-Instituto Politécnico de Lisboa-

-Escola Superior de Tecnologia da Saúde de Lisboa-

Mestrado em Tecnologias Clínico-Laboratoriais

Caracterização do Microambiente Tumoral de Carcinoma do Colón, com foco nas células Natural Killers

Characterization of the Colon Adenocarcinoma Microenvironment with a Focus on Natural Killer Cells

Mastering: Hasti António Meggi Calá

Supervisor: Mireia Castillo-Martín, M.D., Ph.D. – Fundação Champalimaud

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Lisbon, July 2023

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Lisbon, July 2023

"Sometimes we feel that what we do is just a drop of water in the sea. But the sea would be smaller if it lacked a drop."

(Madre Teresa de Calcutá)

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Abstract

Colorectal Cancer (CRC) is the third most common cancer worldwide. In patients with advanced CRC, standard treatment strategies have shown low effectiveness. Adoptive Cell Therapy (ACT) is an immunotherapy approach that relies on the use of autologous T-cells to target tumour cells. More recently, Natural Killer (NK) cells have appeared as an alternative to T-cells due to their lesser secondary effects, strong cytotoxicity, and tumour microenvironment (TME) regulatory capacity. The main goal of this project is to evaluate the immune infiltrates in CRC tissue specimens, with a focus on NK cells, using multiplex immunofluorescence (Mplex IF).

Formalin-fixed paraffin-embedded (FFPE) tumour specimens from 10 patients who underwent surgery for CRC were used to characterise the TME. Multispectral microscopy was used to quantify and analyse the spatial distribution of NK cells, their subtypes and functionality, using antibodies from different animal sources. After initial Mplex optimization, regions of interest were acquired for each specimen using a *Nikon 90i* fluorescence microscope with a multispectral camera. The images were generated with the *Nuance software* and later analysed using the *Fiji/ ImageJ software*.

To evaluate the activation state of NK cells in the CRC TME a Mplex was developed, with the following biomarkers: NCAM1 (NK Cells), CD16 (cytotoxic NK cells), NKG2D (activated NK cells), CD3 (T cells) and Pan-Cytokeratin which was used to label the tumour cells to localize the immune cells with respect of the neoplastic glands.

Different tumour compartments: stroma on the periphery of the tumour, stroma between the tumour glands and intraepithelial were analysed and correlated with clinicopathological patient characteristics.

The results obtained demonstrate that NK cells there was a significant association between high number of NK cells a low stages of tumour progression. Notably, lower number of infiltrating NK cells CRC is associated with a higher risk of mortality.

Keywords: Colorectal Cancer, Natural Killer Cells, Tumour Microenvironment, Immunofluorescence, Multispectral Microscopy, Biomarkers

Resumo

O Cancro Colorretal (CCR) é o terceiro tipo de cancro mais comum em todo o mundo. Em doentes com CCR avançado, as estratégias de tratamento padrão têm pouca eficácia. A Terapia Celular Adotiva (ACT) é uma abordagem de imunoterapia que se baseia na utilização de células T autólogas para atingir as células tumorais. Mais recentemente, as células Natural Killer (NK) têm surgido como uma alternativa às células T devido aos seus menores efeitos secundários, forte citotoxicidade e capacidade reguladora do microambiente tumoral (TME). O principal objetivo deste projeto é avaliar os infiltrados imunitários em amostras de tecido de CCR, com foco nas células NK, utilizando imunofluorescência multiplex (Mplex IF).

Neste trabalho, foram utilizadas amostras de tumores fixadas em formalina e embebidas em parafina (FFPE) para caracterizar o TME de 10 doentes submetidos a cirurgia de CCR. A microscopia multiespectral foi utilizada para quantificar e analisar a distribuição espacial das células NK, os seus subtipos e a função, utilizando anticorpos de diferentes origens animais. Após a otimização inicial da Mplex, foram adquiridas regiões de interesse para cada amostra utilizando um microscópio de fluorescência *Nikon* 90i com uma câmara multiespectral. As imagens foram geradas com o *software Nuance* e posteriormente analisadas com o *software Fiji/Image J.*

Para avaliar o estado de ativação das células NK no TME do CCR, foi desenvolvida uma Mplex com os seguintes biomarcadores NCAM1 (células NK), CD16 (células NK citotóxicas), CD3 (células T), NKG2D (células NK ativadas) e a Pan-Citoqueratina que foi utilizada para marcar as células tumorais de modo a localizar as células imunitárias em relação às glândulas neoplásicas.

Diferentes compartimentos tumorais foram analisados, nomeadamente o estroma na periferia do tumor, o estroma entre as glândulas tumorais e intra-epitelial e correlacionados com as características clinicopatológicas dos pacientes.

Os resultados obtidos demostram que existe uma associação significativa entre o elevado número de células NK e baixos estádios de progressão do tumor. Nomeadamente, um menor número de células NK infiltradas no CCR está associado a um maior risco de mortalidade.

Palavras-chave: Cancro Colorretal; Células Natural Killer; Microambiente Tumoral; Imunofluorescência; Microscopia multiespectral; Biomarcadores

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Glossary of abbreviations

A

ACT – Adoptive Cell Transfer

ADCC – Antibody-Dependent Cellular Cytotoxicity

ADT – Androgen Deprivation Therapy

APC – Adenomatous Polyposis Coli

AWD – Alive With Disease

AWOD – Alive Without Disease

B

BRAF – V-Raf Murine Sarcoma Viral Oncogene Homolog B1

BSA – Bovine Serum Albumin

C

CAFs -– Cancer Associated Fibroblasts

CRC – Colorectal Cancer

CD16 – Cluster Differentiation 16 or FCγ receptor III

CD3 – Cluster Differentiation 3

CD56 – Cluster Differentiation 56

CD226 – Cluster Differentiation 56

CIN – Chromosomal Instability

CIMP – CpG Island Methylator Phenotype

CMS – Consensus Molecular Subtypes

CRC – Colorectal Cancer

CT – Computed Tomography

CTLs – Cytotoxic T Lymphocytes

D

DCs – Dendritic Cells

DFS – Disease-Free Survival

dMMR – Mismatch Repair-Deficient

DNA – Deoxyribonucleic acid

DNAM1 – DNAX Accessory Molecule-1

DOD – Dead of Disease

E

EGFR – Epidermal Growth Factor Receptor

F

5-FU – 5-Fluorouracil

FAP – Familial Adenomatous Polyposis

FBS – Fetal Bovine Serum

FFPE – Formalin-Fixed Paraffin-Embedded

G

GI – Gastrointestinal Tract

GLOBOCAN – Global Cancer Observatory

H

H&E – Hematoxylin & Eosin

HNPCC – Hereditary Non-Polyposis Colon Cancer

I

IBD – Inflammatory Bowel Disease

IF – Immunofluorescence

IFN-γ – Interferon-gamma

IHC – Immunohistochemistry

K

KRAS – Ki-ras2 Kirsten Rat Sarcoma Viral Oncogene Homolog

L

LGR – Large Granular Lymphocytes

M

m – Meters

MAPK – Mitogen-associated protein kinase

mCRC – Metastatic CRC

MHC – Major Histocompatibility Complex

MMR – Mismatch Repair

mPlex IHC/IF – Multiplex Immunohistochemistry/ Immunofluorescence

MRI – Magnetic Resonance Imaging

MSI – Microsatellite Instability

MSI-H – Microsatellite Instability-High

MSS – Microsatellite Stability

MYC – MYC gene

MYH – (MYH)-associated polyposis

N

NCAM1 – Neural Cell Adhesion Molecule 1

NK Cells – Natural Killer Cells

NKG2D – Natural Killer Group 2 Member D

NKp46 – Natural Killer Cell p46

NKp44 – Natural Killer Cell p44

O

OS – Overall Survival

P

Pan-Ck – Pan-Cytokeratin

PBMCs – Peripheral Blood Mononuclear Cells

PBS – Phosphate Saline Buffer

PET – Positron Emission Tomography

PI3K – Phosphatidylinositol 3-Kinase

R

ROIs – Regions of Interest

RT – Room Temperature

T

TAMs – Tumour Associated Macrophages

TCR – T-Cell Receptor

TGF-β – Transforming Growth Factor Beta

TH1 – T Helper 1

TILs – Tumour-Infiltrating Lymphocytes

TME – Tumour Microenvironment

TMN – Tumour, Nodes, Metastasis Classification

TNF-α – Necrosis Factor-alpha

V

VEGF – Vascular Endothelial Growth Factor

W

WNT – WNT (Wingless Related Integration Site) pathway

1. Introduction and Literature Review

1.1. Colon Anatomy and Histology

The large intestine (colon and rectum) is an organ that makes part of the digestive tract. The colon is approximately 1.5 meters (m) that corresponds to one-fifth of the gastrointestinal (GI) tract. The colon is divided into five parts, it includes in the right side the cecum and ascending colon, in the middle the transverse colon, and in the left side the descending colon and the sigmoid colon (Figure 1) (Azzouz & Sharma, 2023).

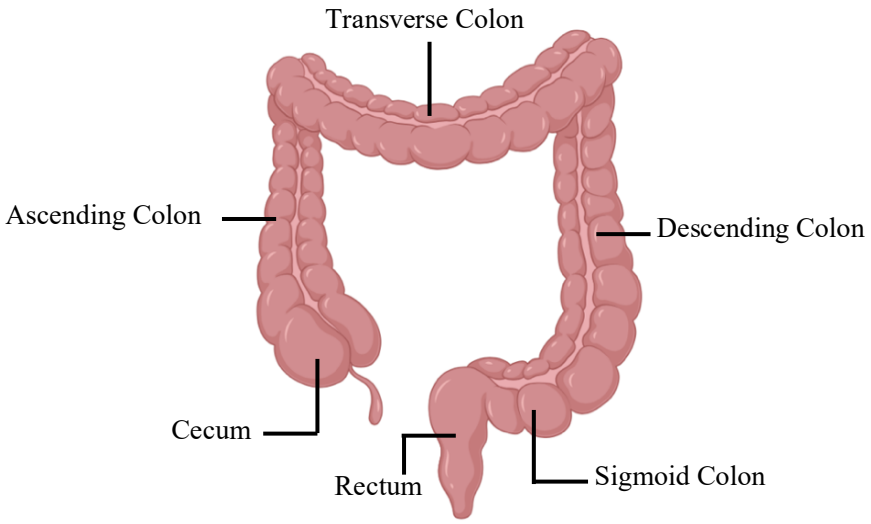


Figure 1 – Anatomic Segments of Colon. *In the right side includes the cecum and ascending colon, in the middle the transverse colon, and in the left side the descending colon and the sigmoid colon (adapted from (Mahul B. Amin et al., 2017). The figure was created using (Biorender) (accessed on 29 April 2023).*

The mucosa of the colon is coated by a layer of epithelial cells arranged in invaginations called the crypts of Lieberkühn that are supported by the lamina propria (Figure 2). There are more layers of the colon, the base of mucosa is separated from the submucosa by a thin layer of muscularis mucosa, and the layer between submucosa and the adipose layer is the muscularis propria, and in the final, the serosa that is the outermost layer of the colon (Mahul B. Amin et al., 2017).

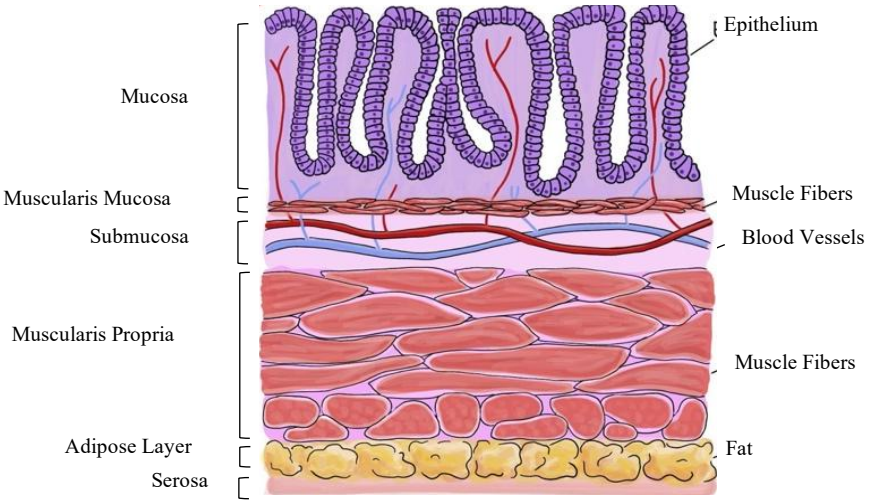


Figure 2 – Normal Colon Layers *(adapted from (My Pathology) (accessed on 20 April 2023).*

1.2. Colon Cancer

A tumour is an abnormal growth of cells that can multiply and can become a malignant or benign tumour. The malignant tumours can spread to other organs of the body, unlike the benign ones (Wang et al., 2018).

1.2.1. Epidemiology

Cancer is one of the main causes of death in the world. Globally, it is estimated that in 2023 more than one million people will be diagnosed with cancer. Cancer is a disease that can spread to other organs, and it originates when healthy cells grow up uncontrolled and become cancer cells. It can occur when healthy cells undergo mutations and multiply abnormally (National Cancer Institute).

Globally, about one in five people will develop cancer during their lifetime. At least about 40% of all cancer cases with effective primary prevention measures could be prevented, and further mortality could be reduced through early detection of tumours (World Health Organization).

According to the World Health Organization (WHO) Colorectal Cancer (CRC) is the third most common cancer type globally, almost two million cases were diagnosed in 2020, being considered the second most common cancer death, and it leads to almost one million deaths per year (Figure 3).

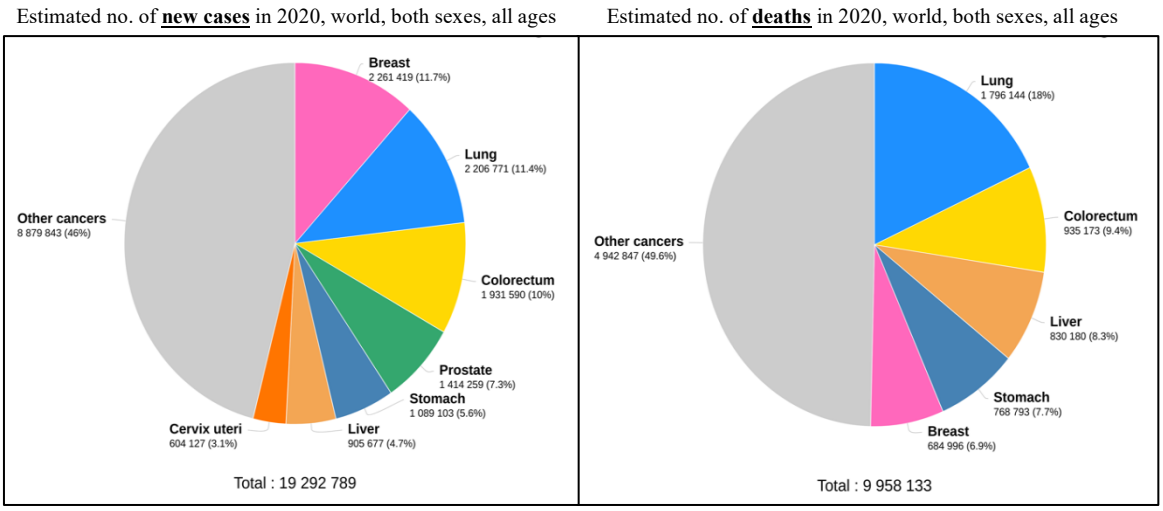


Figure 3 – Distribution of new cases and deaths for the most common cancers in 2020 for both sexes. *CRC has and incidence of 10% and mortality of 9,4% ((Global Cancer Observatory) (accessed on 24 April 2023).*

An increase of 2.2 million new cases and 1.1 million deaths by cancer is predicted by 2030 (Rawla et al., 2019).

CRC has become a predominant cancer in Western countries. Is a cancer with more incidence in industrialized countries, and the epigenetic and genetic factors are inherently involved in its pathogenesis (Zeuner et al., 2014).

1.2.2. Colorectal Cancer Carcinogenesis

CRC is a disorder that occurs by abnormal proliferation of glandular epithelial cells, when these cells acquire a series of genetic and epigenetic alterations. It has been described that CRC is one of the mutated cancers, so it can be hypermutated or non-hypermutated (Hossain et al., 2022).

