

# The clinicopathological implication of biomarker expression diversity in specific regions of colorectal tumours and their corresponding metastases

Ph.D. thesis

**Emese Irma Ágoston M.D.**

Doctoral School of Pathological Sciences  
Semmelweis University



Supervisor: Attila Marcell Szász M.D., Ph.D.  
Consultant: László Harsányi M.D., Ph.D.

Official reviewers: Zoltán Kukor M.D., Ph.D.  
László Landherr M.D., Ph.D.

Head of the Complex Examination Committee:  
Gábor István M.D., Ph.D.

Members of the Complex Examination Committee:  
Tibor Glasz M.D., Ph.D.  
Péter Nagy M.D., Ph.D.

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## List of abbreviations

5FU	5-fluorouracil
AKT	protein kinase B
ANXA10	annexin A10
APC	Adenomatous polyposis coli
BRAF	v-raf murine sarcoma viral oncogene homolog B1
CEA	carcinoembryonic antigen
CIMP	CpG island methylator phenotype
CIMP-H	CpG island methylator phenotype-high
CIMP-L	CpG island methylator phenotype-low
CIN	chromosomal instability
CLDN18	claudin 18
CMS	consensus molecular subtype
CRC	colorectal cancer
c-Src	Proto-oncogene tyrosine-protein kinase Src
CSS	cancer specific survival
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CTSE	cathepsin E
DFS	disease free survival
DSS	disease-specific survival
EGFR	epidermal growth factor receptor
HER2	human epidermal growth factor receptor 2
HNPCC	hereditary nonpolyposis colon cancer
H-score	Histochemical scoring assessment
IFL	irinotecan, fluorouracil, leucovorin
JAK1	Janus tyrosine kinase 1
KRAS	Kirsten rat sarcoma viral oncogene homolog
LOH	loss of heterozygosity
Lot No	Lot number
LV	leucovorin
MEK	Mitogen-activated protein kinase
MLH1	MutL homolog 1

MMR	mismatch repair
MSH2	MutS homolog 2
MSH6	MutS homolog 6
MSI	microsatellite instability
MSI-H	microsatellite instability-high
MSI-L	microsatellite instability-low
MSS	microsatellite stable
mTOR	mammalian target of rapamycin
MUC5AC	mucin 5AC, oligomeric mucus/gel-forming
MUC6	mucin 6, oligomeric mucus/gel-forming
NRAS	neuroblastoma ras viral oncogene homolog
PD-1	programmed cell death protein 1
PD-L1	programmed cell death ligand 1
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PMS2	PMS1 Homolog 2
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
Ras	rat sarcoma virus
Raf	rapidly accelerated fibrosarcoma
RFS	relapse-free survival
RR	response rate
RTU	ready to use
SSA/P	sessile serrated adenoma/polyp
OS	overall survival
TCGA	The Cancer Genome Atlas
TFF2	trefoil factor 2
TNM	tumour (T), nodes (N), and metastases (M)
TP53	tumour protein p53
VSIG2	V-set and immunoglobulin domain containing 2
WHO	World Health Organisation

# 1 Introduction

Colorectal cancer (CRC) is regarded as a clinically and biologically heterogeneous disease group, presently characterised by genetic and epigenetic diversity (1). Advancements in molecular biology have expanded our understanding of tumorigenesis, growth, invasion, and migration during tumour progression. In addition to the classical clinical and pathological parameters, there is now an increasing opportunity to investigate gene defects, defective gene products, microenvironmental specificities, and immunological factors that play crucial roles in these processes. The discovery and characterization of biomarkers, combined with an improved understanding of tumour biology, has the potential to enable clinicians to select the most effective therapy for individual patients in clinical practice.

## 1.1 Heterogeneity of colorectal cancer

CRC can be classified into subtypes based on various characteristics (Table 1). One possible subdivision distinguishes molecular subtypes defined by the presence of microsatellite instability (*MSI*), CpG island methylator phenotype (*CIMP*) and somatic mutations in v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) and Kirsten rat sarcoma viral oncogene homolog (*KRAS*) genes. Jass et al. subdivided CRC into five molecular subgroups: 1. *CIMP-H/MSI-H/BRAF* mutation, 2. *CIMP-H/MSI-L* or *MSS/BRAF* mutation, 3. *CIMP-L/MSI-L* or *MSS/KRAS* mutation, 4. *CIMP-0/MSS*, 5. *MSI-H/CIMP-0* (Lynch syndrome) (2). The Cancer Genome Atlas project (TCGA) classified CRC by genomic analysis. Based on that, a hypermutated (around 16%) and a non-hypermutated (around 84%) CRC subtypes are established (3). The Consensus Molecular Subtypes (CMS) Consortium evaluated data from 6 research groups. The authors utilized gene expression patterns to delineate four distinct subtypes of CRC and investigated potential relationship among them. The resulting CRC subtypes were classified as MSI-immune (CMS 1, comprising 14% of cases), canonical (CMS 2, comprising 37% of cases), metabolic (CMS 3, comprising 13% of cases), and mesenchymal (CMS 4, comprising 23% of cases). In addition, a mixed and unclassified group, accounting for cases that did not fit into any of the aforementioned categories, was also identified, making up 13% of the cases (4). Their efforts have been regarded as one of the most comprehensive and contemporary categorizations in the field.

Table.1: Classification of colorectal cancer into different subtypes based on molecular, genome and gene expression analysis.

<b>Molecular classification</b>	<b>Gene expression pattern-based classification</b>	<b>Genomic analysis-based classification</b>
CIMP-H/MSI-H/ <i>BRAF</i> mutation	MSI-immune CMS 1	Hypermethylated
CIMP-H/MSI-L or MSS/ <i>BRAF</i> mutation	Canonical CMS 2	Non-hypermethylated
CIMP-L/MSI-L or MSS/ <i>KRAS</i> mutation	Metabolic CMS 3	
CIMP-0/MSS	Mesenchymal CMS4	
MSI-H/CIMP-0 (Lynch-syndrome)	Mixed, non-specified	

Abbreviations: *CIMP-H*: CpG island methylator phenotype-high; *CIMP-L*: CpG island methylator phenotype-low; *MSI-H*: microsatellite instability-high; *MSI-L*: microsatellite instability-low; *MSS*: microsatellite stable; *BRAF*: v-raf murine sarcoma viral oncogene homolog B1; *KRAS*: Kirsten rat sarcoma viral oncogene homolog; *CMS*: Consensus Molecular Subtypes.

## 1.2 Molecular pathways involved in colorectal cancer

### 1.2.1 The classical pathway

The classical pathway represents the earliest recognized genetic paradigm wherein the sequence of adenoma-dysplasia-carcinoma is an ordered succession of genetic modifications initiated from normal mucosa, resulting in the formation of benign adenoma, high-grade dysplastic adenoma, and eventually invasive colorectal adenocarcinoma. (5). This pathway is characterised by inactivation of the Adenomatous polyposis coli (*APC*) tumour suppressor gene, activation of the Wnt signalling pathway, oncogenic mutations in *KRAS*, loss of the 18q21 region (heterozygosity) and loss-of-function mutations in the *TP53* gene (6). Constituting roughly 60-70% of all colorectal cancers, these tumours are characterised by chromosomal instability (CIN) and exhibit notable variation in gene copy numbers. (7). Typically, these tumours demonstrate CIMP-0, MSI-L or MSS subtype, with approximately 40% of cases bearing *KRAS* mutations (2). Tumours with chromosomal instability can be further classified into three CRC subgroups based on their gene expression patterns, namely CMS 2-4. (8).



## 1.2.2 The serrated pathway

The development of colorectal cancer through the serrated pathway is instigated by a succession of genetic and epigenetic events. This pathway accounts for approximately 10-30% of all colorectal tumours. (9). According to the molecular categorization, serrated pathway tumours typically exhibit CIMP-H/MSI-H/*BRAF* mutation, CIMP-H/MSI-L or MSS/*BRAF* mutation, or CIMP-L/MSI-L or MSS/*KRAS* mutation characteristics. (2). This encompasses both the hypermutated subgroup, identified via genomic analysis (3) as well as the CMS1 subtype, differentiated by gene expression patterns (4). Apart from their molecular attributes, lesions originating from the serrated pathway diverge from typical lesions in terms of endoscopic macroscopic characteristics, histological features, malignant potential, and likelihood of progression. They exhibit a higher level of aggressiveness, typically localised in the right colon, macroscopically sessile or flat lesions, with minimal protrusion from the mucosa. They have an indistinct border; an asymmetric shape and their colour closely resembles that of the surrounding mucosa. Under microscopic examination, these lesions exhibit a serrated pattern on longitudinal sections, and a star-shaped pattern on latitudinal sections. This characteristic morphology results from the accumulation of non-proliferating cells within the crypts due to inhibition of apoptosis (10). It is noteworthy, however, that not all adenocarcinomas that develop via the serrated pathway ultimately display serrated histological features (11).

## 1.3 Commonly studied biomarkers in CRC

### 1.3.1 Molecular markers

#### 1.3.1.1 p53 protein

The p53 protein is encoded by the tumour suppressor gene *TP53* located on the short arm of chromosome 17. It plays a crucial role in controlling cell growth, DNA repair, apoptosis, and sustaining cellular homeostasis. *TP53* mutations are detected in around 50-70% of colorectal tumours (12). Such mutations led to a dysfunctional protein that plays a fundamental role in tumour carcinogenesis. Based on observation, these mutations are less prevalent in tumours located in the proximal colon (34%) than those in the distal colon (45%), however the exact mechanism remains unclear (13). p53 mutations

can impact not only the tumour cells themselves, but also the tumour microenvironment. Tumours with p53 mutations are abundant in neoantigens that can be exploited for checkpoint-based therapies (14).

#### 1.3.1.2 *APC* gene

The *APC* tumour suppressor gene encodes a protein that participates in cellular migration, adhesion, transcriptional activation, and apoptosis. It functions as a primary negative regulator of the Wnt signalling pathway (15). Inherited mutations in the *APC* gene are accountable for familial adenomatous polyposis (FAP) syndrome. *APC* gene mutations are also detected in 60-80% of sporadic colorectal cancers, and they occur early in the process of colorectal carcinogenesis (16).

#### 1.3.1.3 *KRAS* oncogene

It plays a role in signal transduction, growth factor propagation, cell division, cell differentiation, and apoptosis. It is frequently mutated in CRC, occurring in about 30-40% of cases, and can be observed in both classical and serrated pathway tumours (17). *KRAS* mutation in the serrated pathway is less common than *BRAF* mutation, and is usually associated with CIMP-L type (18). The clinical significance of *KRAS* mutations in colorectal cancer is that it negatively regulates epidermal growth factor receptor (EGFR) signalling. When a tumour carries an activating *KRAS* mutation, it becomes resistant to therapy targeting EGFR. Therefore, *KRAS* mutation testing has become a routine clinical practice in metastatic colorectal cancer to determine eligibility for anti-EGFR therapy. In addition, a broader *RAS* panel is often used to identify additional mutations that may also confer resistance to EGFR-targeted therapy (19). Furthermore, the *KRAS* G12C mutation has been observed in approximately 1-3% of colorectal cancer cases (20). This mutation holds significant clinical importance as it represents a potential target for combination therapy utilising both anti-*KRAS* and anti-EGFR inhibitors, which are currently being investigated in clinical settings (21).

#### 1.3.1.4 The *PTEN* tumour suppressor gene

It encodes a protein consisting of 403 amino acids with dual phosphatase activity. This protein is expressed in both the cytoplasm and nucleus, with distinct functions in each location. In the cytoplasm, the protein is primarily responsible for maintaining the homeostasis of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B

(AKT)/mammalian target of rapamycin (mTOR) signalling pathway by inhibiting the activity of PI3K (22). *PTEN* functions as a direct regulator of cell growth by inhibiting cell cycle progression, promoting apoptosis, regulating cell growth signals, and playing an indirect role in inhibiting angiogenesis. Within the nucleus, the *PTEN* plays a role in maintaining genome stability and regulating the cell cycle (23). Inactivation of this gene occurs in approximately 20-40% of colorectal carcinomas through various mechanisms for instance loss of heterozygosity, promoter hypermethylation, point mutation, and chromosome deletion (24). Loss of function has been correlated with two alterations, one affecting the PI3K/AKT pathway and the other involving the PD-L1 pathway. Accordingly, the loss of function triggers the activation of the PI3K/AKT signalling pathway, which promotes cell proliferation and inhibits apoptosis. Moreover, studies have shown that the PI3K/AKT pathway can modulate immune cell activities, impacting the effectiveness of cancer immunotherapy. Furthermore, the loss of *PTEN* has the potential to induce overexpression of PD-L1 in diverse cancer types potentially leading to resistance in conventional chemotherapy, targeted therapies, and immunotherapy (25). Despite numerous studies, the prognostic role of *PTEN* in CRC remains controversial.

#### 1.3.1.5 The Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (*PIK3CA*) oncogene

*PIK3CA* is responsible for encoding the alpha catalytic subunit of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), and represents a pivotal gene subject to alteration in diverse cancer types including CRC. Mutations in the *PIK3CA* gene have been identified in approximately 10-20% of colorectal cancers (26). Notably, a decreasing trend in *PIK3CA* mutations has been observed from the proximal colon (21-25%) towards the distal colon (8-9%), with a mucinous phenotype being common (27, 28). PI3Ks are lipid kinases that play crucial roles in the regulation of cell division, survival migration, and angiogenesis. These processes are intricately governed through the PI3K/AKT/mTOR/PTEN signalling pathway. Abnormal activation of this pathway can lead to the augmentation of cell growth, proliferation, survival, and counteract the chemotherapeutic-induced apoptosis, providing a survival signal for cancer cells (29). Therefore, various clinical trials are currently exploring the use of PI3K, AKT, mTOR, or dual inhibitors in combination with endocrine or chemotherapy to target this pathway (30).

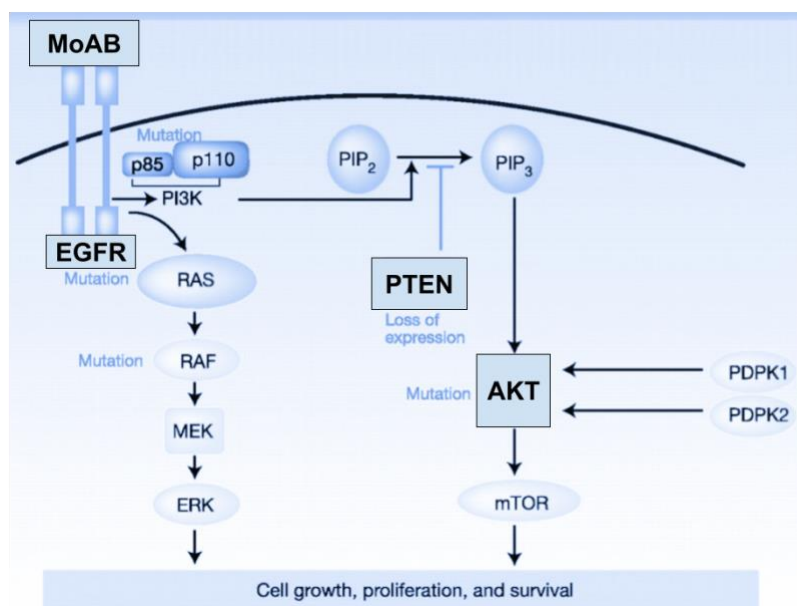


Figure 1: The signalling cascades of the PI3K/PTEN/Akt/mTOR and Ras/Raf/MEK (31).

