 1:50, clone F7.2.38, Dako, Italy), CD68 (dilution 1:200, clone KP-1, Dako, Italy), *FOXP3* (dilution 1:100, clone 236/E7, Abcam, Cambridge, UK), or with mouse IgG (Dako, Milan, Italy) as negative controls. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min at room temperature. Primary antibodies were applied for 1 h at room temperature. Reactive sites were identified by exposure to a MACH 4 Universal HRP-Polymer (Biocare, Space Import-Export, Italy) for 30 min at room temperature. Immunoperoxidase staining was then obtained by using diaminobenzidine as a chromogen (DAB^+ chromogen X-50, ChemMate, Dako Cytomation, Carpinteria, CA, USA).

The slides were subsequently counterstained with haematoxylin (Harris Hematoxylin, DiaPath, Microstain Division, Martinengo, Bergamo, Italy).

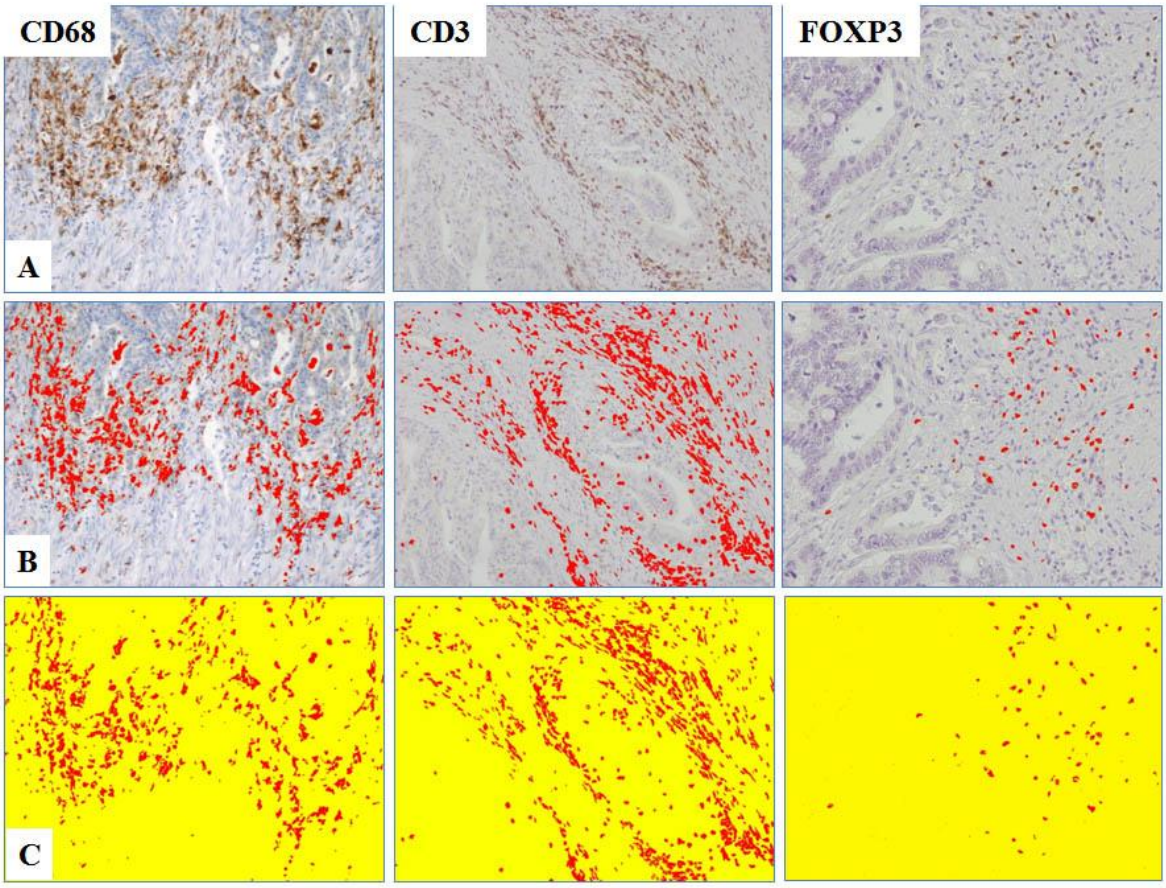
Computer assisted image analysis

Slides were digitized using a computer-aided image analysis system (Olympus DotSlide, Olympus, Italy). Assessment of $CD68^+$ TAM, $CD3^+$ and $FOXP3^+$ TILs density was quantified. An expert pathologist, who was blinded to any patient clinical data, selected three randomly selected non-contiguous and not overlapping microscopic areas located at the tumour invasive front, on tissue sections previously immunohistochemically treated with anti-$CD68^+$ TAM, $CD3^+$ and $FOXP3^+$ TILs antibodies. In each selected area, the cancer tissue had to represent approximately 50% of the entire microscopic field. To measure the extent of $CD68^+$ TAM, $CD3^+$ and $FOXP3^+$ TILs immunoreactivity, we used a computer-aided image analysis system with ad-hoc software able to discriminate the immunostained area on the basis of red, green, and blue (RGB) colour segmentation, and to calculate the per cent immunoreactive area as a fraction of the total area digitally captured (figure 1 Material and methods). For each histological section, the mean values obtained in three different regions were calculated and used for the subsequent statistical analysis as previously described from our group [92]. Results from each of the three selected area were fairly homogenous in most tumours (data not shown), showing a good concordance among the three measurements. Examples of increasing $CD68^+$ TAM, $CD3^+$ and $FOXP3^+$ TILs IRA% are shown in supplementary appendix Figures 6 to 8.

Metastatic Lymph-nodes

In addition to the primary tumour tissues specimens we also obtained matched tissue specimen from metastatic lymph-nodes of 135 out of 209 stage III CRC available in our institution. The tumour area in the lymph-node had to be sufficient to select three fields of interest not overlapping to be stained for CD68 with the same methodology we employed for the primary tumour. In addition, the immunoreactive area was quantified with the same methodology previously described for the primary tumour. A representative image of an immunoreactive surface covered by CD68+ cells in a whole partially metastatic lymph-node is shown in appendix figure 9.

Figure 1 Material and methods. A computer-aided image analysis was used for quantifying infiltrating cells at the tumor front of invasion in primary colorectal cancer tissues. Tissue sections were treated with antibodies raised against CD68, CD3 and FoxP3, and subsequently three not-overlapping and not-contiguous fields were chosen for each cell-type at the tumour invasive front and digitized (A). The immune-reactive surface covered by the infiltrating cells was specifically and automatically selected on the basis of the RGB color segmentation (B). The immune-reactive surface was automatically obtained by the ratio between the immunoreactive surface area and the unstained tissue surface (C). Each patient is characterized by a unique value, for each cell-type, given by the mean values of the three-fields randomly chosen at the tumour invasive front. In the present study has only been evaluated the cells infiltrating the tumor-stromal interface, with a ratio of nearly 1:1 between tumoral and stromal compartments.



Statistical analysis.

The association between the extent of $CD68^+$ TAM, $CD3^+$ and $FOXP3^+$ TILs, patient's baseline characteristics and tumour features was estimated by linear regression analysis. The distribution of immune cells densities in CRC at the tumor invasive front we studied was not resembling a normal one, but had a tendency to be skewed toward low values. For this reason, we represented the distribution of each immune cells type with categorical values in order to generate a qualitative interpretation to score data. A Cox proportional hazards model was developed to assess the role of $CD68^+$ TAM, $CD3^+$ and $FOXP3^+$ TILs density and other clinico-pathological features, in predicting the occurrence of disease specific survival (DFS). The detection of tumour recurrence or death was computed from diagnosis until data were censored on May 30, 2010. Recursive partitioning was referred to as CART analysis that was also used to identify optimal cut points in the data. The default tree was generated from the unmanipulated recursive partitioning CART algorithm. The partitioning of patients into groups with different prognosis using clinical variables available generates a tree-structured model that can be analysed to assess its clinical utility. Each tree's structure depended on the selected split value of the chosen variable. CART analysis is inherently non-parametric and no assumptions were made regarding the underlying distribution of values of the predictor variables. Thus, CART can handle numerical data that are highly skewed as it is the case of immune cells distributions. Differences in median values of $CD68^+$ TAM, $CD3^+$ and $FOXP3^+$ TILs density between subsets of CRC and according to DFS were tested by the Mann–Whitney U test and by Cuzick's trend test. Kaplan-Meier curves of DFS and DSS were plotted, while log-rank test was used to compare the curves of each subgroup of CRC patients. The mean follow-up period was 4,66 years (SD = 2.58 years) for DFS. The mean follow-up period was 5.13 years (SD = 2.25 years) for DSS. A time-dependent receiver operating characteristic (ROC) curve was constructed to define the optimal cut-off value of CD3 and *FOXP3* immunoreactive TILs area for predicting patients relapse in stage II cancers. A time-dependent receiver operating characteristic (ROC) curve was constructed to define the optimal cutoff value of CD68 immunoreactive TAM area for predicting patients relapse in stage III and stage III MSS cancers that received 5-FU adjuvant treatment (Supplementary appendix figure 1 to 5). For each test, only two-sided *P* values lower than 0.05 were considered statistically significant. To test whether $CD68^+$, $CD3^+$ and $FOXP3^+$ immune cells abilities to predict CRC patients prognosis might be modified by any variables we assessed, it is highly relevant to assess statistical interaction effects. Accordingly, by entering into a logistic regression model the categorical densities of immune values, each of the variables we

assessed and a product term consisting of the multiplication of the variables included in the model we tested any statistical interaction when predicting the risk of patient's outcome. All the analyses were done using Epi Info (Version 3.4.3), StatsDirect Statistical software (Version 2.5) and GraphPad Prism software (Version 4.1).

Results

Chapter 4. Densities of CD68+, CD3+ and Foxp3$^+$ immune cells in the primary tumour

4.1 Distribution of CD68$^+$, CD3$^+$ and Foxp3 $^+$ immune cells immunoreactive area at the tumour invasive front and their correlation with clinicopathological features at the time of surgery.

The percentage of immunoreactive area (IRA) calculated represented the density of each population of immune cells in the selected areas. However, the definition of density as the relationship between the numerical count of cells and the tissue surface on which they are distributed, is not respected. In fact, IRA has been calculated as the area occupied by the immune cells for each marker studied, as compared to the whole image of stained tissue. Given that an accurate optical count of immune cells was not feasible in the presence of agglomerates, adjacent cells or long cellular bodies, as it is the case for macrophages, we did not make the attempt to convert area values into number of cells. The distribution of densities of each immune cell population did not resemble a normal one, but was skewed toward low values. We performed the analysis of immune cells densities in 425 CRC patients, with stage II and stage III. In appendix table 1 are shown the distribution of patients according to demographics, clinical and histopathological variables that we took in account. In the entire population of CRC patients the densities of CD68$^+$TAM, CD3$^+$ and Foxp3$^+$TILs we studied ranged from 0% to 44.99%, from 0% to 20.04% and from 0% to 5.70% respectively. The median value of the distribution of CD68$^+$TAM, CD3$^+$TILs and Foxp3$^+$TILs densities in the overall population of cancers was 4.12%, 2.33% and 0.37% respectively. The first quartile and the third quartile of CD68$^+$TAM, CD3$^+$TILs and Foxp3$^+$TILs densities was 1.54% and 9.61%, 0.92% and 5.78%, 0.13% and 0.68% respectively. The densities of CD68$^+$ TAM were the highest at the tumour invasive front among the population of CRC patients we studied (Figure 1). TAMs at the tumour invasive front represent the principal and prevailing population compared to TILs.

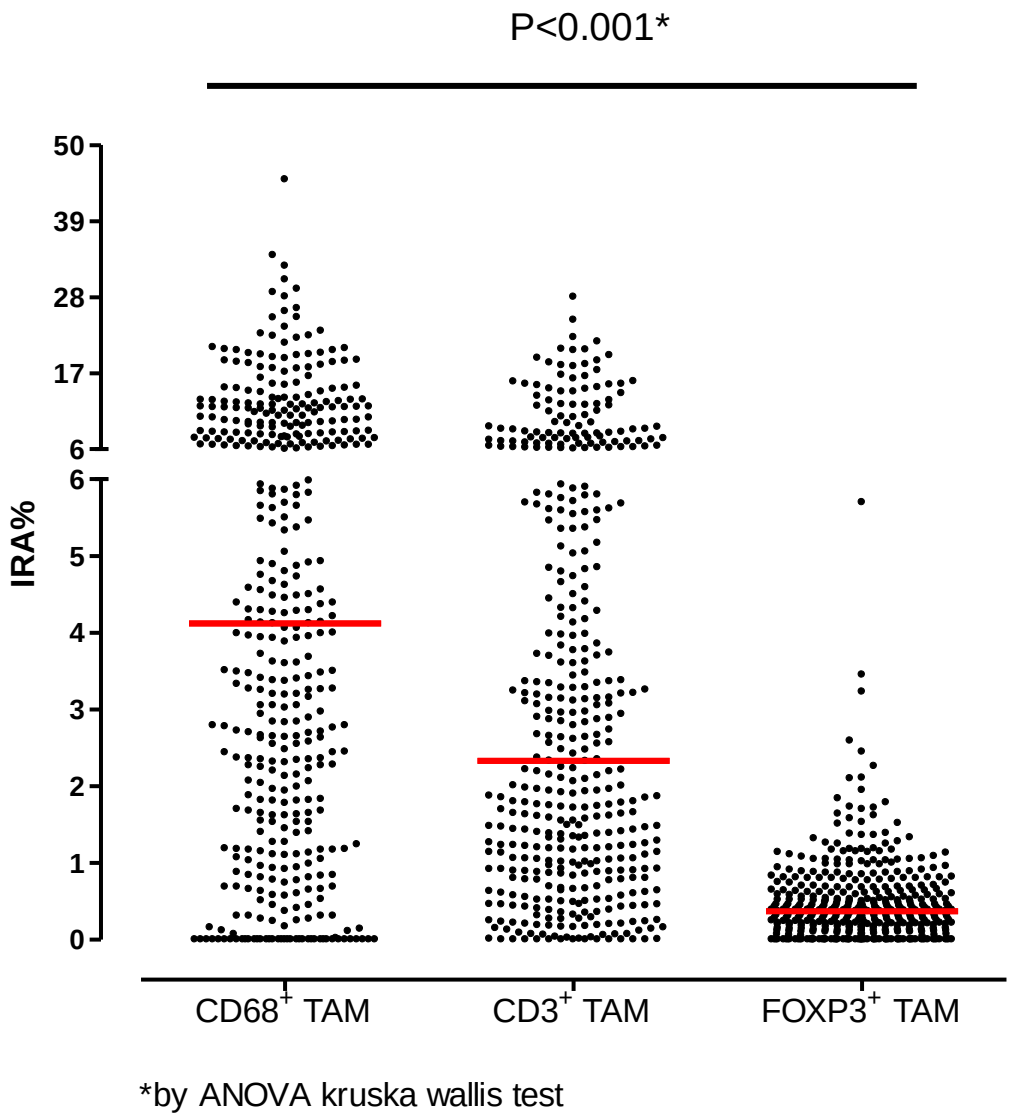


Figure 1. Distribution of CD68^{+}TAM, CD3^{+}TILs and Foxp3^{+} TILs densities at the tumour invasive front in the overall CRC population studied.

The relationship at uni and multivariate analysis between percentage of IRA of each immune cell population, demographics and histopathologic characteristics of patients recruited in this study is shown in table 2-4. CD68IRA% distribution was not changing according to the type of genetic instability and other CRC features (table 1).

Table 1. CD68-Immunoreactive area (IRA, % of microscopic field) at the Invasive Margin of 425 Stage II and III Colorectal Cancers.

		Median Value	2^{nd}-3^{rd} quartile	Univariate* P
Patient Age °	<68 yrs	4.06	1.26 - 9.75	0.83
	≥68 yrs	4.20	1.81 - 9.88	
Patient Gender	Male	3.51	1.65 - 9.79	0.98
	Female	4.49	1.29 - 9.45	
Microsatellite Status	MSS	4.12	1.65 - 9.75	0.98
	MSI	4.18	1.10 - 10.81	
Tumor Site	Colon Dx	3.94	1.45 - 8.48	0.12
	Colon Sx	3.56	1.17 - 9.93	
	Rectum	5.72	2.65 - 10.59	
Tumor Stage	II	4.33	1.58 - 10.19	0.18
	III	3.67	1.41 – 3.67	
Tumor Grade	G1/G2	4.28	1.64 - 9.89	0.09
	G3	2.09	1.16 - 8.21	
Tumor Cell Type	ADC	4.27	1.66 - 9.77	0.18
	Variants	2.31	0.37 - 8.59	
Vascular Invasion	No	4.14	1.39 - 10.02	0.34
	Yes	4.03	1.68 - 9.34	
5-FU Adjuvant Therapy				
Stage II	No	4.29	1.19 – 11.79	0.98
	Yes	4.37	2.20 – 9.66	
Stage III	No	3.47	1.96 – 6.63	0.88
	Yes	3.95	1.24 – 9.79	

* Linear Regression Analysis. "CD68-IRA%" was entered as a dependent, continuous variable.

° Age entered as a continuous variable

Moving to T-lymphocytes we found that $CD3^+$ densities were significantly increasing in CRC patients with MSI compared to those with MSS, thus confirming data in the literature (Table 2). Patients with CRC located in the right colon retained an high CD3IRA%, although this result was dependent by MSI status since MSI patients are more likely to occur in the right colon (data not shown).

Table 2. CD3-Immunoreactive area (IRA, % of microscopic field) at the Invasive Margin of 425 Stage II and III Colorectal Cancers.

		Median Value	2nd-3rd quartile	Univariate* P	Multivariate* P
Patient Age °	<68 yrs	2.42	0.89 - 6.11	0.07	
	≥68 yrs	2.17	0.92 - 5.69		
Patient Gender	Male	2.24	0.89 - 5.16	0.37	
	Female	2.55	1.02 - 6.12		
Microsatellite Status	MSS	1.94	0.83 - 5.03	<0.001	<0.001
	MSI	5.69	2.47 - 10.88		
Tumor Site	Colon Dx	3.38	1.13 - 7.40	<0.001	
	Colon Sx	1.96	0.94 - 5.46		
	Rectum	1.72	0.62 - 3.34		
Tumor Stage	II	2.41	1.01 - 6.09	0.30	
	III	2.24	0.87 - 5.59		
Tumor Grade	G1/G2	2.21	0.89 - 5.69	0.20	
	G3	3.24	1.02 - 5.93		
Tumor Cell Type	ADC	2.34	0.91 - 5.73	0.66	
	Variants	2.32	0.66 - 5.80		
Vascular Invasion	No	2.37	0.89 - 5.16	0.12	
	Yes	2.40	1.02 - 6.12		
5-FU Adjuvant Therapy					
Stage II	No	2.33	1.01 - 5.80	0.67	
	Yes	2.56	1.01 - 6.13		
Stage III	No	2.04	0.86 - 5.35	0.18	
	Yes	2.32	0.87 - 5.93		

* Linear Regression Analysis. "CD3-IRA%" was entered as a dependent, continuous variable.

° Age entered as a continuous variable

Moving to T-Reg lymphocytes, FoxP3IRA% was significantly lower in MSI patients compared to MSS patients, independently from other variables studied (Table 3). Thus, with respect to TILs MSI was positively associated with high $CD3^+$ densities, and negatively with low $FoxP3^+$ densities.

Table 3. Foxp3-Immunoreactive area (IRA, % of microscopic field) at the Invasive Margin of 413 Stage II and III Colorectal Cancers.