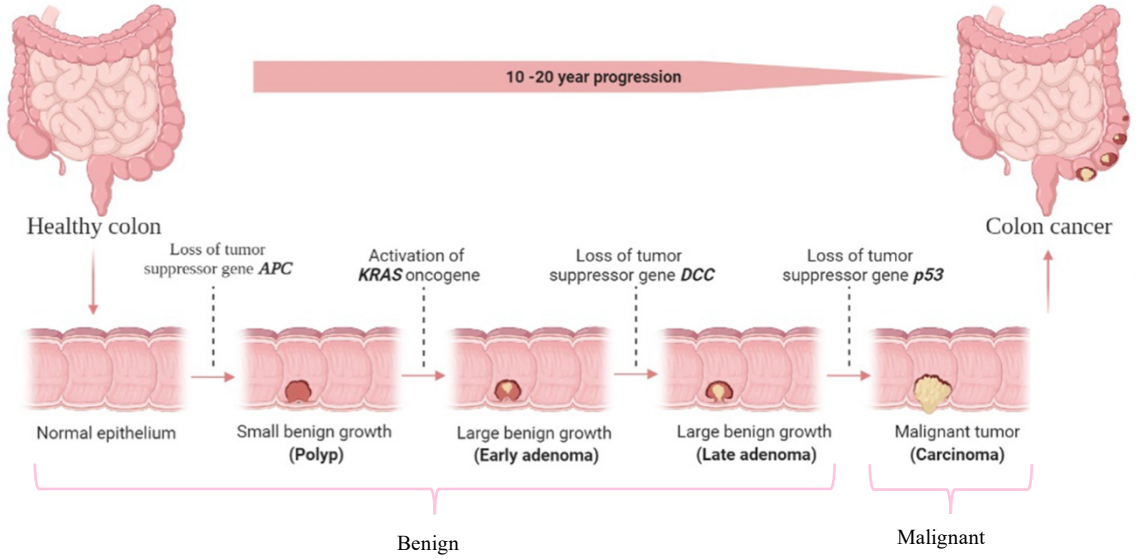


Figure 4 – Colorectal Cancer Carcinogenesis and Stages. *There are four stages in the development of CRC carcinogenesis: initiation, promotion, progression, and metastasis (adapted from (Hossain et al., 2022)).*

CRC development can be divided into four distinct stages: initiation, promotion, progression, and metastasis. Initiation marks the initial genetic alterations that predispose normal cells to potential malignancy. Promotion involves the stimulation of these initiated cells, leading to their clonal expansion and the formation of precursor lesions. Progression refers to the acquisition of additional genetic changes that drive the malignant transformation of these precursor lesions into invasive CRC (Figure 4) (Markowitz & Bertagnolli, 2009).

The gradual progression of polyps to cancer, as observed in the general population, presents an opportunity for early detection and removal of the polyps before their transformation into malignancy. The well-known tumour progression model proposed by Fearon and Vogelstein follows a classic pattern, involving sequential steps in the development of malignancy. It begins with an initiation step, giving rise to benign neoplasms such as adenomas and sessile serrated polyps. Subsequently, a promotion step drives the progression of these neoplasms into more histologically advanced forms. Finally, a transformation step leads to the development of invasive carcinoma (Grady & Markowitz, 2015).

CRC mostly develops from a polyp that is considered a percussor lesion involved in initiation of a neoplastic process, with an estimated period of progression into colorectal cancer of 10-

15 years (Dekker et al., 2019). Most colorectal cancers are sporadic, approximately three-quarters of patients do not have a family history (Kuipers et al., 2015).

While every tumour exhibits a unique set of genetic alterations, CRC commonly presents certain mutations that are considered characteristic of disease. These recurrent somatic mutations involve key cancer-related genes, including Adenomatous Polyposis Coli (APC) and TP53 tumour suppressor genes. Additionally, mutations in oncogenes such as KRAS, BRAF, and PIK3CA are frequently observed. Inactivation of Mismatch Repair (MMR) genes, abnormalities in chromosomal numbers, DNA methylation of CpG dinucleotides in gene promoters are also prevalent in CRC. These shared genetic modifications are regarded as hallmarks of the disease (Battaglin et al., 2018; Grady & Markowitz, 2015; Nojadeh et al., 2018).

The identification and characterization of genetic alterations in tumours have played a crucial role in the diagnosis and classification of CRC. This knowledge has paved the way for the development of more personalized treatment approaches that target the specific genetic profile of each patient's tumour. By understanding the unique genetic alterations present in an individual's tumour, tailored therapies can be designed to effectively target the cancer, leading to improved outcomes and potentially better overall patient care (Grady & Markowitz, 2015).

The molecular mechanisms underlying the development of CRC have been extensively studied and are well understood. The most prevalent genetic alterations in this disease can be overall categorized into three main types:

A) Chromosomal Instability (CIN) – CRC often shows chromosomal instability, leading to widespread genomic alterations and structural changes in chromosomes.
B) Microsatellite Instability (MSI) – Another common genetic alteration observed in CRC is microsatellite instability, which results from defects in the DNA mismatch repair system. This leads to the accumulation of errors in representative DNA sequences called microsatellites.
C) CpG Island Methylator Phenotype (CIMP) – CRC is also associated with CpG island methylation, which involves the abnormal addition of methyl groups to CpG dinucleotides in the DNA. This epigenetic alteration can result in the silencing of tumour suppressor genes and contribute to tumour development.

By understanding these key molecular mechanisms and their impact on CRC, researchers and clinicians can gain insights into the underlying biology of the disease and develop targeted strategies for diagnosis, prognosis, and treatment (Battaglin et al., 2018; Nojadeh et al., 2018).

CIN refers to the presence of structural abnormalities or alterations in the number of copies of chromosomes. It is a common characteristic observed in CRC, with up to 85% of CRC cases exhibiting CIN (Ewing et al., 2014; Grady & Markowitz, 2015). The development of CIN has been linked to the loss of function of tumour-suppressor genes, such as APC, which typically play a crucial role in supressing tumorigenesis (Caldwell & Kaplan, 2009; Ewing et al., 2014). The use of diverse methods to assess CIN highlights a significant challenge in the research field. The absence of a validated and universally agree-upon set of criteria for identifying CIN in CRC hinders the comparability of studies, particularly those aiming to correlate CIN with clinical outcomes (Geigl et al., 2008; Grady & Markowitz, 2015).

Microsatellites, also known as short tandem repeats (STRs), are repeated sequences of more frequently (2-7) nucleotides that are scattered through the entire genome (Ewing et al., 2014). MSI CRC, which make up approximately 15% of CRC cases, are typically considered distinct from CIN tumours. Typically, they show a near-diploid karyotype and carry a unique set of gene mutations that distinguish them from CIN CRC. However, it is important to note that there is a subset of CRC that exhibit both MSI and CIN characteristics, indicating some overlap between the two molecular subtypes (Grady & Markowitz, 2015; Walther et al., 2009).

In contrast to CIN, the mechanisms underlying MSI are relatively well understood and often involve the inactivation of DNA MMR genes through either aberrant DNA methylation or somatic mutations (Grady, 2004). Individuals with Lynch Syndrome, a hereditary cancer syndrome previously known as Hereditary Non-Polyposis Colon Cancer (HNPCC), predominantly develop MSI CRC due to germ line mutations in MMR genes, including MLH1, MSH2 (about 70%), MSH6 and PMS2 (about 30%) (Ewing et al., 2014; Grady & Markowitz, 2015; Singh et al., 2021). Recently, germline deletion mutations in the EPCAM gene have been identified as a novel cause of Lynch Syndrome. This mechanism involves the disruption of the 3’ end of EPCAM, which subsequently leads to epigenetic silencing of the neighbouring MSH2 DNA MMR gene (Ewing et al., 2014; Ligtenberg et al., 2009).

Epigenetic instability in CRC is characterized by two main features: hypermethylation of loci containing CpG islands and global DNA hypomethylation. Aberrant DNA methylation is observed in nearly all CRC, but a distinct subset of CRC (approximately 10-20%) exhibits an exceptionally high proportion of aberrantly methylated CpG loci (Grady & Markowitz, 2015). For instance, a significant proportion of sporadic MSI CRC arise from the CIMP condition. In Table 1, the different examples of genomic instability in CRC are summarized.

Table 1 – Examples of genomic instability in CRC *(adapted from (Ewing et al., 2014)).*

Nature of genomic instability	**Example gene mutations**	**Syndrome associated with germline mutation**	**Notes**
Chromosomal instability	Loss of function mutation of APC gene	Familial adenomatous polyposis (FAP)	Somatic *APC* mutation found in 85% of sporadic CRC
Microsatellite instability	Mismatch repair genes MLH1, MSH2, MSH6 and PMS2	Lynch syndrome	Somatic inactivation of mismatch repair genes found in 15% sporadic CRC
DNA base excision repair defect	MYH gene	MYH-associated polyposis	No recognised somatic equivalent

In Table 2 are summarized some examples of gene mutations implicated in CRC.

Table 2 – Examples of gene mutations implicated in CRC.

Gene or group of genes	Description	Mechanism for mutation increasing CRC risk	Notes
APC	Tumour suppressor gene	Inactivating mutation causes loss of regulation of spindle microtubules during mitosis	APC mutations cause chromosomal instability
TP53	Tumour suppressor gene	Inactivating mutation causes loss of regulation of cell-cycle arrest and cell death	Inactivation may coincide with malignant transformation of adenomas
RAS	Oncogene	Activating mutations drive cell growth through MAPK* pathway	KRAS mutation occurs as early event in adenoma-carcinoma sequence: concordance of primary tumour and metastases
BRAF	Oncogene	Activating mutations drive cell growth through MAPK* pathway	—
PIK3CA	Oncogene	Activating mutation upregulates PI3 K pathway, enhancing prostaglandin E2 synthesis and inhibiting apoptosis	Aspirin is a novel therapeutic agent for mutated PIK3CA tumours
MLH1, MSH2, MSH6, PMS2	MMR genes	Inactivating mutation impairs ability to repair strand slippage within nucleotide repeat	MMR gene mutations cause microsatellite instability
EPCAM	Codes for transmembrane glycoprotein epithelial cell adhesion molecule	Deletion of 3' end of EPCAM leads to epigenetic silencing of MSH2	Novel cause of Lynch syndrome
MYH	Base excision repair gene	Germline inactivating mutation of MYH leads to somatic mutation of APC	Somatic mutations of MYH not described

*MAPK – Mitogen-associated protein kinase

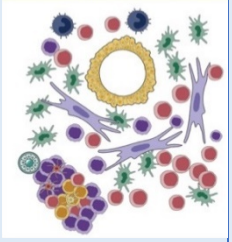
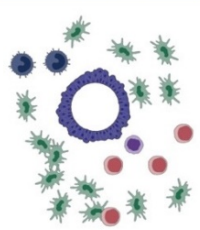
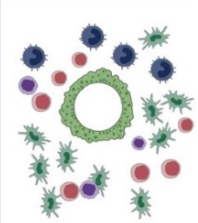
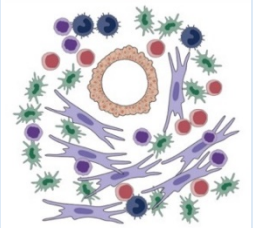
Recently, the advancement of genome-wide techniques has enabled the determination of gene expression signatures in colorectal tumours. Subsequently, the utilization of bioinformatic clustering on the expression profiles has emerged as an additional approach for identifying distinct subtypes of CRC (Jordan, 2018). This has led to the establishment of a consensus molecular classification, which allows for the classification of the majority of tumours into four distinct and reliable subtypes. The four consensus molecular subtypes (CMS) groups currently provide the most comprehensive description of CRC heterogeneity based on gene expression (Dienstmann et al., 2017).
The CMS subtypes are:

A) CMS1 accounts approximately 14% of early-stage tumours; is characterized by distinct features such as hypermutation, hypermethylation, a high prevalence of $BRAF^{V600E}$ mutations, and robust infiltration of immune cells within the tumour microenvironment.
B) CMS2, which represents the canonical subtype and accounts for approximately 37% of early-stage tumours.
C) CMS3 tumours are considered to be metabolic tumours and represents approximately 13% of early-stage tumours.
D) CMS4 tumours have an inflamed, complement-rich, suppressive, and highly angiogenic TME that can be targeted with combination therapies, accounts 23% of early-stage tumours.

CMS1 and CMS4 are subtypes characterized by significant presence of stromal tissue and they are associated with the poorest survival rates and unfavourable prognoses in terms of disease-free survival (DFS) and overall survival (OS) (Gallo et al., 2021). There is a significant presence of $CD8^+$ cytotoxic T Lymphocytes (CTLs), $CD4^+$ T helper 1 (TH1) cells and NK cells in CMS1 subtype (Dienstmann et al., 2017; Gallo et al., 2021). Notably, Cancer Associated Fibroblasts (CAFs) are abundant in the CMS4 subtype (Gallo et al., 2021).
CMS2 encompasses tumour with APC mutations, CIN, and frequent gene amplification or deletion. On the other hand, CMS3 is characterized by KRAS mutations and a mixed status or MSI and CIN (Jordan, 2018).

The CMS subtypes are summarized in Table 3.

Table 3 – Pathological, molecular, and genomic features of each consensus molecular subgroup (CMS) of CRC *(adapted from (Gallo et al., 2021; Jordan, 2018)).*

	CMS1 Hypermutated	CMS2 Canonical	CMS3 Metabolic	CMS4 Mesenchymal
				
Frequency	14%	37%	13%	23%
Tumour Location	Proximal	Distal	Proximal or Distal	Distal
Precursor Lesion	Sessile Serrated	Adenomatous	Serrated or Adenomatous	Adenomatous
DNA Sequence Stability	MSI	MSS	MSS or MSI	MSS
DNA Methylation	CIMP-H	No CIMP	CIMP-L	No CIMP
Chromosome number	Stable	CIN	Stable or CIN	CIN
Mutated genes	BRAF	APC, TP53	KRAS	—
Pathway Signature	Immune Activation	WNT and MYC	Metabolic Deregulation	TGF-β, Mesenchymal

1.2.3. Risk Factors

Several factors are associated with an increased risk to develop CRC. There are two types of risks, such as non-modifiable and modifiable risk factors. The first type is associated with race and ethnicity; sex; age; hereditary mutations; inflammatory bowel disease (IBD); abdominal radiation; cystic fibrosis; cholecystectomy and androgen deprivation therapy. These risks are due to personal or familial history of colorectal polyps or CRC, hereditary conditions such as Lynch syndrome, a personal history of IBD, racial and ethnic background and the presence of

2 type diabetes. The second type (modifiable risks) comprise obesity and physical inactivity; diet; smoking; alcohol; medications, and diabetes and insulin resistance (Rawla et al., 2019).

Several studies have reported that intake of high quantities of dietary fiber, green leaf vegetables, folate, and calcium are protective against the development of CRC (Balchen & Simon, 2016).

Most colorectal malignancies occur over 50 years, but young adults or teenagers are still at risk to develop CRC. Despite both genders are capable to develop CRC, development in males is more frequent than females (Hossain et al., 2022).

The majority of colon cancer are sporadic, and about 75% of patients have a negative family history. There is more risk related for individuals with a first-degree family member with colon cancer and those who are diagnosed at 50-70 years of age, in these cases the chances double, however the risks triple for individuals with first-degree relatives above 50 years of age at diagnosis (Kuipers et al., 2015).

About 5-7% of colorectal cancer patients are affected by well-defined hereditary CRC syndrome. Also, patients with a previous history of colon cancer, those with a long-standing IBD, or adenomas are more susceptible for colon cancer and require adequate observation. There are two groups of hereditary CRC syndromes (about 5-10% of all patients) that can be subdivided as non-polyposis, that can be Lynch syndrome, and familial CRC, in the other way the polyposis syndromes, which can be recognised by the number of polyps (Dekker et al., 2019; Kuipers et al., 2015).

1.2.4. Colon Cancer Symptoms and Diagnosis

Most of cases of CRC are asymptomatic until they reach advanced stages, but the normal symptoms that can affect people with CRC include abdominal pain, changes in bowel habits, anaemia, and occult rectal bleeding (Dekker et al., 2019).

The clinical evaluation of a patient is based on medical history (if patient has some history of this anomaly), physical examination (abdominal evaluation), radiology (that demonstrates the presence or absence of metastasis), and endoscopy with biopsy, in case of lesion. There are different types of radiologic exams that can be relevant for clinical classification of CRC patients that include chest radiographs; computed tomography (CT) of chest, abdomen, and

pelvis area; magnetic resonance (MR) imaging; positron emission tomography (PET) or fused PET/CT scans (Mahul B. Amin et al., 2017).

Colon cancer can occur as multiple or synchronous (2.5%) with identical or different histological patterns and stages of development. Patients with synchronous primary tumours have the same prognosis as patients with single site colon cancers. Metachronous primary tumours arise in up to 3% during five years after surgery, and the incidence increases up to 9% after several decades in long-term survivors (Labianca et al., 2013).

1.2.5. Metastatic Colon Cancer

Colorectal carcinomas can metastasize to any organ, but the liver and lungs are the most commonly affected. Then the ovaries and nonregional lymph nodes.

Most commonly this process occurs by five means: direct extension, lymphatic spread, portal venous spread to liver, peritoneal dissemination, and vascular spread to distant organs including lung, bone, and brain (Stewart et al., 2018).

Approximately 50% of CRC patients will develop liver metastasis during the course of the disease. Liver metastases can be resectable or non-resectable, and depending on this, patients with CRC have different prognoses (Vatandoust, 2015).