#### 1.3.1.6 Microsatellite instability

Mismatch-repair (*MMR*) genes are used to detect and repair nucleotide errors in DNA. Microsatellite instability is a type of genome instability resulting from mutations in *MMR* genes. It occurs in approximately 10-15% of CRC cases (2). Tumours exhibiting high levels of MSI (MSI-H) show distinct clinical, pathological, and molecular features compared to those with low MSI/microsatellite stability (MSI-L/MSS). Typically, MSI-H tumours are less aggressive, preferentially located proximal to the splenic flexure, characterized by poorly differentiated mucinous and mixed histology, accompanied by peri- and intratumoural infiltration of lymphoid cells, and associated with a more favourable prognosis. (32). Their prevalence is higher in Caucasian ethnic groups (33). The enhanced immune activity that is typical of MSI tumours has rendered them a subject of interest in scientific investigations involving immune checkpoint inhibitors (34).

#### 1.3.1.7 The *BRAF* protooncogene

The *BRAF* protooncogene is involved in the regulation of cell proliferation, differentiation, cell migration and apoptosis via the rat sarcoma virus (Ras)/rapidly accelerated fibrosarcoma (Raf)/ mitogen-activated protein kinase (MEK) signalling pathway as a negative regulator of *KRAS*. The occurrence of *BRAF* mutation has been observed in approximately 5-15% of colorectal tumours. These mutations are commonly

linked with female gender, tend to be located on the right side, have a mucinous histology, and are often found in advanced stages of the disease. Additionally, *BRAF* mutations are strongly associated with defective mismatch repair and are specific to the serrated pathway (35). The involvement of *BRAF* mutation in the serrated pathway is commonly associated with molecular types characterized by high MSI-H and CIMP-H (36). While the presence of *BRAF* mutation in MSS colorectal tumours indicates a significantly worse prognosis, its role in MSI-H tumours remains a subject of debate. It is hypothesized that *BRAF* mutations do not inherently confer a poor prognosis, but rather their impact on prognosis may depend on the genetic pathway through which they arise (36).

#### 1.3.1.8 HER2

HER2 is a membrane tyrosine kinase that overexpressed/amplified in approximately 2-5% of CRC (37). Its normal function is to activate the MEK–AKT pathway which regulates cell growth, proliferation, survival, mobility, and invasion. HER2 amplification leads to uncontrolled tumour growth. It has a negative predictive role in the resistance to anti-EGFR therapy (38).

#### 1.3.1.9 The CpG island methylation phenotype

Excessive epigenetic methylation of genetic loci that contain CpG islands are named as the CpG island methylation phenotype (CIMP). The CpG islands are found in the promoter regions in approximately 50% of the human genes and are typically maintained in an unmethylated state. (39). To define CIMP, a selected panel of 5 markers is typically used, and at least 3 of these loci must display methylation. CIMP-positive tumours can be stratified into "high" or "low" based on the number of positive markers for methylation (18). It can affect an increasing number of genes with age, increasing the genetic instability and the likelihood of tumour development (40). CIMP is present in approximately 30% of CRC. It is more frequent in proximal colon tumours (30-40%) than in distal ones (5-15%), including left colon and rectum. (41). Notably, nearly 90% of CIMP-positive tumours carry *BRAF* or *KRAS* mutations (42).

### 1.3.1.10 DNA polymerase epsilon (*POLE*)

*POLE* plays a vital role in DNA replication and repair, with its primary function being to maintain accurate DNA replication and prevent mutations (43). Somatic mutations in *POLE* can give rise to tumours that are extremely hypermutable and is apparently microsatellite stable unless both alleles of a DNA MMR gene become mutated (39). *POLE* mutant CRC is characterised by high infiltration of CD8<sup>+</sup> T cells (44) and have been reported as a promising marker in immunotherapy (45). In The Cancer Genome Atlas classification system, *POLE*-mutant tumours exhibit improved PFS) (46).

## 1.3.2 Immune biomarkers

### 1.3.2.1 Immune cell markers

Understanding the role of the immune system in tumour development has been the focus of numerous studies over the past decade. Tumour-infiltrating immune cells and cytokines have been shown to be promising prognostic markers, and intratumoural T cell infiltration (especially CD3<sup>+</sup> and CD8<sup>+</sup>) has been discovered to be an independent prognostic factor in CRC (47-50). A classification system based on tumour immune cell infiltration, called ‘immunoscore’ (IS), has provided reliable results as a clinically useful prognostic marker at all stages (51). Furthermore, its prognostic value appears to be superior to that provided by the traditional AJCC/UICC TNM classification system. Patients with high IS demonstrates better clinical outcome, lower risk of recurrence, longer OS and DFS (52). The predictive role of the immune system is less discovered yet. In two trials, IS appeared to be able to predict and guide therapeutic decision in stage III CRC (53, 54). Table 2 displays the immune cell types that are frequently investigated in CRC.

Table 2: Most commonly detected immune cell biomarkers in CRC and their roles

<b>IMMUNE CELL BIOMARKER</b>	<b>SITE OF EXPRESSION</b>	<b>FUNCTION</b>
<b>CD3</b>	pro-thymocytes, mature T cells, Purkinje cells	Tc and Th activation
<b>CD4</b>	Th, monocytes, macrophages, dendritic cells	sending signals to other immune cells (e.g., Th cells) by releasing cytokines
<b>CD8</b>	Tc, CD3 negative NK cell	destruction of damaged/cancerous cells

<b>CD20</b>	B cells	B cell activation and growth
<b>CD23</b>	mature B cells, activated macrophage, eosinophil, follicular dendritic cell, platelet	B cell activation and growth
<b>CD45</b>	leukocyte, fibrocyte	T cell activation
<b>CD56</b>	NK cell, $\alpha\beta$ T cell, $\gamma\delta$ T cell, dendritic cell, monocyte	cell-cell/cell-matrix adhesion

*Abbreviations: CD3: Cluster of differentiation 3; CD4: Cluster of differentiation 4; Cluster of differentiation 8; CD20: Cluster of differentiation 20; CD23: Cluster of differentiation 23; CD45: Cluster of differentiation 45; CD56: neural cell adhesion molecule 1; Th: T helper cells; Tc: T cytotoxic cells; NK cell: natural killer cell;  $\alpha\beta$ T cell: alpha beta T cell;  $\gamma\delta$ T cell: gamma delta T cell*

### 1.3.2.2 Immune checkpoint markers

As negative regulators of the immune system, immune checkpoints are responsible for keeping the immune response under control. Therapeutic agents that interrupt these immune checkpoint-regulated processes, such as anti-Cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), anti-programmed cell death protein 1 (PD-1) and anti-programmed cell death ligand 1 (PD-L1), enhance the anti-tumour immune response and facilitate tumour regression (55). Precise mapping of the immune checkpoint signalling pathways, and the use of therapeutic agents acting on these pathways alone or in combination therapy is a subject of many studies recently. Among the various immune checkpoint biomarkers examined in colorectal cancer, CTLA-4, PD-1, and PD-L1 have emerged as the most commonly studied ones.

#### 1.3.2.2.1 CTLA-4

Cytotoxic T-lymphocyte-associated protein-4 is a type 1 glycoprotein expressed on activated T cells and its role is to suppress T-cell function (56). Blocking the CTLA-4 allows the T cells to be active and to fight against tumour cells. The identification of CTLA-4 has been regarded as an indicator of immune surveillance in colorectal cancers. Several studies have presented evidence of immunomodulatory and immunotherapeutic approaches involving the application of anti-CTLA-4 antibodies in the context of colorectal carcinoma (57).

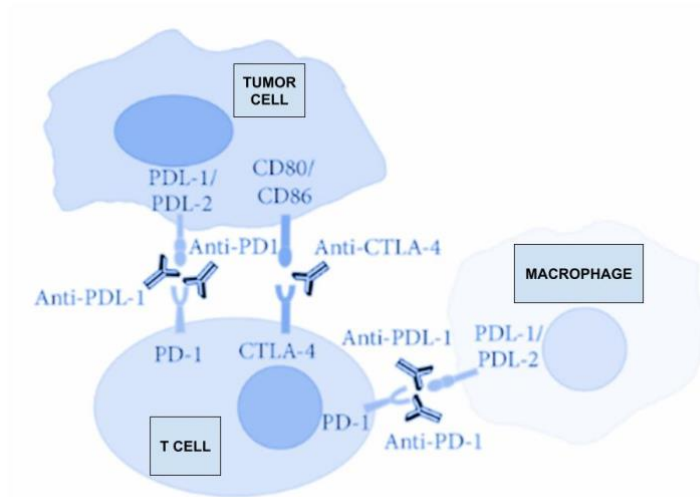
#### 1.3.2.2.2 PD-1

PD-1 is a cell surface receptor present on activated T cells, pro-B cells, and macrophages. It exerts an inhibitory effect on T cell proliferation, diminishing the

secretion of cytokines, including interferon gamma (IFN- $\gamma$ ), tumour necrosis factor-alpha (TNF- $\alpha$ ), and interleukin-2 (IL-2). It also curtails the lifespan of T cells (58). PD-1 predominantly regulates previously activated T cells during the later phase of immune response, primarily in peripheral tissues. (59).

### 1.3.2.2.3 PD-L1

PD-L1 is a transmembrane protein encoded by the *CD274* gene. PD-L1 binds to the PD-1 receptor through an inhibitory signal which reduces antigen-specific T cell proliferation in lymph nodes and induces apoptosis in suppressor T cells (60). The expression of PD-L1 in colorectal carcinoma is linked to clinicopathological and molecular characteristics like the serrated pathway of colorectal carcinogenesis (61).



*Figure 2: The different immune checkpoint inhibitors and their mechanism of action (62).*

## 1.4 The role of the immune-stroma microenvironment (ISM)

The ISM is composed of stromal cells, extracellular matrix (ECM) components, exosomes, and various infiltrating cells of the innate and adaptive immune system. This latter includes various T cells, B cells, natural killer cells, tumour-associated macrophages, myeloid-derived suppressor cells, cancer associated fibroblasts, and endothelial cells, including their mediators (63). The ISM, through various mechanisms, can interact with cancer cells, which may either promote or impede the progression of tumours. (64). The role of the ISM has been the subject of intense research in recent years, with a particular focus on its role in protecting against cancer development through



various mechanisms. In MSI-H CRC, chemokine-mediated activation of cytotoxic T cells, DCs, and NK cells can establish an antitumour effect. In contrast, MSI-L/MSS CRC has a tumour microenvironment rich in vascular endothelial growth factor (VEGF), which promotes the conversion of M2-like macrophages and regulatory T cell ( $T_{reg}$ ) proliferation, leading to the release of different inhibitory cytokines that suppress immune cells and promote tumour survival (34). Additionally, high levels of macrophage-derived matrix metalloproteinase-9 in CRC tissue have been shown to be an independent predictor of metastasis and poor outcome (65). Chronic inflammation has also been identified as a contributing factor to neoplastic formation, with proinflammatory signals produced by tumour necrosis attracting immune cells (66).

CRC has a high complexity of ISM, reflecting genomic, host immunity and environmental diversity. According to the CMS subtypes the CMS1 represents an immunogenic ISM and has favourable outcome, inversely, the CMS4 subtype characterise by an immunosuppressive ISM and has poor prognosis compared to other CMS subtypes (67).  $CD3^{+}/CD8^{+}$  cell infiltration within the tumour microenvironment has been shown to be a prognostic biomarker (68). Another validated prognostic marker is the tumour-stroma ratio. Higher stromal percentage associated with poorer prognosis (69).

Immunohistochemistry with validated semiquantitative scoring systems and RNA sequencing can be utilised to examine peritumoural immune cells. Digital imaging and machine learning algorithms are also viable methods for estimating cell density in clinically annotated tumour slides, though there remains significant variability in scoring methodologies (70).

## **1.5 The prognostic significance of biomarkers**

Besides the anatomical extent of the tumour and the degree of histological differentiation, molecular biological features also carry prognostic impact (71). Multiple studies have demonstrated that CIMP-H/MSI-L/MSS tumour subtype has the poorest prognosis in regard to the overall survival (OS) and disease-free survival (DFS), and in some studies, also for cancer-specific survival (CSS) (72). The MSI-H phenotype alone has been shown to carry a favourable prognosis. It is believed that the prognostic significance of the *BRAF* mutation in CRC is dependent on the pathway through which

the cancer developed (36). The prognostic significance of CIMP phenotype remains a topic of debate. While CIMP has been demonstrated to be an independent negative prognostic factor in various CRC subtypes, its impact may be modulated by co-occurring genetic factors, such as MSI and *KRAS/BRAF* status. (73). *KRAS* codon 12 mutations have been linked to tumour progression, as supported by previous research, however, in stage II-III colorectal cancer, such mutations were not found to have a prognostic role (36, 74). Recent studies indicate that tumours with a high level of immune cell infiltration are associated with a better prognosis. In particular, the infiltration of T cells within the tumour has been identified as an independent prognostic factor for colorectal cancer (47, 48). Colorectal tumours with MSI are particularly affected by significant lymphocyte infiltration, a dominant Th1/cytotoxic T lymphocyte immune microenvironment, and increased expression of HLA and immune checkpoint markers, such as PD-1, PD-L1, and CTLA-4 (75).

## **1.6 Biomarker-based therapeutic options**

Currently available therapeutic options for the treatment of CRC are dependent on the stage and type of cancer. These options include surgical resection, cytotoxic chemotherapy, radiotherapy, targeted therapy, and immunotherapy. (76). In recent years, the identification of colorectal cancer subtypes based on transcriptional compartmentalisation and the growing knowledge of the functions of tumour, stroma, and immunological components have led to an increasing use of a "multimolecular, combination chemotherapy" strategy. (8). At present, there is no standard clinical practice for treating non-metastatic colorectal cancer based on specific subtypes. However, for metastatic colorectal cancer, the presence of a *KRAS* mutation serves as an independent predictive biomarker for anti-EGFR therapy (77). Additionally, promising results have been observed in pre-treated metastatic colorectal cancer through the use of a combination of *KRAS*<sup>G12C</sup> inhibitors and anti-EGFR agents. (78). The practice of using immunohistochemical and/or PCR testing of colorectal cancer to screen for microsatellite instability is in clinical practice to identify the presence of Lynch syndrome. (79). Studies have demonstrated that in stage II colorectal cancer, the presence of deficient DNA mismatch repair (dMMR) is a robust negative predictive biomarker of 5-fluorouracil (5-FU) efficacy (80). However, a meta-analysis indicated significant variability in the

response of this patient group to 5-FU-based treatment (81). MSI CRC is strongly associated with *BRAF*<sup>V600</sup> mutation (82). Monotherapy with selective *BRAF* inhibitors has been found to produce unsatisfactory outcomes in metastatic colorectal cancer. However, a combination therapy approach that targets the *BRAF*, EGFR, and ERK signalling pathways may prove to be a more effective treatment strategy in patients with *BRAF*<sup>V600</sup> mutant metastatic colorectal cancer. (83-85). In *TP53/RAS* mutant CRC Adavosertib seemed to improve progression-free survival (PFS) (86). Different outcomes have been obtained for additional biomarkers, some of which are still controversial and cannot yet be reliably applied in routine clinical practice (87).

In the past few years, immune checkpoint inhibitors (ICIs) have been studied with favourable results in various tumours such as breast, melanoma, and small cell lung cancer. ICIs have been shown to be beneficial in MSI CRC, and this patient group responded well to anti-PD-1 therapy compared to MSS tumours (88). In May 2017, the FDA authorised the use of pembrolizumab monoclonal anti-PD-1 antibody as the first immune biomarker based treatment in solid MSI CRC tumour patients (89). In 2020, it was further approved as a first-line treatment for patients with unresectable or metastatic MSI-H CRC (90). Nevertheless, complete loss of JAK1 function and mutations in genes implicated in IFN $\gamma$  signalling can influence the efficacy of the treatment (91). Over the past few years, other immune checkpoint inhibitors (such as nivolumab and ipilimumab) have been approved, and their combination with a standard treatment regime or in combination with other ICIs are currently under investigation in various ongoing studies (92). The expansion of immunotherapy to hyper-mutated phenotypes (such as POLE-mutated MSS) and its application in neoadjuvant treatment are under investigation and have produced promising results (93-96). Apart from the biomarker studies, exploring transcriptional patterns, distinct immune activation profiles, and microenvironmental factors in CRC may offer additional prospects for targeted therapies (8).