		Median Value	2nd-3rd quartile	Univariate* P	Multivariate* P
Patient Age °	<68 yrs	0.38	0.18 - 0.80	0.54	
	≥68 yrs	0.32	0.10 - 0.61		
Patient Gender	Male	0.35	0.11 - 0.64	0.20	
	Female	0.38	0.16 - 0.73		
Microsatellite Status	MSS	0.39	0.16 - 0.73	0.002	0.01
	MSI	0.21	0.00 - 0.46		
Tumor Site	Colon Dx	0.35	0.12 - 0.61	0.43	
	Colon Sx	0.34	0.12 - 0.80		
	Rectum	0.40	0.20 - 0.71		
Tumor Stage	II	0.34	0.12 - 0.62	0.11	
	III	0.38	0.14 - 0.73		
Tumor Grade	G1/G2	0.37	0.14 - 0.70	0.27	
	G3	0.31	0.10 - 0.51		
Tumor Cell Type	ADC	0.38	0.16 - 0.70	0.004	0.03
	Variants	0.14	0.00 - 0.41		
Vascular Invasion	No	0.38	0.14 - 0.69	0.12	
	Yes	0.31	0.10 - 0.54		
5-FU Adjuvant Therapy					
Stage II	No	0.32	0.08 - 0.61	0.33	
	Yes	0.37	0.15 - 0.68		
Stage III	No	0.26	0.10 - 0.57	0.44	
	Yes	0.42	0.20 - 0.80		

* Linear Regression Analysis. "Foxp3-IRA%" was entered as a dependent, continuous variable.

° Age entered as a continuous variable

Table 4 shows linear regression coefficients between adaptive and innate immune densities. CD68IRA% was significantly increasing in patients with higher densities of CD3IRA% (P<0.001) and FOXP3IRA% (P=0.009), independently by other variables studied. Therefore, the recruitment of $CD68^+$ TAM in the primary tumour positively correlates with the recruitment of both $CD3^+$ and $Foxp3^+$ cells at the tumour invasive front. Differently, CD3IRA% did not correlate with the density of Foxp3IRA% (P=0.24) (Table 4).

Table 4.Correlation between tumour infiltrating immune cells at the tumour invasive front in 413 Stage II and III Colorectal Cancers.

	CD68IRA%	CD3IRA%	FOXP3IRA%
CD68IRA%	- -	r=0.21 p<0.001	r=0.12 p=0.009
CD3IRA%	r=0.21 p<0.001	- -	r=0.05 p=0.24
FOXP3IRA%	r=0.12 p=0.009	r=0.05 p=0.24	- -

4.2 CART analysis of densities of CD68$^+$, CD3$^+$ and Foxp3 $^+$immune cells at the tumour invasive front to explore their correlation with patient's outcome.

By taking advantage of CART methodology we aim to test the ability of CD68$^+$, CD3$^+$ and Foxp3$^+$ immune cells densities in predicting disease progression by splitting such continuous distributions into subsets identified by an attributed threshold value. This process is repeated on each derived subset in a recursive manner (i.e. recursive partitioning). The recursion is completed when splitting no longer adds value to prediction. CART was performed by including in the analysis all the demographics, clinical and histopathological variables that we assessed and the densities of CD68$^+$, CD3$^+$ and Foxp3$^+$ immune cells. A default tree was generated by allowing the CART program to determine the variable with the optimal first split (Figure 2). The results for trees generated on 425 samples indicated that TNM staging was chosen as the initial split with a percentage of patients developing disease progression of 34.9% for stage III (n=216) and 14.9% for stage II (n=209) CRC (Figure 2). The next split node showed that among stage II CRC patients, the densities of Foxp3$^+$ TILs and then CD3$^+$TILs immune cells were selected by CART analysis, as a predictor of patient's outcome at an optimum cut-off value of 1.86% for CD3IRA% and 0.23% for FOXP3IRA% (Figure 2). In stage II subset of patients, densities of FOXP3IRA% below the value of 0.23% (n=85) identified CRC with a percentage of disease recurrence of 28.2%, while patients with densities of FOXP3IRA% in the tumour higher than 0.23% (n=131) had a percentage of disease recurrence of 6.1%. Therefore, densities of FOXP3IRA% were selected by CART analysis as a best predictor of disease relapse among stage II CRC. Similarly, in the next split densities of CD3IRA% below 1.86% identified CRC with a percentage of 39.1% of disease recurrence, while patients with densities of CD3IRA% higher than 1.86% selected patients with a percentage of disease recurrence of 14.3%. In stage III CRC node a cut-off value of 12.13% for CD68IRA%, between the variables analysed, was found by CART analysis to be the optimal split to discriminate patient's progression (Figure 2). Accordingly, a higher (>12,13%) density of CD68$^+$ TAM identified patients (n=35) with a percentage of disease progression of 14.3% while patients with a lower (<12.13%) density of CD68$^+$ TAM (n=174) had a percentage of disease recurrence of 39.1%. (Figure 2)

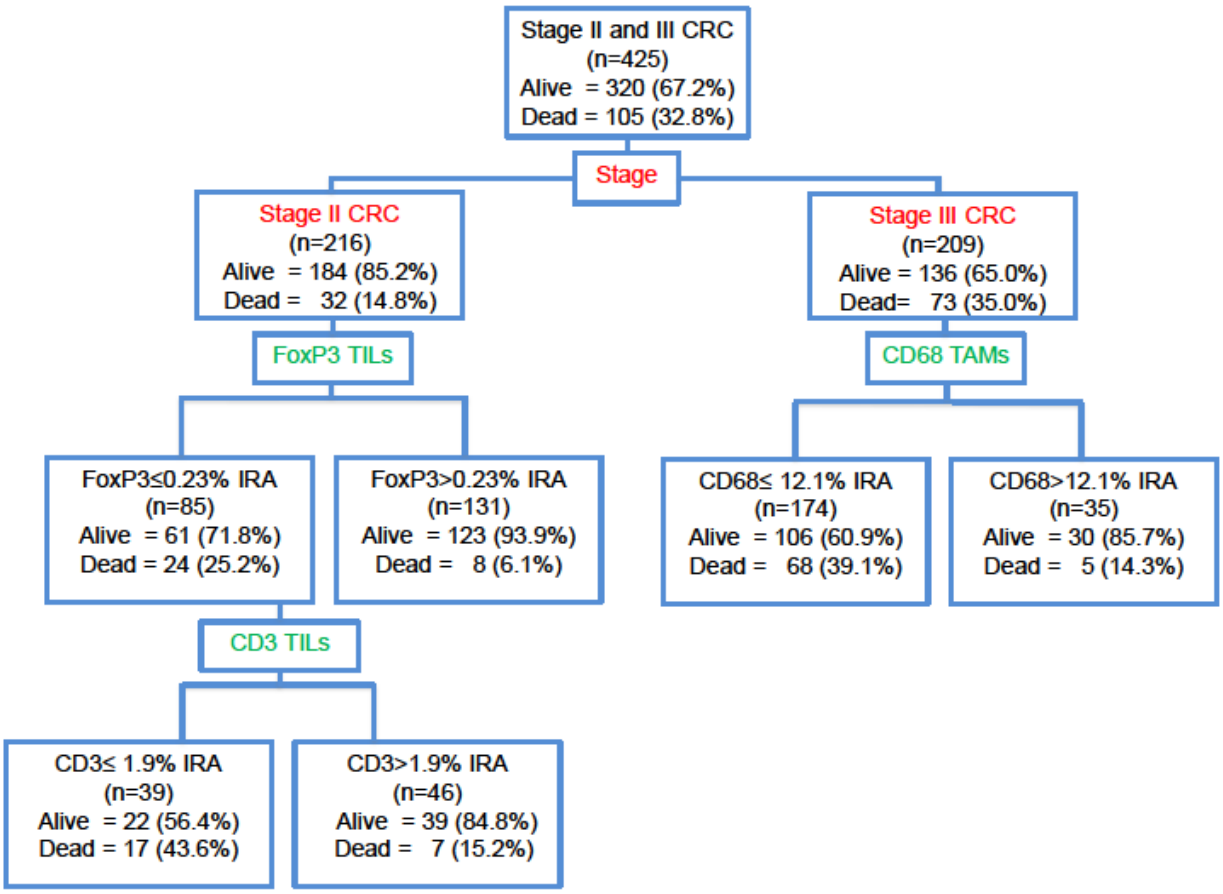


Figure 2. CART hierarchical recursive tree analysis in the overall CRC population studied.

4.3 Densities of CD68$^+$, CD3$^+$ and Foxp3 $^+$ cells at the tumour invasive front and patient's outcome

As shown in table 5 we performed univariate and multivariate Cox regression analysis of the densities of CD68$^+$, CD3$^+$ and Foxp3 $^+$ immune cells. The outcome was patients disease relapse. We recorded 105 events of CRC relapse among 425 stage II and III CRC patients. At univariate analysis, increasing values of CD68$^+$ [HR=0.77 95%CI (0.64-0.93), P=0.007], CD3$^+$ [HR=0.75 95%CI (0.60-0.94), P=0.01] and Foxp3$^+$ [HR=0.76 95%CI (0.63-0.90) P=0.002] IRA were strongly associated with better patient outcome (Table 5). Other features associated with higher risk of disease recurrence were rectal site (P=0.03), TNM stage (P=0.001), grade of differentiation (P=0.02), and vascular invasion (P=0.001). Interestingly, we found an additive negative interaction between 5-FU adjuvant treatment and TNM stage [5-FU treated vs untreated in stage III patients p=0.051] in predicting patient's disease relapse. Accordingly, among stage III CRC patients 5-FU adjuvant treatment had a tendency to be associated with a lower risk of disease relapse [HR=0.63, 95%CI (0.39-1.03), P=0.06]. In parallel, 5-FU adjuvant treatment was irrelevant in predicting prognosis among stage II CRC patients [HR=1.00, 95%CI (0.50-2.02), P=0.99]. This data is in accordance with data reported in the literature, since 5-FU adjuvant treatment is well known to be effective only among stage III CRC but not among stage II CRC. By systematically considering all the factors that may interact in determining patient prognosis, including the densities of immune cell populations, we found a significant interaction between the density of CD68$^+$ cells and 5-FU treatment in patients with stage III CRC when predicting patient's outcome(P=0.03). Differently, the interaction between categorical densities values of CD68$^+$ TAM and 5-FU adjuvant treatment in predicting the risk of patients outcome was not significant in stage II CRC patients (P=0.50). These results suggest that 5-FU adjuvant treatment modifies the ability of CD68$^+$ TAM densities in predicting patient outcome in patients with stage III CRC. On the other hand when testing statistical interaction between categorical densities of CD3$^+$ and Foxp3$^+$ cells and 5-FU adjuvant treatment in predicting the risk of patient's outcome we couldn't find any significant interaction in both stage II (P=0.88, P=0.48) and stage III (P=0.84, P=0.79) CRC patients respectively. Therefore, 5-FU chemotherapy did not modify the ability of adaptive immunity to predict the risk of patient's outcome and vice versa. The interaction between the density of CD68 and 5-FU treatment in patients with stage III CRC was not the only significant one. In fact we also found that adaptive TILs, either CD3$^+$ or FoxP3$^+$, interacted with TNM stage (P=0.02 and P=0.02 respectively) (Table 5). The detected roles of effect modifier exerted by 5-FU therapy with CD68$^+$TAM in stage III patients and by

TNM staging with $CD3^+$ and $FoxP3^+$ TILs in predicting patients prognosis suggested that we should proceed with separate regression models of the prognostic value of TAM and TILs according to interacting variables.

Table 5. Predictive factors for postsurgical relapse and their significant interactions in 425 patients with stage II and III colorectal cancer.

Tumour characteristics		Relapse		Univariate Analysis		Interaction Model
		No (n=320)	Yes (n=105)	HR (95%CI)	P	P<0.05
CD68-IRA	0-4%	149	61	1.00 ref.		
	4%-8%	65	24	0.92 (0.58-1.48)	0.74	**X 5-FU therapy (α)**
	8%-12%	37	10	0.66 (0.34-1.29)	0.22	**in stage III**
	>12%	69	10	0.41 (0.21-0.82)	0.01	
CD3-IRA	0-1%	77	37	1.00 ref.		
	1-5%	140	44	0.72 (0.46-1.11)	0.14	
	5-10%	63	20	0.74 (0.43-1.28)	0.28	**x TNM Stage (β)**
	>10%	69	10	0.26 (0.09-0.73)	0.01	
FoxP3-IRA	0-0,2%	89	40	1.00 ref.		
	0,2-0,4%	65	31	1.03 (0.64-1.65)	0.30	
	0,4-0,7%	77	13	0.40 (0.21-0.75)	0.005	**x TNM Stage (γ)**
	>0,7%	80	18	0.51 (0.29-0.90)	0.02	
Patients Age (years, mean+SD)				1,01 (0,99-1,03)	0.12	
Gender	Male	184	61	1.00 ref.		
	Female	136	44	0.99 (0.67-1.92)	0.95	
Site	Colon Dx	129	36	1.00 ref.		
	Colon Sx	127	36	0.88 (0.55-1.42)	0.60	
	Rectum	64	36	1.66 (1.04-2.64)	0.03	
MS-Status	MSS	264	95	1.00 ref.		
	MSI	56	10	0.66 (0.34-1.26)	0.21	
TNM Stage	II	184	32	1.00 ref.		**x (β)**
	III	136	73	2.62(1.73-4.00)	<0.001	**x (γ)**
Grade	G1/G2	268	79	1.00 ref.		
	G3	52	26	1.68 (1.08-2.61)	0.02	
Cell Type	ADC	296	92	1.00 ref		
	Variants	24	13	1.60 (0.90-2.86)	0.11	
Vascular Invasion	No	258	69	1.00 ref		
	Yes	62	36	1.97 (1.32-2.95)	0.001	
5-FU Adjuvant Therapy						
Stage II	No	107	18	1.00 ref		
	Yes	77	14	1.00 (0.50-2.00)	0.99	
Stage III	No	36	26	3.66 (2.00-6.68)	§	**x (α)**
	Yes	100	47	2.77 (1.31-3.91)		

[§]Expected H.R., 3.66, additive negative interaction; 5-FU chemotherapy in stage III CRC patients, yes vs no, H.R. 0.62 (0.38-1.00), p=0.051

α The interaction between CD68-IRA and 5-FU CHT in stage III (α) is statistically significant when CD68-IRA is entered as a categorical variable (α, P=0.03).

β The interaction between CD3-IRA and TNM staging (β) is statistically significant when CD68-IRA is entered as a categorical variable (β, P=0.020).

γ The interaction between Foxp3-IRA and TNM staging (γ) is statistically significant when CD68-IRA is entered as a categorical variable (γ, P=0.021).

In clinical practice, significant differences exist between patients who receive or not chemotherapy treatment, particularly with regard to age, co-morbidities, and provider/patient preferences. To address selection bias and to find whether CD68IRA% covariates with any demographic, clinical and histo-pathological characteristics owing to non-random treatment assignment we checked for clear group differences of CD68IRA% distribution by 5-FU chemotherapy treatment. Table 6 shows increasing values of CD68IRA% in stage III according to 5-FU adjuvant treatment on demographic clinical and histopathological characteristics. Increasing $CD68^{+}$TAM densities did not covariate with any other variables assessed (table 6).

Table 6. CD68-Immunoreactive area (IRA, % of microscopic field) at the Invasive Margin of 209 Stage III Colorectal Cancers according to adjuvant treatment.

		Adjuvant treatment			
		No chemotherapy		5-FluoroUracyle	
CD68IRA%		Median Value	P	Median Value	P
Patient Age	≤68 yrs >68 yrs	2.11 3.57	0.33	3.99 3.44	0.41
Patient Gender	Male Female	3.41 4.42	0.53	4.01 3.93	0.22
Microsatellite Status	MSS MSI	3.47 3.22	0.35	3.97 3.26	0.70
Tumor Site	Colon Dx Colon Sx Rectum	3.15 3.45 4.28	0.30	3.25 3.08 6.02	0.43
Tumor Grade	G1/G2 G3	4.12 2.65	0.10	3.98 3.78	0.85
Tumor Cell Type	ADC Variants	4.12 1.52	0.05	4.13 2.31	0.16
Vascular Invasion	No Yes	3.42 4.39	0.77	3.87 3.95	0.89

4.4 Prognostic value of CD68$^+$TAM at the tumour invasive front according to 5-FU adjuvant treatment and TNM stage

We inspected the effect modification exerted by 5-FU adjuvant treatment and CD68$^+$ TAM densities in predicting the risk of patient's relapse according to TNM tumour staging, by performing subgroup analysis. The predictive value of CD68$^+$ TAM in CRC patients who received or not 5-FU chemotherapy in stage II and III subgroups of CRC patients is shown in table 6. Among stage II CRC patients increasing values of CD68$^+$ TAMs densities were not associated with prognosis in both 5-FU adjuvant treated [n=147 HR=0,93; 95%CI (0,57-1,52); P=0.77] and chemotherapy un-treated patients [n=62 HR=0,77 95% CI (0,50-1,19); P=0.24]. However, when considering stage III subgroup of CRC patients we found that increasing values of CD68$^+$ TAM densities were associated with a lower risk of disease progression only among CRC patients receiving 5-FU adjuvant treatment [n= HR=0,64; 95%% CI (0,47-0,87) P=0.005], but not among patients that didn't receive any adjuvant treatment [n= HR=1,07; 95% CI (0,74-1,54) P=0.71].

Table 7. Prognostic value of CD68$^+$TAMs densities in 425 stage II and III CRC according to TNM stage and 5-FU adjuvant therapy.

	Tumour Stage							
	Stage II				**Stage III**			
	Relapse				Relapse			
CD68 IRA	No	Yes	HR (95%CI)	P	No	Yes	HR (95%CI)	P
5-FU Therapy = No								
0-4%	49	12	1.00 Ref		20	13	1.00 Ref	
4-8%	17	2	0.55 (0.12-2.47)	0.44	9	7	1.09 (0.43-2.74)	0.84
8-12%	16	1	0.28 (0.04-2.16)	0.22	3	2	0.76 (0.17-3.41)	0.72
>12%	25	3	0.61 (0.17-2.16)	0.44	4	4	1.39 (0.45-4.30)	0.55
5-FU Therapy = Yes								
0-4%	34	6	1.00 Ref		46	30	1.00 Ref	
4-8%	20	4	1.14 (0.32-4.05)	0.83	19	11	0.87 (0.44-1.75)	0.71
8-12%	10	3	1.59 (0.39-6.43)	0.51	8	4	0.76 (0.27-2.17)	0.61
>12%	13	1	0.46 (0.05-3.87)	0.47	27	2	0.14 (0.03-0.61)	0.008

Scatter plots of $CD68^+$ TAM densities according to CRC disease recurrence and adjuvant therapy in stage III or in stage II are shown in figure 3 and 4, respectively. When considering stage III CRC patients, among 5-FU adjuvant treated cancers CD68IRA% was significantly lower (P=0.008) in patients with evidence of disease relapse (n=100; median=4,21%; second-third quartile=1.81% – 12.25%) compared to patients with no evidence of tumour progression (n=47; median=2,37%; second-third quartile=0.41% – 5.82%) (Figure 3). Conversely, CD68IRA% was not different in stage III CRC patients who did not receive chemotherapy adjuvant treatment (P=0.89) with (n=36; median=3.47%; second-third quartile=2.26% – 6.52%) or without (n=26; median=3.64%; second-third quartile=0.93% – 7.66%) evidence of disease progression (Figure 3).