1.2.6. Tumour Staging and Grading

There are many types of systems used to stage cancer, the most common and currently used system is the TNM, and it is used to describe the tumour (T), node (N), and metastasis (M) categories (Global Cancer Observatory):

- T describes the depth of invasion of the primary tumour (Figure 5).
- N describes whether the cancer has spread to the regional lymph nodes.
- M describes whether it has spread to a different part of body.

The TMN is normally used to determine the CRC prognostic and helps the clinicians to decide the best treatment for each patient. This is an international system of classification for malignant tumours (Cancer Research UK).

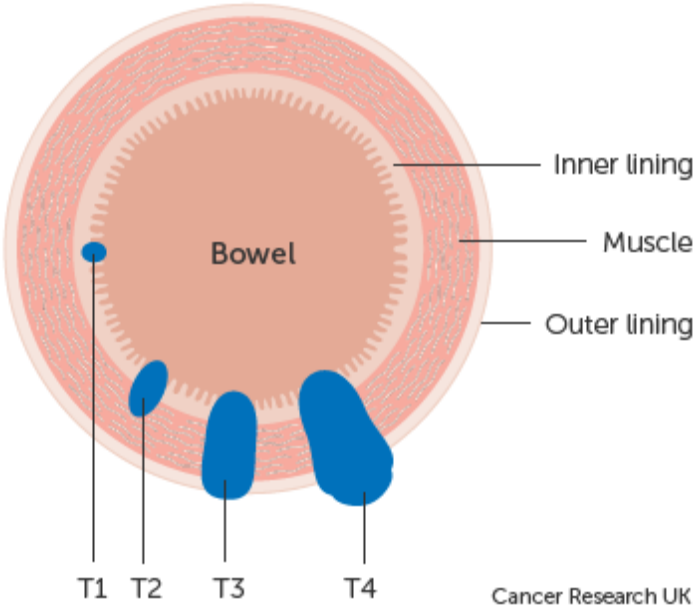


Figure 5 – Colon Cancer TMN stages. *Representation of different stages according to the colon layers (adapted by Cancer Research UK).*

Using these classifications, the TNM stage is determined, ranging from stage 0 (carcinoma *in situ*) to stage IV (metastatic disease). The TNM stage is often combined with other factors, such as histological grade and molecular characteristics to guide treatment decisions (Mahul B. Amin et al., 2017; National Comprehensive Cancer Network).

The classifications used by clinicians are summarized in Table 4, according to the definitions of primary tumour (T), the Regional Lymph Nodes (N) and the Distant Metastasis (M).

Table 4 – Definitions of Primary Tumour (T), Regional Lymph Node (N) and Distant Metastasis (M) *(adapted from* (Mahul B. Amin et al., 2017)*).*

Classification	Definition
T Category	**T Criteria**
TX	Primary tumour cannot be measured
Tis	Tumour cells are only growing *in situ* (involvement of lamina propria with no extension thought muscularis mucosae)
T0	No evidence of a primary tumour
T1	Tumour cells are only in the submucosa
T2	Tumour invades the muscularis propria
T3	Tumour invades through the muscularis propria into pericolorectal tissues
T4	Tumour invades the visceral peritoneum or invades or adheres to adjacent organ or structure
T4a	Tumour invades through the visceral peritoneum (including gross perforation of the bowel through tumour and continuous invasion of tumour through areas of inflammation to the surface of the visceral peritoneum)
T4b	Tumour directly invades or adheres to adjacent organs or structures
N Category	**N Criteria**
NX	Regional lymph nodes cannot be assessed
N0	No lymph nodes metastasis
N1	One to three regional lymph nodes are positive
N1a	One regional lymph node is positive
N1b	Two or three regional lymph nodes are positive
N1c	No regional lymph nodes are positive, but there are tumour cells in the tissue near the tumour (subserosa, mesentery or mesorectal tissues)
N2	Four or more regional nodes are positive
N2 a	Four to six regional lymph nodes are positive
N2 b	Seven or more regional lymph nodes are positive
M Category	**M Criteria**
M0	No evidence of tumours in distant sites or organs
M1	Metastasis to one or more distant sites or organs or peritoneal metastasis is identified
M1a	Metastasis to one site or organ is identified without peritoneal metastasis
M1b	Metastasis to two or more sites or organs is identified without peritoneal metastasis
M1c	Metastasis to the peritoneal surface is identified alone or with other site or organ metastases

1.2.7. Prognostic Groups

The prognosis of patients with CRC is dependent on various factors: clinical, pathological, and biological. There is an objective and easily determined pathological prognostic parameter in CRC to help in selection of patients for further treatments such as the adjuvant treatment. This classification in CRC is the most widely used and has been shown a reduction in recurrence and mortality as well as increase free survival (Table 5) (Petersen et al., 2002).

Table 5 – AJCC Prognostic Stage Groups *(adapted from (Mahul B. Amin et al., 2017)).*

When T is…	And N is…	And M is…	Then the stage group is…
Tis	N0	M0	0
T1 T2	N0	M0	I
T3	N0	M0	II A
T4a	N0	M0	II B
T4b	N0	M0	II C
T1-T2	N1/ N1c	M0	III A
T1	N2a	M0	III A
T3-T4a	N1/ N1c	M0	III B
T2-T3	N2a	M0	III B
T1-T1	N2b	M0	III B
T4a	N2a	M0	III C
T3-T4a	N2b	M0	III C
T4b	N1-N2	M0	III C
Any T	Any N	M1a	IV A
Any T	Any N	M1b	IV B
Any T	Any N	M1c	IV C

1.2.8. Treatment for Colorectal Cancer

The standard treatment for CRC patients is based on a combination of surgery, chemotherapy, and radiotherapy, sometimes with low efficacy in advanced stages (Kekelidze et al., 2013). Surgery plays a crucial role in the management of metastatic CRC (mCRC). It is commonly used for primary tumour resection and can also be employed for the removal of isolated metastases, particularly in the liver or lungs. Surgical procedures aim to remove as much tumour as possible to alleviate symptoms, avoid complications, and improve overall outcomes (Chiappa et al., 2016; Nordlinger et al., 2009). To improve CRC patients' survival time, primary and adjuvant therapy can be used. Currently, there are different novel approaches, including immunotherapy and gene therapy for treatment of CRC patients (Hossain et al., 2022).

The systemic treatment of CRC patients has substantially developed over the past two decades, with major improvements in the neoadjuvant setting for CRC. Adjuvant treatment is used for CRC patients, and the cure rate by surgery alone for T3, T4a, T4b and N0M0 colon cancers, is high although only approximately 5% of patients benefit from adjuvant chemotherapy (Kuipers et al., 2015; Labianca et al., 2013). Systemic chemotherapy is typically administered in combination regimens that include various cytotoxic drugs. Commonly used chemotherapy agents for mCRC, include fluorouracil (5-FU), capecitabine, oxaliplatin, and irinotecan (Grothey et al., 2018; Van Cutsem et al., 2016).

Several targeted therapies have been developed to specifically target certain molecular alterations in CRC cells. These include anti-EGFR (epidermal growth factor receptor) antibodies, such as cetuximab and panitumumab, which are effective in tumours with wild-type KRAS genes. Additionally, anti-VEGF (vascular endothelial growth factor) agents, such as bevacizumab and aflibercept, can be used to inhibit angiogenesis and tumour growth. These targeted therapies are often used in combination with chemotherapy to enhance treatments outcomes (Douillard et al., 2010; Van Cutsem et al., 2009, 2012).

Mostly for metastatic disease immune checkpoints inhibitors have modernised cancer treatment, for those patients with little recourse. Patients who respond positively to the therapy, in which the benefits more often last for years rather than months, have been led to potential cures for cancer diseases (Wilky, 2019). Immune checkpoints inhibitors, such as pembrolizumab and nivolumab, have shown promise in a subset of patients with microsatellite

instability-high (MSI-H) or mismatch repair-deficient (dMMR) mCRC. These agents help to unleash the immune system's ability to recognize and target cancer cells. Immunotherapy is generally reserved for specific patients who meet the molecular criteria (André et al., 2020; Le et al., 2017; Overman et al., 2017, 2018).

1.3. Tumour Microenvironment

The tumour microenvironment (TME) is an environment extremely complex in which tumours cells co-exist with immune cells (Figure 6). TME include NK cells, macrophages, mast cells, T and B lymphocytes, dendritic cells (DCs), polymorphonuclear leukocytes and non-immune cells, such as stromal and endothelial cells, and have interactions with them (Giraldo et al., 2019; Giraldo & Taube, 2018). Generally, human tumours are infiltrated by inflammatory cells, although these infiltrates of inflammatory cells can vary in their composition and size from tumour to tumour, and their presence or absence has been taken in consideration on tumour progression (F. Balkwill & Mantovani, 2001; Mihm et al., 1996; Whiteside, 2008). The role of TME involve proliferative signalling, resisting cell death, evading growth suppressors, inducing angiogenesis, enabling replicative immortality, reprogramming energy metabolism, evading immune destruction, and activating invasion and metastasis. The genetic diversity underlying these hallmarks is generated by genome instability, which accelerates the acquisition of genetic diversity, and inflammation, which promotes multiple hallmark functions (Hanahan & Weinberg, 2011). The different cell types and their functions on TME are described in Table 6.

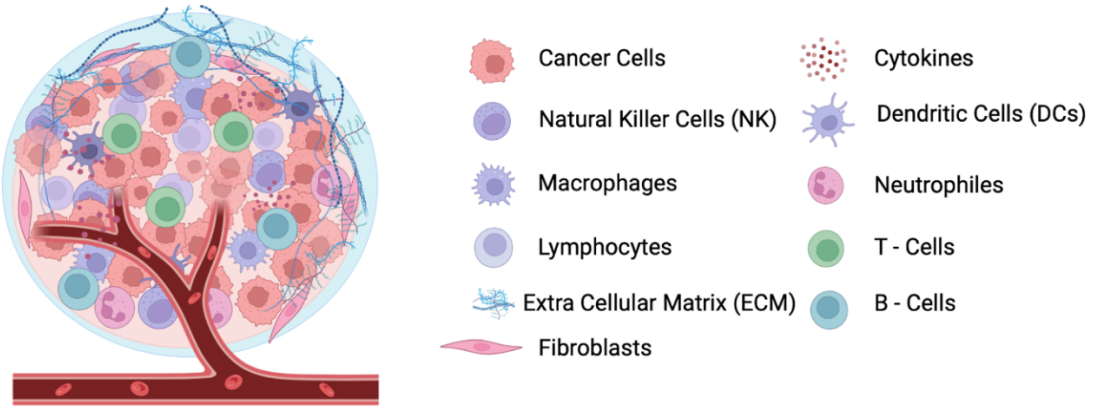


Figure 6 – Tumour Microenvironment with different immune cell types. *The Tumour Microenvironment is composed of various infiltrating cells of the innate and adaptive immune system and their associated mediators. The figure was created using Biorender (accessed on 19 April 2022).*

Table 6 – Different cell types and function on TME and their role on cancer. *(Adapted from (F. R. Balkwill et al., 2012; Bingle et al., 2002; Fridman et al., 2012; Lin et al., 2006; Tachibana et al., 2005; Zumsteg & Christofori, 2009)).*

Cell Type	Function of Cell
B Cells	These cells can be found at the invasive margin of tumours, but there a more present in draining lymph nodes and lymphoid structures adjacent to the TME.
T Cells	There are many different T cell populations within the TME that infiltrate the tumour areas, at the invasive tumour margin and in draining lymphoid organs. High number of T cells in TME also correlate with a good prognosis.
NK Cells	Innate cytotoxic lymphocytes, also infiltrate the tumour stroma, but are not found in contact with tumour cells. For many cancers, such as colorectal cancer, they appear to predict a good prognosis.
Tumour Associated Macrophages (TAMs)	TAMs produce angiogenic factors and accumulate in hypoxic and necrotic areas of TME. There is pre-clinical and clinical evidence that abundance of TAMs in the TME is associated with poor prognosis.
Cancer Associated Fibroblasts (CAFs)	They are in abundance in TME. The induction of CAFs can also cause organ fibrosis which enhances the risk of cancer development.
Dendritic Cells	They have important functions in antigen processing and presentation. The DCs are found in the TME through to be detective.
Tumour Associated Neutrophils (TANS)	They contribute to primary tumour growth and metastasis, increasing degradation of the extracellular matrix
Vascular Endothelial Cells	The tumour vasculature is abnormal in almost every aspect of the structure and function, they increase hypoxia and facilitates metastasis.

Stromal cell populations exhibit a wide range of functional properties that significantly contribute to the fundamental and emerging hallmarks of cancer. The dynamic interplay between stromal cells and cancer cells plays an essential role in shaping the tumour microenvironment and influencing tumour progression, highlighting the importance of understanding the complex interactions within the stromal compartment for developing effective therapeutic strategies (Bissell et al., 1982; Flier et al., 1986; Folkman, 1974).

The immune cells present within the tumour microenvironment exert a significant impact on the growth and evolution of cancerous cells. The complex interplay between immune cells and cancer cells influences various aspects of tumour biology, including tumour initiation, progression, metastasis, and response to treatment. Immune cells can exhibit both tumour-promoting and tumour-suppressive functions, with their activation or suppression being induced by various factors (Korneev et al., 2017; Wang et al., 2018).

Understanding of tumours has undergone a paradigm shift due to the continually evolving knowledge about the intricate complexity of the cancer niche and the dynamic interactions among its various components (Giraldo et al., 2019).

1.3.1. Tumour Microenvironment of Colorectal Cancer

The TME, which refers to the surrounding tissue environment of a tumour, exerts a crucial influence on the development and prognosis of colon cancer. Furthermore, characteristics of the TME can influence treatment response and patient outcomes. Recognizing the fundamental role of the TME in CRC is crucial for advancing our understanding of the disease and developing targeted therapeutic approaches (Chen et al., 2021).

The presence of specific immune cell subsets, such as cytotoxic T cells or TAMs, can either enhance or supress anti-tumour immune responses. Similarly, the abundance of stromal cells, such as CAFs, can impact tumour progression and therapy resistance through the secretion of various factors. Understanding the dynamic interactions between these cell populations in the TME is essential for predicting and optimizing therapeutic responses and developing more effective treatment strategies for cancer patients (Chen et al., 2021; Quail & Joyce, 2013; Wood et al., 2014).

The influence of the TME on outcomes and treatment responses in CRC holds significant importance and can serve as a valuable base and reference for clinical management and targeted therapy. Understanding the complex interplay between tumour cells and the surrounding microenvironment is crucial for predicting disease progression, evaluating prognosis, and designing personalized treatment approaches. By unravelling the complex dynamics of the TME in CRC, clinicians can make informed decisions regarding personalized therapies and optimize patients care to improve clinical outcomes (Chen et al., 2021).

The multistep progression from normal colonic epithelium to an adenomatous polyp and, eventually, to an invasive colon carcinoma in CRC is closely associated with and influenced by the TME (Peddareddigari et al., 2010).

In the past decade, there has been significant progress in our comprehension of the TME, adding to the identification of crucial participants in the immune response against tumours. Among these, tumour-infiltrating lymphocytes (TILs) have emerged as particularly important and prognostically relevant in CRC. TILs constitute heterogenous populations of T lymphocytes present within the TME. Their presence and characteristics play a vital role in shaping the tumour immune response and have implications for the clinical prognosis of CRC patients (Anitei et al., 2014; Barbosa et al., 2021; Wirta et al., 2017).

The NK cells possess receptors that can detect changes in the expression of molecules on the surface of tumour cells. When NK cells recognize these alterations, they can directly kill cancer cells through the release of cytotoxic granules containing perforin and granzymes. This recognition and elimination process by NK cells helps to control tumour growth and prevent metastasis (Lanier, 2008; Smyth et al., 2005; Vivier et al., 2008).

Several studies have indicated that the presence of $CD8^+$ T lymphocytes is associated with a good prognosis in patients diagnosed with CRC (Ko & Pyo, 2019). This specific population of T lymphocytes exerts anti-tumour activity through mechanisms such as antigen-specific cytotoxicity and the production of anti-tumour cytokines, including Interferon-gamma (IFN-γ) and Tumour Necrosis Factor-alpha (TNF-α). These T lymphocytes play a crucial role in mounting an immune response against tumour cells and contribute to the favourable prognosis observed in patients with CRC (Barbosa et al., 2021; Maimela et al., 2019; Martínez-Lostao et al., 2015).

1.3.2. Natural Killer Cells

Natural Killer (NK) cells are a subset of large granular lymphocytes that constitutes approximately 10-15% of the peripheral blood lymphocyte population. They play a crucial role in providing rapid immune responses to viral infections and actively participate in anti-tumour immune surveillance. With their innate ability to recognize and eliminate target cells, including virus-infected cells and tumour cells, NK cells contribute significantly to the body´s defence against infections and the prevention of tumour growth (Boudreau & Hsu, 2018; Franks et al., 2020).