## 2 Objectives

In the colorectal cancer working group of the Department of Pathology, Forensic and Insurance Medicine, Semmelweis University, we aimed to perform a detailed analysis of selected CRC samples and consecutive metastases to evaluate the frequency, heterogeneity, prognostic, and predictive potential of various biomarkers.

My PhD thesis focused on the following biomarkers:

- MSI
- PTEN
- tumour infiltrative immune cells
- (CD3, CD4, CD8, CD20, CD23, CD45, CD56)
- immune checkpoint markers
- (CTLA-4, PD-1, PD-L1)

The study aimed to provide insights into the following questions:

### MSI:

1. What is the frequency, intratumoural heterogeneity of MSI in our colorectal cancer surgical specimens?
2. Is there a difference in the expression of MMR markers between primary tumour regions and their corresponding liver metastases?
3. What is the predictive value of the clinicopathological factors available in our cohort?
4. Is there a difference in the expression of MMR markers between distinct regions of primary tumours and lymph node metastases?
5. Is the MMR status prognostic for DFS and OS in our patients after surgical resection?
6. Is the MMR status predictive for the effectiveness of 5-FU-based chemotherapy regimens (including oxaliplatin or irinotecan) or chemotherapy regimens in combination with targeted biological therapy (bevacizumab or cetuximab, or panitumumab)?
7. Do stage II and stage III MSI colon tumours respond differently to 5-FU-based treatment or are the outcomes of stage II and III patients after 5-FU treatment unfavourable compared to MSS tumours of the same stage?

### PTEN:

1. How is the PTEN expression and their intracellular staining pattern with Dako, CellSignaling and Neomarker antibodies?
2. What is the degree of variation in PTEN expression based on the utilisation of various scoring methodologies?
3. What is the expression pattern of the three antibodies in the chosen tumour regions and metastatic sites?
4. Are there any discernible variations in PTEN expression based on the anatomical location of the tumour within the colon?
5. What is the relationship between PTEN expression and clinicopathological characteristics?

### IMMUNE BIOMARKERS:

1. How is the distribution of the different immune cells and checkpoint markers in the main tumour mass and metastases?
2. Does tumour localisation in the large bowel shows any differences in the IC and ICI pattern?
3. How is the distribution of the immune markers with the lymph node status?
4. How is the distribution of the immune markers within the liver metastases?
5. How is the distribution of markers in different areas of CRC and in metastatic lymph nodes?
6. Which genes show different expression between the main tumour mass and the metastases and what is their prognostic significance?

## **3 Methods**

### **3.1 Patient information**

The tumour samples in this study consisted of CRC and lymph node (LN) metastases from 55 patients, metastatic CRC (mCRC) samples from 56 patients (including 33 samples of primary tumours and liver metastases from patients treated with Cetuximab, and 23 samples from patients treated with Bevacizumab). These patients were diagnosed between 1987 and 2011 and were chosen randomly from the database of the Department of Pathology, Forensic and Insurance Medicine at Semmelweis University, Budapest, Hungary. The surgical procedures were conducted at the Department of Surgery, Transplantation and Gastroenterology at Semmelweis University, and the postoperative oncological treatment was carried out at the Oncology Department of the United St. István and St. László Hospital, Budapest, Hungary. An additional control group of 34 patients was selected from the Uzsoki Teaching Hospital, Budapest, Hungary who had survived for more than five years without progression of their disease. Approval for the investigation was obtained from the Regional and Institutional Committee of Science and Research Ethics at Semmelweis University (SE-TUKEB 207/2011). We included 122 patients in the study of microsatellite instability, out of the 122 patients, 89 patients had CRC and 33 were selected from the control group. Furthermore, paired liver metastases from 69 patients were also analysed. We selected 55 patients for the PTEN assessment, and 137 individuals were included in the investigation of immune-based biomarkers and immune checkpoint marker distribution. Additionally, 12 primary tumours and 12 liver metastases (as 11 paired samples, as well as an additional primary tumour and metastasis sample) were selected for the immune panel gene expression assay. The clinicopathological characteristics and survival data of all patients were available and systematically organised in our database.

### **3.2 Sampling and sample processing**

Tissue microarrays (TMA) were compiled from formalin-fixed and paraffin-embedded (FFPE) tissue samples using a structured core punching algorithm with the Tissue Microarray Builder instrument (Histopathology Ltd., Pécs, Hungary). The cores with a diameter of 2 mm, were obtained from the normal colorectal mucosa (NORMAL), main tumour mass (MAIN), the tumour-normal interface (BORDER), deepest infiltrative

area (FRONT), lymph node metastasis (LN) and/or liver metastasis (MET). To analyse the biomarkers, 4 micrometre thick sections were cut from TMA blocks and mounted on adhesive glass slides (SuperFrost UltraPlus from Gerhard Menzel Ltd., Braunschweig, Germany). The sidedness of the tumour was defined as follows: a tumour originating from the cecum, ascending colon, or the proximal two-thirds of the transverse colon was classified as right-sided, meanwhile, a tumour arising from the distal one-third of the transverse colon, descending colon, sigmoid colon, or rectum was considered left-sided. Tumour staging was determined based on the American Joint Committee on Cancer (AJCC) grouping system (97), which involved both histopathological analysis of surgical samples and imaging investigations.

### **3.3 Immunohistochemistry**

Immunohistochemical assays were performed on these sections to assess the following biomarkers: microsatellite instability (MSI), phosphatase and tensin homolog deleted on chromosome 10 (PTEN), tumour infiltrative immune cells (IC) and immune checkpoint markers (ICIs).

For MSI staining we used four different antibodies, namely MLH1, MLH-1 (M1)-VENTANA Lot No.: G03827 RTU (ready to use)-dilution, MSH2, MSH-2 (G219-1129) Cell Marque Lot No: V0000153 RTU-dilution, MSH6 MSH-6 (44)-VENTANA 790-4455 Lot No.: C01829 RTU dilution and PMS2 PMS-2 (EPR3947) VENTANA 760-4531 Lot No.: 1127701B RTU dilution.

The immunohistochemical analysis of PTEN involved using three anti-PTEN antibodies including PTEN Clone 6H2.1 (Code M3627, Dako) at a 1:100 dilution, PTEN 138G6 antibody (9559, Cell Signaling) at a 1:40 dilution, and PTEN Ab6 28H6 (MS1797, Neomarkers) at a 1:100 dilution.

In the immune study we investigated the following biomarkers: CD3 (a pan-T cell marker), CD3 (a pan-T cell marker)-Novocastra NCL-CD3-SP1 Lot No.: L114128 1:100 dilution, CD4 (a helper T cell marker)-Novocastra NCL-CD4-1F6 1:50 dilution, CD8 (a cytotoxic T cell marker), CD8 – Cell Marque Lot No.: 1308003D 1:75 dilution, CD20 (a B cell marker), CD20cy – Dako M 0755 Lot No.: 00014636 1:250 dilution, CD23 (a mature B cell and activated macrophage marker), CD23 – Dako M 0763 Lot No.: 051(101), 1:200 dilution, CD45 (a leukocyte and LCA marker), LCA - Dako M 0701 Lot

No.: 00009497 1: 150 dilution, and CD56 (a natural killer cell marker) CD56 – Dako M 7304 Lot.:0004147 1: 80 dilution, as well as immune checkpoint markers CTLA-4, CTLA 4 (F-8) sc-376016 Lot No.: AO616 1:100 dilution, PD-L1,(28-8) Abcam ab205921 1:100 dilution, and PD-1 Diagnostic BioSystems DBM 15.5 Lot No.: E790-NB 1:100 dilution.

The tissue sections were processed for immunohistochemistry in an automated immunostainer (Ventana Benchmark XT, Roche, Tucson, AZ, USA), using the manufacturer's recommended solutions and settings. The process of routine dewaxing and antigen retrieval was carried out by immersing the samples in either a pH 6.0 Target Retrieval Solution, or a pH 9.0 buffer of 0.01 M Tris-0.1 M EDTA and heating them to approximately 105 °C for a duration of 30 minutes, utilising an electric pressure cooker (Avair Ida, YDB50-90D, Biatlon Ltd., Pécs, Hungary). To block the activity of endogenous peroxidase, Sections underwent a 20-minute treatment with a 0.5% hydrogen peroxide methanol solution, followed by a 10-minute treatment with the protein blocking reagent included in the Novolink kit at room temperature in a humidifying chamber. The sections were left to incubate overnight with primary antibodies, suitably diluted in 1% bovine serum albumin within Tris Buffered Saline (TBS). Washing occurred between all incubation steps for 3 minutes in TBS supplemented with 0.01% Tween-20. Following this, the sections were exposed to the post-primary reagent from the Novolink kit for 30 minutes, succeeded by an additional 30-minute exposure to the Novolink Polymer Detection Systems kit. Enzymatic activity was visualised utilising a hydrogen peroxide/DAB solution at pH 4.5 for a duration of 3.5 minutes, with internal controls (neural elements and endothel) utilised for each reaction. The slides underwent counterstaining with haematoxylin.

### **3.4 Immune panel gene expression assay**

RNA was extracted from each sample. Five 5-micron-thick sections were obtained from formalin-fixed paraffin-embedded (FFPE) blocks, and total RNA was extracted using the High Pure FFPE RNA Isolation Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. The RNA concentration was determined utilising the Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA samples with sufficient concentration were subjected to hybridisation with the nCounter® PanCancer Immune Profiling Panel (NanoString), which contains 770 genes, for a duration of 16



hours utilising a thermocycler. The specimens were subsequently processed using the nCounter Prep Station (NanoString).

### 3.5 Digital analysis

Digital images of the stained slides were obtained using a Pannoramic P250beta slide scanner (3DHistech Ltd., Budapest, Hungary). The evaluation of the images was performed in a semiquantitative manner supported by the Pannoramic Viewer software, which utilised the TMA and Histoquant modules for analysis (3DHistech).

The sections assessing the MSI were evaluated according to the following scheme: intensity (0-3), frequency (0-5).

The analysis of PTEN expression patterns included the following dimensions: intracellular localisation (nuclear, cytoplasmic, nuclear, and cytoplasmic), intensity (0: none, 1: weak, 2: intermediate, 3: strong expression), and proportion (0: none, 1: 0-1 %, 2: 2-10 %, 3: 11-33 %, 4: 34-66 %, 5: 67-100 % of respective cells stained). The scores acquired from duplicate regions were averaged, and the unprocessed data was employed for statistical analysis. Three different scoring systems were used to calculate the Histochemical scoring assessment (H-score) for a tumour region based on the intensity and frequency of staining. The H1-score multiplied the intensity and frequency resulting in a range of 0-15, the H2-score summed the intensity and frequency resulting in a range of 0-8, and the H3-score was biased towards intensity, resulting in a scale ranging from 0 to 15 (Table 3).

*Table 3: The H3-score is computed by evaluating the intersection of the corresponding row and column, resulting in the determination of the H3-score value (98).*

	H3-score	0-1%	1-10%	10-33%	33-66%	67-100%
Intensity	1+	1	2	3	4	5
	2+	6	7	8	9	10
	3+	11	12	13	14	15

For the immune study IHC reactions were assessed and analysed using computer-assisted image analysis with the QuantCenter digital analyser. This method allowed for the calculation of the count of positive cells for each annotation, where each annotation aligning with the surface area of a core cylinder measuring 3.14 mm<sup>2</sup>.

Gene expression profiles were digitalised using the nCounter Digital Analyser and measured utilising nSolver 4.0 Analysis Software (NanoString).

### **3.6 Statistical analysis:**

For the MSI study the statistical evaluation was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Non-parametric variables were compared using Fisher's exact test. Prognostic and predictive tests were performed using Kaplan-Meier analysis, with log-rank statistical support.

In the PTEN prognostic investigation, data from respective regions of the tumours were averaged to yield a final value representing the staining for a given case. The comparison of PTEN expression across heterogeneous tumour areas and different antibodies was carried out using the Chi-square test, Friedman test, and Wilcoxon test. To assess the relationship between PTEN expression and conventional prognostic markers, Kruskal-Wallis and Mann-Whitney tests were employed. PTEN expression was dichotomized through ROC analysis. Staining attributes were compared using Cohen's kappa. Prognostic significance, supported by the Kaplan-Meier method and evaluated through the log-rank test, was visually presented. Statistical analyses were performed using SPSS 22, with all tests being two-sided and p-values less than 0.05 considered as statistically significant.

In the immune study the data was analysed in R for Windows (v4.1.2) utilising mixed-effect linear models incorporating patient IDs as the random factor (nlme package, v3.1-155). Tukey's method was employed for post-hoc comparisons of parameters with more than two factor levels.

NanoString data was analysed utilising the RUVSeq method (RUVSeq package, v1.26.0), followed by differential expression analysis and gene set enrichment analysis using DESeq2 (v1.32.0) and univariate/multivariate Cox regression models for survival analysis (survival package, v3.3-0 and coxme package, v2.2-16). Results were visualised using ggplot2 and ComplexHeatmap packages. Statistically significant values were defined as  $p < 0.05$  for all of our studies.

## 4 Results

In our cohort immunohistochemical assessment was performed for MSI (122 patients), PTEN (55 patient), immune cell and immune checkpoint marker distribution (137 patients). An additional 11 paired primary tumour and metastasis samples were selected for immune panel gene expression assay.

### 4.1 MSI

#### 4.1.1 The frequency of MSI tumours with immunohistochemistry

In our cohort of 122 specimens, 14 tumours showed MSI phenotype. For the investigation we applied immunohistochemistry with four different antibodies (Table 4). Single marker loss in 7.8%, double marker loss in 2.6% and triple marker loss in 0.9% of all tumours were identified. 108 (88.5%) tumours expressed all the four markers to some extent (Figure 3).

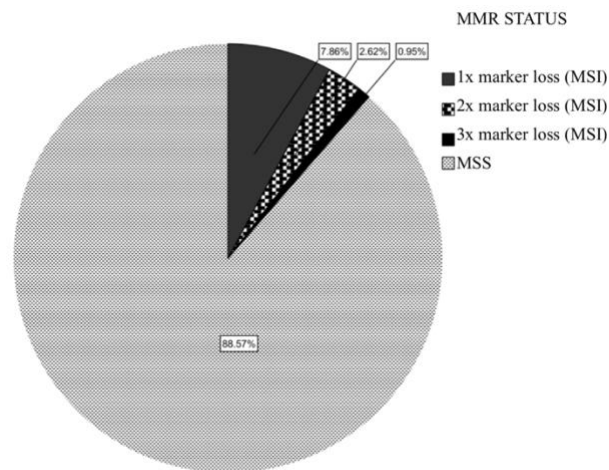


Figure 3: Distribution of the CRC tumours by MMR status: 88.5% MSS and 11.5% MSI (99).

Table 4: Details of the primary antibodies applied in our study (99).

Antibody	Clone	Identifier	Manufacturer	Program	Dilution
MLH1	G168-728	760-4264	Ventana	864	Ready-to-use
MSH2	G219-1129	760-4265	Ventana	880	Ready-to-use
MSH6		790-4455	Ventana	866	Ready-to-use
PMS2	EPR3947	760-4531	Ventana	879	Ready-to-use

#### 4.1.2 Expression of the MSI markers in the selected primary tumour regions and lymph node metastases

For detailed investigation, surgical specimens have taken from 56 patients. The following regions were analysed: normal colon mucosa, normal mucosa - tumour margin (border), main tumour mass, invasive front, and lymph node metastasis (265 regions in total). The MSI markers' expression demonstrated variations in their magnitude, but statistically significant differences (i.e., complete disappearance or appearance) were not observed between the distinct regions ( $p = 0.873$ ) (Table 5).