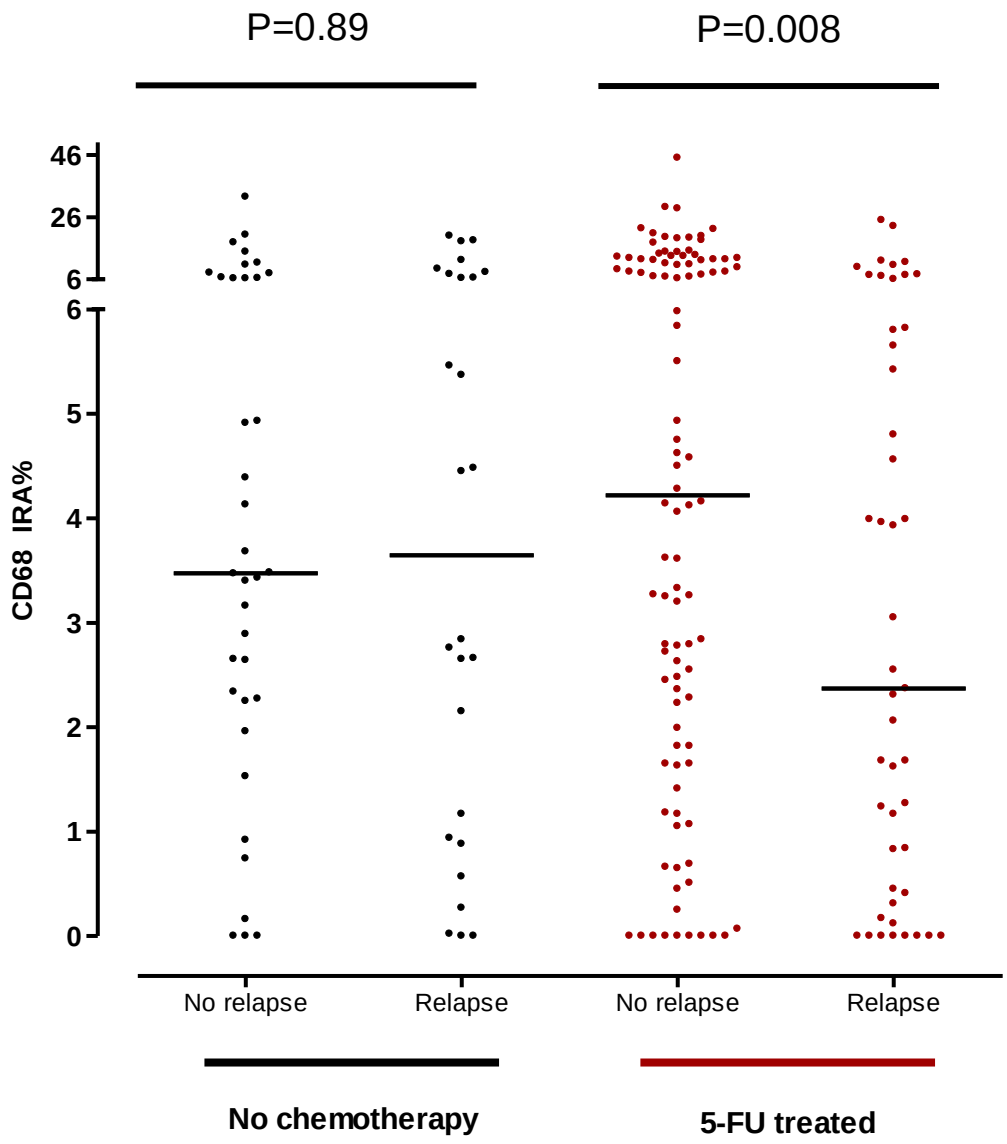


Figure 3. Distribution of CD68^{+}TAM densities at the tumour invasive front in <u>Stage III CRC</u> patients according to occurrence of disease progression and adjuvant chemotherapy treatment.

Among Stage II CRC patients CD68IRA% densities were not differing when comparing patients with or without evidence of tumour progression, in both 5-FU adjuvant treated (P=0.36) or untreated patients (P=0.74) (Figure 4).

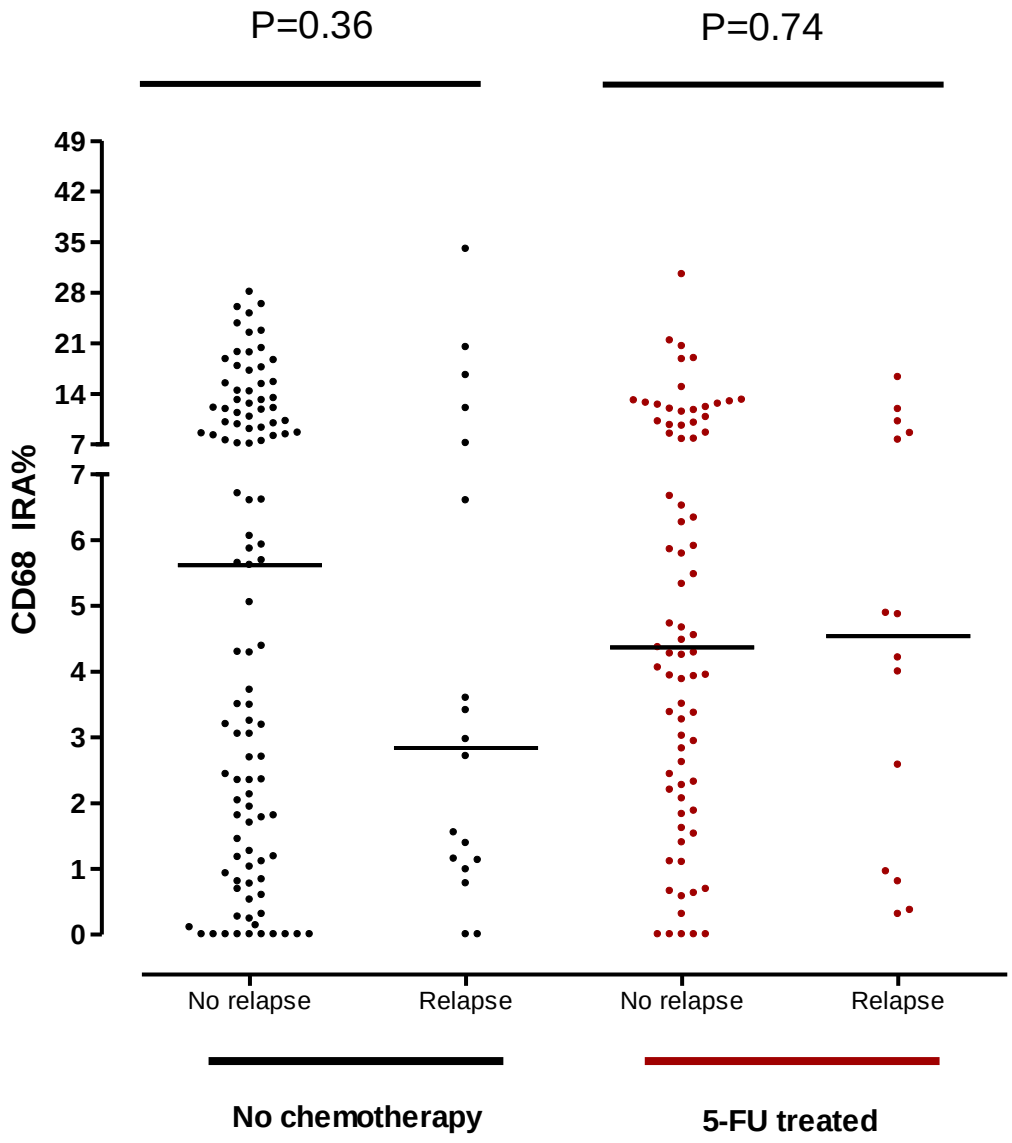


Figure 4. Distribution of CD68^{+}TAM densities at the tumour invasive front in <u>Stage II CRC</u> patients according to occurrence of disease progression and adjuvant chemotherapy treatment.

Figure 5a shows Kaplan Meyer survival curves of stage III CRC patients sub-grouped by values above ("high") or below ("low") the median density of $CD68^+$ TAMs. Patients who received 5-FU treatment and had a high density of $CD68^+$ TAMs had a better outcome (P=0.02) (figure 5a). On the contrary, the prognosis of stage III CRC patients that didn't receive any chemotherapy treatment was not affected (P=0.75) by densities of $CD68^+$ (figure 5a).

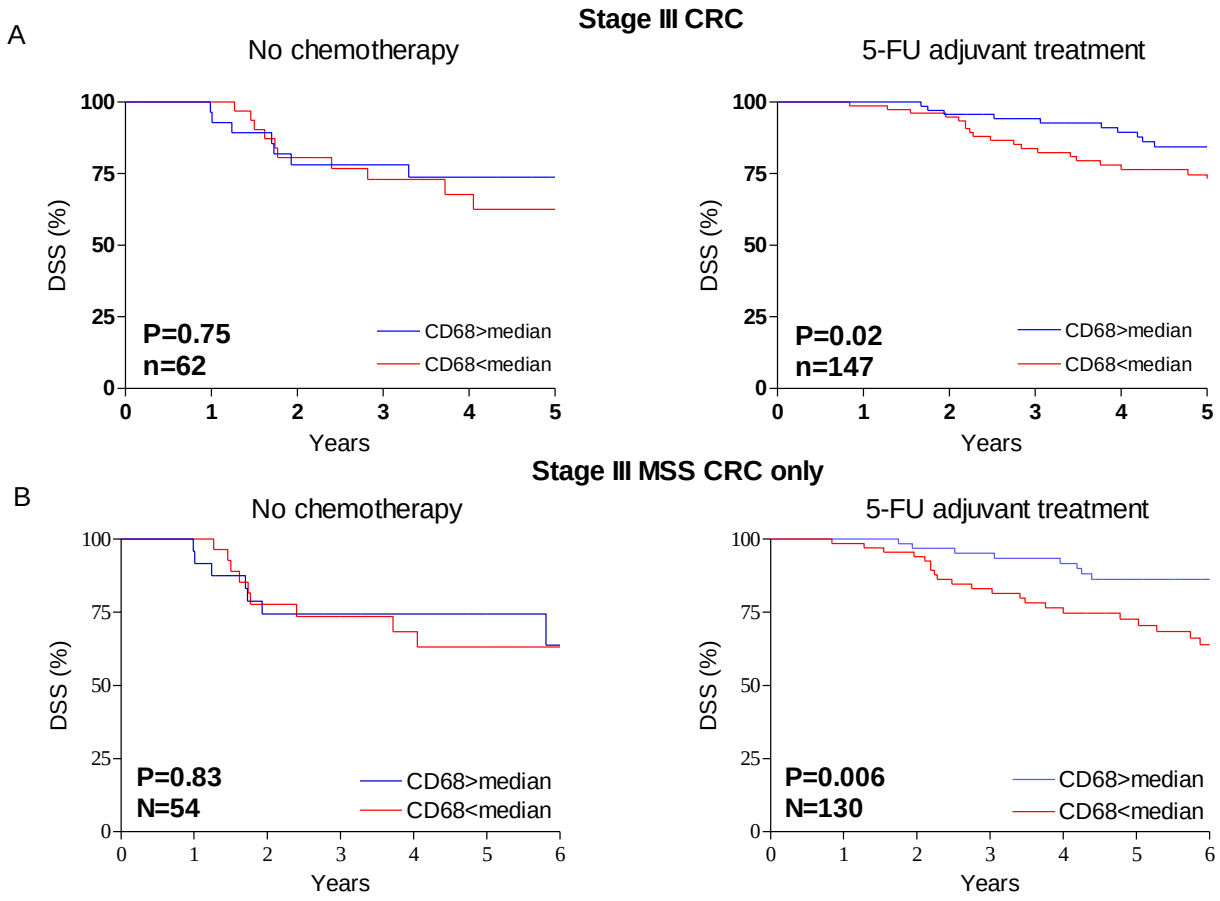


Figure 5A. Kaplan-Meyer survival curves showing disease specific survival (DSS) for <u>stage III CRC</u>. The median value (4.12%) of $CD68^+$ TAMs in the overall population was used to define high $CD68^+$TAMs.

Figure 5B. Kaplan-Meyer survival curves showing disease specific survival (DSS) for <u>stage III MSS CRC</u>. The median value (4.12%) of $CD68^+$ TAMs in the overall population was used to define high $CD68^+$TAMs.

4.5 Predictive value of CD68$^+$TAM density in response to 5-FU treatment is enhanced in stage III chromosomal instable tumours.

Ms-Status is an important determinant of CRC responsiveness to 5-FU adjuvant treatment, (i.e., predictive factor). For this reason, we tested whether the type of genetic instability might influence the ability of CD68$^+$ TAMs to predict the prognostic advantage of 5-FU adjuvant treatment in CRC patients. Of note, it is relevant to acknowledge that the low prevalence of MSI CRC patients translated in to only modest power to detect a statistically significant finding for an interaction effect with this variable. When MSI patients were removed from the model, among stage III CRC the interaction between increasing values of CD68$^+$ TAMs densities and 5-FU adjuvant treatment in predicting disease progression increased its statistical power (P=0.01). For that reason, we suspect that the type of Ms-status might modify the ability of TAMs to predict chemotherapy efficacy. Considering only stage III MSS CRC, increasing values of CD68$^+$TAM were associated with improved prognosis [n=130, HR=0.55; 95%CI (0.39-0.80); P=0.001] among patients that received 5-FU chemotherapy treatment. On the contrary, increasing values of CD68$^+$TAM densities in stage III MSI CRC were irrelevant to predict patient's prognosis [n=54 HR=1.05; 95%CI (0.73-1.52); P=0.77] among patients that didn't receive chemotherapy adjuvant treatment. Figure 5b shows Kaplan Meyer cancer related survival curves of stage III MSS CRC grouped by the density of CD68$^+$ TAMs. In the subset of stage III CRC patients, when we removed MSI patients from the analysis, an higher (>4,12%) density of CD68$^+$ TAM was associated with better cancer related survival (P=0.006) among patients that received 5-FU adjuvant chemotherapy treatment (Figure 5b). Conversely, the cancer related survival of stage III CRC patients who didn't receive chemotherapy did not change (P=0.84) according to CD68$^+$ TAM density (>median) (Figure 5b).

In table 8 are shown the predictive abilities of $CD68^+$ TAMs densities in stage III CRC patients with or without microsatellite instability stratified by 5-FU adjuvant treatment. Among stage III MSS CRC a higher density (>median, 4.12%) of $CD68^+$ TAMs was associated with a lower risk of disease progression only among patients that received 5-FU adjuvant treatment [HR=0.43, 95%CI (0.22-0.81), P=0.01] but not among patients that didn't received any chemotherapy treatment [HR=1.10, 95%CI (0.49-2.46), P=0.80] (Table 8). Conversely, among stage III MSI CRC the density of $CD68^+$ TAMs was not associated with patient's outcome, in both 5-FU receiving or not CRC patients (Table 8). Importantly, among stage III MSS CRC patients with higher density of $CD68^+$ TAMs at the tumour invasive front, 5-FU adjuvant treatment was associated with a lower risk of disease progression [HR=0.34, 95%CI (0.16-0.75), P<0.001] (Table 8). Conversely, among stage III MSS CRC patients with a lower (<median) density of $CD68^+$ TAMs the risk of disease relapse was not affected [HR=0.89, 95%CI (0.45-1.74), P=0.73)] by 5-FU adjuvant treatment (Table 8). Therefore, our data evidence that in stage III CRC MSS patients, 5-FU chemotherapy treatment seems to be effective only when the primary tumour retains a relevant number of macrophages at the tumour invasive front.

Table 8. Likelihood of disease relapse in stage 209 III CRC, by postsurgical adjuvant therapy and TAM density in the primary tumour.

		5-FU Chemotherapy								
		No				Yes				Yes *vs* No
		Relapse		HR (95%CI)	P	Relapse		HR (95%CI)	P	HR (95%CI)*	P
		No	Yes			No	Yes				
Stage III CRC		143	44			177	61				
MSS											
TAM	***Low***	17	12	1.10 (0.49-2.46)	0.80	38	29	0.43 (0.22-0.81)	0.01	0.89 (0.45-1.74)	0.73
	High	13	12			49	14			0.34 (0.16-0.75)	<0.01
MSI											
TAM	***Low***	3	1	1.15 (0.07-18.59)	0.91	9	1	5.43 (0.55-52.91)	0.14	0.42 (0.02-6.74)	0.54
	High	3	1			4	3			1.63 (0.16-15.87)	0.67

*Risk of relapse with respect to 5-FU treatment within each subgroup

4.6 Prognostic value of CD3$^+$ and Foxp3$^+$Tils densities at the tumour invasive front according to TNM stage of disease

Like CD68$^+$ TAMs, we tested the effect modification of TNM staging by separately assessing the prognostic abilities of CD3$^+$ and Foxp3$^+$ TILs in stage II and III CRC patients. Accordingly, the prognostic value of CD3$^+$ and Foxp3$^+$ TILs in stage II and stage III CRC patients is shown in table 9. Among stage II CRC patients, we found that a lower risk of disease progression was associated with increasing values of CD3$^+$ TILs [HR=0,49; 95% CI (0,31-0,77); P=0.002] and Foxp3$^+$ TILs densities [HR=0.50; 95% CI (0,34 - 0,74); P<0.001]. Conversely, among stage III CRC patients increasing values of CD3$^+$ TILs [n= HR=0.94; 95% CI (0.73-1.22); P=0.64] and Foxp3$^+$ TILs [n= HR=0.85; 95% CI (0,69 - 1,04); P=0.10] were irrelevant in predicting patient prognosis.

Table 9. Prognostic value of CD3$^+$TILs and FoxP3$^+$Tils densities as a predictor of disease relapse according to TNM stage.

	Tumour Stage							
	Stage II				Stage III			
	Relapse				Relapse			
	No	Yes	HR (95%CI)	P	No	Yes	HR (95%CI)	P
CD3 IRA								
0-1%	37	16	1.00 Ref		40	21	1.00 Ref	
1-5%	83	12	0.40 (0.12-2.47)	0.02	57	32	1.06 (0.61-1.85)	0.81
5-10%	38	2	0.15 (0.04-0.66)	0.01	25	18	1.35 (0.72-2.55)	0.34
>10%	26	2	0.22 (0.05-1.00)	0.05	14	2	0.32 (0.07-1.40)	0.13
FoxP3 IRA								
0-0,2%	52	18	1.00 Ref		37	22	1.00 Ref	
0,2-0,4%	40	10	0.72 (0.33-1.56)	0.40	25	21	1.35 (0.74-2.45)	0.33
0,4-0,7%	44	2	0.15 (0.03-0.65)	0.01	33	11	0.57 (0.28-1.18)	0.13
>0,7%	43	2	0.14 (0.03-0.63)	0.009	37	16	0.72 (0.38-1.37)	0.32

Scatter plots of individual densities of $CD3^+$ and $Foxp3^+$ TILs are shown in figure 6 and 7. In the subgroup of stage II CRC patients CD3IRA% (P<0.001) and Foxp3IRA% (P<0.001) were significantly lower in patients with evidence of disease relapse (CD3IRA%: n=32, median=1.09, second-third quartile=0.38-2.46; Foxp3IRA%: n=32, median=0.12, second-third quartile=0.00-0.28) compared to patients with no evidence of disease relapse (CD3IRA%: n=184, median=2.88, second-third quartile=1.23-6.41; Foxp3IRA%: n=179, median=0.39, second-third quartile=0.17-0.69). Conversely, in stage III CD3IRA% (P=0.60) and Foxp3IRA% (P=0.18) did not differ in CRC patients with (CD3IRA% : n=70; median=2.32; second-third quartile=0.78-5.59; n=70; Foxp3IRA%: median=0.29; second-third quartile=0.12-0.66) or without (CD3IRA%: n=136; median=2.23; second-third quartile=0.89-5.59; Foxp3IRA%: n=132; median=0.42; second-third quartile=0.17-0.79) evidence of disease progression.