NK cells are classified as group I members of innate lymphoid cells, recognized for their prominent role in producing IFN-γ and proinflammatory cytokines (Coppola et al., 2015). Two subsets of NK cells have been identified based on the expression intensity of neural cell adhesion molecule 1 (NCAM1), also known as CD56, on their cell surface. The first subset, called $CD56^{dim}$ NK cells, is characterized by low levels of CD56 expression in approximately 90% of FCγ receptor III (CD16) NK cells. Conversely, the second subset, referred to as $CD56^{bright}$ NK cells, has high levels of CD56 expression in around 10% of $CD16^{-}$ NK cells (Cooper et al., 2001; Coppola et al., 2015; Lanier et al., 1986).

The $CD56^{bright}$ NK cell population primarily produces regulatory cytokines, while the $CD56^{dim}$ subset is responsible for mediating natural and antibody-dependent cellular cytotoxicity (ADCC). While CD56 is widely used as a marker of NK cells, it is important to note that it is also expressed by other cell types, including NK T cells, DCs and a small subset of human monocytes (Coppola et al., 2015; Phillips & Lanier, 1986; Sconocchia et al., 2005). Therefore, it is important to recognize that the CD56 antigen is not an exclusive marker for the phenotypic and functional characterization of human NK cells (Coppola et al., 2015). CD16, serves as a primary activating receptor on NK cells, enabling both natural cytotoxicity through interactions with unknown ligand (s) and ADCC.

NKG2D is an important activation marker expressed on the surface of NK cells. It is an essential receptor involved in the recognition and elimination of solid tumour cells by NK cells. It plays a critical role in mediating the killing of tumour cells by NK cells through its interactions with specific ligands expressed on the surface of the tumour cells. There are some markers of NK cells activation, these can be DNAM1 (DNAX Accessory Molecule-1), which also known by CD226 (Cluster Differentiation 226); NKp46 (Natural Killer Cell p46), NKp44 (Natural Killer Cell p44), and many more (Bauer et al., 1999; Coppola et al., 2015).

NK T cells are a distinct subset of lymphocytes that exhibit a unique phenotype, expressing receptors commonly found on conventional T cells as well as receptors typically associated with NK cells (Wilson & Kaer, 2003). They are recognized as significant contributors to tumour immunosurveillance. Their ability to recognize and respond to tumour cells enables them to participate actively in the immune surveillance against cancer, further emphasizing their importance in the overall immune response (Coppola et al., 2015).

During the early stages of tumour development, NK T cells play a crucial role in initiating effective anti-tumour immune responses. However, as tumour progression ensues, NK T cells can become overstimulated and anergic, leading to the deletion of a subset of NK T cells in cancer patients. This phenomenon highlights the complex interplay between NK T cells and tumour progression, underscoring the significance of maintaining a functional NK T cell population for mounting effective anti-tumour immunity (Krijgsman et al., 2018).

1.4. Multiplex Immunofluorescence

It is becoming increasingly crucial to not only measure the average expression of molecules in homogenized tissue but also assess their spatial distribution by preserving cellular and tissue architectural features. Given the colocalization of signals of interest, achieving high resolution molecular imaging becomes technically challenging (Mansfield, 2014).

Multiplex Immunohistochemistry/ Immunofluorescence (mIHC/ IF) is a technology that allows the simultaneous detection of multiple markers to quantify numerous proteins on a single tissue section, that has been introduced and adopted in research and clinical purposes in response to increased demand of improved techniques (Eng et al., 2022; Tan et al., 2020). mIF is a dependable and efficient high-throughput technique enabling the identification of multiple markers on tumour cells and tumour associated immune cells at a cellular level (Parra et al., 2021). In recent years, several advanced tissue imaging technologies with high multiplexing capabilities have surfaced, enabling comprehensive investigations into cell composition, functional state, and cell-cell interactions. These advancements hold the promise of enhanced diagnostic benefits (Tan et al., 2020). The benefit of using this powerful tool is that it allows us to use more than two biomarkers and can permitted identification of different cell populations within the TME (Viratham Pulsawatdi et al., 2020). These days understanding

the cellular composition and spatial distribution in tissue sections, termed “spatial biology”, is particularly valuable in the age of immunotherapy (Hoyt, 2021).

In various contexts, the labelling of dozens of targets within intact sections has become necessary to identify crucial cell types and accurately measure their spatial relationships. These advanced techniques not only enable a comprehensive understanding of the molecular landscape within tissues but also provide insights into the complex cellular interactions and organization that underline physiological and pathological process (Eng et al., 2022).

In the era of personalized medicine, the stratification of cancer patients based on molecular expression profiles will be of paramount importance. The availability of viable biomarkers will play a key role in ensuring optimal patient selection and care (Gevaert et al., 2020).

2. Aims and Hypothesis

The main goal of this study is to evaluate the immune infiltrates in CRC tissue specimens, with a focus on NK cells. It has been previously reported that NK cells can mediate anti-tumour responses, so we hypothesize that the number and activation status of NK cells in the CRC TME may have prognostic significance.

The specific aims of this project are:

- ⇒ Optimize the multiplex immunofluorescence with five biomarkers.
- ⇒ Analyse and quantify the distribution of NK cells in CRC tissue specimens.
- ⇒ Analyse the activation state of NK cells in the tissues.
- ⇒ Correlate NK cells subpopulations with patients' clinicopathological characteristics.

3. Material and Methods

In this study, it was combined different biomarkers to validate a multiplex immunofluorescence, to study the distribution of NK cells and their subtypes on TME in CRC in FFPE tissue.

3.1. Biological Samples

3.1.1. Ethical Committee Approval

This project has Champalimaud Foundation's Ethics Committee approval since Formalin-Fixed Paraffin Embedded (FFPE) archived blocks with human biological material were used during the development of the experimental (investigational) work. Tissue samples were collected from the archives of the Service of Anatomic Pathology of the Champalimaud Clinical Centre and were classified by a pathologist, according to the following criteria: samples with a diagnosis of colon adenocarcinoma; no previous treatment; presence of consent signed by the patient and the diagnosis between 2014 and 2016 (to have enough follow-up for statistical analyses). All the human samples and the corresponding necessary clinical data were received after being de-identified through the Champalimaud Foundation Biobank (CFB). Clinical information from the patient, such as, age at diagnosis; sex; tumour stage; molecular alterations of the tumour; follow-up time; tumour recurrence/ progression and end-stage of the patient were also used for clinicopathological correlations.

3.1.2. FFPE Blocks of Human Colorectal Cancer

The 10 FFPE blocks from each patient who underwent surgery for CRC, with different stages from stage I to stage IIIC, without any treatment before surgery were used for this study.

The summary of patients' clinicopathological information is summarized in Table 7. The most common gender in this study was men, around 70% of the cohort, with the mean age at the diagnosis of 66.4 years, and the mean time of clinical follow up was 60.18 months. Different colon locations were observed, namely the right and left colon, the right includes the cecum and ascending, while the left includes the hepatic flexure, sigmoid and rectum-sigmoid.

According to the MMR there are two patients with MSI and seven with MSS, unfortunately there is one patient for whom the MMR status is unknown.

After surgery, three patients had to undergo adjuvant treatment and two patients whose disease had progressed.

Table 7 – Clinicopathological characteristics of a cohort of 10 CRC patients.

Number of Patients	10
Gender	
Male	7 (70%)
Female	3 (30%)
Age at Diagnosis (years)	
Mean	66.4
Range	(37.2 – 81.5)
Stage	
I	2 (20 %)
II A	4 (40%)
III B	3 (30%)
III C	1 (10%)
Clinical Follow up (months)	
Mean	60.18
Range	(20.83 – 81.27)
Status of Follow up (%)	
AWD	1 (10 %)
AWOD	8 (80 %)
DOD	1 (10 %)
Localization	
Right Colon	4 (40 %)
Left Colon	6 (60 %)
MMR *	
MSI	2 (22.22%)
MSS	7 (77.78%)
Adjuvant Treatment	
Yes	3 (30 %)
No	7 (70 %)
Progression (Yes)	2 (20%)

* 1 case unknown

AWD – Alive with disease; AWOD – Alive without disease; DOD – Dead of disease; MMR – Mismatch Repair; MSI – Microsatellite Instability; MSS – Microsatellite Stability

3.2. Optimization of antibodies by IF

A colon adenocarcinoma cell line, namely HKe3, was cultured in DMEM medium, supplemented with 10% FBS and antibiotics at 37ºC. After expansion of HKe3 cells, a pellet of these cells and a pellet of Peripheral Blood Mononuclear Cells (PBMCs) from one CRC patient were used to obtain a positive control to optimize the antibodies as a single IF. HistoGel™ (Epredia, HG-4000-012) was used to create a matrix to support the cell pellets and the gel was put in a tissue cassette and fixed in 10% formalin for 24h. The two types of cells were processed as FFPE blocks, and then were cut into a thickness of 5 µm using a Leica RM2255 microtome (Leica Microsystems) by the Histology Platform of the Champalimaud Centre for the Unknown, and put together in the same slide to have two types of control cells in a single slide.

Later, FFPE tissue blocks of different tissues (spleen, normal colon; lymph nodes; appendix and colon adenocarcinoma) were used as positive controls for optimization of IF. Tissue sections were cut at a thickness of 5µm similar to the cell pellet FFPE blocks. The IF protocol included a first deparaffinization step, followed by antigen retrieval and finally antibody incubation. Briefly, for the deparaffinization, xylene was used, followed by a series of graded ethanol (100%, 95% and 70% ethanol) for re-hydration and finally a washing step using distilled water was performed. The antigen retrieval buffer was done using Tris-EDTA buffer pH = 9.0 (Epitope Retrieval Solution, RE7119, Novocastra, Leica Biosystems), which was pre-heated for 30 minutes and then the slides were immersed into the container filled with antigen retrieval for an additional 30 minutes. After the slides were cooled down in the buffer at room temperature (RT), they were rinsed with distilled water and next in 1X Phosphate Saline Buffer (PBS) (Mediatech, Corning) and permeabilized by quick dips with 0.2% TritonX (Fisher Scientific, Jassen Pharmaceuticalaan, #9002-93-1) in 1X PBS at RT. Bovine Serum Albumin (BSA) containing 0.1% Tween 20 (Fisher Scientific, Jassen Pharmaceutical) in 1XPBS was used for 30 minutes at RT, to initiate background reduction. After 30 minutes, this blocking serum was aspirated and then the primary antibodies against the immune markers were used on slides, after diluting the appropriate volume at specific dilutions in blocking serum and, being incubated for two hours.

The antibodies used for this project are summarized in the Table 8.

Table 8 – ***Description of the primary antibodies.*** *List of all antibodies used, and their respective dilutions, including all tested and the dilution selected.*

Biomarker	Clone	Company	Reference	Dilution	Animal Source
NCAM1 (Anti-NCAM1 Alexa Fluor® 488 conjugated antibody)	[EP2567Y]	Abcam	ab237455	**1:100** 1:200 1:500	Rabbit IgG
Pan-CK (Anti-Pan Cytokeratin Alexa Fluor® 555 conjugated antibody)	[C-11]	Abcam	ab279324	1:50 **1:100** 1:200	Mouse IgG1
CD16 (Anti-CD16 antibody)	[2H7]	Gene Tex	GTX7539	**1:50** 1:80 1:100	Mouse IgG2a
CD3 (Anti-CD3 antibody)	[CD3-12]	Abcam	ab11089	1:50 **1:100** 1:200	Rat IgG
NKG2D (Anti-NKG2D antibody)	[EPR24072-342]	Abcam	ab302907	1:2000 1:1000 **1:500**	Rabbit IgG

After the incubation, the slides were washed with distilled water and then three times with 1X PBS. Then, an incubation of one hour at RT with secondary antibodies conjugated with Alexa Flour® was performed (Table 9). Some of the primary antibodies were already labelled with Alexa Flour®, thus no secondary antibody was needed to add to these.

Table 9 – ***Description of secondary antibodies.*** *List of secondary antibodies conjugated with Alexa Fluor® used for each not conjugated primary antibody.*

Primary Antibody	Secondary Antibody
CD16	Alexa Fluor® 568 Goat anti-Mouse IgG2a (A21134, *Termofisher*)
CD3	Alexa Fluor® 594 Donkey anti-Rat IgG (A21209, *Termofisher*)
NKG2D	Alexa Fluor® 647 Zenon Alexa Fluor anti-Rabbit IgG (Z25308, *Termofisher*)

At the end of the incubations the slides were washed 3 times in 1X PBS, then were counterstained with ProLong™ Gold antifade reagent with DAPI (P36935, Invitrogen) and covered with a coverslip. The slides were placed for 20 minutes at -20°C before visualization under the microscope.

3.3. Duplex and Triplex IF Staining

After each antibody was optimized for the single staining, the biomarkers were validated by Triplex IF and Duplex IF in two different sets. The first set was composed by a cocktail of primary antibodies, CD16 + NKG2D + CD3, followed by the other cocktail of respective secondary Alexa Fluor® antibodies, and the last set with a cocktail of primary conjugated antibodies NCAM1 and Pan-CK. For this step, a FFPE block of colon adenocarcinoma tissue was used as positive control to identify all the biomarkers that were expressed with a good expression.

3.4. Multiplex IF Staining

For the mPlex IF staining was used the same protocol as a single, duplex or triplex antibody, but instead of 1X PBS solution, 1X BOND Wash Solution was used (AR 9590, Leica Biosystems) and in the end of the staining the slides were fixed with 100% Ethanol and 4% Formalin for 10 minutes in each. Then the slides were placed in a dark drawer at RT overnight to allow them to dry and, later, put at -20ºC for 20 minutes before visualization under the microscope.

3.5. Multispectral Imaging

3.5.1. Image Acquisition

CRI Nuance Multispectral camera mounted on a *Nikon 90*i automated fluorescence microscope was used to acquire fluorescence images (at 200x magnification) and controlled by *MetaMorph* software. The spectral library was built using the *Nuance* software using an individual emission peak of each biomarker, and the images from the different regions were unmixed, using the spectral library.

The different biomarkers were recorded taking into consideration the different wavelengths of absorption and emission peaks of the biomarkers optimized (Table 10).

Table 10 – ***Different wavelengths of absorption and emission peaks of Alexa Fluor Secondary antibodies.*** *Five different biomarkers were distinguished by different colours and corresponding peak of absorption and emission.*

Secondary Antibody	λ / nm Absorption	λ / nm Emission	Filter	Color
Alexa Fluor 488	495	519	FITC	Green
Alexa Fluor 555	555	565	555	Yellow
Alexa Fluor 568	578	603	M1	Orange
Alexa Fluor 594	590	617	M1	Red
Alexa Fluor 647	650	668	M2	Pink

3.5.2. Spectral Library building

First, for each antibody the single immunofluorescence staining was used to build a spectral library that contains the emission peaks of each fluorophore, plus DAPI. To create a clean library, the background of each individual fluorophore was eliminated (Figure 7).

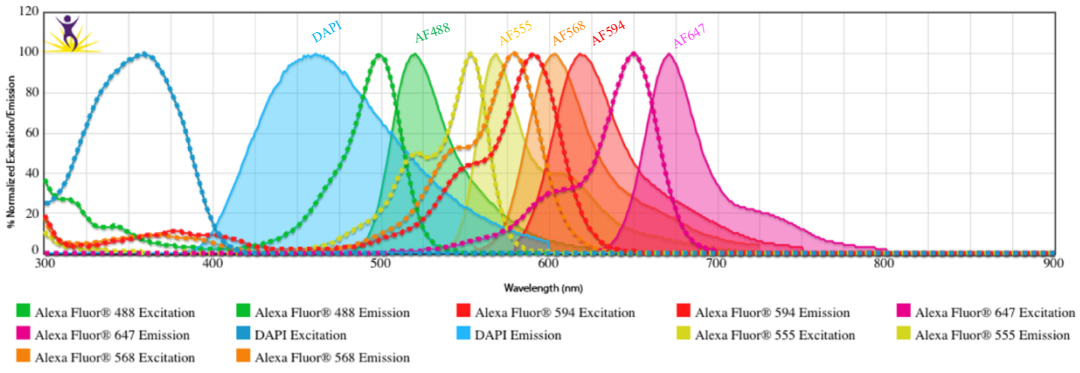


Figure 7 – Spectra of Excitation and Emission peaks of the Alexa Fluor Antibodies. *Alexa Fluor 488 (NCAM1), Alexa Fluor 555 (Pan-CK), Alexa Fluor 568 (CD16), Alexa Fluor 594 (CD3), Alexa Fluor 647 (NKG2D) (Adapted from (Biolegend Spectra Analyzer) (accessed on 10 February 2022).*

3.5.3. Analysis of CRC Cases

Each specimen was analysed in the same way: Five representative areas – Regions of Interest (ROIs) – were randomly chosen from both the periphery and the tumour area at 200X magnification (Figure 8).