*Table 5: Based on markers expressed in each tumour region ( $n = 265$ ) MSI status was found to be constant ( $p = 0.873$ ) (99).*

	Normal mucosa	Border	Main tumour mass	Invasive front	Lymph node
MSS	42	41	79	67	17
MSI	1	3	6	6	1
Total	43	44	85	73	18

#### 4.1.3 Comparison of the MMR status of primary tumours and their liver metastases

In 69 patients, we investigated the correlation of the MMR status between the primary tumours and their corresponding liver metastases. A clear disappearance or appearance of the marker has been statistically analysed. In 14 (20.2%) cases the primary tumour and the metastasis were classified as different in the MMR status (Figure 4).

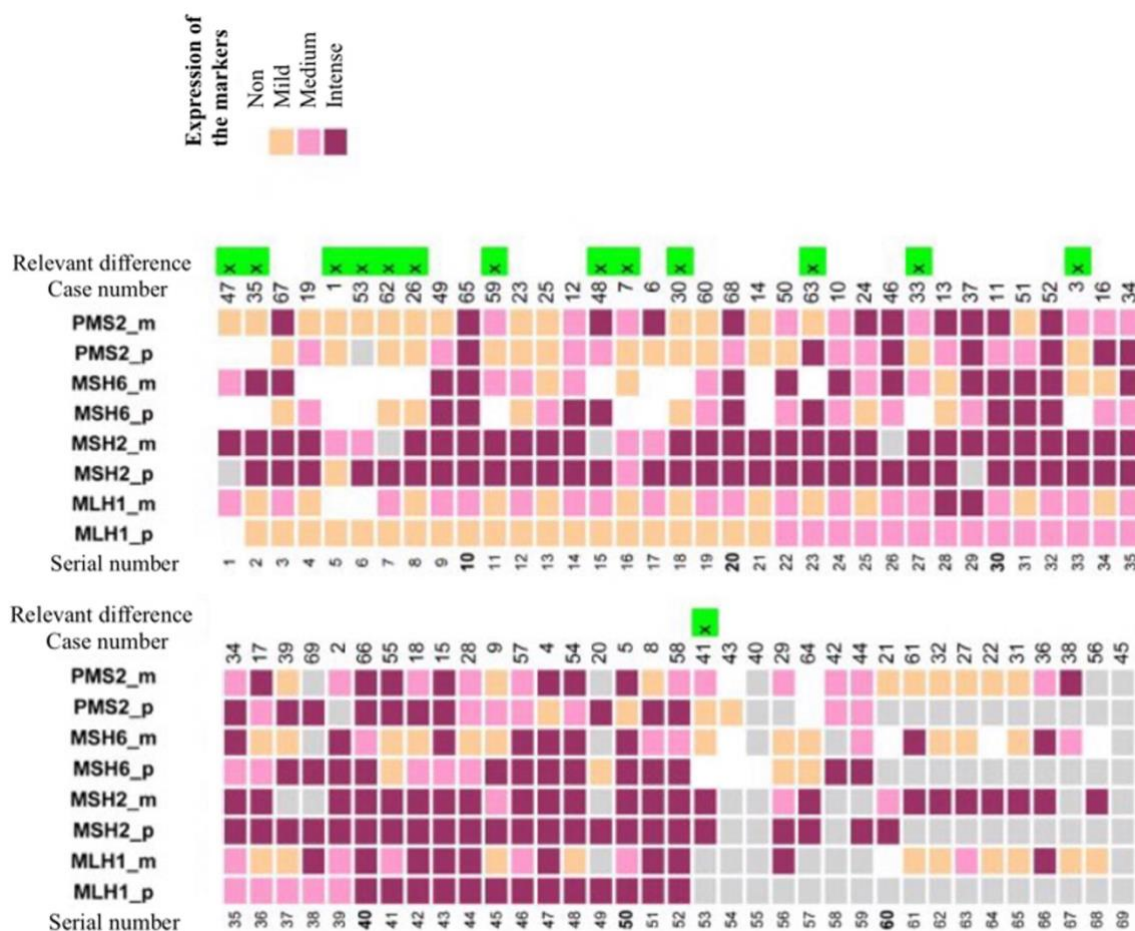


Figure 4: Comparison of primary tumours (MARKER\_p) and their metastases (MARKER\_m) for each marker. The expression of markers increases with the intensity of the colour scale, white colour indicates marker loss. Patients with relevant differences are marked in separate rows on the right (99).

#### 4.1.4 Clinicopathological features and their predictive potential

The average age of our patients was  $68,3 \pm 10,7$  years. Enrolment consisted of 68/122 (56%) males and 54/122 (44%) females. Their clinicopathological characteristics are shown in Table 6. In relapse-free survival (RFS), Dukes classification and clinical stage both showed significant predictive power ( $p = 0.001$ ). The latter was prognostic to OS too ( $p = 0.009$ ) while the other clinicopathological factors showed a tendency only for OS. Regarding sidedness, it was found that there was only a trend in PFS ( $p = 0.072$ ), where patients with left colon tumours having the most favourable outcome, followed by patients with rectal and right colon tumours.

Table 6: Clinicopathological characteristics of the patients included in the prognostic study (n = 122) (99).

Gender	Male	68
	Female	54
Age (Mean±SD)	68,3±10,7	
KRAS	Wild type	61
	Mutated	28
Grade	1	3
	2	78
	3	24
	Unknown	13
Dukes	A	8
	B	37
	C	51
	D	21
	Unknown	3
AJCC TNM Stage (97)	1	11
	2	36
	3	50
	4	21
	Unknown	4

<sup>1</sup>The absence of case numbers arose due to the unavailability of patient data in both the SE database and medical system, as well as within the pathology department.

#### 4.1.5 The prognostic value of the MSI status for RFS and OS after surgical resection

In our investigation of 122 patients, the MMR status was not prognostic for RFS nor OS (p/RFS/= 0.437, p/OS/= 0.907) (Figure 5).

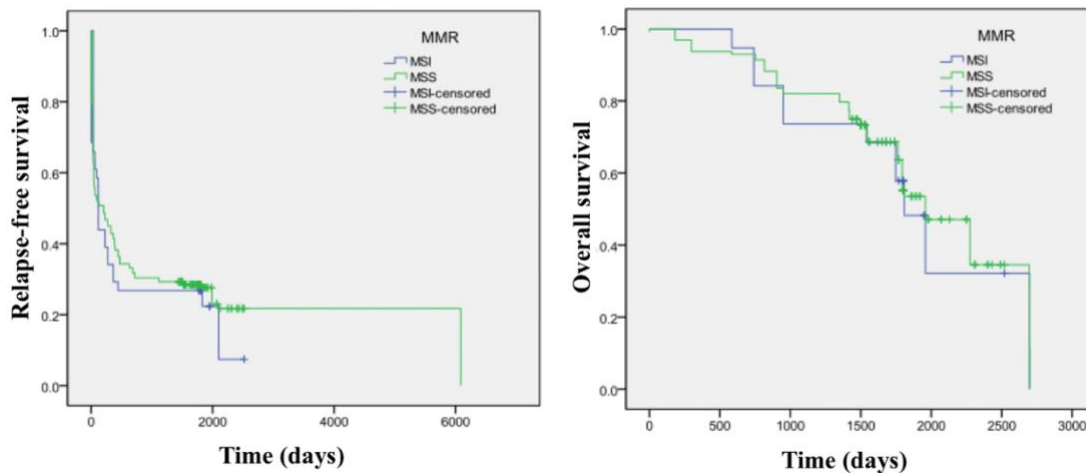


Figure 5: No significant difference found between relapse-free (left) and overall (right) survival of MSI and MSS tumours (99).

#### 4.1.6 The predictive role of MSI status for a given systemic oncological treatment

The relationship between the postoperative treatment and molecular findings were investigated in 97 patients (Table 7). The MSI status was not predictive for the OS with 5-FU-containing regimens ( $p = 0.968$ ), and similar results were obtained for oxaliplatin ( $p = 0.936$ ), irinotecan ( $p = 0.609$ ), bevacizumab ( $p = 0.561$ ), cetuximab ( $p = 0.755$ ), and panitumumab ( $p = 0.617$ ). Similarly, the MSI status was not predictive for PFS with any of the given oncological treatments (Table 8).

Table 7: The table represents the association between different treatments and the MMR status of the patients (99).

		MSI		Total
		No	Yes	
5-FU	No	26	4	30
	Yes	57	10	67
Total		83	14	97

		MSI		Total
		No	Yes	
Bevacizumab	No	55	11	66
	Yes	28	3	31
Total		83	14	97

		MSI		Total
		No	Yes	
Oxaliplatin	No	40	4	44
	Yes	43	10	53
Total		83	14	97

		MSI		Total
		No	Yes	
Cetuximab	No	67	6	73
	Yes	16	8	24
Total		83	14	97

		MSI		Total
		No	Yes	
Irinotecan	No	52	6	58
	Yes	31	8	39
Total		83	14	97

		MSI		Total
		No	Yes	
Panitumumab	No	79	10	89
	Yes	4	4	8
Total		83	14	97

Table 8.  $p$ -values of Cox regression survival models investigating the relationship between MSI/MSS status and routine oncological treatments. The  $p$ -values of the survival models (OS and PFS) of microsatellite instability (MSI) predictive potential with respect to various therapeutic agents. The MSI status was not predictive for PFS and OS with any of the given oncological treatments (99).

	5FU	Oxaliplatin	Irinotecan	Bevacizumab	Cetuximab	Panitumumab
OS	0.968	0.936	0.609	0.561	0.755	0.617
PFS	0.945	0.897	0.961	0.946	0.910	0.951

#### 4.1.7 The efficacy of 5-fluorouracil (5-FU) based therapy in treating stage II and III colon tumours with MSI

The examinations were performed separately on stage II and III patients looking for any different responses that the MSI tumours might have in these stages, and whether patients with stage II disease have an unfavourable outcome after 5-FU treatment. The study was able to enrol only 12 patients with stage II tumours and 35 patients with stage III tumours. In the stage II group, no significant differences were seen in RFS or OS. Similar trends were observed in stage III cases (Figure 7).

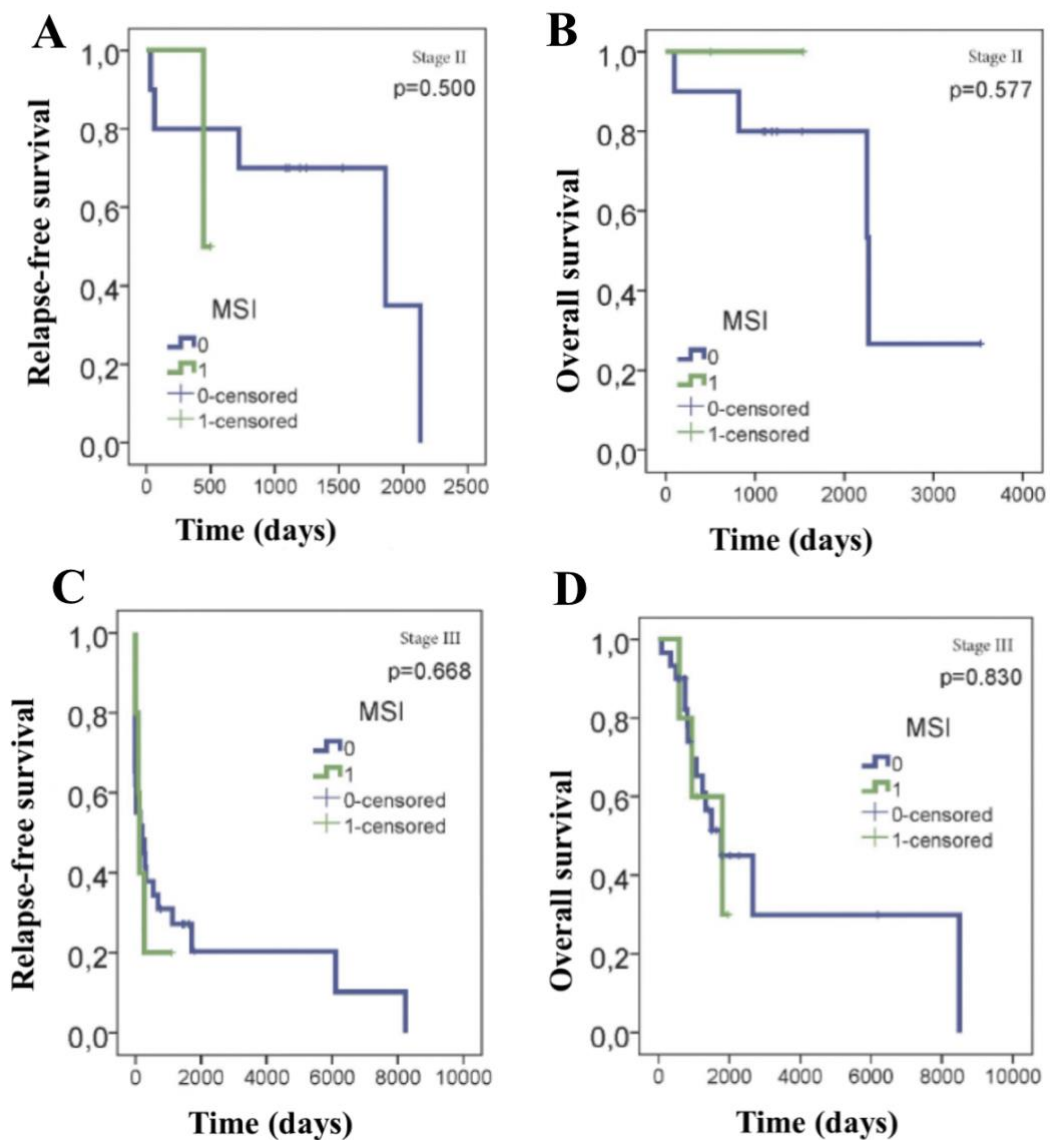


Figure 7: The response of stage II and III MSI CRC to 5-FU treatment (99).



## 4.2 PTEN study

The average age of the participants was  $63.45 \pm 9.9$  years. Enrolment consisted of 28/55 (51%) males and 27/55 (49%) females. Only tumours with documented *KRAS* status were considered for the study, with the intention of including 51% wild-type and 49% mutated. The clinicopathological data of the patients shown in Table 9.