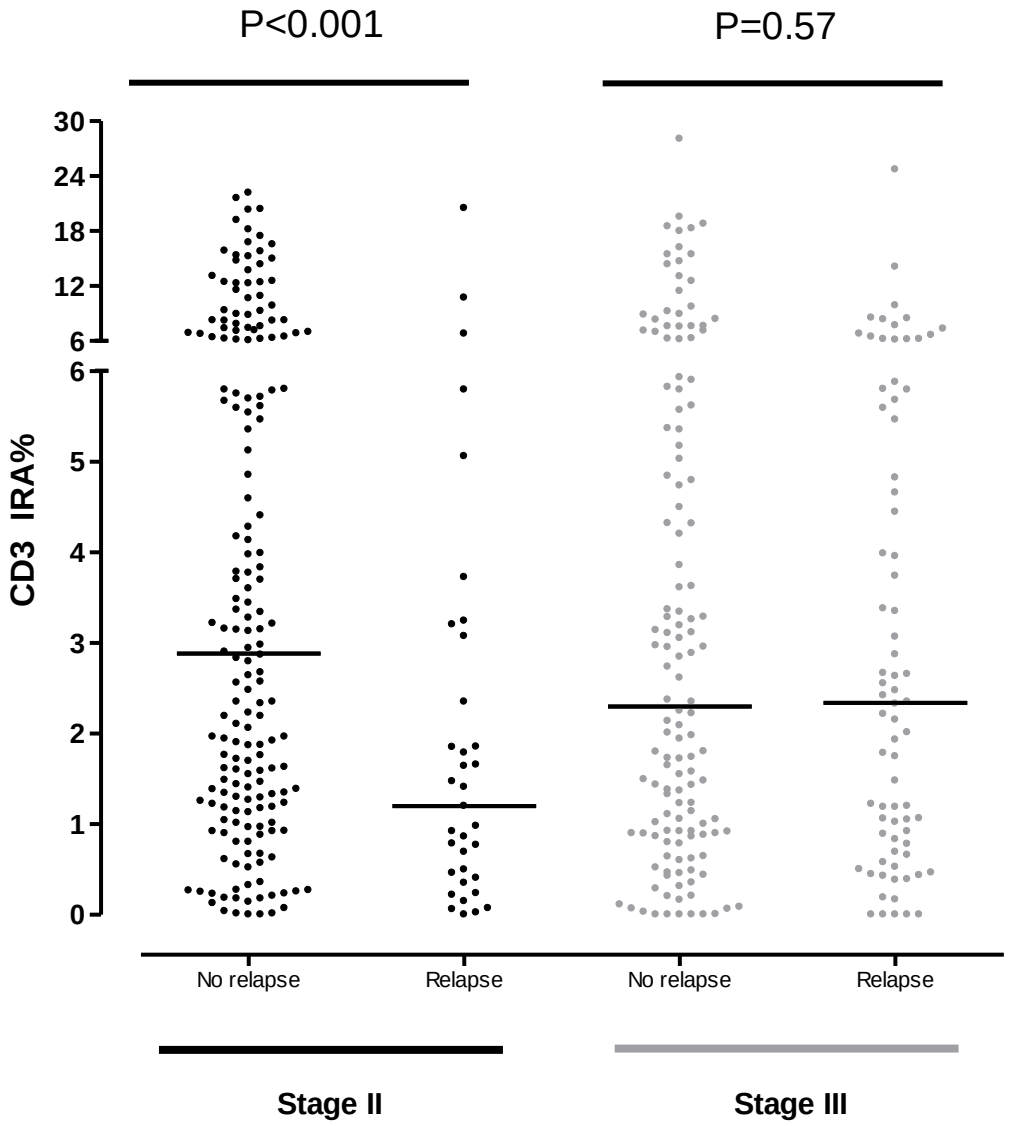


Figure 6. Distribution of $CD3^+$ Tils densities at the tumour invasive front in stage II and III CRC according to occurrence of disease progression.

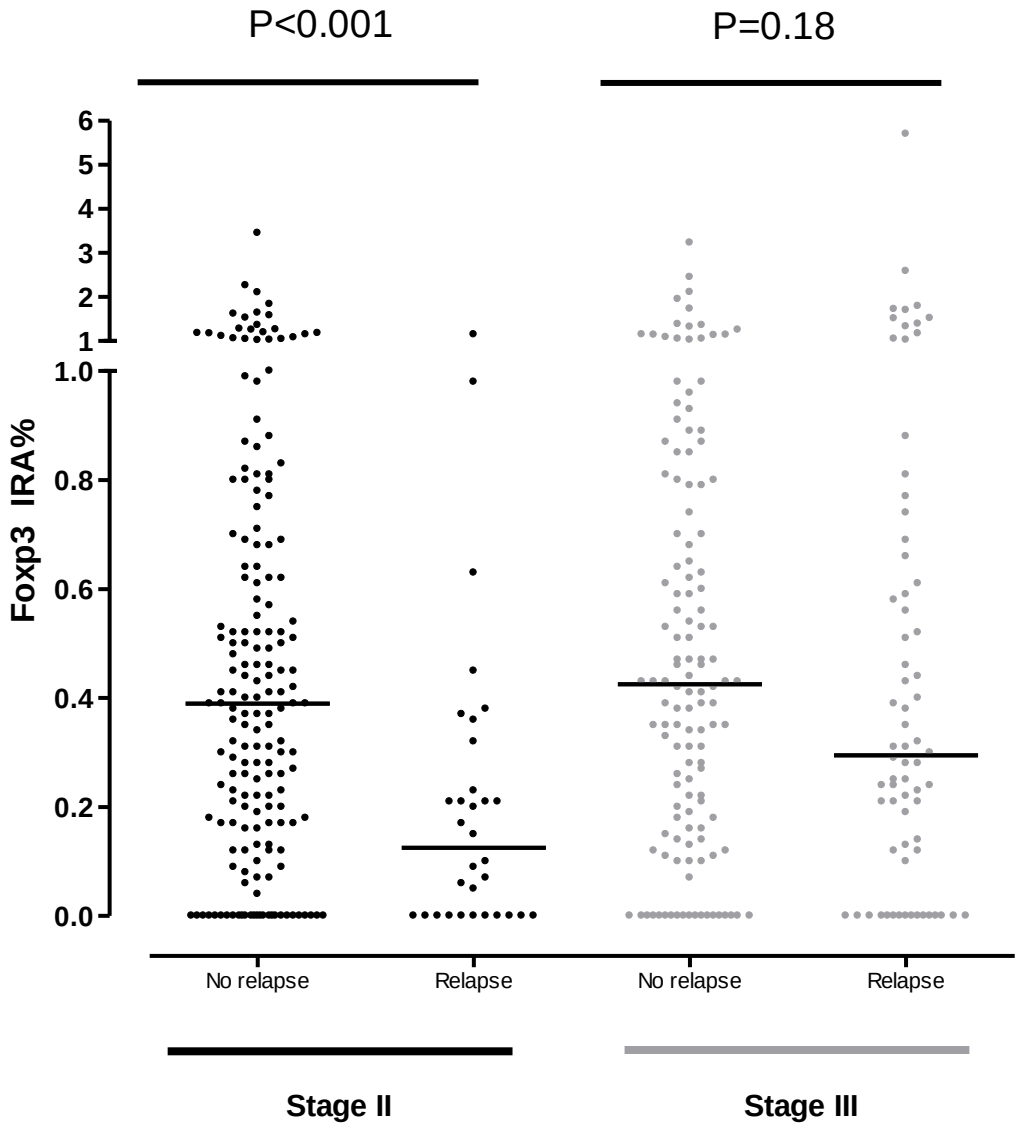


Figure 7. Distribution of FoxP3^{+} Tils densities at the tumour invasive front in stage II and III CRC according to occurrence of disease progression.

Figure 8 shows Kaplan Meyer prognostic curves of CRC patients studied sub-grouped by TNM tumour stage and $CD3^+$ and $FoxP3^+$TILs densities. Among patients with stage II CRC, those with a high density of $CD3^+$ and $FoxP3^+$ cells (>1.86%, >0.23% respectively) had a lower risk of tumour progression (P<0.001, P<0.001 respectively) compared to those with a lower density of $CD3^+$ and $FoxP3^+$ cells, while the prognosis of stage III CRC patients was not affected by the density of $CD3^+$ and $FoxP3^+$ cells (P=0.95, P=0.21 respectively) (figure 8).

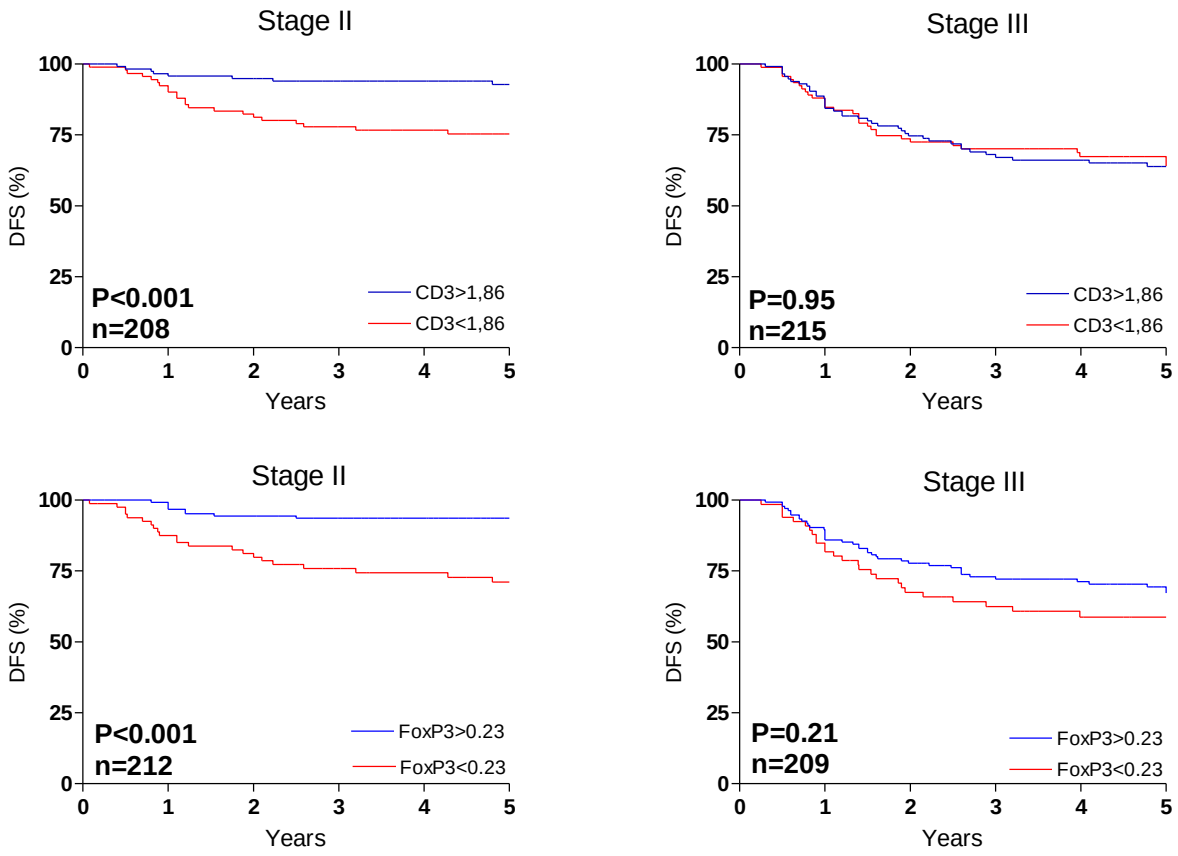


Figure 8. Kaplan-Meyer outcome curves showing disease free survival (DFS). The cut-off values generated from CART analysis (1.86% and 0.23%) were used to define high $CD3^+$ and $FoxP3^+$TILs respectively.

In figure 9 and 10 are shown the distribution of $CD3^+$ and $FoxP3^+$TILs according to the extent of lymph-node metastasis. Patients with ≤ 4 metastatic pericolic (or perirectal) lymph-nodes have N1, and patients with > 4 have N2 CRC. Of patients with no evidence of disease progression (n=320), the density of $CD3^+$ cells was decreasing (P=0.01) along with the severity of lymph-nodal tumour involvement. Conversely, among patients who experienced tumour relapse (n=105) the density of $CD3^+$ cells was increasing (P=0.04) along with extent of lymph-nodal tumour metastasis. Thus, among CRC patients that experienced relapse only those without any evidence of lymph-node metastasis had a lower density of $CD3^+$ TILs (P<0.001) compared to those that didn't experience relapse. (Figure 9)

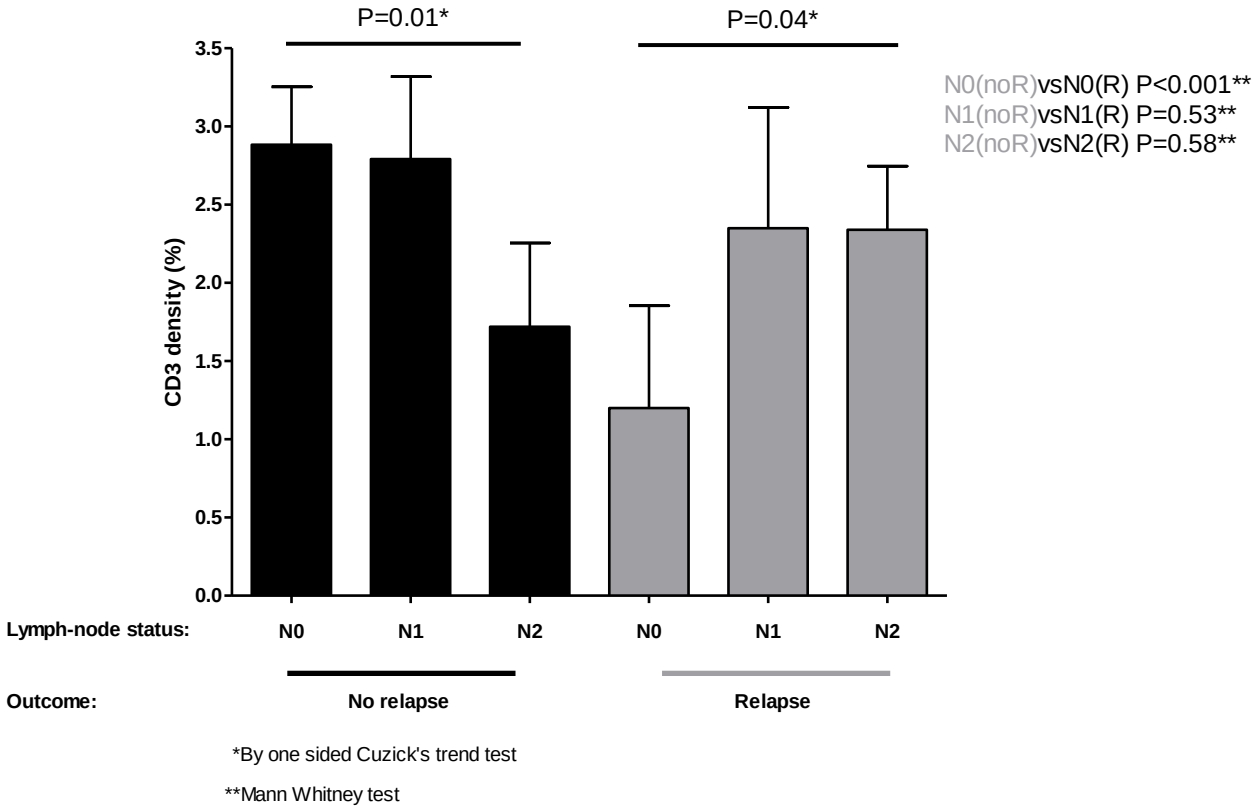


Figure 9. Distributions of $CD3^+$TILs according to the extent of lymph-nodes metastasis and patient's disease relapse.

Taking in consideration Foxp3^{+} cells, in CRC patients with no evidence of disease progression (n=311), the density Foxp3^{+} TILs did not change (P=0.34) according to the extent of lymph-nodal involvement. Conversely, among cancer patients that experienced tumour relapse (n=102) the density of Foxp3^{+} TILs increased (P=0.04) along with the extent of lymph-node tumour metastasis. (Figure 10) .

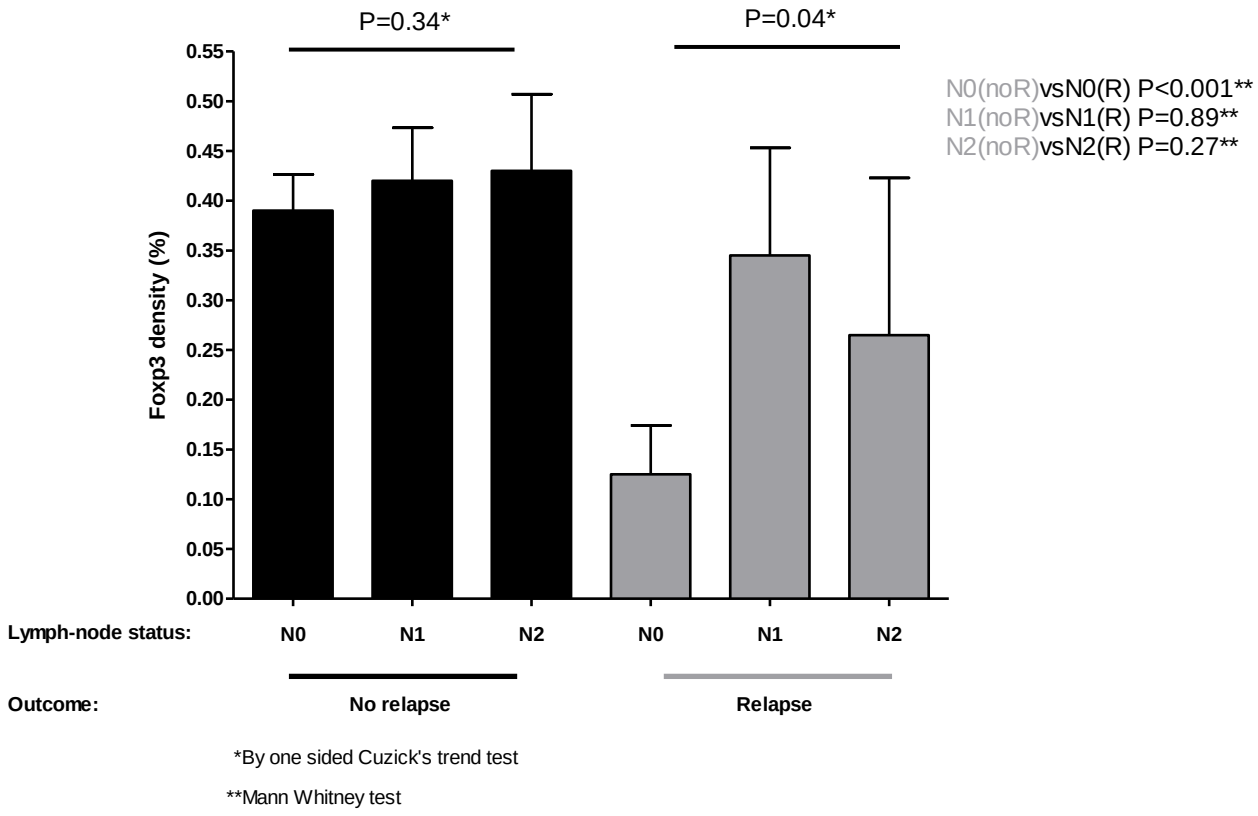


Figure 10. Distributions of FoxP3^{+}TILs according to the extent of lymph-nodes metastasis and patient's disease relapse.

Chapter 5. CD68$^+$ TAM densities in metastatic lymph-nodes

5.1 Density of CD68$^+$TAM in the tumour margin of metastatic lymph-nodes and its correlation with clinicopathological features at the time of surgery.

The ability of CD68$^+$TAM in CRC primary tumor to predict response to 5-FU was detected only in stage III CRC patients. We investigated CD68$^+$ TAM density at the tumor margin in metastatic lymph-nodes from stage III CRC patients. In the entire population of stage III CRC patients the densities of CD68$^+$TAM M-LN we studied ranged from 0% to 13.52 %. The median value of the distribution of CD68$^+$TAM M-LN densities was 1.77% and the first and the third quartile were 0.57% and 4.56% respectively. The distribution of CD68$^+$ TAM-IRA M-LN according to demographics, clinical and histopathologic characteristics in the population of CRC patients recruited in this study is shown in appendix table 2. CD68IRA% M-LN did not correlate with any of the variables assessed in this study.

5.2 Density of CD68$^+$TAM in the tumour margin of metastatic lymph-nodes and its correlation with patient's outcome

To validate the predictive ability of CD68$^+$ TAM density in the primary tumor we investigated whether CD68$^+$ TAM density in M-LN also correlates with the prognosis of CRC patients. As shown in table 10, we performed univariate and multivariate Cox regression analysis of the densities of CD68$^+$ TAM M-LN according to the outcome of CRC patients. We recorded 49 events of CRC disease relapse among 135 stage III CRC patients. At univariate analysis increasing densities of CD68$^+$ TAM M-LN [HR=0.65 95%CI (0.49-0.86), P=0.002] densities were strongly associated with better patient outcome. Other features associated with higher risk of disease recurrence were rectum site (P=0.03) and vascular invasion (P=0.003). To confirm whether 5-FU adjuvant treatment modifies the abilities of CD68$^+$TAM densities in metastatic lymph-nodes to predict CRC patients prognosis, we performed interaction analysis. Accordingly, by entering into a logistic regression model the categorical density values of CD68$^+$ TAM M-LN, 5-FU adjuvant treatment and a product term consisting of the multiplication of the above variables we found a statistical interaction when predicting the risk of patient's outcome (P=0.03). This result indicates that 5-FU adjuvant treatment modify the ability of CD68$^+$ TAM densities in metastatic lymph-nodes in predicting the risk of patient's outcome, thus confirming results obtained in the primary tumour. (Table 10).