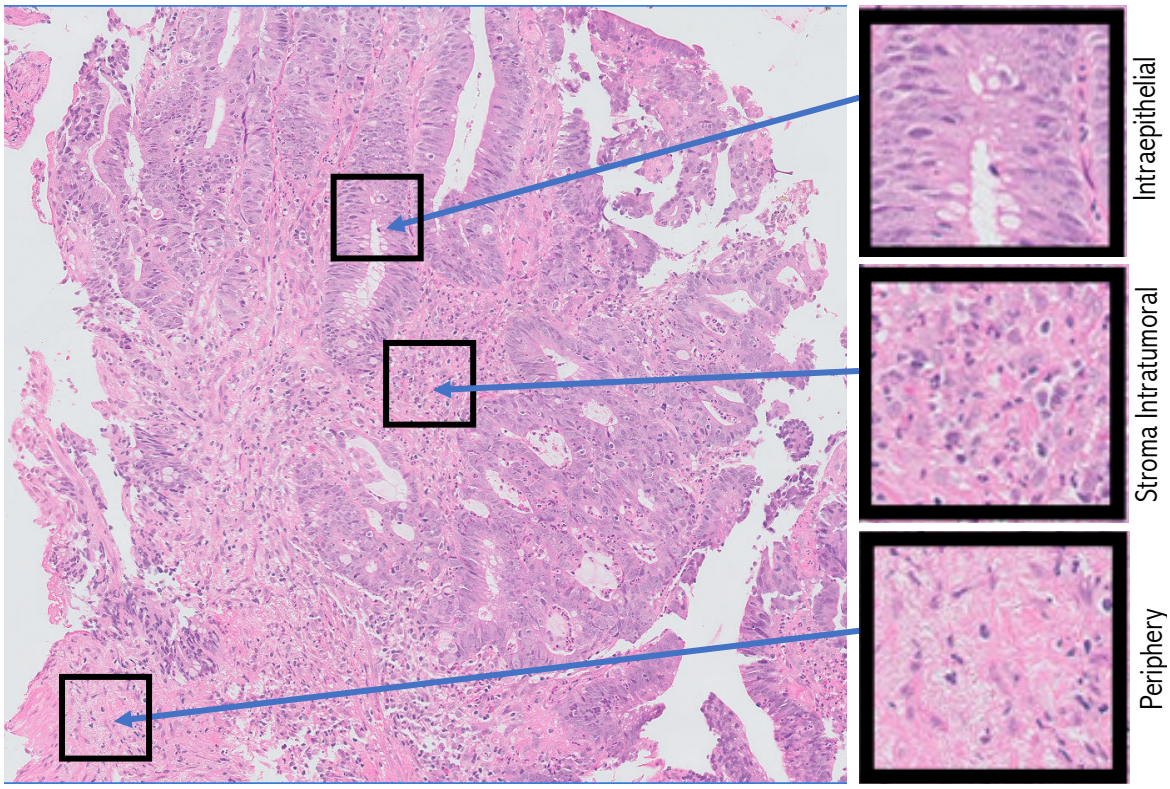


Figure 8 – Representation of the interested regions of interest in Haematoxylin & Eosin (H&E). *The regions of interest: Intraepithelial, Stroma Intratumoral and the Periphery.*

All image analysis was guided using *Fiji/ Image J* software. The images for analysis were configurated at 8-bit, and the threshold was chosen at the same intensity value for a specific staining and converted to binary format. Each ROI analysed corresponded to 0.3468 mm^2 of tissue.

For all tumoral tissue, the localization of the different immune cells was determined in the different areas. First the intratumoral stroma was analysed which is identified between the tumour glands, then the periphery that was the stroma far from the tumour, and also the intraepithelial cells, which were localised inside the malignant epithelium. The Pan-CK signal were used to evaluate the number of cells that was intratumoral or intraepithelial. The population of cells was counted using the Particles Analysis functionality in each ROI. The number of cells/mm2 was calculated by counting the number of cells identified in each compartment and dividing it by the total area of that compartment (as depicted in Figure 9). The region of the epithelium in the image with DAPI and Pan-CK was carefully drawn as an area around the epithelium, then after marking the epithelial region, then manually count the cells that exhibit each biomarker inside (intraepithelial) and outside the epithelium (intratumoral stroma). To facilitate the counting of cells, an image that only represent DAPI and the biomarker of interest were opened (Figure 9).

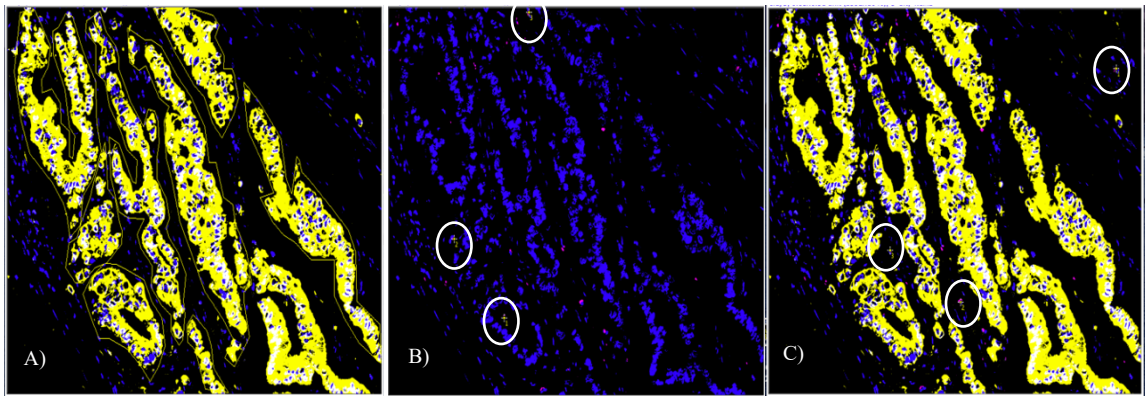


Figure 9 – Representation of each compartment to count cells. *A) Area of DAPI and Pan-CK marked; B) Cells counted inside the epithelium; C) Cells counted outside the epithelium.*

To determine the NK cells was considered only the cells with positive signal with NCAM1 and negative signal with CD3. The CD16 signal was used to determine which NK cells are or not cytotoxic being the cytotoxic NK cells NCAM1 and CD16 positive, and CD3 negative. The number of regulatory NK cells was determined considering only the positive signal of NCAM 1, and negative signal for both CD16 and CD3. Then the number of active NK cells was only considered when the cells have the positive signal of NCAM1, CD16 and NKG2D, with negative signal of CD3.

Also, the number of T cells was counted based on CD3 positive signal and NCAM1 negative signal in the same cell. The type of different cells analysed in this study and the classification that corresponded are summarised in the Table 11.

Table 11 – Classification of Cells according to the Biomarker Expression.

Classification of Cells	Biomarker Expression|
NK Cells	$NCAM1^{+}$ $CD3^{-}$
T Cells	$NCAM1^{-}$ $CD3^{+}$
NK T Cells	$NCAM1^{+}$ $CD3^{+}$
Cytotoxic NK Cells	$NCAM1^{+}$ $CD16^{+}$ $CD3^{-}$
Regulatory NK Cells	$NCAM1^{+}$ $CD16^{-}$ $CD3^{-}$
Activated NK Cells	$NCAM1^{+}$ $CD16^{+}$ $NKG2D^{+}$ $CD3^{-}$

3.5.4. Statistical Analysis

Excel was used for data organization, and GraphPad Prism 9.5.1 (*GraphPad Software, LLC*) was used for construction of graphs and to perform statistical analyses. The data is presented as mean ± standard deviation (SD). First, a normality test was performed in order to know if the population had a normal distribution or not, and in these cases as the population was not normal the non-parametric tests, which was Kruskal-Wallis, that allowed for the comparison of more than two independent groups. A p-value greater than 0.05 was considered statistically non-significant. A p-value <0.05 is statistically significant (*), p-value <0.01 (**) is very statistically significant and p-value <0.001 (***) or p-value <0.0001 (****) are extremely significant. Kaplan-Meier curves were constructed to depict the percentage of patient survival, stratifying the patient samples based on the median expression of the biomarkers.

4.1. Optimization of Single IF

In this section, with the aim to define the localization of different types of immune cells and compare with the tumoral stroma, specific biomarkers were chosen to indicate the presence of those biomarkers in CRC TME. The biological functions of each biomarker used in this study are summarized on Table 12. Five biomarkers were used to identify the expression in CRC tissue specimens, using Alexa Fluor dyes. Two of the primary antibodies were already conjugated with Alexa Fluor dyes, whereas three were not conjugated, so it was necessary to add the secondary antibodies to label properly all the cells. For this purpose, different colours to label them according to the animal source of the primary antibodies were used (Table 9). The optimization process of each biomarker for the staining protocol for each, was carefully tested in different conditions. Antigen Retrieval solutions of pH 6.0 and pH 9.0 were tested for all biomarkers, and for all of them the successful was pH 9.0, getting more specific and with higher intensity in comparison with pH 6.0. The dilution of each antibody was optimized, according to the information of the datasheet, trying always three different dilutions, including above, the same and below the information described in the datasheet. It was possible to have a good and specific signal for all biomarkers tested.

Table 12 – Biological functions of biomarkers analyzed in CRC in TME.

Biomarker	Biological Function
NCAM1	Transmembrane-anchored glycoprotein, about 90% of the NK cells are positive for CD16, excellent marker for detection of NK cells.
Pan- Cytokeratin	Recognizes epitopes present in most human epithelial tissues. It facilitates to distinguish of normal, and neoplastic cells.
CD16	Expressed on large granular lymphocytes (LGR) of NK cells and T-cells.
CD3	Makes the T-cell receptor (TCR), which is found in all mature T-cells.
NKG2D	Is an activating receptor expressed by all NK cells and sub-sets of T cells, essentially all $CD56^{+}$ $CD3^{-}$ NK cells.

The images of the optimization are represented on Figure 10. The NKG2D was optimized using Alexa Fluor® 594 as a secondary antibody for the control, but in the CRC final cases the Alexa Fluor® 647 secondary antibody was used.

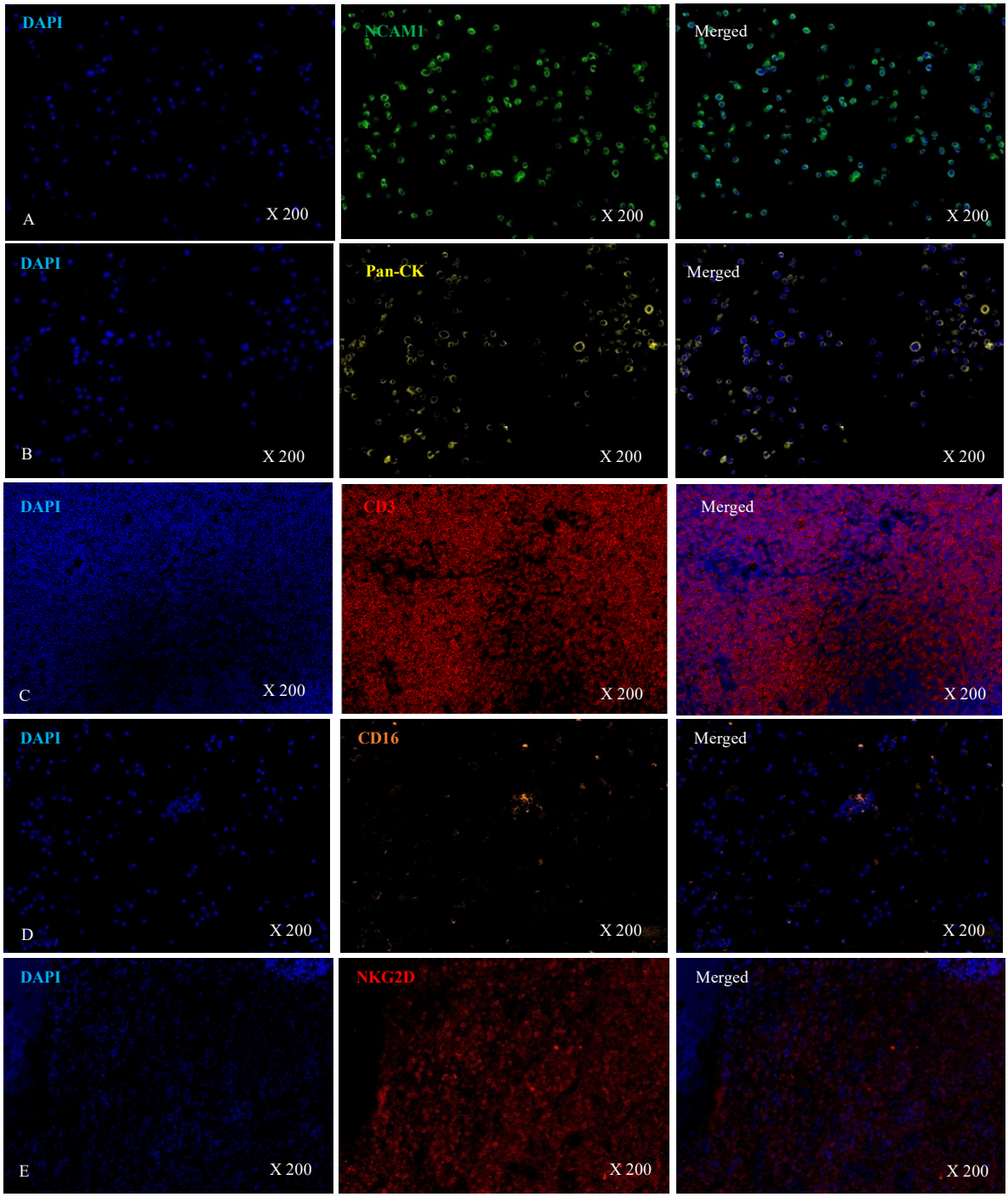


Figure 10 – Optimization of single Immunofluorescence and expression of each biomarkers respective images. (A) NCAM1 staining (green) in PBMCs. (B) Pan-CK staining (yellow) in HKe3 cell line. (C) CD3 staining (*red) in tonsil tissue. (D) CD16 staining (orange) in HKe3 cell line. (E) NKG2D staining (red) in lymph node. DAPI was used to label the nuclear DNA. The images represented were made with Multispectral Microscope with CRI multispectral camera at 200x magnification.*

4.2. Optimization of Duplex IF

After the optimization of the single IF of each antibody and determination of the best dilution, for the optimization of duplex IF FFPE blocks of colon adenocarcinoma as positive control were used, and different antibody combinations were tested in order to verify possible competitions between them. Although in the single IF they worked very well, it is necessary to go through this step in order to demonstrate that the antibodies show the same signal as in the single IF. Competitions may occur between antibodies in case they are from the same animal source or, if they are from different sources, the signal may not be observed in this case. The possible cause is a competition to get the antigen of the added antibody to bind immediately to the tissue epitope. When combined, NCAM1 and Pan-CK, since they both were already conjugated it was not necessary to add secondary antibodies in order to obtain the desired colouration, in the same cocktail, a good signal was obtained, and it was easily distinguished since they had two completely distinguishable colours and they mark different areas of the tissue, the NCAM1 only stained the NK cells and the Pan- CK was able to mark all the epithelium of the tissue. The images of the duplex optimization are represented on Figure 11.

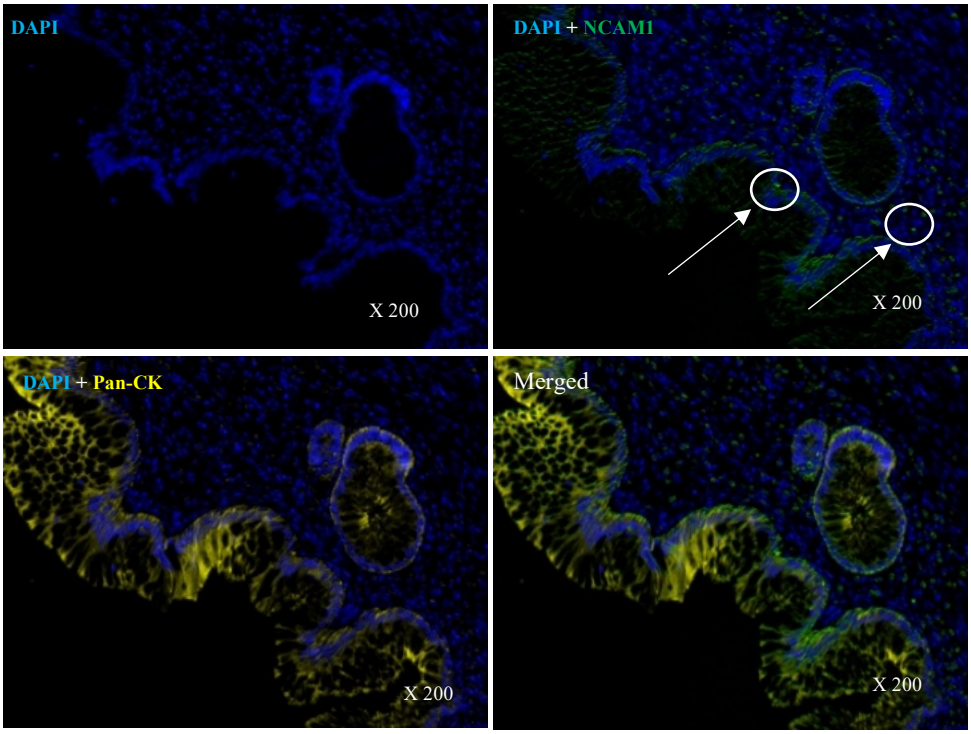


Figure 11 – Optimization of Duplex Immunofluorescence and expression of each biomarkers respective images*. Immunofluorescence expression of NCAM1 (green) and Pan-CK (yellow) in colon adenocarcinoma as positive control. DAPI was used to label the nuclear DNA. The images represented were made with Multispectral Microscope with CRI multispectral camera at 200x magnification.*

4.3. Optimization of Triplex IF

To optimize the Triplex IF of the remaining antibodies, and after, paying special attention to the animal source of each antibody, and since they were all from different sources, CD16, CD3 and NKG2D were put all antibodies together in a cocktail, this way it was possible to see if there was competition, or loss of signal intensity of each antibody. In this case it was possible to observe all three signals, and there was no negative interaction between them. The images of the triplex optimization are represented on Figure 12.