*Table 9: Clinicopathological characteristics of the patients in the PTEN study (98).*

(*n*=55)

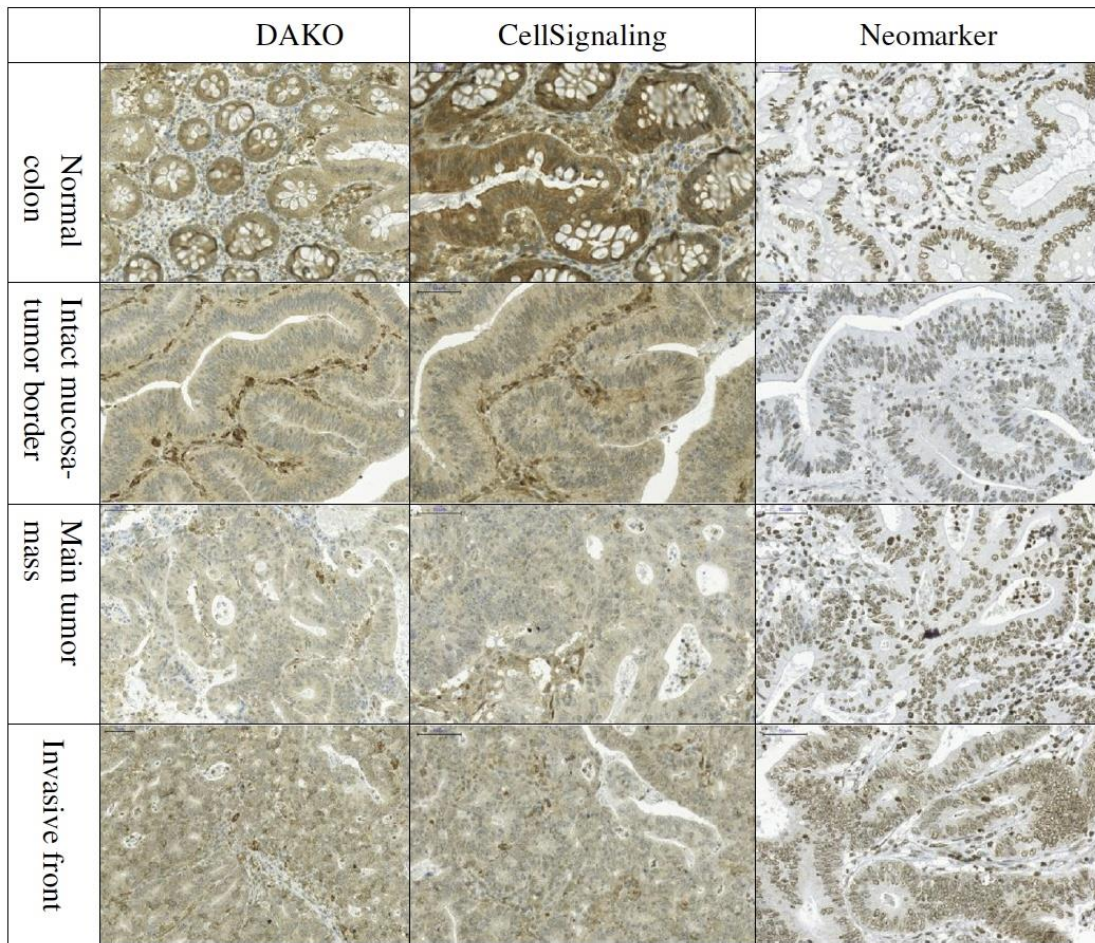
Parameter	Number of Observation/Mean $\pm$ SD
Age (year)	60.58 $\pm$ 11.01
Sex (Male: Female)	27:28 (49%:51%)
Location of the CRC	
- Coecum	9
- Ascending colon	8
- Hepatic flexure	5
- Transverse Colon	4
- Splenic flexure	4
- Descending colon	2
- Sigmoid colon	11
- Rectosigmoid	5
- Rectum	7
Grade of the tumour <sup>1</sup>	
- Low Grade	30
- High Grade	17
- Unknown	8
pT—extent of the tumour	
- T2	3
- T3	31
- T4	10
- Unknown	11
pN—lymph node status	
- N0	13
- N1	15
- N2	14
- Unknown	13
AJCC TNM Stage(97)	
- Stage I	1
- Stage II	13
- Stage III	20
- Stage IV	10
- Unknown	11
Dukes	
- A	1
- B	12
- C	21
- D	10
- Unknown	11
mAC	
- B1	1

- B2 9	9
- B3 3	3
- C1 3	3
- C2 12	12
- C3	6
- D	10
- Unknown	11
MMR (IHC)	
- MSS	40
- MSI	3
- Unknown	12
KRAS	
-Wild type	28
-Mutated	27

<sup>1</sup>The absence of case numbers arose due to the unavailability of patient data in both the SE database and medical system, as well as within the Pathology department.

#### 4.2.1 PTEN expression and intracellular staining pattern with various antibodies

Initially, we performed an assessment of PTEN expression using Dako, CellSignaling and Neomarker antibodies in all tumour regions to obtain a comprehensive technical comparison (Figure 8). The frequency, intensity, and localisation of PTEN expression were evaluated in all stained core samples and recorded in Table 10. Our findings revealed that all antibodies exhibited a relatively homogeneous staining pattern in terms of the frequency of PTEN-positive cells. However, the intensity of staining was observed to be lower for the Dako and Cell-Signaling antibodies, but higher for the Neomarkers antibody. Notably, the Neomarkers antibody exhibited specific staining of cell nuclei, whereas the remaining two antibodies demonstrated concurrent nuclear and cytoplasmic staining in most specimens. The staining patterns exhibited a moderate level of agreement between the Dako and Cell-Signaling antibodies in terms of frequency, intensity, and localization, whereas the Neomarkers antibody exhibited only marginal concurrence with the other two antibodies.



*Figure 8: Immunohistochemical images of the studied regions, which were probed with the corresponding PTEN antibody. The Dako, CellSignaling, and Neomarkers antibodies were used to stain the normal colon, tumour-normal border, main tumour mass, and invasive front of the colorectal tumours at 20x magnification (98).*

Table 10: The PTEN expression as determined by the three antibodies (98).

DAKO				Neomarkers			Cell Signaling		
	Frequency	Valid Percent		Frequency	Valid Percent		Frequency	Valid Percent	
Frequency	0	12	5.6	0	2	.8	0	7	3.0
	1 = 0-1 %	13	6.0	1 = 0-1 %	3	1.3	1 = 0-1 %	4	1.7
	2 = 2-10 %	12	5.6	2 = 2-10 %	27	11.4	2 = 2-10 %	2	.8
	3 = 11-33 %	8	3.7	3 = 11-33 %	37	15.6	3 = 11-33 %	5	2.1
	4 = 34-66 %	23	10.6	4 = 34-66 %	27	11.4	4 = 34-66 %	15	6.3
	5 = 67-100 %	148	68.5	5 = 67-100 %	141	59.5	5 = 67-100 %	204	86.1
	Total	216	100.0	Total	237	100.0	Total	237	100.0
Statistics	DAKO vs. NEOM	Value	p	NEOM vs. CELLS	Value	p	CELLS vs. DAKO	Value	p
	Kappa	.067	.066	Kappa	.056	.066	Kappa	.264	.000
	Chi-square		0.064	Chi-square		.286	Chi-square		.000
Intensity	0	12	5.6	0	2	.8	0	7	3.0
	1 = +	158	73.1	1 = +	41	17.3	1 = +	146	61.6
	2 = ++	29	13.4	2 = ++	61	25.7	2 = ++	33	13.9
	3 = +++	17	7.9	3 = +++	133	56.1	3 = +++	51	21.5
	Total	216	100.0	Total	237	100.0	Total	237	100.0
Statistics	DAKO vs. NEOM	Value	p	NEOM vs. CELLS	Value	p	CELLS vs. DAKO	Value	p
	Kappa	.051	.051	Kappa	.082	.014	Kappa	.305	.000
	Chi-square		.024	Chi-square		.260	Chi-square		.000
Location	0	11	5.0	0	2	.8	0	8	3.2
	1 = nucl	5	2.3	1 = nucl	241	99.2	1 = nucl	2	.8
	2 = cytop	44	19.9	2 = cytop	0	.0	2 = cytop	22	8.7
	3 = nucl+cytop	161	72.9	3 = nucl+cytop	0	.0	3 = nucl+cytop	220	87.3
	Total	221	100.0	Total	243	100.0	Total	252	100.0
Statistics	DAKO vs. NEOM	Value	p	NEOM vs. CELLS	Value	p	CELLS vs. DAKO	Value	p
	Kappa	-.001	.712	Kappa	-.005	.000	Kappa	.390	.000
	Chi-square		.480	Chi-square		.000	Chi-square		.000

#### 4.2.2 Comparison of the scoring methods

The H-scores (H1, H2, H3) were computed by integrating the intensity (0–3) and frequency (0–5) values. Notably, these scoring schemes exhibited a robust correlation (Spearman's  $\rho = 0.854-0.948$ ), and no statistically significant disparity was observed among them (Cohen's  $\kappa$ :  $p = 0.228-0.666$ ).

#### 4.2.3 The intratumoural distribution of the PTEN expression

In order to evaluate the PTEN protein expression across specific intratumoural areas, we utilized various scoring systems. However, statistical analysis was only presented for the H1-score as no significant differences were observed among the different systems. Utilising both Dako and Cell-Signaling antibodies, we observed a gradual decrease in PTEN expression spanning from normal colon mucosa to tumour progression (tumour-

normal border → main mass → invasive front → lymph node metastasis), although these differences were not statistically significant. Notably, PTEN H-scores were significantly lower in all tumour areas examined when compared to normal colon mucosa (Dako normal vs. lymph node  $p = 0.109$ , vs. main mass  $p = 0.005$ , vs. for all others: border, invasive front, and lymph node metastasis  $p < 0.001$ , respectively; Cell-Signaling normal vs. all tumour regions  $p < 0.001$ ). While the Neomarkers antibody displayed a similar pattern, it did not demonstrate any notable distinction in PTEN expression between normal colon and tumour areas. (Figure 9).

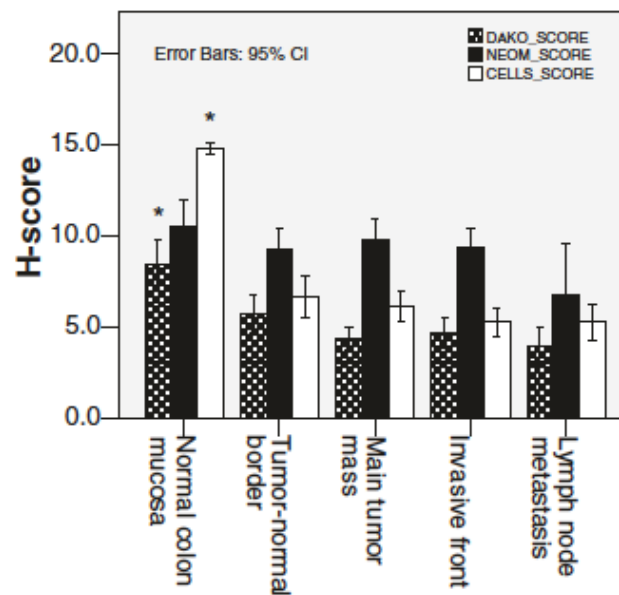


Figure 9: The PTEN expression levels in normal colon mucosa, and different areas of the primary tumour, and metastases. PTEN immunohistochemistry (IHC) was performed using antibodies from Dako, CellSignaling, and Neomarkers on specimens from different tumour regions, including normal colon, tumour-normal border, main tumour mass, and invasive front of colorectal tumours. H-scores were computed using a combination of staining intensity and frequency. The mean PTEN H-score values, with 95% confidence intervals, were determined using the three antibodies and for the different tumour regions. Significant differences were observed between the normal colon and all detected tumour regions using both the Dako and CellSignaling antibodies ( $p < 0.001$  for all regions except the normal vs. border region detected by the Dako antibody with  $p = 0.005$ ). No significant differences were observed based on the staining achieved with the Neomarkers antibody. The results are presented in a data chart (98).

#### 4.2.4 The evaluation of PTEN expression based on the anatomical location of the tumour

The study evaluated the PTEN expression levels across different regions of the colorectum, including the cecum, ascending colon, hepatic flexure, transverse colon, splenic flexure, descending colon, sigmoid colon, and rectum and colon sides such as left colon, right colon, and rectum. However, the results showed no significant variations in PTEN expression levels among these regions and colon sides when analysed with any of the three antibodies.

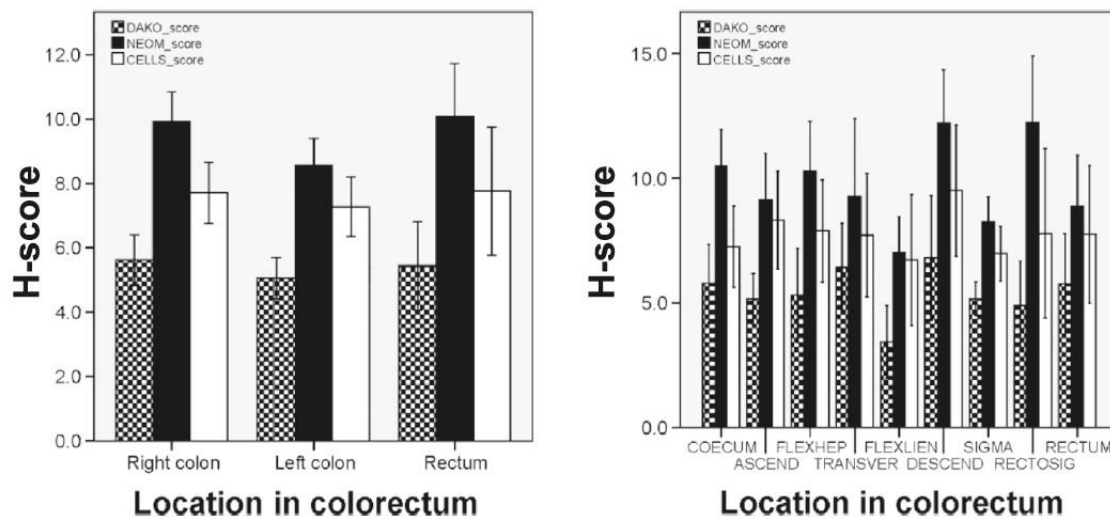


Figure 10: PTEN expression in relation to the anatomic location of the tumour in the large bowel. The mean expression of PTEN ( $\pm 95\%$  confidence interval) was analysed using three different antibodies (Dako, CellSignaling, and Neomarkers) according to the location of the tumours in the colorectum (98).

#### 4.2.5 Clinicopathological evaluation

In the investigation of PTEN protein expression within selected tumour areas (as shown in Figure 9), we did not observe any significant variations. Consequently, we employed the following formula to determine the average score for each individual case:  $H\text{-score}_{\text{case}} = (H1\text{-score}_{\text{tumour-normal border}} + H1\text{-score}_{\text{main tumour mass}} + H1\text{-score}_{\text{invasive front}}) / 3$ . Subsequently, we examined the relationship between clinicopathological data and the  $H\text{-score}_{\text{case}}$  (Figure 10). Our findings revealed that none of the tested antibodies, including Dako, CellSignaling, and Neomarkers exhibited any significant association between PTEN expression and pT. Similarly, there was no significant relationship observed between Dukes, Dukes-MAC, and clinical stage with



respect to PTEN expression. However, *KRAS* status was found to be correlated with PTEN expression only when using the Neomarkers antibody for staining. Specifically, PTEN expression detected by the Neomarkers antibody was lower in *KRAS* mutant (*mKRAS*) tumours with the mutation in exon 13. The staining results obtained using the CellSignaling antibody showed an opposite trend compared to the Neomarkers antibody, but this trend was not statistically significant. Specifically, the staining results indicated lower PTEN expression in *mKRAS* tumours with the mutation in exon 12 and in WT tumours, as compared to the few *mKRAS* tumours with the mutation in exon 13 carcinomas. In relation to the association with tumour grade, similar pattern was observed only with Neomarkers, indicating higher PTEN expression in high grade tumours, while equal distribution was found with Dako and CellSignaling. PTEN expression was not found to associate with MMR status with any of the antibodies (Table 11).

*Table 11: The statistical significance levels (p-values) indicating the correlation between PTEN expression and the clinicopathological characteristics of the tumours (98).*

	pT	Dukes	Dukes-MAC	Clinical stage	<i>KRAS</i> status	Tumour grade	MMR status
Dako	0.817	0.454	0.718	0.806	0.713	0.832	0.731
CellSignaling	0.611	0.824	0.990	0.727	0.062	0.099	0.679
Neomarker	0.175	0.896	0.728	0.984	0.029	0.040	0.315

*Abbreviations: pT: pathological T stage, MAC: modified Astler-Coller, MMR: mismatch repair, KRAS: Kirsten rat sarcoma viral oncogene homolog*

To assess the prognostic power of PTEN immunohistochemistry on patient survival, a dichotomizing/binary system was utilized based on two methods: *method A* assigned PTEN-normal expression when the PTEN expression of the tumour was similar to surrounding normal mucosa, whereas PTEN loss was determined in cases where tumour regions exhibited notably decreased PTEN expression compared to the adjacent normal mucosa. This method was in accordance with previously published approaches (100-103). A more objective approach was utilized with *Method B*, where ROC analysis was employed to identify the most suitable threshold for PTEN expression, as detected through immunohistochemistry.. The cut-off point of PTEN expression yielded a sensitivity of 61% and specificity of 70%. The analyses described above did not reveal

any prognostic power of any of the antibodies in any setting. Data on the clinicopathological characteristics of the patients presented in Table 8.