Table 10. Predictive factors for postsurgical relapse and their significant interactions in 135 CRC patients of stage III colorectal cancer.

	Relapse		Univariate Analysis		Interaction Model
	No (n=86)	Yes (n=49)	HR (95%CI)	P	P<0.05
CD68-IRA M-LN					
0-1%	24	25			
1-3%	23	14	0.69 (0.35 – 1.32)	0.26	**x 5-FU therapy (α)**
3-5%	15	4	0.32 (0.11 – 0.92)	0.03	
>5%	24	6	0.30 (0.12 – 0.75)	0.009	
Age (years, mean+SD)			1,01 (0,99-1,03)	0.12	
Gender					
Male	54	29	1.00 ref.		
Female	32	20	1.16 (0.65-2.06)	0.59	
Tumor Site					
Colon	69	30	1.00 ref.		
Rectum	17	19	1.85 (1.04-3.30)	0.03	
Tumor MS Status					
MSS	72	47	1.00 ref.		
MSI	14	2	0.31 (0.07-1.27)	0.10	
Tumor Grade					
G1/G2	62	31	1.00 ref.		
G3	24	18	1.43 (0.80-2.57)	0.21	
Tumor Cell Type					
ADC	74	41	1.00 ref		
Variants	12	8	1.13 (0.53-2.42)	0.74	
Tumor Vascular Invasion					
No	65	25	1.00 ref		
Yes	21	24	2.28 (1.30-4.01)	0.003	
5-FU Adjuvant Therapy					
No	20	11	1.00 ref		x **(α)**
Yes	66	38	0.79 (0.40-1.56)	0.50	

° Age entered as a continuous variable

The interaction between CD68-IRA M-LN and 5-FU CHT is statistically significant when CD68-IRA M-LN is entered as a categorical (α, P=0.03) or a continuous variable (α, P=0.009).

5.3 Prognostic ability of CD68$^+$TAM in the tumour margin of metastatic lymph-nodes according to 5-FU adjuvant treatment.

Considering the significantly interacting variables only, we tested the effect modification of 5-FU adjuvant treatment on the ability of densities of CD68$^+$ TAM in metastatic lymph-nodes in predicting the risk of patient's relapse by performing subgroup analysis. The predictive value of CD68$^+$ TAM in CRC patients that received 5-FU chemotherapy or not is shown in table 11. Among 5-FU adjuvant treated CRC patients increasing values of CD68$^+$ TAMs densities in the metastatic lymph-nodes were associated with better prognosis [n=104; HR=0.54; 95%CI (0.39-0.76); P<0.001]. Conversely among chemotherapy un-treated CRC patients increasing values of CD68$^+$ TAMs densities were not associated with prognosis [n=31; HR=1.13; 95% CI (0-67-1.90); P=0.64]. (Table 11)

Table 11. CD68$^+$TAM density at the tumor margin in metastatic lymph-nodes as a predictor of disease relapse according to 5-FU adjuvant therapy.

	Adjuvant Treatment*							
	5-FU Therapy				**No chemotherapy**			
	Relapse				Relapse			
	No	Yes	HR (95%CI)	P	No	Yes	HR (95%CI)	P
CD68-IRA M-LN*								
0-1%	16	20	1.00 ref		8	5	1.00 ref	
1-3%	17	13	0.75 (0.37 – 1.51)	0.42	6	1	0.34 (0.04 – 2.96)	0.33
3-5%	12	2	0.18 (0.04 – 0.80)	0.02	3	2	0.89 (0.17 – 4.62)	0.89
>5%	21	3	0.16 (0.04 – 0.56)	0.004	3	3	1.47 (0.34 – 6.32)	0.60

*The interaction between CD68-IRA M-LN and 5-FU CHT is statistically significant when CD68-IRA M-LN is entered as a categorical (α, P=0.03) or a continuous variable (α, P=0.009).

Figure 12 shows scatter plots of individual CD68^{+} TAM densities in metastatic lymph-nodes according to CRC disease recurrence. Among 5-FU adjuvant treated patients CD68IRA% M-LN was significantly lower (P<0.001) in patients with evidence of disease relapse (n=38; median=2.92%; second-third quartile=1.04% – 5.74%) compared to patients with no evidence of tumour progression (n=66; median=2,37%; second-third quartile=0.41% – 5.82%). Conversely, CD68IRA% was not differing in patients that did not receive chemotherapy adjuvant treatment (P=0.96) with (n=11; median=2.03%; second-third quartile=0.11% – 5.72%) or without (n=20; median=1.76%; second-third quartile=0.33% – 3.32%) evidence of disease progression (Figure 12).

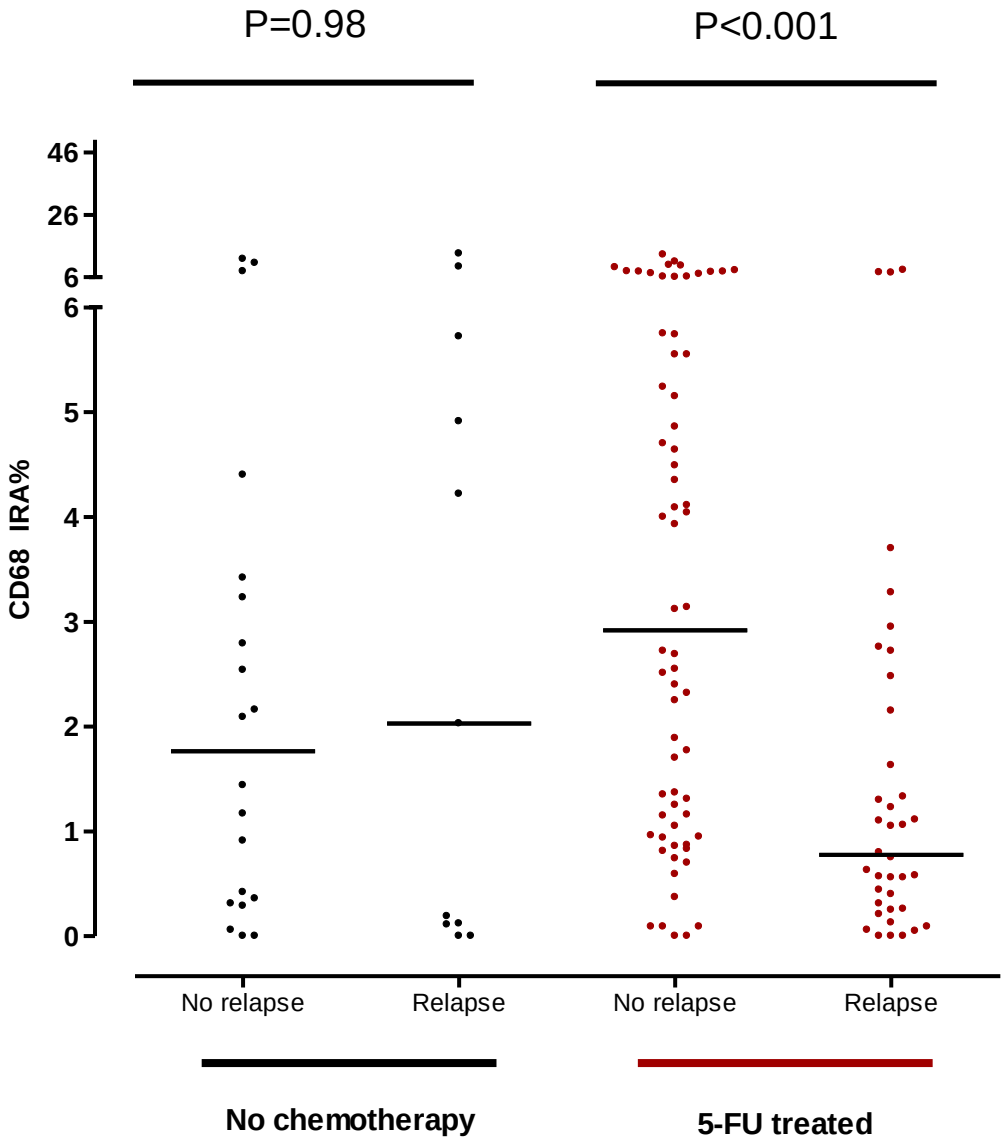


Figure 12. Distributions of CD68^{+}TAM densities at the tumour margin of metastatic lymph-nodes according to occurrence of disease progression and chemotherapy adjuvant treatment.

Figure 13 shows Kaplan Meyer survival curves sub-grouped by (>median) $CD68^+$ TAMs densities in metastatic lymph-nodes and 5-FU adjuvant treatment. Among patients that received 5-FU adjuvant treatment those with a high (>median) density of $CD68^+$ TAMs M-LN had a better DSS (P<0.001) than those with a low density (figure 13). Again, the DSS of CRC patients who didn't receive chemotherapy was not affected (P=0.86) by densities of $CD68^+$ TAMs M-LN (figure 13). Therefore, the predictive effect of $CD68^+$TAM on the effectiveness of 5-FU chemotherapy treatment was confirmed in metastatic lymph-nodes indicating that $CD68^+$TAM effect modification on chemotherapy is maintained in tissues other than the intestine.

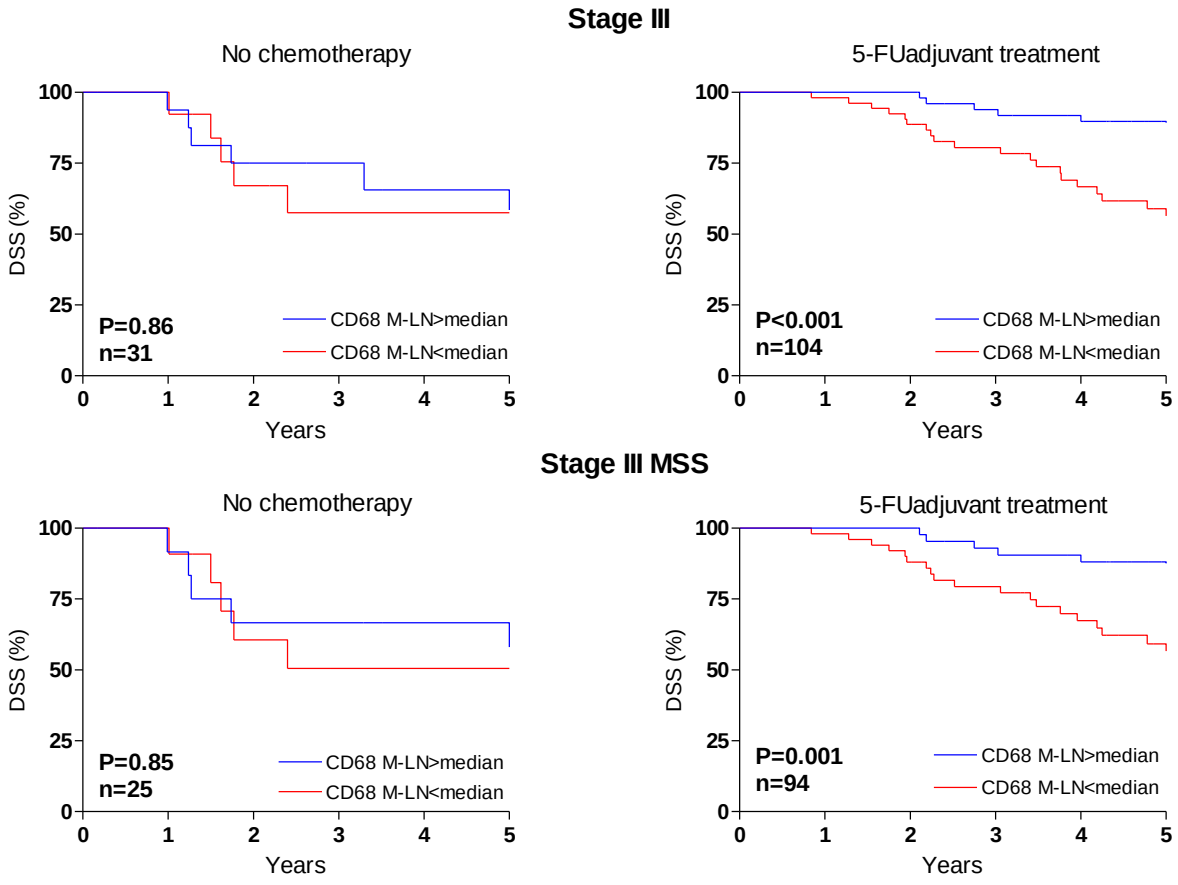


Figure 13. Kaplan-Meyer survival curves according to chemotherapy adjuvant treatment. $CD68^+$TAM median density in metastatic lymph-nodes was used to define high $CD68^+$TAM.

Supplementary appendix

Appendix Table 1: Patient demographics, tumour pathological features and microsatellite status of 425 stage II and III colorectal cancers.

		N (%)
Patient Age	≤69 yrs	228 (53.6%)
	>69 yrs	197 (46.4%)
Patient Gender	Male	245 (57.7%)
	Female	180 (42.4%)
Tumor Site	Colon	321 (75.5%)
	Rectum	104 (24.5%)
Microsatellite status	MSS	359 (84.5%)
	MSI	66 (15.5%)
Tumor Stage	II	216 (50.8%)
	III	209 (49.2%)
Tumor Grade	G1/G2	347 (81.6%)
	G3	78 (18.4%)
Tumor Cell Type	ADC	388 (91.3%)
	Variants	37 (8.7%)
Vascular Invasion	No	327 (76.9%)
	Yes	98 (23.1%)
5-FU Adjuvant Therapy		
Stage II	No	125 (57.9%)
	Yes	91 (42.1%)
Stage III	No	62 (29.7%)
	Yes	147 (70.3%)

Appendix Table 2. CD68-Immunoreactive area (IRA, % of microscopic field) at the tumour margin of metastatic lymph-nodes in 135 Stage III Colorectal Cancers.

		Median Value	2nd-3rd quartile	Univariate* P
Patient Age °	≤69 yrs	2.02	0.57 - 4.66	
	>69 yrs	1.39	0.38 - 4.67	0.42
Patient Gender	Male	2.31	0.58 - 4.90	
	Female	1.23	0.48 - 3.75	0.19
Microsatellite Status	MSS	1.44	0.55 - 4.49	
	MSI	2.55	0.90 - 6.26	0.25
Tumor Site	Colon	1.89	0.70 - 4.69	
	Rectum	1.39	0.35 - 3.18	0.46
Tumor Grade	G1/G2	2.09	0.74 - 4.63	
	G3	1.10	0.31 - 4.49	0.33
Tumor Cell Type	ADC	2.03	0.58 - 4.63	
	Variants	1.37	0.18 - 4.51	0.42
Vascular Invasion	No	2.11	0.74 - 4.49	
	Yes	1.17	0.44 - 4.90	0.55
5-FU Adjuvant Therapy	No	2.03	0.19 – 4.40	
	Yes	1.73	0.72 – 4.66	0.50

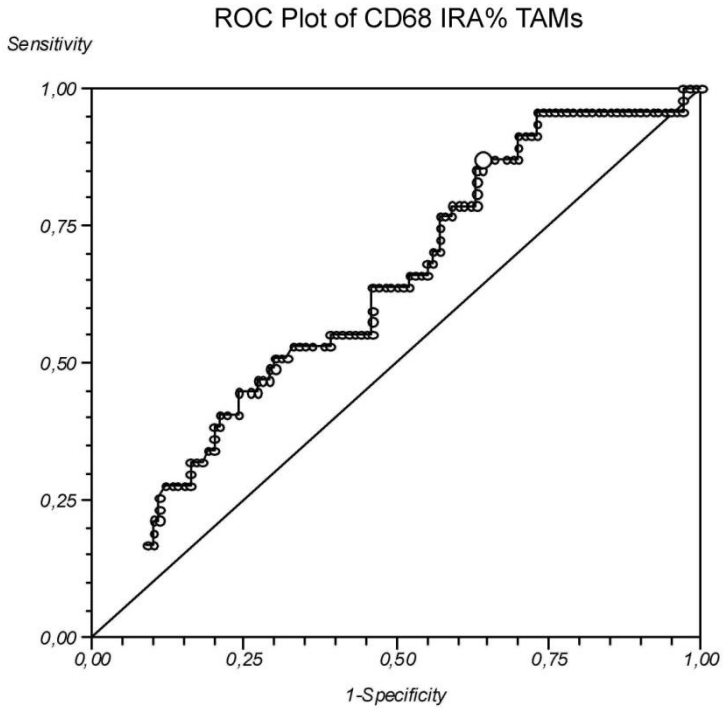
* Linear Regression Analysis. "CD68-IRA%" was entered as a dependent, continuous variable.

° Age entered as a continuous variable

CD68-IRA% values of in patients whose age was below or above the median age of the entire series, are shown only for a descriptive purpose.

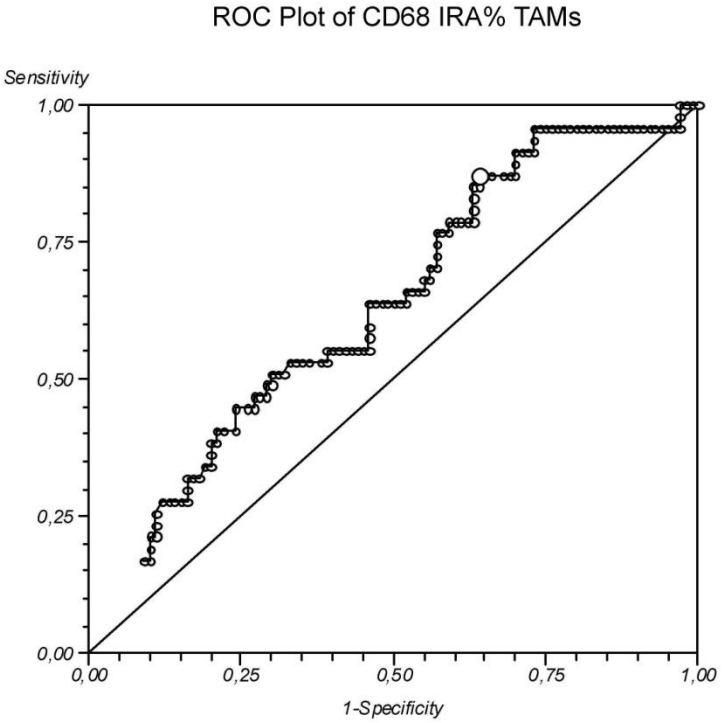
Appendix Figure 1.

Receiver Operating Characteristic (ROC) analysis of $CD68^+$ IRA% TAMs as a predictor of prognosis in patients with stage III CRC 5-FU adjuvant treated. Area under the curve 0.63. At a cut-off value of 7.59 %, sensitivity was 0.87, specificity 0.36.



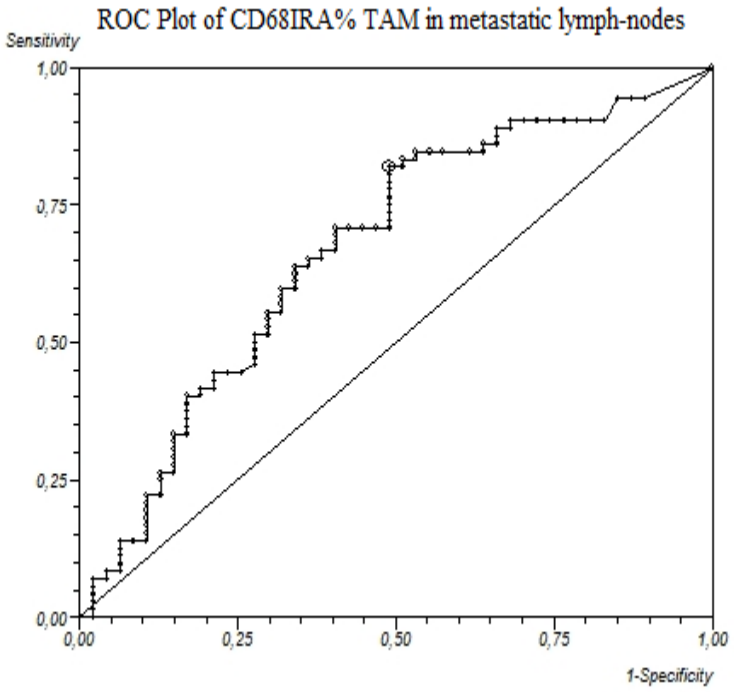
Appendix Figure 2.