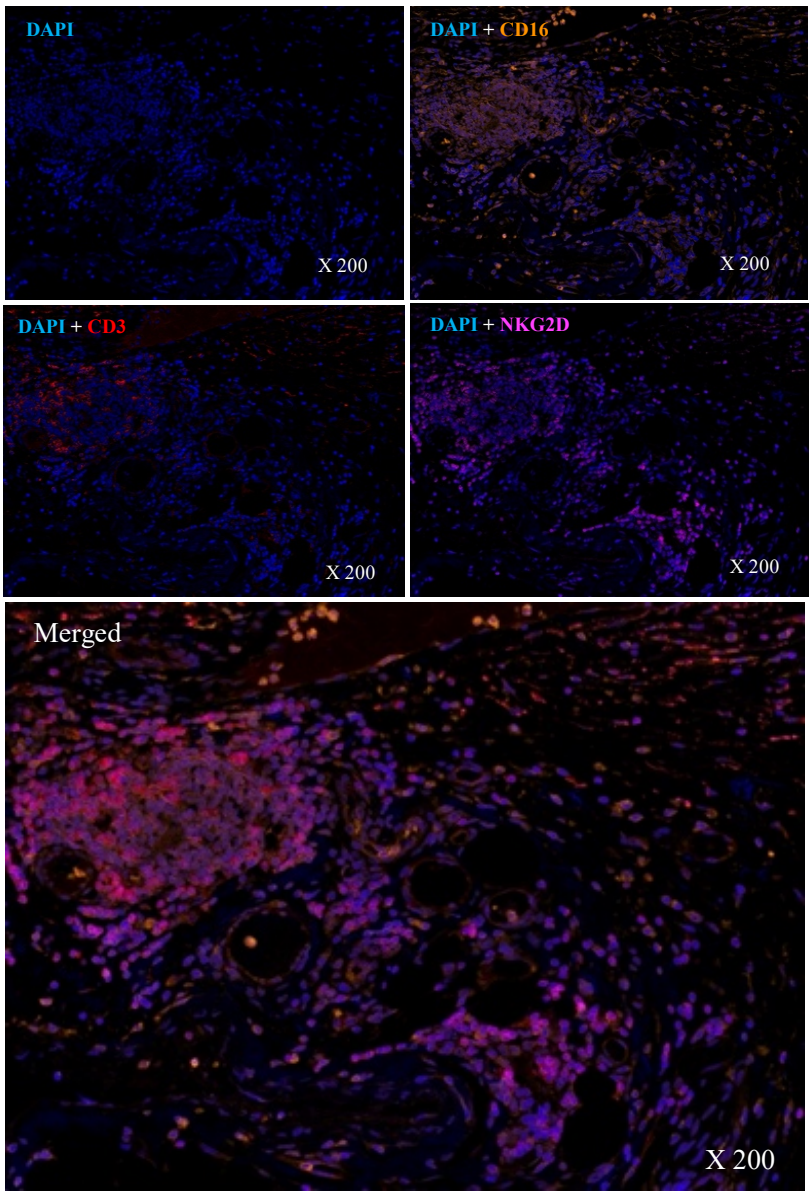


Figure 12 – Optimization of Triplex Immunofluorescence and expression of each biomarkers respective images. Immunofluorescence expression of *CD3 (red), CD16 (orange) NKG2D (pink) in colon adenocarcinoma. DAPI was used to label the nuclear DNA. The images represented were made with Multispectral Microscope with CRI multispectral camera at 200x magnification.*

4.4. Optimization of Mplex

After optimization of duplex and triplex IFs, all antibodies were tested in the same sample, with two different incubations. First cocktail was composed with three antibodies that were tested in triplex CD16, CD3 and NKG2D, and then the respective secondary antibodies to label with the right colours chosen previously. Finally, the second cocktail was composed with two antibodies that were already conjugated. The images of the multiplex optimization are represented on Figure 13.

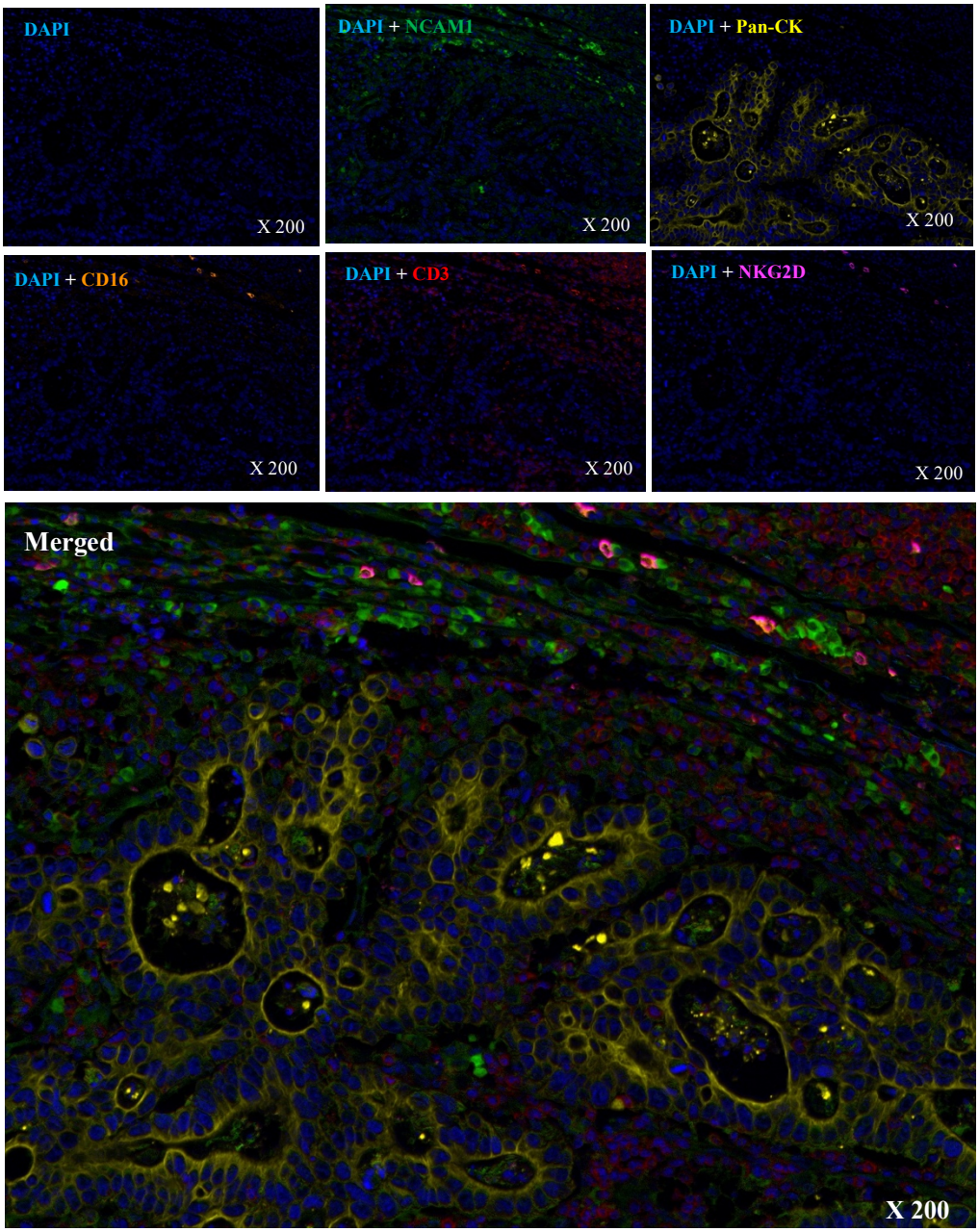


Figure 13 – Multiplex Staining Optimization. *FFPE of colon adenocarcinoma. NCAM1 (green) and Pan-CK (yellow), CD3 (red), CD16 (orange) and NKG2D (pink) DAPI was used to label the nuclear DNA. The images represented were made with Multispectral Microscope with CRI multispectral camera at 200x magnification.*

The final phase of the optimization protocol primarily centred around adjusting the image acquisition parameters, specifically the exposure times, considering the specific wavelengths of emission and excitation for each dye used. Different times were evaluated to achieve a distinct and clear signal for each biomarker (Table 13).

Table 13 – Image acquisition parameters in Multispectral Microscope with CRI Multispectral Camera. *The acquisition information, specifically the exposure time was determined for each biomarker and DAPI.*

Filters	**Colour**	**Wavelengths**	**Excitation**	**Emission**	**Peaks**	**Exposure Time**	**Primary Antibody**	**Secondary Antibody**
DAPI	Blue	460-480	—	—	470	250	—	
FITC	Green	510-560	496	519	530-540	200	NCAM1	Alexa Fluor 488
M1	Orange	580-650	578	603	610	300	CD16	Alexa Fluor 568
M1	Red	580-650	590	617	620	300	CD3	Alexa Fluor 594
M2	Pink	640-710	650	665	680	200	NKG2D	Alexa Fluor 647
555	Yellow	560-630	555	569	580-610	200	Pan-CK	Alexa Fluor 555

4.5. Multispectral Microscopy and Image Acquisition

Advancements in the design and application of biological models, coupled with the development of novel fluorescent probes, have raised the expectations for molecular imaging systems. These systems are now required to offer improved sensitivity, accurate quantitation, and the capability to detect and distinguish multiple simultaneous signals. However, sensitivity is often limited, particularly in the visible spectral range, due to the presence of widespread autofluorescence signals. Separating these autofluorescence signal from those emitted by targeted fluorophores is crucial for achieving reliable and accurate molecular imaging (Levenson et al., 2008).

The utilization of multispectral imaging enabled the differentiation of five fluorophores, with each signal quantified and visualized independently. This approach maximizes the potential for multiplexing while effectively mitigating the impact of autofluorescence on detectability and accurate quantitation (Levenson et al., 2008; Mansfield, 2014).

5 biomarkers were used to stain FFPE tissue specimens (described above), the samples were examined under the microscope, and ten ROIs were randomly selected within each specimen as described in the materials and methods section. The selection criteria aimed to choose five regions within the stromal areas located between the tumour glands and five regions at the periphery of the tumour nests. The microscope utilized in this study is equipped with five long-pass filters. For each filter, the captured spectra are recorded, allowing the complete spectral characteristics of each individual signal to be effectively utilized, for spectral unmixing. The *Nuance software* was employed to visualize the raw images, construct libraries, and perform the unmixing of scanned images.

The optimization of the multispectral libraries was carried out using control slides specific for each biomarker. The multispectral library is essential as it ensures that the spectrum of interest for each marker is captured independently. Subsequently, the library facilitates the accurate merging of all images into a final composite image. Ultimately, a multispectral library was successfully constructed, enabling the unmixing of all scanned images and facilitating precise image analysis and quantification of the antibodies used (Figure 14).

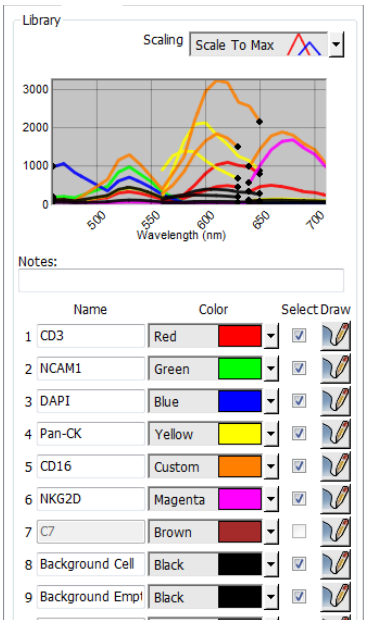


Figure 14 – Multispectral library spectrum peaks for the biomarkers studied and DAPI.

4.6. Biomarker Expression

All the molecular data obtained from the staining was acquired and processed using the functionalities of the *Fiji* software. A specific image analysis protocol was designed based on the project objectives and the available tools within the software. *Fiji* was extensively used to perform the necessary image processing and analysis tasks as required by the project.

4.6.1. Subtypes of NK cells

NK cells are a critical component of innate immune system and play a crucial role in the surveillance and elimination of tumours cells. When a high number of NK cells are observed within a tumour, it can have significant implications for the TME and patient's prognosis (Takanami et al., 2001).

The analysis of NK cells in three distinct compartments revealed an increased presence of diverse types of NK cells within the intratumoral region, located between the tumour glands. Specifically, three subtypes of NK cells were observed within the tumour: cytotoxic NK cells, characterized by positive expression of NCAM1 and CD16 and negative expression of CD3; activated NK cells, identified by positive expression of NCAM1, CD16, and NKG2D, and negative expression of CD3; and regulatory NK cells, displaying positive expression of NCAM1 and CD16, but negative expression of CD3. Only a small number of NK T cells was detected, as these cells are present in limited quantities in humans. NK T cells were identified by positive expression of both NCAM1 and CD3. Interestingly, this type of cells was mostly identified in the periphery of the tumour nests. In addition to NK cells, the expression of T lymphocytes, identified by the presence of CD3 positive cells, was also observed within the intratumoral compartment (Figure 15).

When comparing the intraepithelial compartment and the stroma of periphery, significant differences on activated NK cells expression (Figure 15D).

The presence of a high number of NK cells within the tumour suggests that the immune system is mounting an effective response against the tumour. NK cells have the ability to directly recognize and kill tumour cells through various mechanisms, such as releasing cytotoxic molecules like perforin and granzymes, as well as inducing apoptosis in target cell. (Smyth et al., 2002; Toffoli et al., 2021; Vivier et al., 2008).

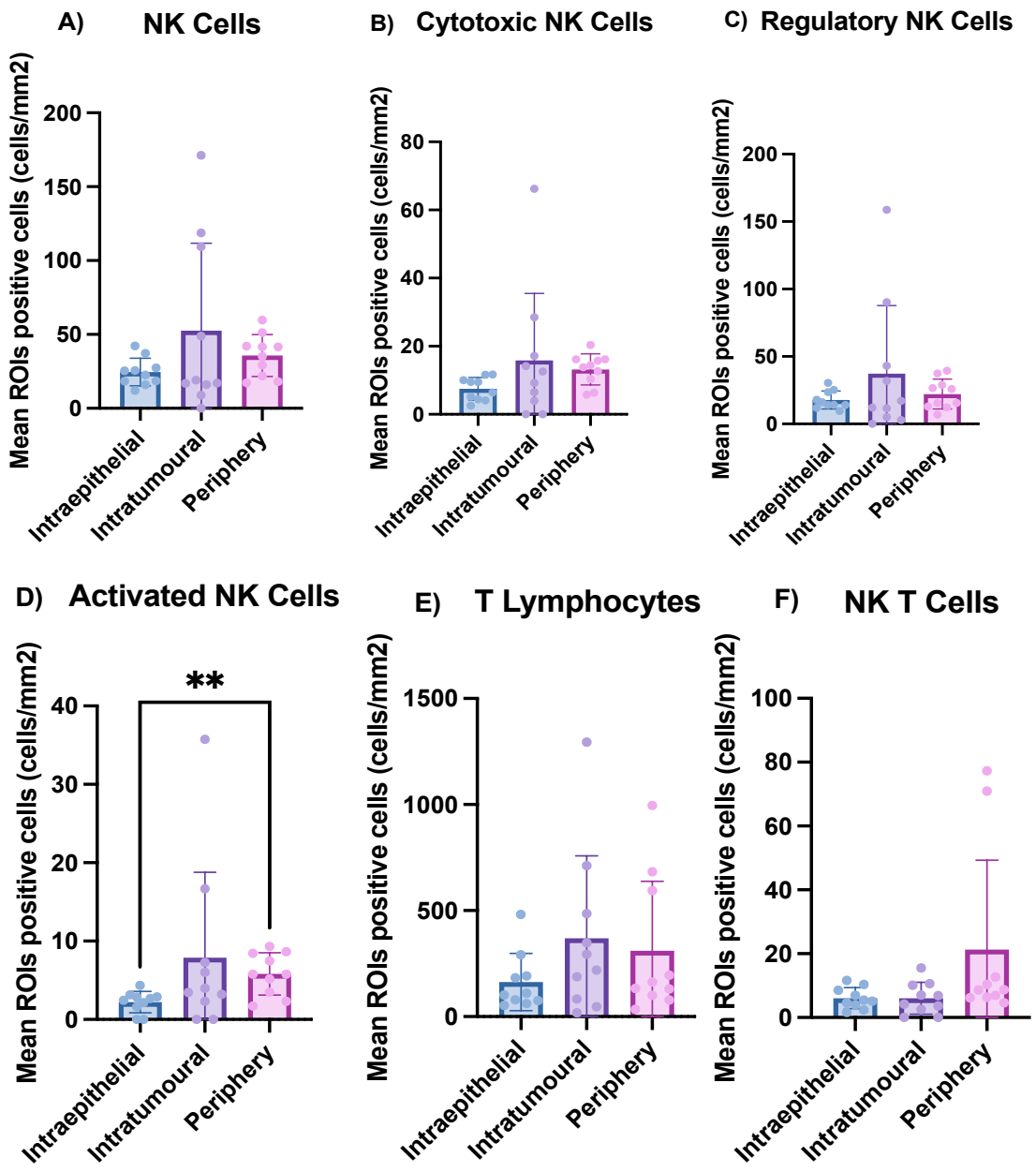


Figure 15 – Expression of different types of NK and T cells in each compartment. *A) Representation of NK cells; B) Representation of Cytotoxic NK cells; C) Representation of Regulatory NK cells; D) Representation of Activated NK cells, the results were very statistically significant that represents a p-values <0.01 (**); E) Representation of T Lymphocytes; F) Representation of NK T cells.*

4.6.2. Analyses of NK cells with tumour progression

We observed differences in the number of NK cells in the different tumour stages in the three analysed compartments, and interestingly, this analysis revealed a decrease in the number of NK cells in more aggressive tumours with advanced stages when focusing in the periphery of the tumour (Figure 16).