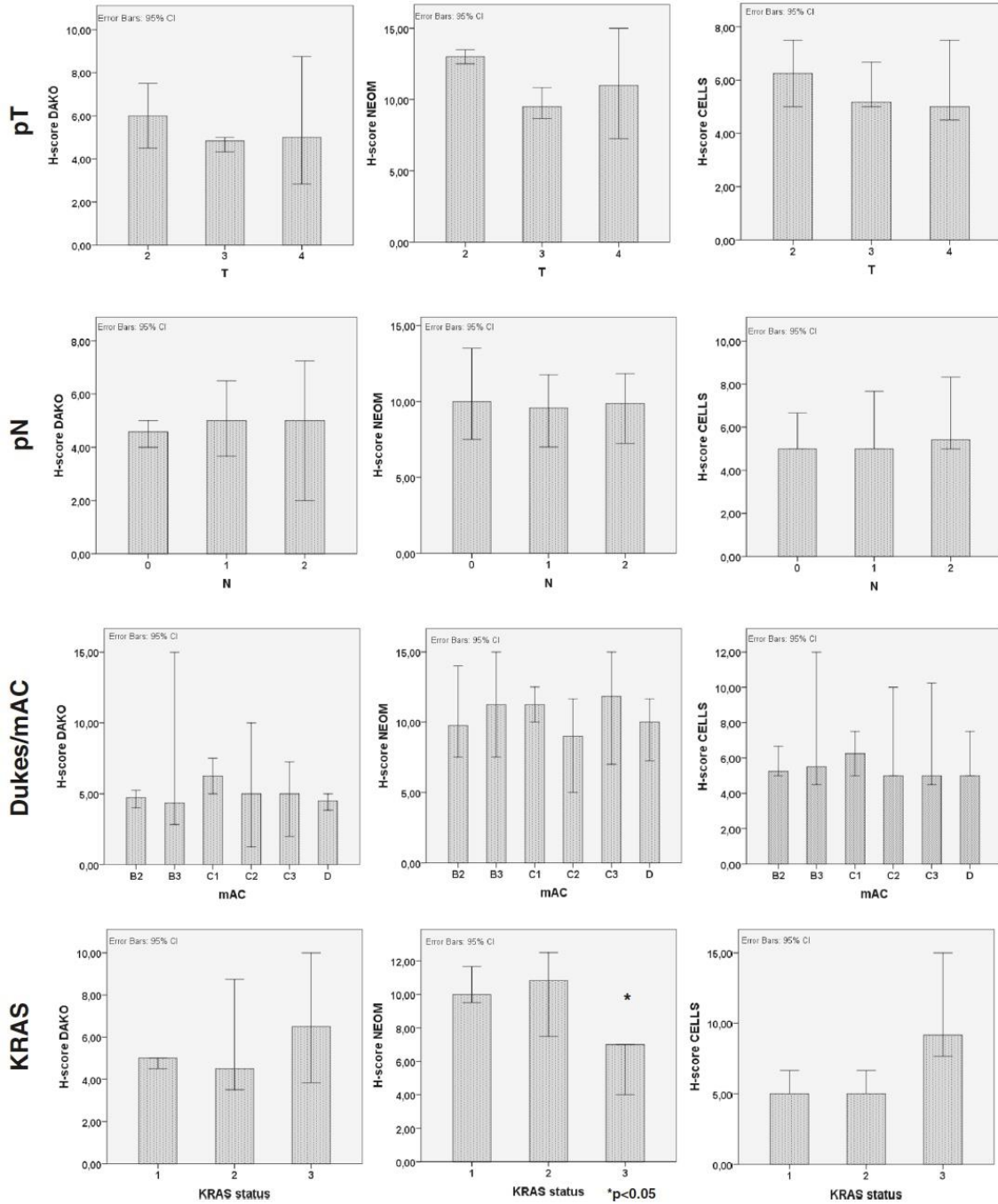


Figure 11: The connection between PTEN expression and clinicopathological parameters. The average PTEN expression level was examined using Dako, Neomarkers and CellSignaling antibodies, stratified by pT, pN, Dukes-mAC stages, and KRAS status (98).



### 4.3 Immune biomarkers

The participants' mean age was  $60.58 \pm 11.01$  years, and the enrolment comprised of 77 (56.2%) males and 60 (43.8%) females. Table 12 displays the clinicopathological attributes of the patients.

Table 12: Clinicopathological data of the patients included in the study (104).

Parameter	Number of Observation/Mean $\pm$ SD
Age (year)	60.58 $\pm$ 11.01
Sex (Male: Female)	77:60 (56.2%:43.8%)
Location of the CRC <sup>1</sup>	
– Coecum	16 (11.7%)
– Ascending colon	16 (11.7%)
– Transverse colon	11 (8%)
– Descending colon	15 (10.9%)
– Sigmoid colon	50 (36.5%)
– Rectum	28 (20.4%)
Sidedness of the tumour	
– Right-sided	45 (32.8%)
– Left-sided	92 (67.2%)
pT—extent of the tumour <sup>1</sup>	
– T1	1 (0.7%)
– T2	11 (8%)
– T3	93 (67.9%)
– T4	27 (19.7%)
pN—lymph node status <sup>1</sup>	
– N0	44 (32.1%)
– N1	48 (35%)
– N2	42 (30.7%)
AJCC [9] staging <sup>1</sup>	
– Stage I	2 (1.5%)
– Stage II	27 (19.7%)
– Stage III	54 (39.4%)
– Stage IV	51 (37.2%)

<sup>1</sup> For one patient, the exact location of the tumour was unknown, except that it was on the right side. Staging information was also unavailable for three patients. The abbreviations AJCC and CRC refer to the American Joint Committee on Cancer and colorectal cancer, respectively. (n=137)

#### 4.3.1 The distribution of the different immune cells and immune checkpoint markers in the main tumour mass and metastatic samples

A statistically significant increase in the number of CD56<sup>+</sup> cells in the primary tumour samples were observed compared to the metastatic samples (p=0.0195, Figure 12A). While the occurrence of CD23<sup>+</sup> (p = 0.1133, Figure 12B) and PD-1<sup>+</sup> (p = 0.1312, Figure 12C) cells in primary tumours was somewhat more frequent. There was no statistically

significant difference in the number of CD3<sup>+</sup>, CD4<sup>+</sup>, CD20<sup>+</sup>, CD45<sup>+</sup>, CTLA-4<sup>+</sup>, and PD-L1<sup>+</sup> cells between the primary (MAIN) and metastatic (MET) samples.

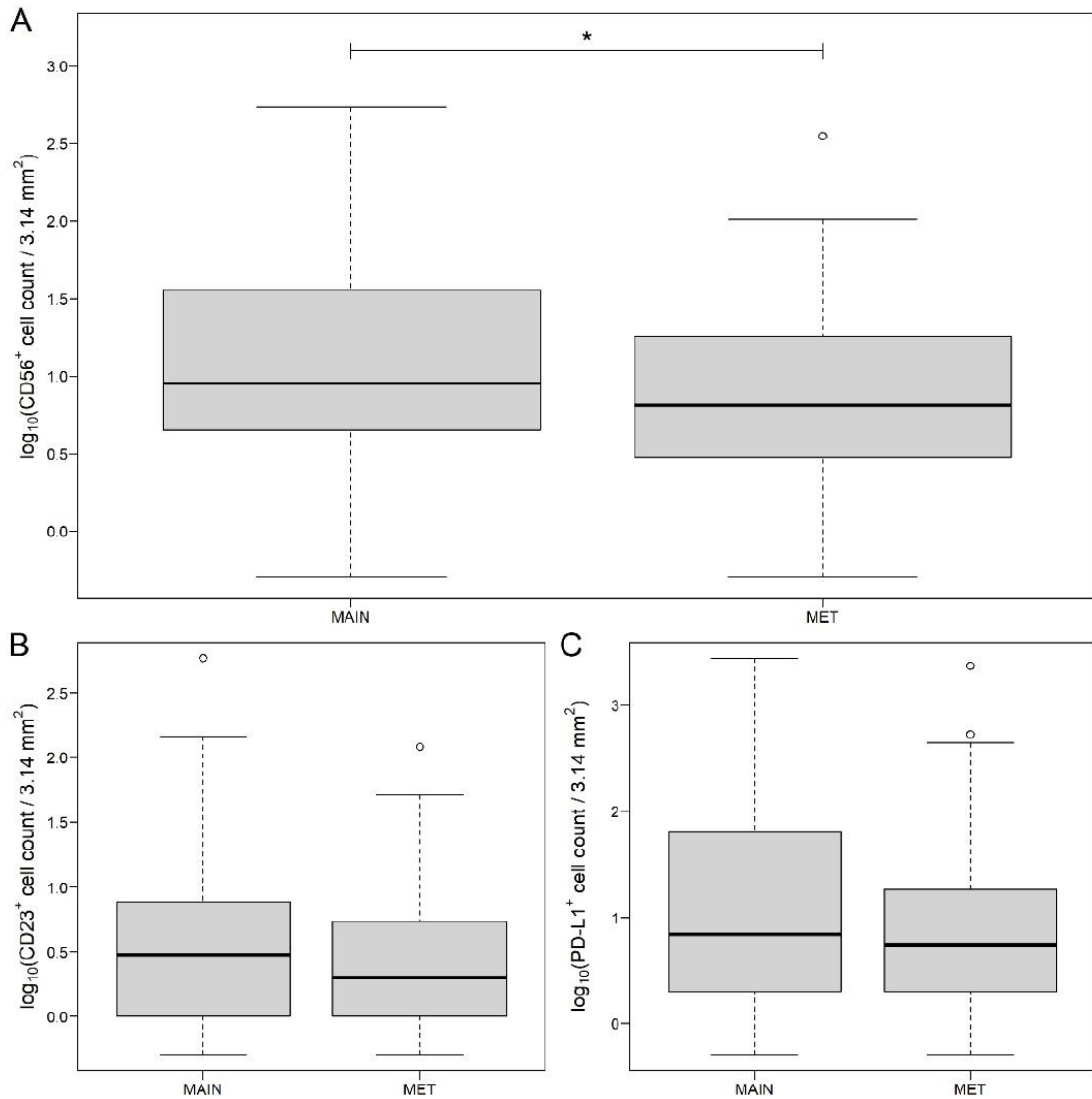


Figure 12: The count of CD56- (A) and CD23-positive (B) immune cells, and programmed death-ligand 1 (PD-L1, C) positivity in both the primary tumour mass (MAIN) and liver metastasis (MET) samples. In the comparison of CD56, CD23, and PD-L1 counts between two sample types, only CD56 showed a significant difference while CD23 and PD-L1 showed only marginal differences. The outliers ( $>1.5$  times the interquartile range above the upper quartile) are represented by hollow black circles and the median value is represented by the thick line. The significance level for CD56 was  $* p < 0.05$  (104).

### 4.3.2 The anatomic localisation of CRC within the colorectum

In the primary tumour samples (MAIN), a statistically significant increase in the number of PD-1<sup>+</sup> cells were observed ( $p=0.0092$ , Figure 13A). Furthermore, there was a tendency towards a higher number of CD45<sup>+</sup> cells in right-sided tumours, although this difference was not statistically significant ( $p=0.1313$ , Figure 13B). Table 9 displays the clinicopathological characteristics of the patients.

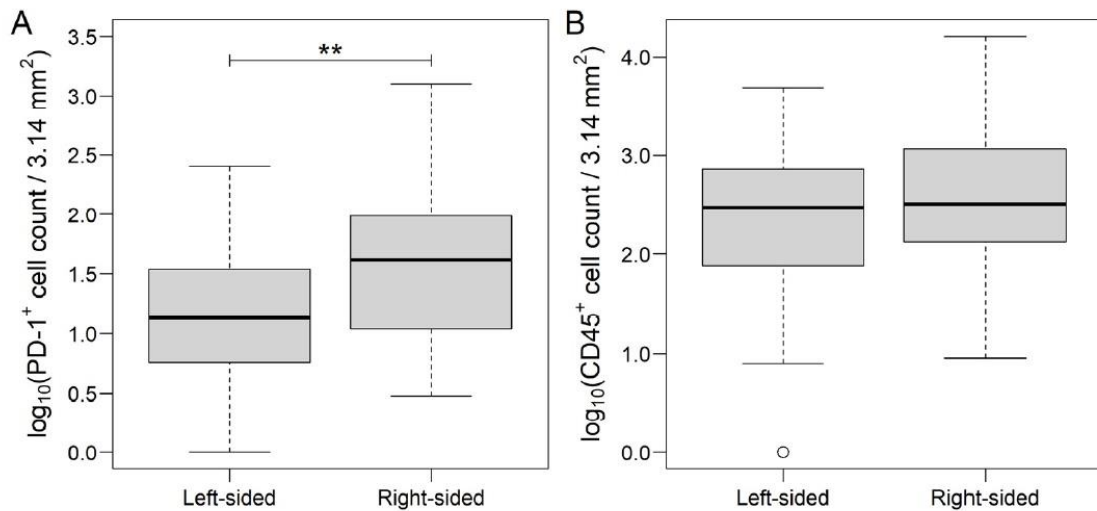


Figure 13: The count data of programmed cell death protein 1- (PD-1, A) and CD45-positive (B) cells in colorectal cancer samples from the left and right colon sides. The expression level of PD1 was found to be significantly different between the two sides, whereas only a marginal difference was observed in the case of CD45. The median value is represented by the thick line. Significance level is denoted as \*\* ( $p < 0.01$ ) (104).

### 4.3.3 The distribution of the immune markers with the lymph node status

A significant association was observed between a greater number of metastatic lymph nodes and a lower count of CD20<sup>+</sup> cells ( $p = 0.0119$  for N0 vs. N2 and  $p = 0.0292$  for N1 vs. N2, Figure 14A). Additionally, a trend towards increased counts of CD3<sup>+</sup> cells ( $p = 0.0587$ , Figure 14B) and CD45<sup>+</sup> cells ( $p = 0.1204$ , Figure 13C) was observed with more advanced lymph node metastasis status.