Receiver Operating Characteristic (ROC) analysis of CD68^{+} IRA% TAMs as a predictor of prognosis in patients with stage III MSS CRC 5-FU adjuvant treated. Area under the curve 0.67. At a cut-off value of 7.59 %, sensitivity was 0.90, specificity 0.37.



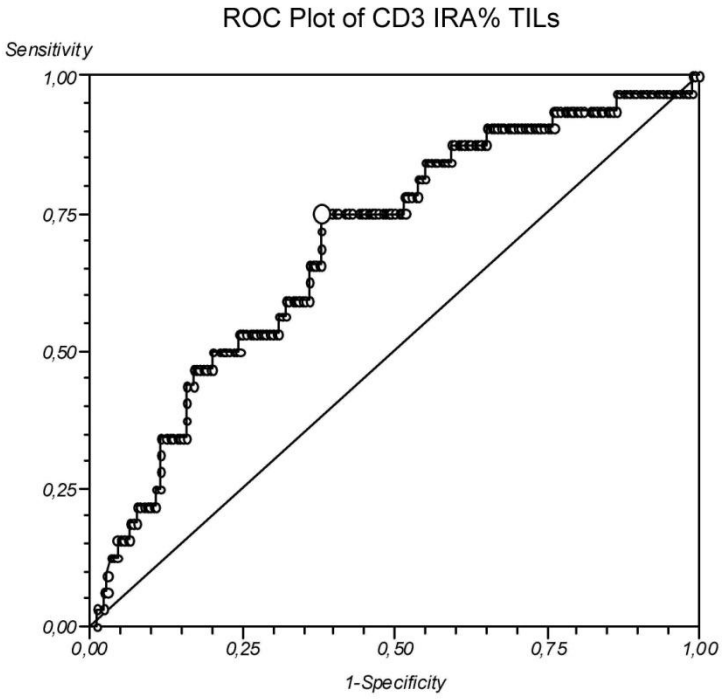
Appendix Figure 3

Receiver Operating Characteristic (ROC) analysis of $CD68^+$ IRA% TAMs in metastatic lymph-nodes as a predictor of prognosis in patients with stage III MSS CRC 5-FU adjuvant treated. Area under the curve 0.67. At a cut-off value of 0.81%, sensitivity was 0.82, specificity 0.51.



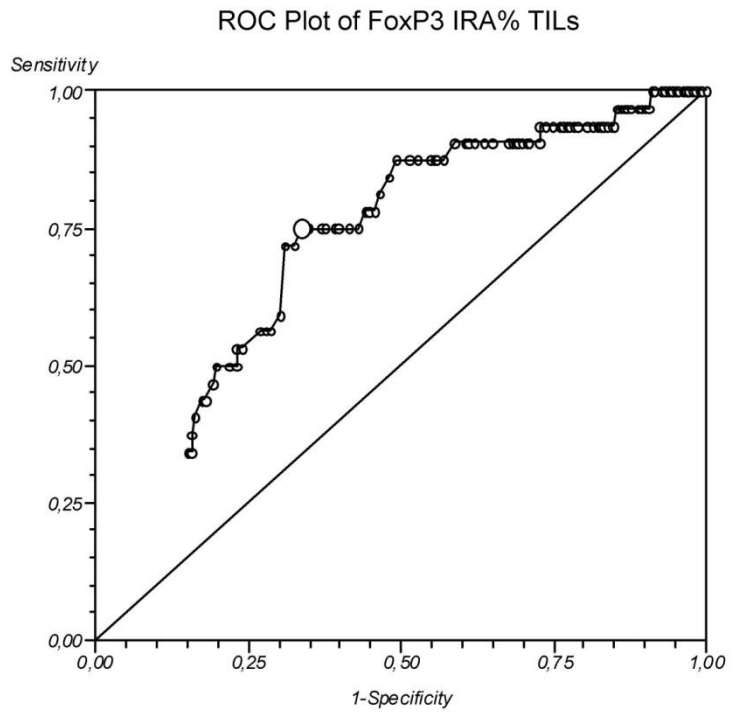
Appendix Figure 4.

Receiver Operating Characteristic (ROC) analysis of $CD3^+$ IRA% TILs as a predictor of prognosis in patients with stage II CRC. Area under the curve 0.69. At a cut-off value of 1.85 %, sensitivity was 0.75, specificity 0.61.



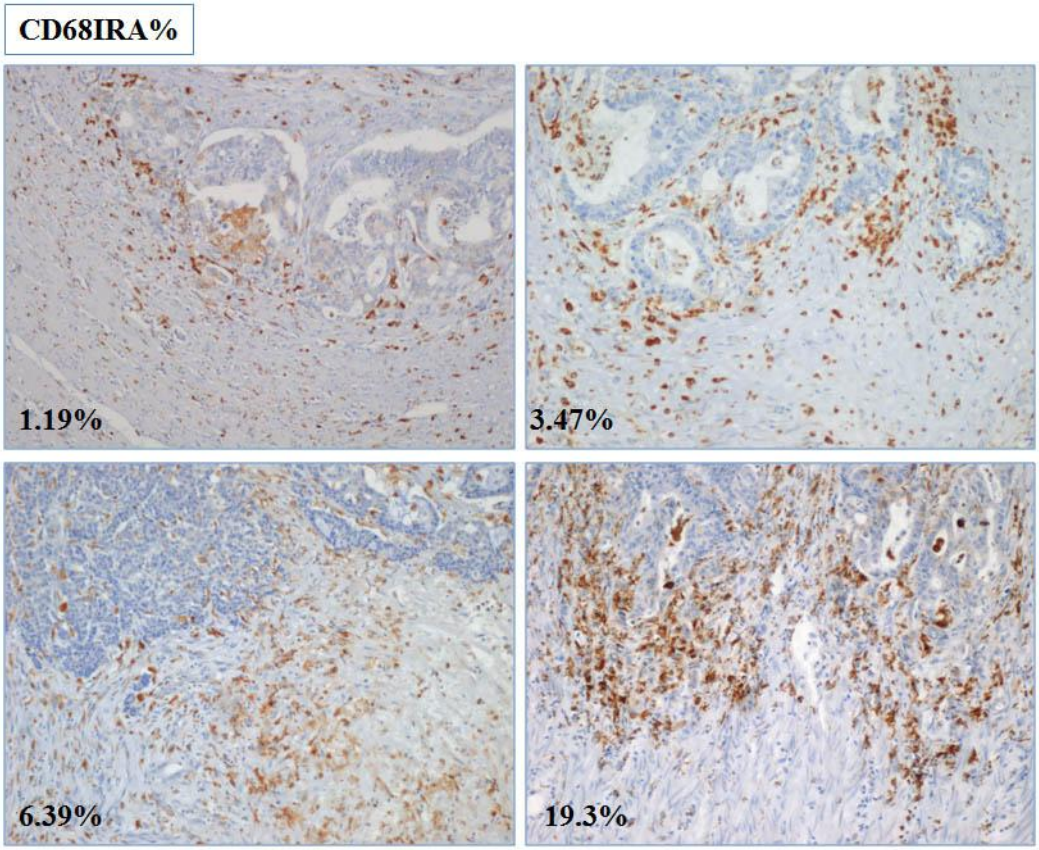
Appendix Figure 5.

Receiver Operating Characteristic (ROC) analysis of FOXP3^{+} IRA% TILs as a predictor of prognosis in patients with stage II CRC. Area under the curve 0.72. At a cut-off value of 0.23 %, sensitivity was 0.75, specificity 0.66.

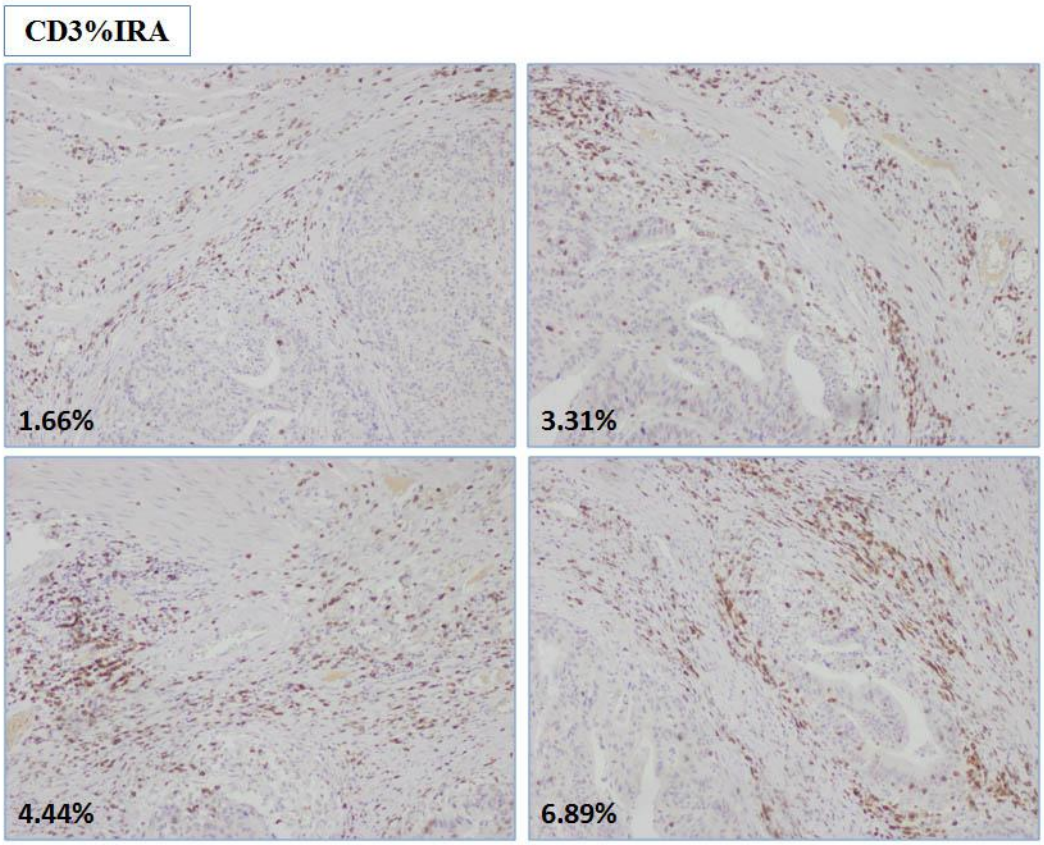


Appendix Figures 6 to 8. Examples of increasing amounts of immunoreactive surfaces covered by CD68IRA%,CDIRA% and FOXP3IRA% cells, detected at the invasive front of CRC by a computer-aided image analysis system. (Objective magnification 10x)

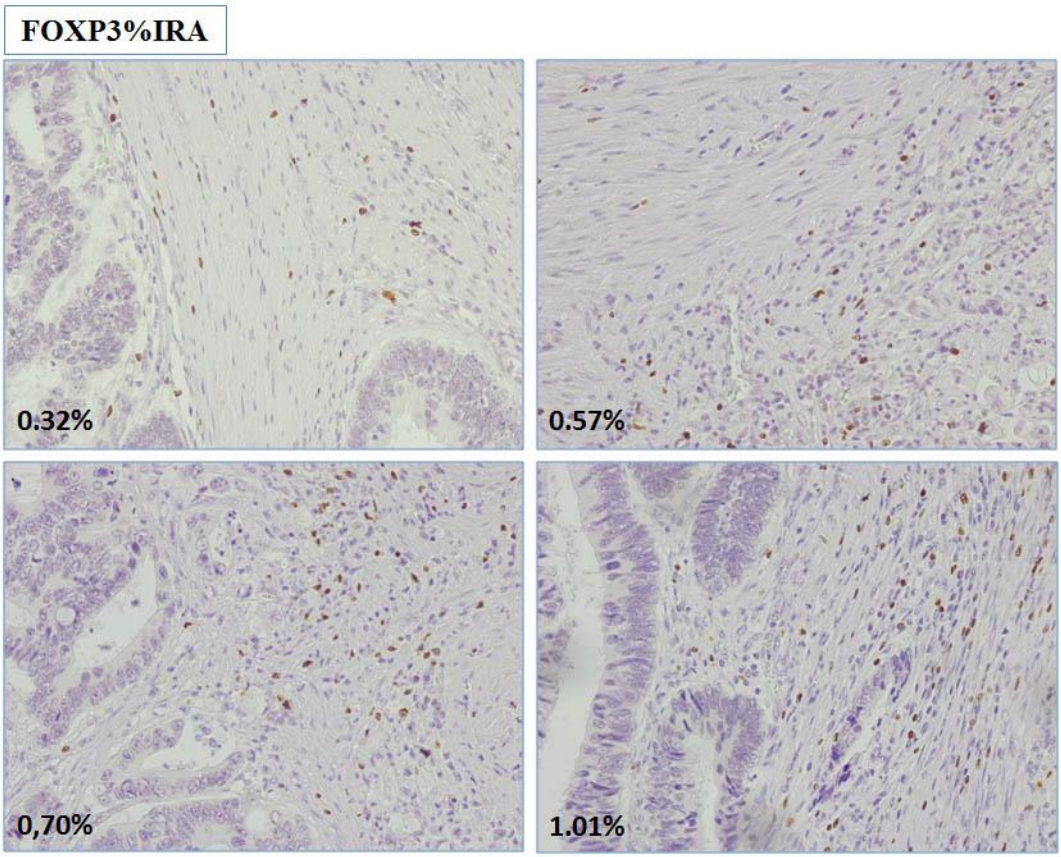
Appendix Figure 6 . Rappresentative images of CD68IRA%



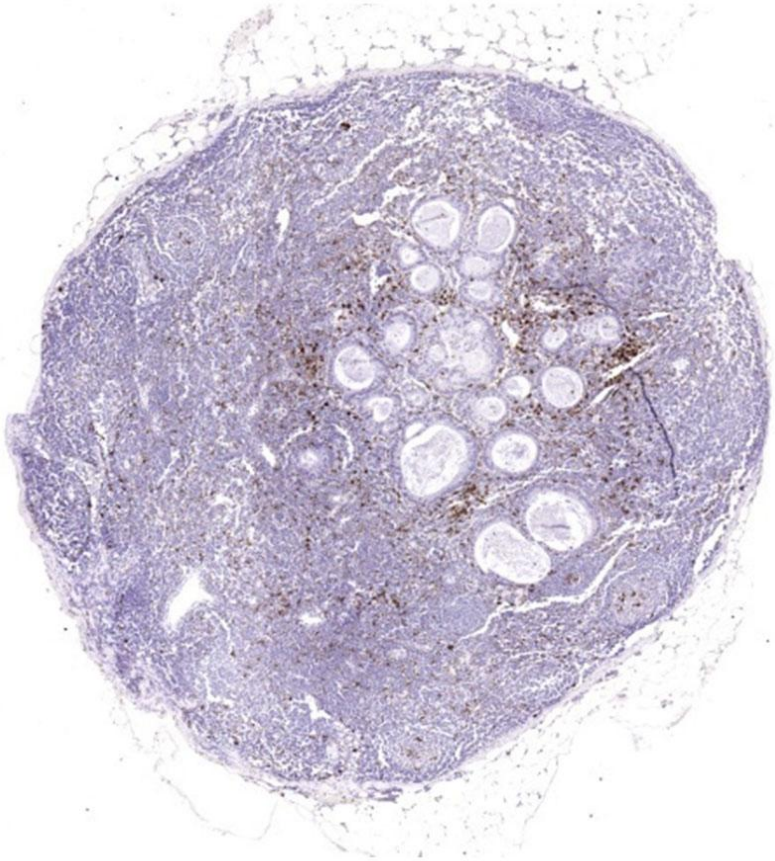
Appendix Figure 7. Rappresentative images of CD3IRA%



Appendix Figure 8. Rappresentative images of FOXP3%IRA



Appendix Figure 9. Rapresentative image of an immunoreactive surface covered by $CD68^+$ cells in a whole lymph-node partially metastatic.



Discussion

This study greatly expands our original observation that the prognostic value of immune cells infiltrating CRC changes with disease progression. We first reported that $CD3^+$ TILs and tumour stage interact in determining patients prognosis [92]. In the present study we detected two other effect modifications associated with CRC infiltrating cells. The first one occurs between $CD68^+$TAM and 5-FU adjuvant chemotherapy in patients with stage III CRC in determining the outcome after adjuvant treatment. The second interaction occurred between *FOXP3*$^+$ TAM and TNM stage and resembles that originally reported for $CD3^+$ TILs.

The most innovative finding of the present study is represented by the identification of TAM as a novel predictive marker of response to 5-FU adjuvant treatment in stage III CRC. In our dataset TAMs modifies the effect of 5-FU adjuvant treatment in exerting antitumor activity. Accordingly, we demonstrated that 5-FU adjuvant treatment in stage III was most efficient in exerting antitumor activity in tumours with high levels of TAM, while in patients with a low density of TAM chemotherapy was not effective in improving patient outcome. These data were obtained in a patient dataset including a "control arm" of chemotherapy untreated patients. Results also exclude that adaptive immunity, represented by $CD3^+$ and *FOXP3*$^+$ TILs might retain any ability to modify 5-FU adjuvant treatment effectiveness in predicting patient prognosis (in both stage II and III patients). To date, most studies relating adaptive immune cell densities and CRC prognosis didn't reveal that any immune effectors was modified by 5-FU adjuvant treatment in their predictive value. Only two studies from Morris and Halama reported association between chemotherapy response and levels of adaptive immune cells in patients prognosis [138,139]. However, it is important to underline that the first study didn't report any effect modification of the predictive abilities of adaptive immune cells by statistical interaction with 5-FU, while the latter lacked of a control group of untreated CRC patients. Differently, very few studies assessed TAM prognostic abilities in CRC, and no one took advantage of computer assisted image analysis to measure immune cell density. This technology has the advantage to provide continuous values for immune cell quantity. Accordingly, data are more informative, detailed and statistically relevant in representing immune cells densities and their relevance on prognosis. A weakness of our retrospective study is represented by the non-random assignment of 5-FU adjuvant treatment. In clinical practice, significant differences exist between patients who receive or not adjuvant treatment, particularly with regard to age. However, to address selection bias owing to non-random treatment assignment we excluded that TAM density may covariate with any demographic

and clinico-pathological variable by performing separate analysis of TAM distributions in patients who received 5-FU adjuvant treatment and those who did not. Since TAM distribution was balanced across different groups of patients, we exclude any need for covariates adjustments in our prognostic models.

Different clinical studies showed that MSI is a negative predictive marker of response to 5-FU in CRC by providing evidence that patients with MSI tumours did not received a survival benefit [128-131,135]. This concept was demonstrated also in experimental studies in vitro and in vivo [125-127]. Our data shows that TAM enhanced their predictive ability in MSS tumours, thus providing partial biological explanation for the unresponsiveness of cancers with MSI molecular features to 5-FU treatment. However, it is relevant to notice that the low prevalence of MSI patients translated into only modest power to detect a statistically significant finding for an interaction effect with this variable. Therefore, it would be relevant to address this issue in larger dataset of patients.

To assess whether the protective effect of high TAM density in stage III patients treated with 5-FU adjuvant therapy is not restricted to the primary tumour, we studied TAM density in metastatic lymph-nodes of stage III CRC patients. The predictive effect of TAM density in metastatic lymph-nodes was clear-cut, results in metastatic lymph-nodes validate TAM predictive effect that we observed in the primary tumour, thus confirming that the presence of a high density of TAM is coupled with clinical response of stage III CRC patients to 5-FU adjuvant therapy. These data also demonstrate that such effect modification is mirrored in a non-colonic tissue microenvironment, different by the one where the tumour arise. Therefore, we speculate that TAM antitumour effect in conjunction with 5-FU might be exerted also on metastatic tumour clones far from the primary site, not clinically detectable. Moreover, the substantial correlation between TAM levels in the primary tumour and those in the lymph-nodes metastasis suggest that pathways involved in TAM recruitment at the tumour border operate in metastatic lymph-nodes.