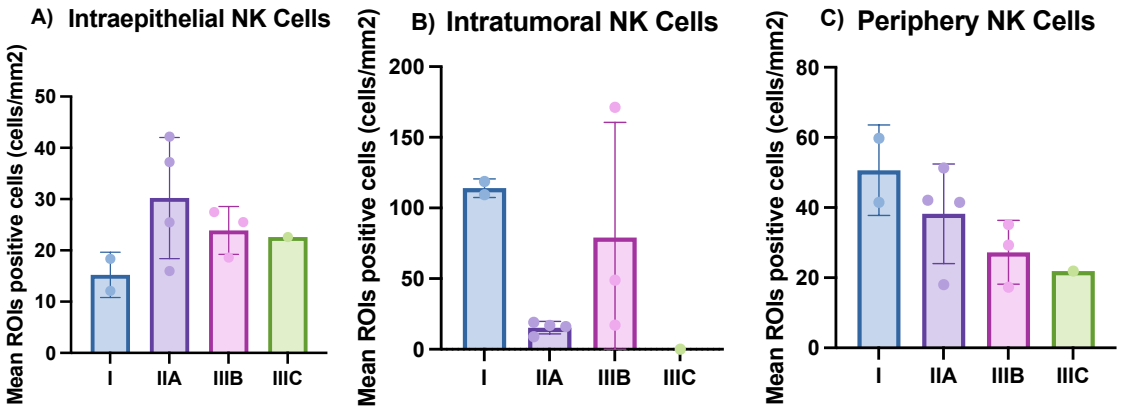


Figure 16 – Expression of NK cells with the tumour stages progression in different compartments. *Expression of NK cells Intraepithelial (A), Intratumoral (B) and in the Periphery (C) according to the tumour stages.*

NK cells pay a crucial role in immune surveillance against tumour cells, as they possess cytotoxic capabilities and the ability to produce immunoregulatory cytokines. However, in advanced stages of CRC, the number of NK cells is reduced. This decline in NK cells abundance can be attributed to multiple factors, including the immunosuppressive TME, alterations in NK trafficking, and evasion mechanisms employed by tumour cells to escape NK cell-mediated cytotoxicity (Chiossone et al., 2018).

When we focused in activated NK cells, we observed that similar to the whole population of NK cells there was a decrease in the number of activated N cells and more importantly (Figure 17C), we observed statistically significant differences also in the intratumoral stroma (Figure 17B). The results obtained with the other types of cells showed a high level of consistency. Therefore, only 6 representative graphs, illustrating two types of NK cells expression are presented here. The remaining graphs can be found in the supplementary data.

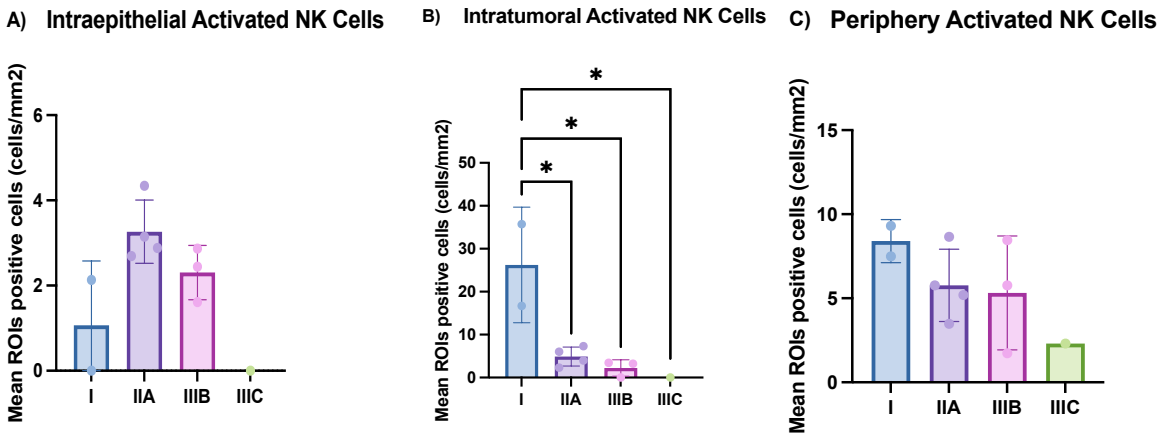


Figure 17 – Expression of Activated NK cells with the tumour stages progression in different compartments. *Expression of Activated NK cells Intraepithelial (A), Intratumoral (B) and in the Periphery (C) according to the tumour stages. The results for Intratumoral Activated NK cells (B) were statistically significant that represents a p-values <0.05 (*).*

4.7. Correlation between TME features and Clinicopathological Data

In order to assess the impact of biomarker on overall survival, the patients were stratified into two groups based on the median number of a cell type as a cut-off. The classification resulted in the formation of high and low groups for each cell type, allowing for an analysis of the survival percentages and the creation of Kaplan-Meier survival curves.

According to the expression of different biomarkers in different compartments (intratumoral stroma, intraepithelial and the periphery stroma) all patients were analysed according to the follow-up time (months).

4.7.1. Correlation of NK cells expression with disease specific survival

The results obtained with the correlation of NK cells were determined using the survival curves that represented a similar data from different compartments. We can observe that the patient who died of the CRC presented low numbers of NK cells in all the compartments, and although this is a very small sample, it could have clinical significance (Figure 18).

The correlation between all NK cells subpopulations with overall survival is provided in the supplementary data.

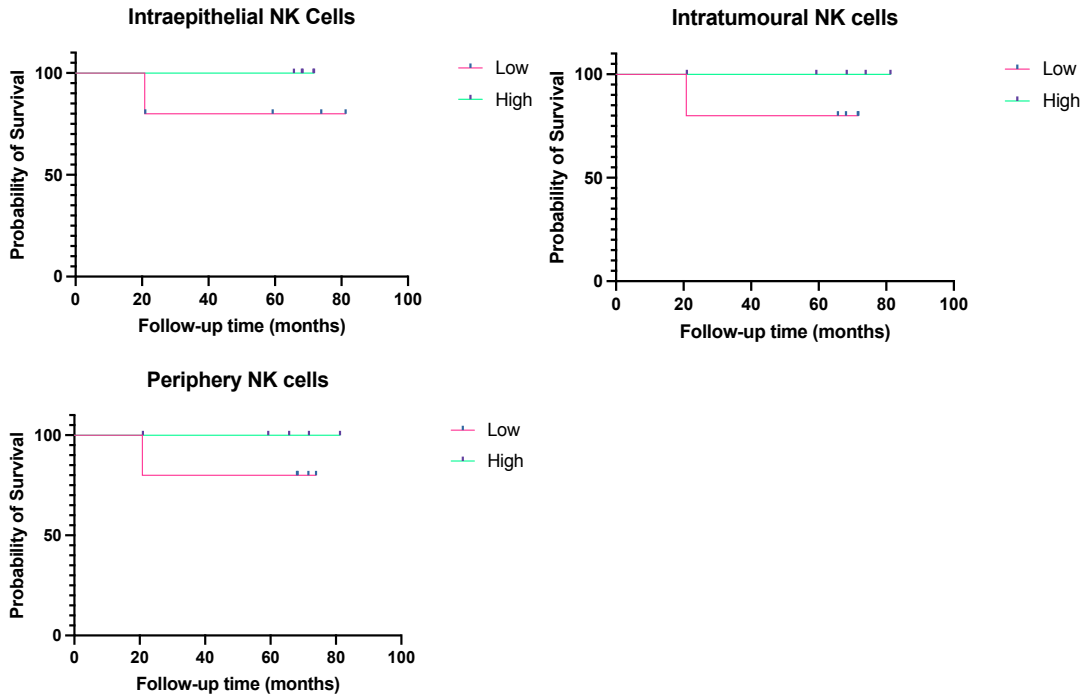


Figure 18 – Correlation of NK cells expression with Clinicopathological data according to the different compartments.

In several cancer types, a higher infiltration of NK cells within the tumour has been associated with improved patient outcomes. Studies have shown that patients with a high density of intratumoral NK cells tend to have better overall survival rates and increased disease-free survival. This suggests that the presence of NK cells can serve as a favorable prognostic indicator, reflecting more effective immune response against the tumour (Carrega et al., 2008; Sconocchia et al., 2014; Villegas et al., 2002).

5. Final Considerations

5.1. Conclusions

The clinical role of NK cells in CRC remains a subject of debate and more studies need to be done in this area. However, the emerging evidence suggesting that NK cells contribute to the anti-CRC response underscores the importance to further investigations in this field. Continued studies focusing on the functional relevance and therapeutic potential of NK cells in CRC may provide valuable insights for the development of novel treatment strategies and optimization of patient outcomes.

mPlex IF enables the precise identification of individual cell phenotypes, distinct cell subpopulations, and even rare cell populations with exceptional accuracy based on the expression of antibodies in a mPlex IF panel. This advanced technique allows for comprehensive and detailed characterization of cellular composition within tissues, facilitating a deeper understanding of complex cellular landscapes and its functional significance in various biological contexts.

This analysis reveals a significant association between high numbers of NK cells and low stages of tumour progression. The presence of a robust NK cell infiltrate within tumours at early stages underscores the potential role of these innate immune cells in restraining tumour growth and suppressing metastasis. The enhanced activity of NK cells in these early-stage tumours may be attributed to their ability to recognize and eliminate cancerous cells without prior sensitization, highlighting their innate cytotoxic capabilities.

The observed correlation between high NK cell counts and low tumour stages suggests that reinforcement the immune response by targeting NK cells could potentially serve as a promising therapeutic strategy to prevent disease progression and improve patient outcomes. Harnessing the potent anti-tumour properties of NK cells through immunotherapeutic interventions may not only inhibit tumour growth but also confer long-lasting protection against recurrence and metastatic dissemination.

Although very limited, the data suggests that the presence or activity of NK cells may play a crucial role in determining patient outcomes and overall survival rates.

5.2. Study Limitations

The main limitation of this study resides in the fact that I have been able to analyse only 10 CRC patients' tissues, and although these patients have a long follow-up time, it limited the statistical analyses very much.

To characterize the CRC TME, mplex IF was extensively used in this project. Although, this is a method with high contrast, high specificity and allowing a quantitative analysis, there are some limitations associated. For reducing autofluorescence in the tissues it was critical to optimise the protocol for obtaining a good staining, to analyse the different markers of interest. Also, different tissues analysed implied adjustments in the spectral library built during the optimization procedure.

The optimization of antibodies was a complex part of this study, that involved too much time for test and put together all markers, trying to put the different animal sources and the different colours to make sure that the signal on the slide was corresponding to the antibody or the background of cells with autofluorescence.

5.3. Suggestions for Future Studies

It is important to highlight that a bigger cohort used in this study could help to have more data to compare and establish a correlation between all the cases stained and to take more conclusions with the corresponding clinical information of each patient. At the beginning of the study I had planned to use a cohort of 60 cases, but due to time constrains, I was not able to accomplish this; however, as soon as possible I intend to realise the cases that were not used in this study so that the results can be published.

It could be interesting to correlate the NK subpopulations with expanded NK cells from fresh tissues of CRC, in order to compare the quantity of NK cells in FFPE blocks and fresh tissues. In addition, it also desired to see different markers on the same tissues to analyse the different functions of NK cells including other activation markers (i.e. CD226, DNAM-1), degranulation markers (i.e. CD107a) and exhaustion markers, amongst others.

Further research is needed to validate these observations and elucidate the underlying biological process that govern the relationship between NK cell expansion and patient survival.

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Expression of Cytotoxic NK cells with tumour stages progression

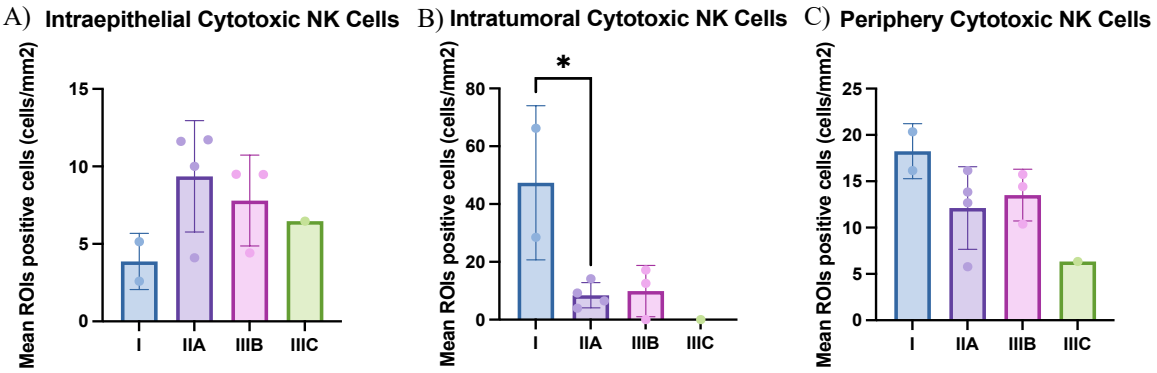

Figure 19 – Expression of Cytotoxic NK cells with the tumour stages progression in different compartments. *Expression of Cytotoxic NK cells Intraepithelial (A), Intratumoral (B) and in the Periphery (C) according to the tumour stages. The results for Intratumoral Cytotoxic NK cells (B) were statistically significant that represents a p-values <0.05 (*).*

Expression of NK T cells with tumour stages progression

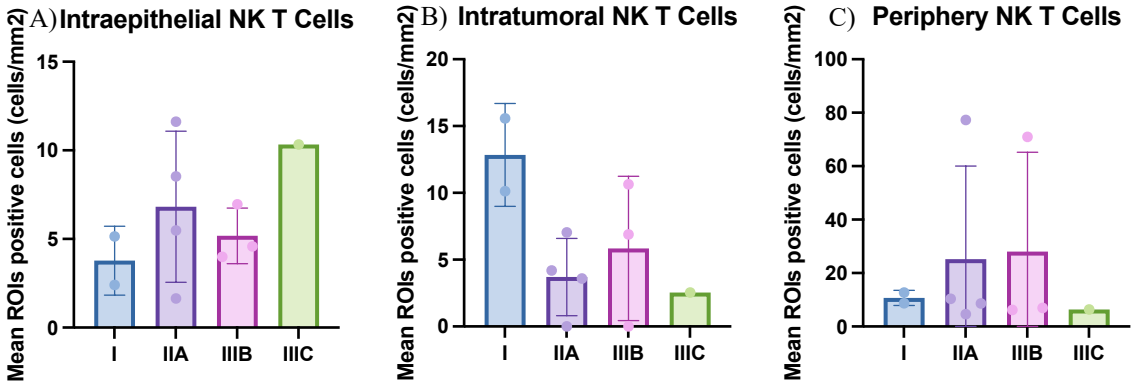

Figure 20 – Expression of NK T cells with the tumour stages progression in different compartments. *Expression of NK T cells Intraepithelial (A), Intratumoral (B) and in the Periphery (C) according to the tumour stages.*