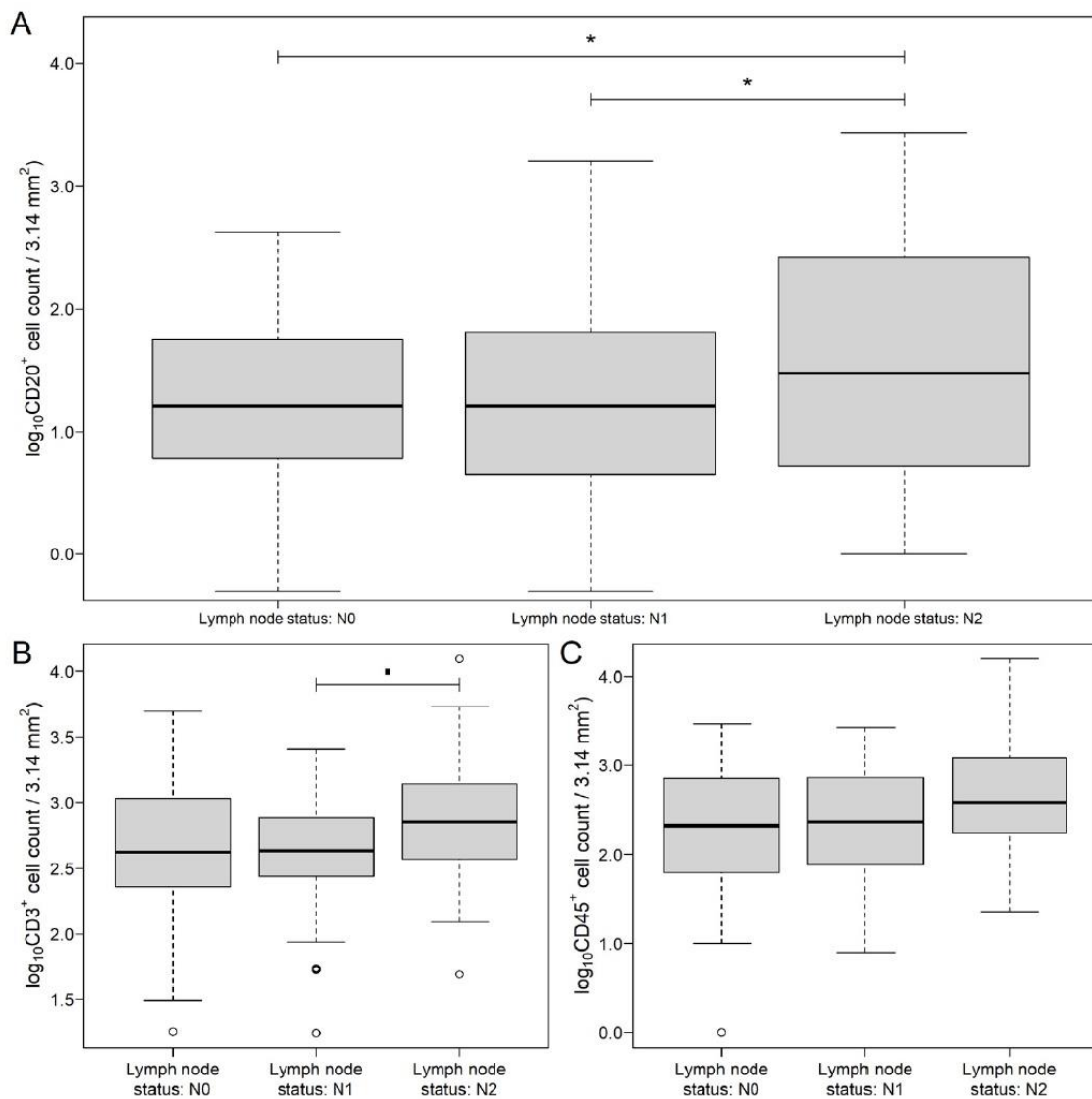


Figure 14: The count data of CD20 (A), CD3 (B), and CD45 (C) positive tumour-infiltrating immune cells in colorectal cancer samples were analysed and grouped according to their lymph node metastasis status. CD20 counts were significantly different between the two groups, while only marginal differences were observed in the case of CD3 and CD45. The thick line represents the median value, and the hollow black circles represent outliers (greater/less than 1.5 times the interquartile range above/under the upper/lower quartile). Statistical significance was indicated by \* for  $p < 0.05$  and for  $0.1 < p < 0.05$  (104).

#### 4.3.4 The distribution of the immune markers within the liver metastases

Within the MET samples, our analysis detected significant variations exclusively in the CD56<sup>+</sup> and CD45<sup>+</sup> cell counts. Notably, the CD56<sup>+</sup> count exhibited a decrease in cases where metastasis was observed since the initial diagnosis of colorectal cancer (CRC), with statistical significance noted for Stage I-II versus IV ( $p = 0.0208$ ) and a non-significant trend observed for Stage I-II versus III ( $p = 0.1056$ ; Figure 15A). Furthermore, a modest elevation was observed in the CD45<sup>+</sup> cell count in Stage IV CRC in comparison to Stage III ( $p = 0.0820$ ; Figure 15B).

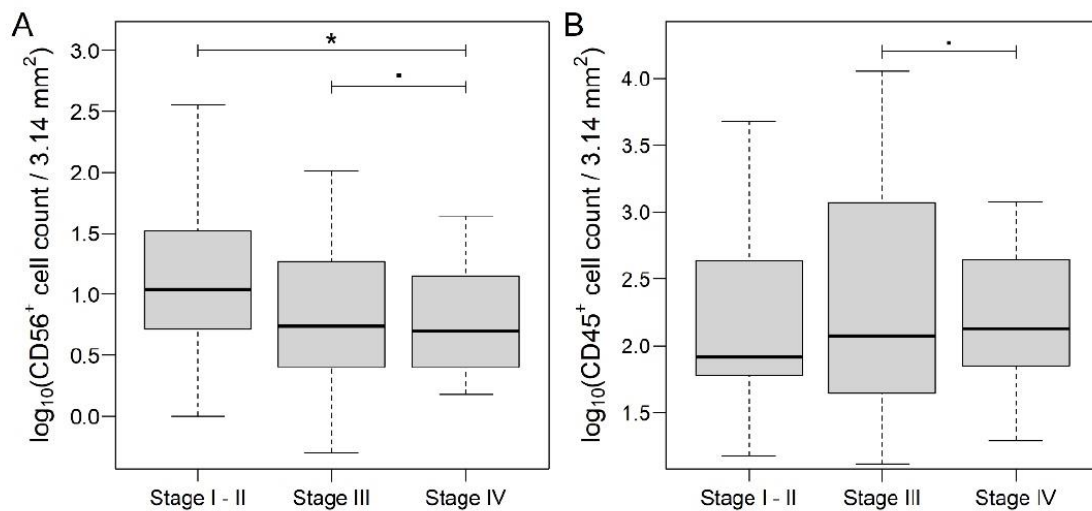


Figure 15: CD56 (A), and CD45 (B) count of liver metastasis samples of CRC patients, grouped by AJCC staging (105). The results showed that the count of CD56 was significantly lower in samples where metastasis was detected at the time of tumour diagnosis, while the count of CD45 exhibited the opposite trend. The median value is represented by the thick line. Statistical significance is indicated by \*  $p < 0.05$  and  $0.1 < p < 0.05$  (104).

#### 4.3.5 The distribution of markers in various anatomical sites of CRC and in metastatic lymph nodes

The distribution of immune cells was assessed across different anatomical sites including normal colon tissue (NORMAL), primary tumour central mass (MAIN), tumour-normal interface (BORDER), deepest infiltrative area (FRONT), and lymph node metastasis (LN). Analysis of the markers CD4<sup>+</sup> and PD-L1<sup>+</sup> revealed no significant

differences in any of the examined regions. However, the expression of CD56 was found to be significantly higher in normal colon tissue as compared to the other regions ( $p < 0.0001$ ). In contrast, CD3, CD8, CD20, CD23, CD45, CTLA-4, and PD-1 exhibited significantly higher expression only in the lymph node metastases ( $p < 0.0001$  as compared to all other sites except for CTLA-4:  $p = 0.0008$  vs. BORDER,  $p = 0.0005$  vs. FRONT,  $p = 0.0004$  vs. MAIN,  $p = 0.0021$  vs. NORMAL; and PD-1:  $p = 0.0022$  vs. BORDER; Figure 16).

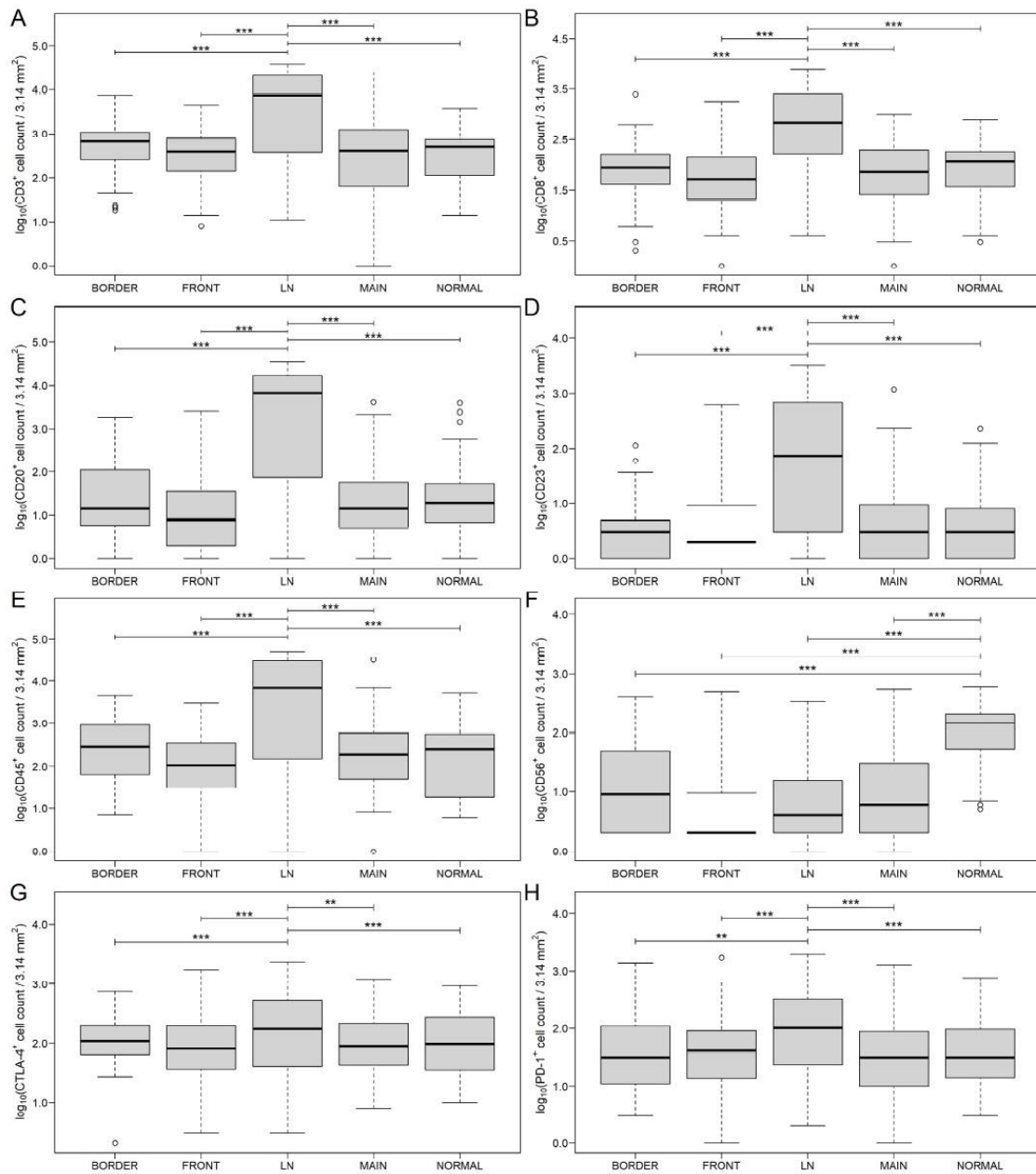


Figure 16: The count data for CD3 (A), CD8 (B), CD20 (C), CD23 (D), CD45 (E), CD56 (F), CTLA-4 (G), and PD-1 (H) in colorectal cancer samples at various tumour sites are

presented. MAIN (main tumour mass), BORDER (tumour normal interface), FRONT (deepest infiltrative area), LN (lymph node metastasis), and NORMAL (normal colon tissue). Outliers with values greater than 1.5 times the interquartile range above the upper quartile are represented by hollow black circles. The median value is indicated by the thick line. Statistical significance is indicated by \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  (104).

#### 4.3.6 Immune Panel Gene Expression Analysis

We selected a set of 12 MAIN and 12 MET samples for analysis using the NanoString nCounter® PanCancer Immune Profiling Panel. These samples were obtained from 13 patients. We conducted differential expression analysis to identify Differentially Expressed Genes (DEGs) between MAIN and MET samples. We found 11 and 29 DEGs to be significantly and marginally different, respectively, between the two sample types (Figure 17). The analysis revealed that among the 11 DEGs, the genes complement C4B (*C4B*), complement factor I (*CFI*), defensin beta 1 (*DEFB1*), interleukin-1 receptor accessory protein (*IL1RAP*), interleukin-27 (*IL27*), mannose binding lectin 2 (*MBL2*), and metallophosphoesterase domain containing 1 (*MPPED1*) were observed to be downregulated. On the other hand, the genes caspase recruitment domain family member 9 (*CARD9*), C-C motif chemokine receptor 7 (*CCR7*), lymphotoxin beta (LTB), and tumour necrosis factor (TNF) receptor superfamily member 8 (*TNFRSF8*) were observed to be upregulated. No statistically significant distinction was detected in the gene expression patterns between tumours located on the left and right colon sides. Gene set enrichment analysis (GSEA) was employed to investigate the over-representation of genes. The study revealed reduced expression of innate immune response genes, with 6 out of the 11 DEGs exhibiting decreased expression (odds ratio (OR): 16.04, 95% confidence interval (CI): 2.44–∞,  $p = 0.0133$ ). Additionally, alterations were observed in pathways related to members of the TNF superfamily and their receptors, with a slight increase in the expression of *LTB* and *TNFRSF8* (OR: 24.38, 95% CI: 2.56–∞,  $p = 0.0659$ ). However, no additional pathways with alterations were identified (Figure 18).

The investigation evaluated the predictive importance of DEGs by employing two distinct forms of survival models. Firstly, standard Cox regression models were used to analyse the 12 MAIN and 12 MET samples separately. Secondly, a mixed effect Cox regression model was used to analyse all 24 samples, with patient's IDs and sample source being used as the random and stratification factors, respectively. The results indicated that

lower MAIN *TNFRSF8* counts were associated with poorer disease-specific survival (DSS) of patients, whereas higher MAIN *DEFB1* counts were significantly linked to poorer progression-free survival. Moreover, worse PFS was associated with lower *C4B*, *CFI*, and *IL1RAP* counts, and higher *CARD9* counts within the MET samples. No additional findings were observed in the stratified, mixed effect models. The standard multivariate survival models did not reveal any significant differences. However, the secondly applied approach showed that *C4B* ( $p = 0.0240$ ), *MBL2* ( $p = 0.0180$ ), *CARD9* ( $p = 0.0160$ ), and *TNFRSF8* (HR: 0.0008; 95% CI: 0.0000 – 0.1723;  $p = 0.0093$ ) significantly impacted DSS, while *IL27* ( $p = 0.0220$ ) and *LTB* ( $p = 0.0350$ ) were prognostic of PFS. Moreover, *DEFB1* was found to significantly affect both DSS ( $p = 0.0390$ ) and PFS ( $p = 0.0420$ ) (Table 13).