Cancer immunoediting theory fits to dynamics of clinical progression of early stages of CRC not associated with CAC. In this case surgery removes macroscopically detectable colon cancer burden by physical excision while adjuvant chemotherapy is administered by assuming that it will kill circulating tumour cells and micrometastasis that spread out in the body. Such cells are not detectable by conventional diagnostic methods and are likely in a dormant state, and later they may give rise to metachronous metastasis, the main cause of death in colorectal cancer. Immune system might play a role in this process by keeping not detectable

micrometastasis in an equilibrium phase, while the evolution of such tumour cells might give them the chance to escape immune system recognition and cause recurrence. Adjuvant chemotherapy gives a clear survival advantage to CRC patients, though its role on immune system micrometastasis recognition and clearance is still unknown. Robust epidemiological evidence suggests that 5-FU adjuvant treatment does not exert any beneficial effect on patient's prognosis among stage II patients. Moreover, in our data 5-FU adjuvant treatment in stage II CRC was not effective in improving patient's relapse, regardless of $CD68^{+}$TAM density. Chemotherapy seems to have beneficial effects only at a stage of disease when tumour clones have spread to metastatic lymph-nodes and perhaps to the body, although not clinically detectable. Despite clinical evidence, the biological and molecular basis of the discrepancies of chemotherapy benefit along CRC progression remain unknown. The current lack of valid experimental models that mirror different stages of CRC patients progression fits at least in part with this lack of knowledge. However, it is tempting to speculate that chemotherapy might overcome immune-escape mechanisms by causing alteration in antigenic properties of tumour clones that might modify tumour cell immunogenicity and recognition by the innate immune system. In this scenario, our data clinically suggest that 5-FU chemotherapy and TAM might play a synergic role in recognition and elimination of tumour clones by immune system with 5-FU being an efficient activator of antitumour immune responses.

Accumulating evidence suggests that certain chemotherapeutic agents can confer to tumour cells immunogenic abilities [110-112]. The National Cancer Institute strategies to select conventional antineoplastic agents with the best ability to kill cancer cells of most solid tumours have been developed to date on murine immunodeficient host based on drugs ability to directly interact with cancer cells and thus to inhibit their growth or induce cell death [102, 103]. However, this strategy completely neglect that the host immune system might have any effect or interaction on tumour eradication in the context of chemotherapy. Accordingly, not much clinical data in CRC support the predictive value of immune effectors in contributing to chemotherapy effects. Since tumour infiltrating immune effectors might contribute to the efficacy of CHT, it is emerging that an immunological link might exists between CHT-driven antitumor activity and patients' prognosis [110-112]. Macrophages are the most prevalent antigen-presenting cells in tumours and in certain cases may account for about 50% of the tumour mass [70]. Both DCs and macrophages have the ability to pick up tumour antigens for cross-presentation on MHC class I molecules [115]**.** In this context it is important to consider that a recent paper from De Visser challenged the idea that adaptive system may increase

chemotherapy-mediated tumour cell death proposed by Zittvogel and colleagues [117]. Understanding why the adaptive immune system does not contribute to chemoresponsiveness may yield to new strategies or new cellular mediators able to enhance chemotherapy-driven antitumor activity. In this setting our study suggest TAM as a new player in chemotherapy driven tumour cell death, thus in vivo functional studies in reliable immunocompetent experimental models of CRC are required to understand TAM behaviour on tumour cells in the context of 5-FU chemotherapy.

We have no experimental explanation for the detected predictive role of TAM. First, it should be underlined that negative predictive role of TAM has been reported in patients with Hodgkin lymphoma (HL) [144]. In that setting it has been found that patients with a higher number of $CD68^+$ TAM were proposed as a new marker for prediction of worst outcome after primary and secondary treatment. The effect modification of TAM and chemotherapy in HL is only speculative due to the lack of a control arm, comprising untreated patients. However, the so called natural history scenario of disease is no longer seen in the 21st century. The situation is different in our dataset, comprising both treated and untreated patients and encompassing different disease stages. However, TAM density was determined at diagnosis, before the administration of any treatment. This clearly demonstrates that TAM density at diagnosis is indeed a modifier of response to chemotherapy, later on. It is important to consider that HL is a lymphoid cancer with haematological origin and thus with a different lineage compared to epithelial cancers such as CRC and receiving different chemotherapy treatments. The opposite TAM predictive effect can be due to the different disease and affected cell of origin or the employed drugs, or both. Recently, in an experimental model of metastatic pancreatic ductal adenocarcinoma (PDAC) has been shown that treatment with CD40 ligand caused tumour regression[145]. In this process TAM were found to be functionally necessary to mediate antitumour activity, while TILs were irrelevant. This exciting and surprising result is the first experimental evidence that TAM directly exert antitumour activity in cancer, even though only when re-educated by CD40 ligand. In this experimental model of PDAC, $CD3^+$ T cells (which are the major contributors of CD40 ligand) were not observed to infiltrate tumours before and after treatment with CD40 as well as in PDAC patients with metastatic disease[145]. In this scenario the variability and the complexity of cancer microenvironment among different patients with the same tumour type suggest that the clinical exploitation of TAM require careful and further analysis. In order to understand the behaviour of this player of inflammation in functional models is crucial to take in account the different tumour types and the different settings of cancer disease progression.

There's general consensus supporting the fact that CRC development is positively influenced by the host immune system (Reviewed in Roxburgh[86] and Table 2.4.2.1). Adaptive immune markers are powerful prognostic markers able to identify CRC patients that are more likely to experience cancer recurrence and thus might facilitate clinical decision-making regarding the necessity for adjuvant systemic therapy. However, this study is the first evidence of TAMs as the most promising candidate as predictive biomarkers in stage III CRC and that adaptive immune cells levels seems not to modify the effectiveness of 5-FU adjuvant therapy.

We confirmed in a larger dataset of CRC patients data that our group have previously published [92]. In this study high levels of $CD3^{+}$TILs identified stage II CRC patients with a very low risk of cancer progression. However, in stage III the recruitment of even very dense TILs is irrelevant to the prognosis of patients. Despite conflicting data in the literature, we suggest that TNM staging still represent the best single prognostic indicator which justify its clinical use. As long as the tumour doesn't gain the ability to invade and macroscopically colonize mesenteric lymph-nodes the extent of T-lymphocytes recruitment in the tumour seems to play an inhibitory effect on tumour progression, for that reason increasing recruitment of TILs in stage II CRC is expected to exert antitumour activity. In this view we speculate that in stage II tumour immunoescape mechanisms seem to act along TILs recruitment, as CRC that doesn't have proper potential to recruit TILs will experience relapse and progression of disease. On the other hand, as long as tumours acquire the ability to spread and colonize mesenteric lymph-nodes TILs doesn't seem to retain any antitumour activity, because tumour progression and relapse of disease will occur regardless of the extent of TILs recruitment. Therefore, in this setting lymph-nodal tumour metastasis might represent an alternative way for tumour immunoescape. Taken together, our data provide the first evidence that tumour immunoescape seems to act through two different strategies. The first feature resides in the ability of tumours to recruit TILs which is likely dependent on the antigenic potential of stage II tumours. The second strategy lies in the potential of T-lymphocytes recruited at the tumour site to exert antitumour activity, which is likely a feature of their cytotoxic activity. Therefore, the antitumour activity of TILs seems to be dependent on the presence of metastasis in the lymph-nodes regardless of their extent of recruitment at the tumour site. This last hypothesis was supported by a study from Koch wherein authors found that the proportion of activated TILs decreased significantly in higher tumour stage (from stage II through stage III to stage IV), giving functional assessment of increasing immune evasion along with more advanced clinical histopathologically staging [62]. In a study from Atreya authors further supported this concept by showing that the proportion of

activated CD8+ TILs is not the only relevant feature in mediating CTL antitumor activity, as their cytolytic abilities is determinant to mediate an effective antitumor activity [63]. Authors demonstrated that eomesodermin, a T-box transcription factor involved in controlling the cytotoxic activity of CD8+ CTLs, is inversely correlated with the presence of lymph node metastasis at diagnosis in CRC patients [63].These data together provide molecular basis for a role of lymph-nodes metastasis in this phenomena. In this new scenario, the development of immuno-therapies strategies aimed to enhance antitumour activities of TILs should take account of such the immunoescape mechanisms along the state of CRC progression at diagnosis.

Another important result of our study regards a subpopulation of T-lymphocytes expressing the transcriptional factor *FOXP3* which we found to retain positive prognostic ability, as demonstrated by other studies. However, this is the first study demonstrating that, alongside $CD3^+$TILs, the recruitment of *FOXP3*$^+$TILs exert antitumour activity only among CRC without lymph-nodal metastasis. These data further corroborate the idea that in stage II CRC T-lymphocytes above a threshold level are implicated in tumour regression processes. In accordance with our data a study from Salama group demonstrated that *FOXP3*$^+$ cells randomly measured in stage II and III CRC tissue were a better positive prognostic marker than $CD8^+$ and $CD45RO^+$ cells [95]. The prognostic advantage of *FOXP3*$^+$ cells was shown by authors to be significant restricting the analysis to stage II CRCs. Our data expand such observation by demonstrating that *FOXP3*$^+$ cells are associated with no antitumour activity in stage III CRC. The idea that a marker of T-reg cells might be associated in immuno elimination processes is counterintuitive and contrasts with data obtained from other cancers, including melanoma[146] and breast[147], ovarian[148], hepatocellular[149,150] and pancreatic[151] cancers. This observation highlight the importance of tumour and tissue type specificity in performing mechanistic studies exploring the role of *FOXP3* in T-cells antitumour activities. The antigen specificity of tumour-infiltrating TRegs (*FOXP3*$^+$) cells has not been established in humans and *FOXP3* transcription factor might also be expressed by activated effector T cells [61]. On the other hand, it's conceivable to speculate that increasing levels of *FOXP3*$^+$ cells at the tumour site may reflect a decrease of the chronic inflammatory response/circuits/microenvironment at the tumour site, which is thought to facilitate tumour progression, while being irrelevant to the acute process that promotes tumour destruction. These last hypotheses are corroborated by the evidence that in our study *FOXP3*$^+$ Tils are not correlating with $CD3^+$ Tils in the overall population of CRC suggesting different pathways of recruitment for those immune cells. It is important to underline that the recruitment of $CD3^+$

and *FOXP3*$^{+}$TILs in our study seems to be positively and inversely correlated with MSI, respectively. However, this result is in contrast with data from Nosho, wherein *FOXP3*$^{+}$TILs densities were significantly higher in patients with MSI cancer than in MSS and from Salama, wherein *FOXP3*$^{+}$ cells densities were not significantly associated with MS-Status [91, 95]. Such discrepancies might be explained by differences in measurement standards among studies. The study from Nosho analyzed the distribution of *FOXP3*$^{+}$ TILs densities only in the tumour epithelial area while in the study from Salama was performed a random analysis, since authors didn't record whether the tumour tissue location measurement was performed at the invading margin or the centre of the tumour [91,95]. Accordingly, this result suggest that *FOXP3*$^{+}$TILs recruitment in tumour with different MS-Status as genetic background might vary whether the analysis is performed at the tumour invasive front or tumour epithelial areas. On the other hand it is interesting to mention that by comparing this study with others, CD3^{+} TILs densities are increased in MSI patients regardless of the fact that measurement was performed at the tumour invasive front or at the tumour centre in epithelial areas. The harmonization of measurement methodologies in immune infiltrate across studies is a relevant issue. Protocol variability of immunohistochemistry in conjunction with inconsistent tissue region selection criteria, combined with differences in qualitative and semi-quantitative criteria to measure immune infiltration, all contribute to the variability of the results obtained among studies and raise the concern that standardization of protocols may be required. It is therefore essential to pursue assay uniformity by collaboration among different groups to reduce these limitations in order to be able to compare results in the future, and for the development of more effective prognostic and predictive markers to improve clinical decision-making and understand behaviour of inflammation in the tumour in different settings and cancers.

This study will contribute to the following publication

Title	Tumor associated macrophages as a potential predictive biomarker of response to 5-Fluoro-Uracyl in patients with microsatellite-stable colorectal cancer
Authors	***G. Di Caro*** *, F. Grizzi, P. Bianchi, V. Torri, G. Celesti, G. Basso, Prof. A. Mantovani, Prof. A. Malesci, L. Laghi*
Journal	JCO**, In preparation**

Publications not directly related to the topic of the thesis

Peer-Reviewed publications

Title	MSH3 Protein Expression and Nodal Status in MLH1-Deficient Colorectal Cancers.
Authors	*L. Laghi , P.Bianchi, G. Delconte, G. Celesti,* ***G. Di Caro****, M. Pedroni, A. Chiaravalli, B. Jung, C. Capella, M. Ponz de Leon, A. Malesci.*
Journal	Clinical Cancer Research Jun 1;18:3142-3153, 2012.

Title	Irrelevance of Microsatellite Instability in the Epidemiology of Sporadic Pancreatic Ductal Adenocarcinoma
Authors	*L Laghi, S Beghelli, A Spinelli, P Bianchi, G Basso,* ***G Di Caro****, A Brecht, G Celesti, G Turri, S Bersani, G Schumacher, C Roecken, I Graentzdoerffer, MG Roncalli, A Zerbi, P Neuhaus, C Bassi, M Montorsi, A Malesci, A Scarpa.*
Journal	Plos one 2012;7(9) Epub 2012 Sep 21.

In preparation publications

Title	Occurrence and prognostic significance of tertiary lymphoid tissue in human colo-rectal cancer
Authors	****G. Di Caro,*** **F. Bergomas, F. Grizzi, A. Doni, P. Bianchi, Prof. A. Malesci, L. Laghi, P. Allavena, Prof. A. Mantovani, F. Marchesi*
Journal	EMBO Mol Med, **Submitted**, *these authours equally contributed to this work

Title	Expression of the fractalkine receptor CX3CR1 is a feature of early neoplastic lesions of the pancreas.
Authors	****Di Caro G,*** **Celesti G, Bianchi P , Grizzi F, Marchesi F, Basso G, Rahal D, Cappello P, Roncalli M, Zerbi A, Montorsi M, Novelli F, Prof. Mantovani A, Allavena P, Prof. Malesci A., Laghi L.*
Journal	Clinical Cancer Research, **Under review**, *these authors equally contributed to this work

Title	Colorectal cancer stroma: tumor cells in diguise.
Authors	*G. Celesti,* ***G. Di Caro,*** *P. Bianchi, F. Grizzi, L. Rubino, G. Basso, F. Marchesi, A. Doni, G. Marra, M. Roncalli, Prof. A. Mantovani, Pro. A. Malesci, L. Laghi.*
Journal	Gastroenterology, **revision requested**

Title	Molecular and Prognostic Heterogeneity of Synchronous Colorectal Neoplasms.
Authors	*A Malesci, G Basso, P Bianchi, L Fini, G Delconte, F Grizzi, G Celesti,* ***G Di Caro****, G Delconte, F Dattola, A Repici, M Roncalli, M Montorsi, L Laghi*
Journal	Lancet Oncology, **Submitted**

Bibliography

1. Siegel R, Naishadham D, Jemal A: Cancer statistics, 2012. CA Cancer J Clin 62:10-29, 2012
2. Ferlay J, Shin HR, Bray F, et al: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127:2893-917, 2010
3. Mejia A, Schulz S, Hyslop T, et al: Molecular staging individualizing cancer management. J Surg Oncol 105:468-74, 2012
4. Chapuis PH, Chan C, Dent OF: Clinicopathological staging of colorectal cancer: Evolution and consensus-an Australian perspective. J Gastroenterol Hepatol 26 Suppl 1:58-64, 2011
5. Ueno H, Mochizuki H, Akagi Y, et al: Optimal colorectal cancer staging criteria in TNM classification. J Clin Oncol 30:1519-26, 2012
6. O'Connell JB, Maggard MA, Ko CY: Colon cancer survival rates with the new American Joint Committee on Cancer sixth edition staging. J Natl Cancer Inst 96:1420-5, 2004
7. Fearon ER, Vogelstein B: A genetic model for colorectal tumorigenesis. Cell 61:759-67, 1990
8. Markowitz S, Wang J, Myeroff L, et al: Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. Science 268:1336-8, 1995
9. Samuels Y, Velculescu VE: Oncogenic mutations of PIK3CA in human cancers. Cell Cycle 3:1221-4, 2004
10. Cunningham D, Atkin W, Lenz HJ, et al: Colorectal cancer. Lancet 375:1030-47, 2010
11. Thibodeau SN, Bren G, Schaid D: Microsatellite instability in cancer of the proximal colon. Science 260:816-9, 1993
12. Hermsen M, Postma C, Baak J, et al: Colorectal adenoma to carcinoma progression follows multiple pathways of chromosomal instability. Gastroenterology 123:1109-19, 2002
13. Grilley M, Holmes J, Yashar B, et al: Mechanisms of DNA-mismatch correction. Mutat Res 236:253-67, 1990
14. Culligan KM, Meyer-Gauen G, Lyons-Weiler J, et al: Evolutionary origin, diversification and specialization of eukaryotic MutS homolog mismatch repair proteins. Nucleic Acids Res 28:463-71, 2000
15. Aquilina G, Hess P, Branch P, et al: A mismatch recognition defect in colon carcinoma confers DNA microsatellite instability and a mutator phenotype. Proc Natl Acad Sci U S A 91:8905-9, 1994
16. Strand M, Earley MC, Crouse GF, et al: Mutations in the MSH3 gene preferentially lead to deletions within tracts of simple repetitive DNA in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 92:10418-21, 1995
17. Malesci A, Laghi L, Bianchi P, et al: Reduced likelihood of metastases in patients with microsatellite-unstable colorectal cancer. Clin Cancer Res 13:3831-9, 2007
18. Kuper H, Adami HO, Trichopoulos D: Infections as a major preventable cause of human cancer. J Intern Med 248:171-83, 2000
19. Parkin DM: The global health burden of infection-associated cancers in the year 2002. Int J Cancer 118:3030-44, 2006
20. Aggarwal BB, Vijayalekshmi RV, Sung B: Targeting inflammatory pathways for prevention and therapy of cancer: short-term friend, long-term foe. Clin Cancer Res 15:425-30, 2009
21. Takahashi H, Ogata H, Nishigaki R, et al: Tobacco smoke promotes lung tumorigenesis by triggering IKKbeta- and JNK1-dependent inflammation. Cancer Cell 17:89-97, 2010
22. Park EJ, Lee JH, Yu GY, et al: Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression. Cell 140:197-208, 2010
23. Hanahan D, Weinberg RA: Hallmarks of cancer: the next generation. Cell 144:646-74, 2011

24. Mantovani A, Romero P, Palucka AK, et al: Tumour immunity: effector response to tumour and role of the microenvironment. Lancet 371:771-83, 2008

25. Terzic J, Grivennikov S, Karin E, et al: Inflammation and colon cancer. Gastroenterology 138:2101-2114 e5, 2010

26. Schottenfeld D, Beebe-Dimmer J: Chronic inflammation: a common and important factor in the pathogenesis of neoplasia. CA Cancer J Clin 56:69-83, 2006

27. Eaden JA, Abrams KR, Mayberry JF: The risk of colorectal cancer in ulcerative colitis: a meta-analysis. Gut 48:526-35, 2001

28. Canavan C, Abrams KR, Mayberry J: Meta-analysis: colorectal and small bowel cancer risk in patients with Crohn's disease. Aliment Pharmacol Ther 23:1097-104, 2006

29. Strober W, Fuss IJ, Blumberg RS: The immunology of mucosal models of inflammation. Annu Rev Immunol 20:495-549, 2002

30. Triantafillidis JK, Nasioulas G, Kosmidis PA: Colorectal cancer and inflammatory bowel disease: epidemiology, risk factors, mechanisms of carcinogenesis and prevention strategies. Anticancer Res 29:2727-37, 2009

31. Chan AT, Ogino S, Giovannucci EL, et al: Inflammatory markers are associated with risk of colorectal cancer and chemopreventive response to anti-inflammatory drugs. Gastroenterology 140:799-808, quiz e11, 2011

32. Greten FR, Eckmann L, Greten TF, et al: IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell 118:285-96, 2004

33. Grivennikov SI, Greten FR, Karin M: Immunity, inflammation, and cancer. Cell 140:883-99, 2010

34. Mantovani A, Allavena P, Sica A, et al: Cancer-related inflammation. Nature 454:436-44, 2008

35. Sakamoto K, Maeda S, Hikiba Y, et al: Constitutive NF-kappaB activation in colorectal carcinoma plays a key role in angiogenesis, promoting tumor growth. Clin Cancer Res 15:2248-58, 2009