Expression of Regulatory NK cells with tumour stages progression

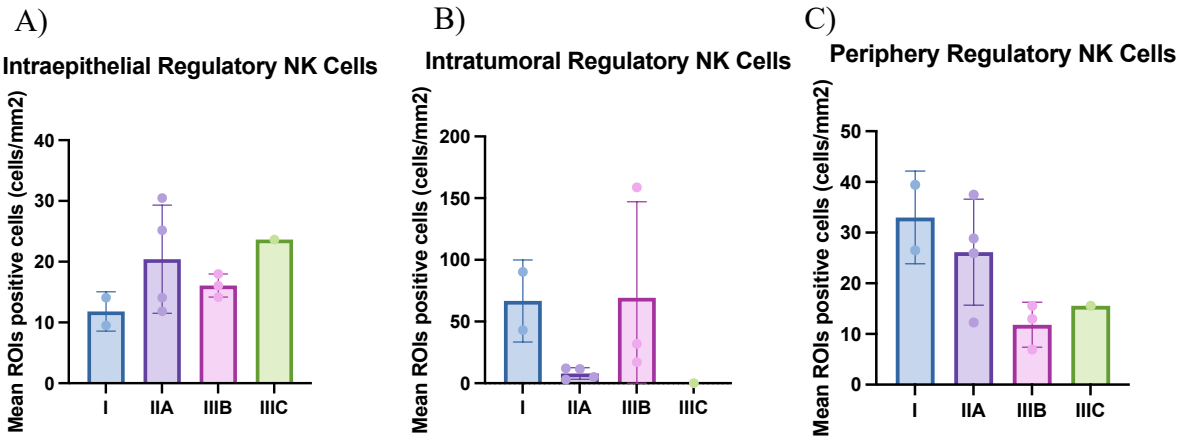

Figure 21 – Expression of Regulatory NK cells with the tumour stages progression in different compartments. *Expression of Regulatory NK cells Intraepithelial (A), Intratumoral (B) and in the Periphery (C) according to the tumour stages.*

Expression of T Lymphocytes NK cells with tumour stages progression

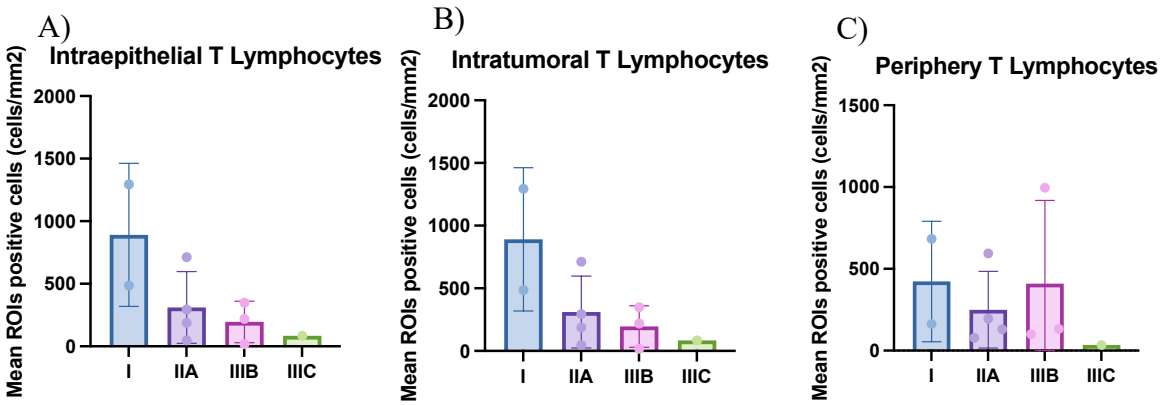

Figure 22 – Expression of T Lymphocytes with the tumour stages progression in different compartments. *Expression of Regulatory NK cells Intraepithelial (A), Intratumoral (B) and in the Periphery (C) according to the tumour stages.*

Correlation of Activated NK cells expression with Clinicopathological data

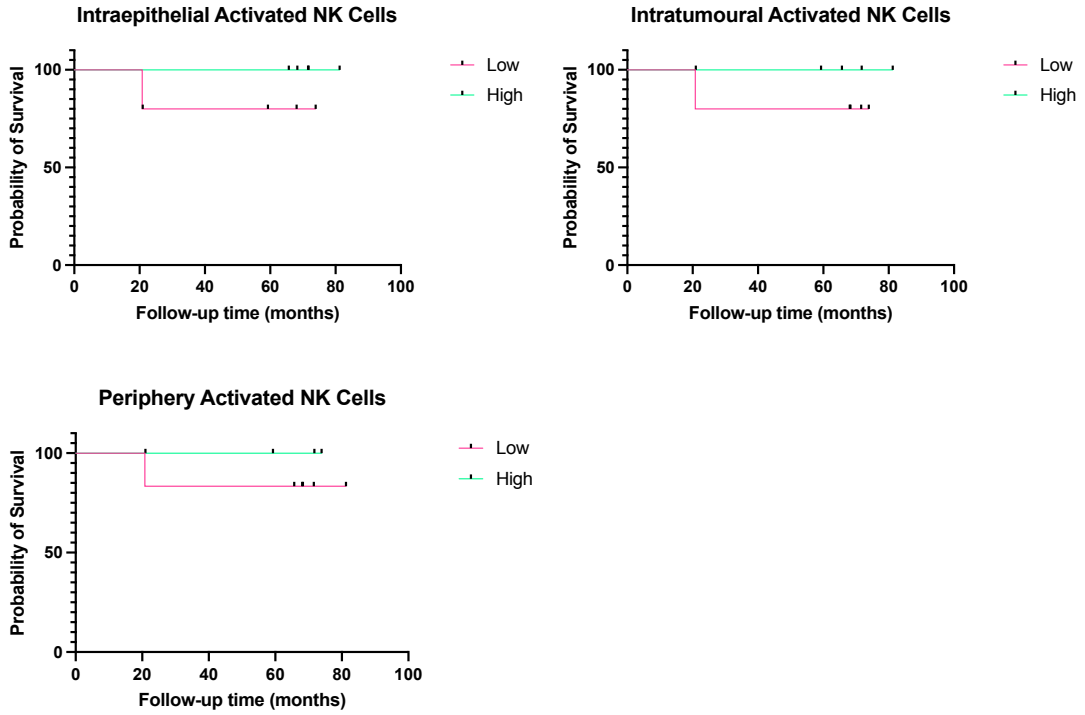

Figure 23 – Correlation of Activated NK cells expression with Clinicopathological data according to the different compartments.

Correlation of Cytotoxic NK cells expression with Clinicopathological data

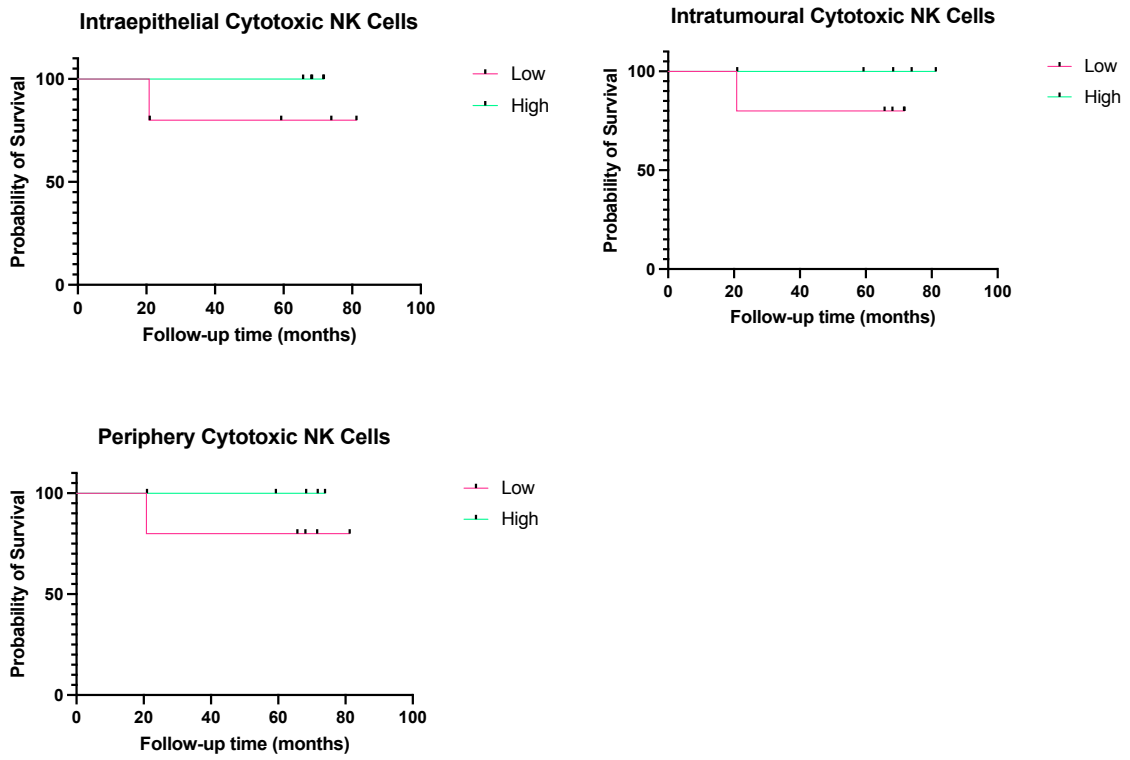

Figure 24 – Correlation of Cytotoxic NK cells expression with Clinicopathological data according to the different compartments.

Correlation of NK T cells expression with Clinicopathological data

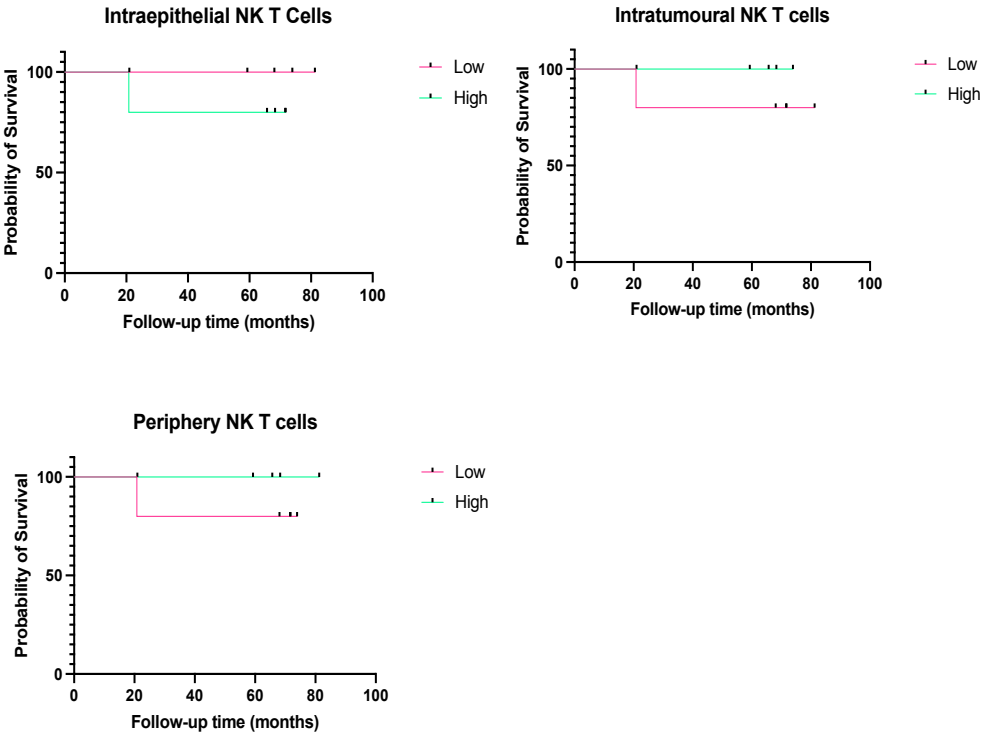

Figure 25 – Correlation of NK T cells expression with Clinicopathological data according to the different compartments.

Correlation of Regulatory NK cells expression with Clinicopathological data

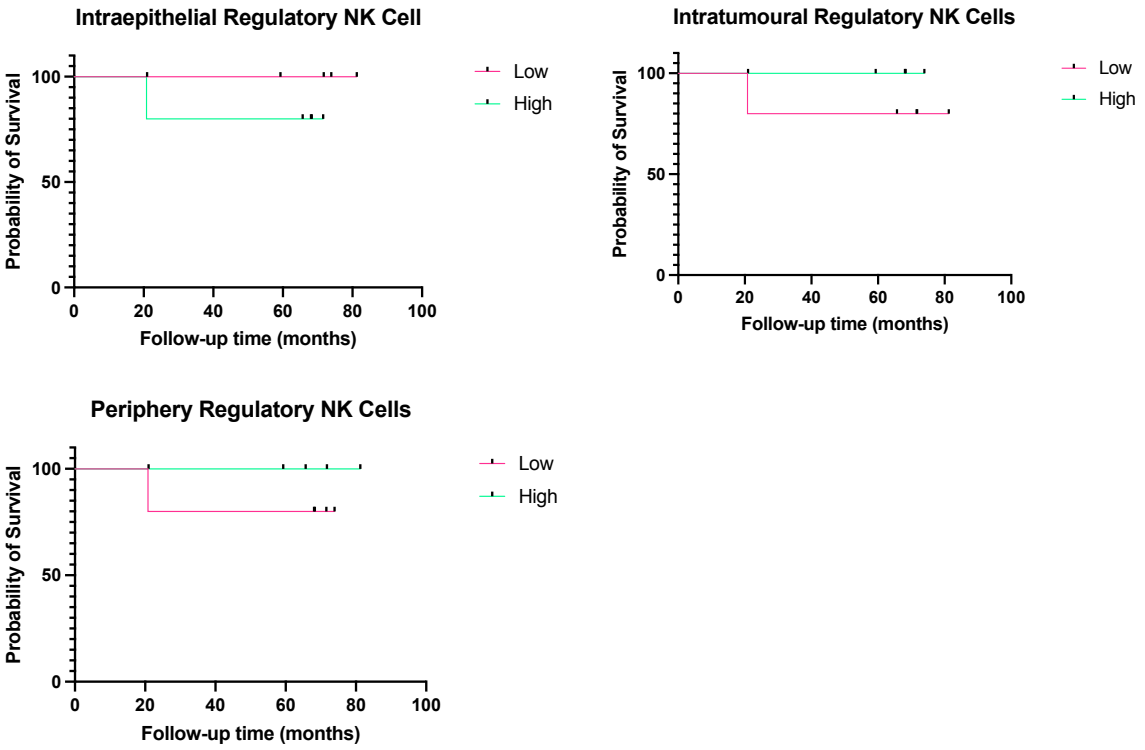

Figure 26 – Correlation of NK cells expression with Clinicopathological data according to the different compartments.

Correlation of T Lymphocytes expression with Clinicopathological data

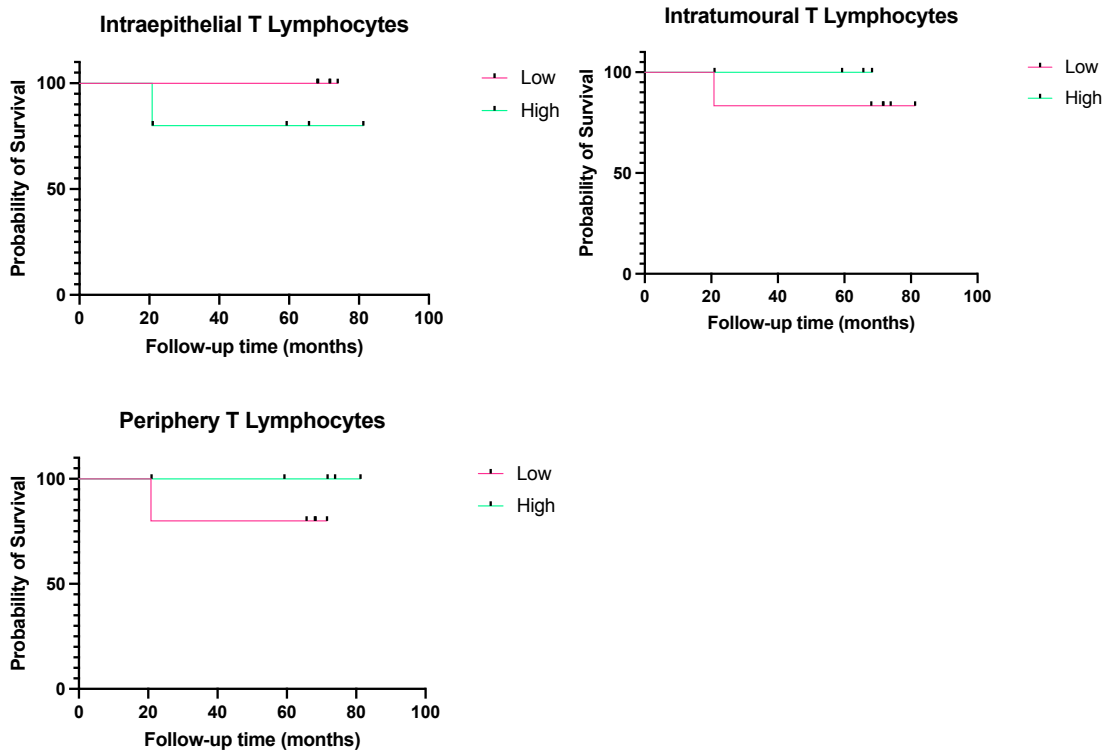

Figure 27 – Correlation of T Lymphocytes expression with Clinicopathological data according to the different compartments.