36. Yu H, Pardoll D, Jove R: STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer 9:798-809, 2009

37. Algra AM, Rothwell PM: Effects of regular aspirin on long-term cancer incidence and metastasis: a systematic comparison of evidence from observational studies versus randomised trials. Lancet Oncol 13:518-27, 2012

38. Flossmann E, Rothwell PM, British Doctors Aspirin T, et al: Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies. Lancet 369:1603-13, 2007

39. Baron JA, Cole BF, Sandler RS, et al: A randomized trial of aspirin to prevent colorectal adenomas. N Engl J Med 348:891-9, 2003

40. Thun MJ, Jacobs EJ, Patrono C: The role of aspirin in cancer prevention. Nat Rev Clin Oncol 9:259-67, 2012

41. Chia WK, Ali R, Toh HC: Aspirin as adjuvant therapy for colorectal cancer--reinterpreting paradigms. Nat Rev Clin Oncol 9:561-70, 2012

42. Liao X, Lochhead P, Nishihara R, et al: Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. N Engl J Med 367:1596-606, 2012

43. Lievre A, Blons H, Laurent-Puig P: Oncogenic mutations as predictive factors in colorectal cancer. Oncogene 29:3033-43, 2010

44. Dighe AS, Richards E, Old LJ, et al: Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. Immunity 1:447-56, 1994

45. Kaplan DH, Shankaran V, Dighe AS, et al: Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. Proc Natl Acad Sci U S A 95:7556-61, 1998

46. Shankaran V, Ikeda H, Bruce AT, et al: IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 410:1107-11, 2001

47. Schreiber RD, Old LJ, Smyth MJ: Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 331:1565-70, 2011

48. Old LJ, Boyse EA: Immunology of Experimental Tumors. Annu Rev Med 15:167-86, 1964

49. Dunn GP, Bruce AT, Ikeda H, et al: Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol 3:991-8, 2002

50. Vesely MD, Kershaw MH, Schreiber RD, et al: Natural innate and adaptive immunity to cancer. Annu Rev Immunol 29:235-71, 2011

51. Smyth MJ, Crowe NY, Godfrey DI: NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. Int Immunol 13:459-63, 2001

52. Street SE, Hayakawa Y, Zhan Y, et al: Innate immune surveillance of spontaneous B cell lymphomas by natural killer cells and gammadelta T cells. J Exp Med 199:879-84, 2004

53. Koebel CM, Vermi W, Swann JB, et al: Adaptive immunity maintains occult cancer in an equilibrium state. Nature 450:903-7, 2007

54. Dunn GP, Koebel CM, Schreiber RD: Interferons, immunity and cancer immunoediting. Nat Rev Immunol 6:836-48, 2006

55. MacKie RM, Reid R, Junor B: Fatal melanoma transferred in a donated kidney 16 years after melanoma surgery. N Engl J Med 348:567-8, 2003

56. Shunyakov L, Ryan CK, Sahasrabudhe DM, et al: The influence of host response on colorectal cancer prognosis. Clin Colorectal Cancer 4:38-45, 2004

57. Titu LV, Monson JR, Greenman J: The role of CD8(+) T cells in immune responses to colorectal cancer. Cancer Immunol Immunother 51:235-47, 2002

58. Dalerba P, Maccalli C, Casati C, et al: Immunology and immunotherapy of colorectal cancer. Crit Rev Oncol Hematol 46:33-57, 2003

59. Zou W: Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol 6:295-307, 2006

60. Hori S, Nomura T, Sakaguchi S: Control of regulatory T cell development by the transcription factor Foxp3. Science 299:1057-61, 2003

61. Fridman WH, Pages F, Sautes-Fridman C, et al: The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer 12:298-306, 2012

62. Koch M, Beckhove P, Op den Winkel J, et al: Tumor infiltrating T lymphocytes in colorectal cancer: Tumor-selective activation and cytotoxic activity in situ. Ann Surg 244:986-92; discussion 992-3, 2006

63. Atreya I, Neurath MF: Immune cells in colorectal cancer: prognostic relevance and therapeutic strategies. Expert Rev Anticancer Ther 8:561-72, 2008

64. Mantovani A, Sica A, Allavena P, et al: Tumor-associated macrophages and the related myeloid-derived suppressor cells as a paradigm of the diversity of macrophage activation. Hum Immunol 70:325-30, 2009

65. Sica A, Mantovani A: Macrophage plasticity and polarization: in vivo veritas. J Clin Invest 122:787-95, 2012

66. Mantovani A, Germano G, Marchesi F, et al: Cancer-promoting tumor-associated macrophages: new vistas and open questions. Eur J Immunol 41:2522-5, 2011

67. Biswas SK, Mantovani A: Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol 11:889-96, 2010

68. Mantovani A, Sica A: Macrophages, innate immunity and cancer: balance, tolerance, and diversity. Curr Opin Immunol 22:231-7, 2010

69. Allavena P, Sica A, Garlanda C, et al: The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance. Immunol Rev 222:155-61, 2008

70. Hagemann T, Balkwill F, Lawrence T: Inflammation and cancer: a double-edged sword. Cancer Cell 12:300-1, 2007

71. Forssell J, Oberg A, Henriksson ML, et al: High macrophage infiltration along the tumor front correlates with improved survival in colon cancer. Clin Cancer Res 13:1472-9, 2007

72. Jadus MR, Williams CC, Avina MD, et al: Macrophages kill T9 glioma tumor cells bearing the membrane isoform of macrophage colony stimulating factor through a phagocytosis-dependent pathway. J Immunol 160:361-8, 1998

73. Oosterling SJ, van der Bij GJ, Meijer GA, et al: Macrophages direct tumour histology and clinical outcome in a colon cancer model. J Pathol 207:147-55, 2005

74. Spratt JS, Jr., Spjut HJ: Prevalence and prognosis of individual clinical and pathologic variables associated with colorectal carcinoma. Cancer 20:1976-85, 1967

75. Zamcheck N, Doos WG, Prudente R, et al: Prognostic factors in colon carcinoma: correlation of serum carcinoembryonic antigen level and tumor histopathology. Hum Pathol 6:31-45, 1975

76. House AK, Watt AG: Survival and the immune response in patients with carcinoma of the colorectum. Gut 20:868-74, 1979

77. Jass JR, Love SB, Northover JM: A new prognostic classification of rectal cancer. Lancet 1:1303-6, 1987

78. Ropponen KM, Eskelinen MJ, Lipponen PK, et al: Prognostic value of tumour-infiltrating lymphocytes (TILs) in colorectal cancer. J Pathol 182:318-24, 1997

79. Halvorsen TB, Seim E: Tumour site: a prognostic factor in colorectal cancer? A multivariate analysis. Scand J Gastroenterol 22:124-8, 1987

80. Kubota Y, Sunouchi K, Ono M, et al: Local immunity and metastasis of colorectal carcinoma. Dis Colon Rectum 35:645-50, 1992

81. Coca S, Perez-Piqueras J, Martinez D, et al: The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. Cancer 79:2320-8, 1997

82. Diez M, Pollan M, Enriquez JM, et al: Histopathologic prognostic score in colorectal adenocarcinomas. Anticancer Res 18:689-94, 1998

83. Klintrup K, Makinen JM, Kauppila S, et al: Inflammation and prognosis in colorectal cancer. Eur J Cancer 41:2645-54, 2005

84. Ogino S, Nosho K, Irahara N, et al: Lymphocytic reaction to colorectal cancer is associated with longer survival, independent of lymph node count, microsatellite instability, and CpG island methylator phenotype. Clin Cancer Res 15:6412-20, 2009

85. Graham DM, Appelman HD: Crohn's-like lymphoid reaction and colorectal carcinoma: a potential histologic prognosticator. Mod Pathol 3:332-5, 1990

86. Roxburgh CS, McMillan DC: The role of the in situ local inflammatory response in predicting recurrence and survival in patients with primary operable colorectal cancer. Cancer Treat Rev 38:451-66, 2012

87. Naito Y, Saito K, Shiiba K, et al: CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. Cancer Res 58:3491-4, 1998

88. Nagtegaal ID, Marijnen CA, Kranenbarg EK, et al: Local and distant recurrences in rectal cancer patients are predicted by the nonspecific immune response; specific immune response has only a systemic effect--a histopathological and immunohistochemical study. BMC Cancer 1:7, 2001

89. Guidoboni M, Gafa R, Viel A, et al: Microsatellite instability and high content of activated cytotoxic lymphocytes identify colon cancer patients with a favorable prognosis. Am J Pathol 159:297-304, 2001

90. Galon J, Costes A, Sanchez-Cabo F, et al: Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 313:1960-4, 2006

91. Nosho K, Baba Y, Tanaka N, et al: Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review. J Pathol 222:350-66, 2010

92. Laghi L, Bianchi P, Miranda E, et al: CD3+ cells at the invasive margin of deeply invading (pT3-T4) colorectal cancer and risk of post-surgical metastasis: a longitudinal study. Lancet Oncol 10:877-84, 2009

93. Canna K, McMillan DC, McKee RF, et al: Evaluation of a cumulative prognostic score based on the systemic inflammatory response in patients undergoing potentially curative surgery for colorectal cancer. Br J Cancer 90:1707-9, 2004

94. Pages F, Kirilovsky A, Mlecnik B, et al: In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. J Clin Oncol 27:5944-51, 2009

95. Salama P, Phillips M, Grieu F, et al: Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. J Clin Oncol 27:186-92, 2009

96. Frey DM, Droeser RA, Viehl CT, et al: High frequency of tumor-infiltrating FOXP3(+) regulatory T cells predicts improved survival in mismatch repair-proficient colorectal cancer patients. Int J Cancer 126:2635-43, 2010

97. Zhou Q, Peng RQ, Wu XJ, et al: The density of macrophages in the invasive front is inversely correlated to liver metastasis in colon cancer. J Transl Med 8:13, 2010

98. Kang JC, Chen JS, Lee CH, et al: Intratumoral macrophage counts correlate with tumor progression in colorectal cancer. J Surg Oncol 102:242-8, 2010

99. Dolcetti R, Viel A, Doglioni C, et al: High prevalence of activated intraepithelial cytotoxic T lymphocytes and increased neoplastic cell apoptosis in colorectal carcinomas with microsatellite instability. Am J Pathol 154:1805-13, 1999

100. Bauer K, Michel S, Reuschenbach M, et al: Dendritic cell and macrophage infiltration in microsatellite-unstable and microsatellite-stable colorectal cancer. Fam Cancer 10:557-65, 2011

101. Saeterdal I, Bjorheim J, Lislerud K, et al: Frameshift-mutation-derived peptides as tumor-specific antigens in inherited and spontaneous colorectal cancer. Proc Natl Acad Sci U S A 98:13255-60, 2001

102. Ishikawa T, Fujita T, Suzuki Y, et al: Tumor-specific immunological recognition of frameshift-mutated peptides in colon cancer with microsatellite instability. Cancer Res 63:5564-72, 2003

103. Markowitz SD, Roberts AB: Tumor suppressor activity of the TGF-beta pathway in human cancers. Cytokine Growth Factor Rev 7:93-102, 1996

104. Ripberger E, Linnebacher M, Schwitalle Y, et al: Identification of an HLA-A0201-restricted CTL epitope generated by a tumor-specific frameshift mutation in a coding microsatellite of the OGT gene. J Clin Immunol 23:415-23, 2003

105. Saeterdal I, Gjertsen MK, Straten P, et al: A TGF betaRII frameshift-mutation-derived CTL epitope recognised by HLA-A2-restricted CD8+ T cells. Cancer Immunol Immunother 50:469-76, 2001

106. You KT, Li LS, Kim NG, et al: Selective translational repression of truncated proteins from frameshift mutation-derived mRNAs in tumors. PLoS Biol 5:e109, 2007

107. Banerjea A, Ahmed S, Hands RE, et al: Colorectal cancers with microsatellite instability display mRNA expression signatures characteristic of increased immunogenicity. Mol Cancer 3:21, 2004

108. Akiyama Y, Iwanaga R, Saitoh K, et al: Transforming growth factor beta type II receptor gene mutations in adenomas from hereditary nonpolyposis colorectal cancer. Gastroenterology 112:33-9, 1997

109. Baker K, Zlobec I, Tornillo L, et al: Differential significance of tumour infiltrating lymphocytes in sporadic mismatch repair deficient versus proficient colorectal cancers: a potential role for dysregulation of the transforming growth factor-beta pathway. Eur J Cancer 43:624-31, 2007

110. Apetoh L, Ghiringhelli F, Tesniere A, et al: The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy. Immunol Rev 220:47-59, 2007

111. Apetoh L, Ghiringhelli F, Tesniere A, et al: Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med 13:1050-9, 2007

112. Tesniere A, Schlemmer F, Boige V, et al: Immunogenic death of colon cancer cells treated with oxaliplatin. Oncogene 29:482-91, 2010

113. Lauber K, Blumenthal SG, Waibel M, et al: Clearance of apoptotic cells: getting rid of the corpses. Mol Cell 14:277-87, 2004

114. Lauber K, Bohn E, Krober SM, et al: Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. Cell 113:717-30, 2003

115. Ardavin C, Amigorena S, Reis e Sousa C: Dendritic cells: immunobiology and cancer immunotherapy. Immunity 20:17-23, 2004

116. Backer R, Schwandt T, Greuter M, et al: Effective collaboration between marginal metallophilic macrophages and CD8+ dendritic cells in the generation of cytotoxic T cells. Proc Natl Acad Sci U S A 107:216-21, 2010

117. Ciampricotti M, Hau CS, Doornebal CW, et al: Chemotherapy response of spontaneous mammary tumors is independent of the adaptive immune system. Nat Med 18:344-6; author reply 346, 2012

118. Sanoff HK, Carpenter WR, Martin CF, et al: Comparative effectiveness of oxaliplatin vs non-oxaliplatin-containing adjuvant chemotherapy for stage III colon cancer. J Natl Cancer Inst 104:211-27, 2012

119. Golfinopoulos V, Salanti G, Pavlidis N, et al: Survival and disease-progression benefits with treatment regimens for advanced colorectal cancer: a meta-analysis. Lancet Oncol 8:898-911, 2007

120. Schrag D, Rifas-Shiman S, Saltz L, et al: Adjuvant chemotherapy use for Medicare beneficiaries with stage II colon cancer. J Clin Oncol 20:3999-4005, 2002

121. Benson AB, 3rd, Schrag D, Somerfield MR, et al: American Society of Clinical Oncology recommendations on adjuvant chemotherapy for stage II colon cancer. J Clin Oncol 22:3408-19, 2004

122. NIH consensus conference. Adjuvant therapy for patients with colon and rectal cancer. JAMA 264:1444-50, 1990

123. Quasar Collaborative G, Gray R, Barnwell J, et al: Adjuvant chemotherapy versus observation in patients with colorectal cancer: a randomised study. Lancet 370:2020-9, 2007

124. O'Connor ES, Greenblatt DY, LoConte NK, et al: Adjuvant chemotherapy for stage II colon cancer with poor prognostic features. J Clin Oncol 29:3381-8, 2011

125. Koi M, Umar A, Chauhan DP, et al: Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces N-methyl-N'-nitro-N-nitrosoguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation. Cancer Res 54:4308-12, 1994

126. Arnold CN, Goel A, Boland CR: Role of hMLH1 promoter hypermethylation in drug resistance to 5-fluorouracil in colorectal cancer cell lines. Int J Cancer 106:66-73, 2003

127. Pocard M, Bras-Goncalves R, Hamelin R, et al: Response to 5-fluorouracil of orthotopically xenografted human colon cancers with a microsatellite instability: influence of P53 status. Anticancer Res 20:85-90, 2000

128. Kim GP, Colangelo LH, Wieand HS, et al: Prognostic and predictive roles of high-degree microsatellite instability in colon cancer: a National Cancer Institute-National Surgical Adjuvant Breast and Bowel Project Collaborative Study. J Clin Oncol 25:767-72, 2007

129. Elsaleh H, Joseph D, Grieu F, et al: Association of tumour site and sex with survival benefit from adjuvant chemotherapy in colorectal cancer. Lancet 355:1745-50, 2000

130. Ribic CM, Sargent DJ, Moore MJ, et al: Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. N Engl J Med 349:247-57, 2003

131. Sargent DJ, Marsoni S, Monges G, et al: Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. J Clin Oncol 28:3219-26, 2010

132. Benatti P, Gafa R, Barana D, et al: Microsatellite instability and colorectal cancer prognosis. Clin Cancer Res 11:8332-40, 2005

133. Jover R, Zapater P, Castells A, et al: The efficacy of adjuvant chemotherapy with 5-fluorouracil in colorectal cancer depends on the mismatch repair status. Eur J Cancer 45:365-73, 2009

134. Popat S, Hubner R, Houlston RS: Systematic review of microsatellite instability and colorectal cancer prognosis. J Clin Oncol 23:609-18, 2005

135. Sinicrope FA: DNA mismatch repair and adjuvant chemotherapy in sporadic colon cancer. Nat Rev Clin Oncol 7:174-7, 2010

136. Halama N, Zoernig I, Grabe N, et al: The local immunological microenvironment in colorectal cancer as a prognostic factor for treatment decisions in the clinic: The way ahead. Oncoimmunology 1:62-66, 2012

137. Zitvogel L, Kepp O, Kroemer G: Immune parameters affecting the efficacy of chemotherapeutic regimens. Nat Rev Clin Oncol 8:151-60, 2011

138. Morris M, Platell C, Iacopetta B: Tumor-infiltrating lymphocytes and perforation in colon cancer predict positive response to 5-fluorouracil chemotherapy. Clin Cancer Res 14:1413-7, 2008

139. Halama N, Michel S, Kloor M, et al: Localization and density of immune cells in the invasive margin of human colorectal cancer liver metastases are prognostic for response to chemotherapy. Cancer Res 71:5670-7, 2011

140. Halama N, Michel S, Kloor M, et al: The localization and density of immune cells in primary tumors of human metastatic colorectal cancer shows an association with response to chemotherapy. Cancer Immun 9:1, 2009

141. Mlecnik B, Tosolini M, Kirilovsky A, et al: Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. J Clin Oncol 29:610-8, 2011

142. Fridman WH, Galon J, Pages F, et al: Prognostic and predictive impact of intra- and peritumoral immune infiltrates. Cancer Res 71:5601-5, 2011

143. Prall F, Duhrkop T, Weirich V, et al: Prognostic role of CD8+ tumor-infiltrating lymphocytes in stage III colorectal cancer with and without microsatellite instability. Hum Pathol 35:808-16, 2004

144. Steidl C, Lee T, Shah SP, et al: Tumor-associated macrophages and survival in classic Hodgkin's lymphoma. N Engl J Med 362:875-85, 2010

145. Beatty GL, Chiorean EG, Fishman MP, et al: CD40 agonists alter tumor stroma and show efficacy against pancreatic carcinoma in mice and humans. Science 331:1612-6, 2011

146. Miracco C, Mourmouras V, Biagioli M, et al: Utility of tumour-infiltrating CD25+FOXP3+ regulatory T cell evaluation in predicting local recurrence in vertical growth phase cutaneous melanoma. Oncol Rep 18:1115-22, 2007

147. Bates GJ, Fox SB, Han C, et al: Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. J Clin Oncol 24:5373-80, 2006

148. Curiel TJ, Coukos G, Zou L, et al: Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 10:942-9, 2004

149. Kobayashi N, Hiraoka N, Yamagami W, et al: FOXP3+ regulatory T cells affect the development and progression of hepatocarcinogenesis. Clin Cancer Res 13:902-11, 2007

150. Gao Q, Qiu SJ, Fan J, et al: Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. J Clin Oncol 25:2586-93, 2007

151. Hiraoka N, Onozato K, Kosuge T, et al: Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. Clin Cancer Res 12:5423-34, 2006

Acknowledgments

I would like to thanks Dr. Luigi Laghi, who designed the present research, gave me invaluable scientific support and trained me in the art of "translational medicine". I am grateful to Dr. Fabio Grizzi for his precious biological and methodological suggestions to realize the present study. Thanks to Dr. Paolo Bianchi who helped me with clinical database and to Dr. Valter Torri who gave support with his statistical knowledge for improving the scientific value of the study. Finally, thanks to Prof. Alberto Mantovani, Prof. Massimo Locati and Dr. Raffaella Bonecchi for their important encouragement in the accomplishment of this phase of my scientific course.