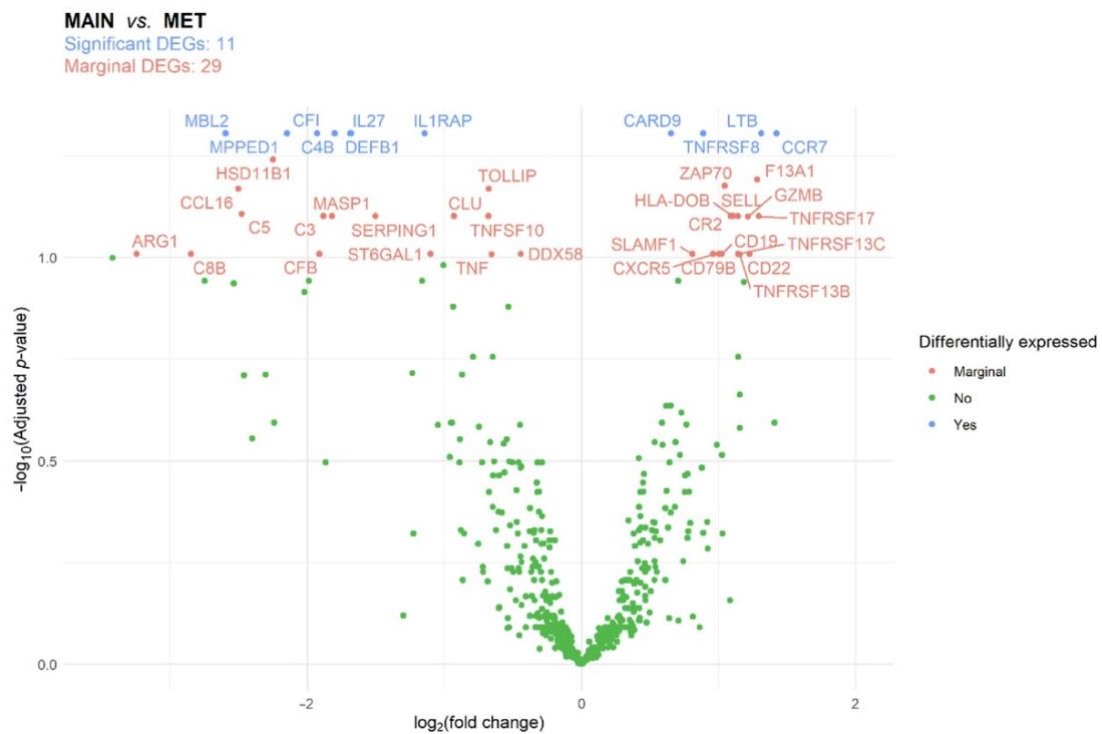


Figure 17: The differentially expressed genes (DEGs) between the main tumour mass (MAIN) and liver metastasis (MET) samples of CRC patients. The  $p$ -values were adjusted using the false discovery rate method (104).



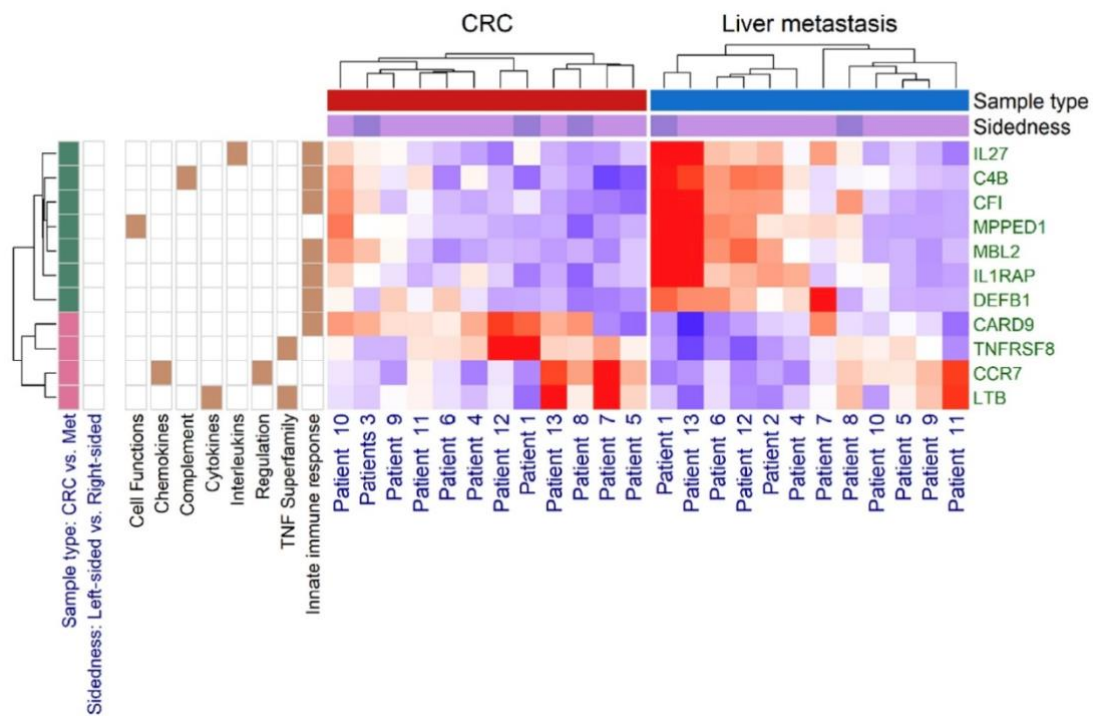


Figure 18. A heatmap was generated to display the significantly different gene expressions between the main tumour mass (CRC) and samples of liver metastasis (MET) samples. The downregulation and upregulation of genes were respectively represented by green and pink boxes. The brown box indicated the enrichment annotation information of the differentially expressed genes (104).

Table 13: The outcomes derived from the survival models applied to disease-specific survival and progression-free survival. The table includes only those p values where an association was found. The analysis was conducted on two subsets: the 12 MAIN samples and the 12 MET samples. For the former, standard Cox regression models were employed, whereas for the latter, a mixed-effects Cox regression model was utilized. In the mixed-effects model, patient identifiers served as the random factor, while the source of the samples was employed as the stratification factor. This approach was employed for the analysis of all 24 samples (104).

DEG	DSS		PFS	
	Univariate model p value	Multivariate model p value	Univariate model p value	Multivariate model p value
MAIN samples				
<i>TNFRSF8</i>	0.0378			
<i>DEFB1</i>			0.0410	
MET samples				
<i>DEFB1</i>			0.0410	
<i>C4B</i>			0.0474	
<i>CF1</i>			0.0449	
<i>IL1RAP</i>			0.0266	
Stratified, mixed effect models				
<i>C4B</i>		0.0240		
<i>CARD9</i>		0.0160	0.0018	
<i>DEFB1</i>		0.0390		0.0420
<i>MBL2</i>		0.0180		
<i>TNFRSF8</i>		0.0093		
<i>IL27</i>				0.0220
<i>LTB</i>				0.0350

Abbreviations: *DEG*: differentially expressed gene; *MAIN*: main tumour mass; *MET*: liver metastasis, *DSS*: Disease specific survival, *PFS*: progression-free survival.

## 5 Discussion

Genomic alterations play a crucial role in the development and progression of cancer, including colorectal cancer. This phenomenon applies not only in the primary tumour but also in the metastatic setting. Besides the classical clinicopathological characteristics (such as TNM, Dukes, modified Astler-Coller classifications, clinical stage, lymphovascular and perineural invasion), the number of biomarkers utilized in diagnostic testing is steadily increasing. The term ‘biomarker’ typically pertains to DNA, RNA, microRNA (miRNA), epigenetic modifications, peptides, or antibodies. Biomarkers presently hold a significant role in identifying and managing individuals with colorectal cancer. In the clinical setting, we utilize *RAS* mutation status to identify patients who are unlikely to respond to EGFR monoclonal antibodies. BRAFV600E mutations are associated with a poor prognosis and predict decreased sensitivity to standard therapies, which highlights the importance of incorporating BRAF inhibitors into targeted combination therapies. Consecutive examination of circulating tumour DNA has the potential to guide forthcoming therapeutic choices in CRC by providing insights into the genetic landscape of tumours and identifying potential drug targets (106). Transcriptomic subtypes and signatures of pathway activation have additionally exhibited prognostic and possibly predictive significance in metastatic CRC, providing insights into the interactions between cancer cells and the stromal and immune microenvironments (107). Our research aimed to provide relevant insights by assessing specific biomarkers in patients with colorectal cancer.

In our patient population we initially studied microsatellite instability and subsequently expanded our investigations to include the PTEN tumour suppressor gene. Ultimately, our research focused on exploring the function of tumour infiltrating immune cells and immune checkpoint markers. The MMR system has long been recognised as an important mechanism of colorectal carcinogenesis (108). MSI serves as a recognized predictive marker. Patients with dMMR MSI-H CRC have been shown to be particularly sensitive to immune checkpoint inhibitors, as these tumours tend to have higher levels of immune infiltration and a more immunogenic profile (109). On the other hand, tumours with a mesenchymal phenotype tend to be more resistant to immunotherapy due to the activation of immunosuppressive cascades. Individuals affected by Hereditary Nonpolyposis Colorectal Cancer (HNPCC) syndrome (also known as Lynch syndrome)

often exhibit a distinctive localization and tissue pattern of tumours that develop as a result of inherited mutations in *MMR* genes (110, 111). It is also recognized that tumours associated with HNPCC syndrome have a distinct prognosis, which is typically more favourable when compared to sporadic tumours with similar location and stage (112). Defects in the MMR system can also arise in sporadic CRC, with approximately 15% of CRCs exhibiting sporadic MSI, as reported in the literature (113).

In our MSI study, we processed surgical specimens from 122 patients with CRC, and we also sought answers to questions that, to our knowledge, had not been previously investigated. We conducted an analysis of different areas of primary tumours and potential lymph node metastases (56 patients) and paired resected liver metastases with primary tumours (69 patients) to examine the expression of MSI-indicating proteins (MLH1, MSH2, MSH6, PMS). We also wanted to determine whether intratumoural heterogeneity in the expression of these proteins could be observed. When evaluating primary tumour-metastasis pairs, we specifically searched for heterogeneity within individual patients.

In our patient population, 14 tumours (11.4%) demonstrated MSI phenotypes, which aligns with data reported in the literature (114). When the selected tumour regions were analysed, MSI marker expression of normal colon, normal tumour border, main tumour mass, invasive front and lymph node metastasis showed differences in intensity, but no statistically significant differences were observed among these areas and between primary tumours and their lymph node metastasis either. To our knowledge, only a limited number of studies in the literature have examined a comparable question to ours in colorectal cancer (115-117), or other types of cancers (118). Regarding sampling, the determination of MSI status from any region of the colorectal tumour can be inferred.

When analysing the MMR status of primary tumours and their corresponding liver metastases, it was observed that 20.2% of the tumours exhibited a discordant MMR status between the primary tumour and its metastasis. Varied research outcomes were obtained concerning changes in MMR status from primary tumours to liver metastases during tumour progression. Some studies have reported a higher proportion of MSI in primary tumours (119-121), while other authors have found higher incidence in the corresponding liver metastasis (122). The inconsistency could be attributed to technical errors, such as variations in staining procedures or factors, or differences in the microsatellite loci

examined (122). Another possible explanation is a genuine biological phenomenon, such as genetic instability that intensifies during tumour progression and clonal selection during metastasis (123, 124). Additionally, there could be other undiscovered reasons. In our study, the discrepancy between primary tumours and their liver metastases is not attributed to technical reasons, and we consider the use of the TMA procedure to be reliable when using appropriate positive and negative controls (125). The potential for technical error in our study is mitigated by utilising a standardized, automated immunohistochemistry technique.

Several studies, both small and large, have been published in recent years regarding the prognostic implications of sporadic MSI in colorectal cancer, with most concluding that a defective MMR status is correlated with better DFS and OS compared to MSS tumours (32, 126-129). However, our findings do not align with this conclusion. After analysing the RFS and OS of our 122 patients with follow-up data, we did not identify a prognostic significance for MSI status. The influence of MSI status on DFS and OS of adjuvant-treated colorectal cancer patients is a clinically important and heavily debated topic in the literature.

The majority of publications have examined the relationship between MSI status and the commonly used 5-FU treatment (130). Some studies have reported a higher sensitivity of MSI tumours to 5-FU treatment (131-133), while others have found no variation in treatment response according to MSI status (134). Recent studies have increasingly suggested that patients with stage II and III MSI colorectal cancer do not benefit from 5-FU-based chemotherapy (80). However, for stage II MSI colorectal tumours, better RFS and OS is maintained even with adjuvant 5-FU treatment, in contrast to MSS tumours (135). Therefore, identifying the MMR status is crucial in determining the most effective treatment strategy for patients with colorectal carcinoma. It is important to consider individual factors when deciding on the administration of adjuvant chemotherapy, and further research is needed to better understand the optimal treatment approach for patients with dMMR tumours (136, 137).

Our analysis of accurate oncological treatment data from ninety-seven patients revealed that MMR status did not serve as a predictive factor for RFS, PFS or OS in the context of various treatments, including 5-FU, oxaliplatin, irinotecan, and newer biological therapies such as bevacizumab, cetuximab, and panitumumab. Furthermore,

our analyses did not detect any statistically significant difference between MSI and MSS tumours in 5-FU-treated stage II and stage III cases.

It is important to note that due to the use of multimodal therapy and multi-line combinations of agents in our study, it was difficult to establish homogeneous patient groups. It is likely that analysing subgroups based on the expression of molecules involved in complex signalling pathways in a larger sample size may increase the statistical power of our study. This approach could potentially lead to a more comprehensive and detailed results.

PTEN loss of function is commonly found in advanced colorectal cancer, and its detection is believed to have prognostic significance and is being investigated as a potential predictor of responsiveness to anti-EGFR therapy. However, despite the widespread use of immunohistochemical assessment of PTEN expression, there is currently a lack of standardization in the field, and the results are often difficult to compare across different publications. Various silencing mechanisms such as mutations, loss of heterozygosity (LOH), promoter hypermethylation, copy number changes, or the interaction with miRNAs may result in decreased PTEN-expression or PTEN-loss.

Immunohistochemistry is a widely used and cost-effective method for detecting PTEN expression, owing to its relative insensitivity to the cause of PTEN loss and its straightforward implementation. The use of IHC for PTEN detection provides a valuable tool for assessing PTEN status in clinical and research settings. However, it is important to note that possible differences in PTEN expression may arise. This phenomenon can manifest during the pre-analytical phase (including aspects like tissue sampling technique, ischemia duration, fixation duration, temperature, and dehydration conditions) as well as throughout the IHC staining procedure (antibody concentration, diluent, variations in antibody sensitivity and specificity between tissue types, and detection method), or during the data evaluation and interpretation (such as applying different methods to consider the staining positive vs. no reaction; histoscore; different cut-off levels for PTEN loss; the intracellular localization of PTEN). Although IHC is used in the majority of recent studies, the absence of a standardized protocol can make it challenging to compare results and draw meaningful conclusions across them. In our work we focused on two critical factors: the antibody type and scoring method used to assess PTEN loss. Although multiple methods have been developed for assessing PTEN

loss, the most informative results have been obtained from the sequential studies by Sangale and colleagues, who conducted a rigorous validation process of ten different anti-PTEN clones on diverse cell lines and samples of known PTEN status. They subsequently identified the most effective clones and demonstrated the prognostic value of PTEN in a cohort of mCRC patients using the selected antibody (102, 138). To evaluate PTEN expression, we adopted their approach by employing the two top-ranked antibodies (Dako and CellSignaling) alongside a commonly used ‘nuclear’ clone (Neomarkers). As there is no widely accepted protocol for PTEN-IHC, we incorporated internal controls as recommended by recent authors. Given the variability in PTEN scoring methods, including differences in the number of positivity classes and the percentage of positive tumour cells used for dichotomization relative to the internal control, comparability across studies with different PTEN classifications and patient populations is challenging. To address this issue, we employed multiple evaluation methods and compared the results on a standardized set of cases, aiming to achieve a more robust and comprehensive understanding of PTEN expression in the studied population.

In our study, we assessed the intracellular and intratumoural heterogeneity of PTEN expression in CRC using the three above-mentioned commercially available antibodies: Dako, Neomarkers, and CellSignaling. We developed and applied three combined scoring methods that incorporated the intracellular localization, intensity, and frequency of PTEN expression, which, to our understanding, have not been applied previously. Our primary objective was to characterize the staining patterns of these antibodies and correlate them with tumour localization and clinicopathological features of CRC. Additionally, we aimed to evaluate the prognostic significance and clinical applicability of PTEN protein expression in this setting.

Our examination revealed a moderate correlation between the application of the Dako and CellSignaling antibodies, concerning both the occurrence and intensity of PTEN expression. However, the Neomarkers antibody exhibited no noteworthy correlation with the aforementioned antibodies. This discordance could be attributed to the nuclear staining preference of the Neomarkers antibody, while the Dako and CellSignaling antibodies primarily produced cytoplasmic staining. This observation is consistent with the recent validation study by Sangale et al., which found that the

Neomarkers antibody exhibited limited sensitivity and specificity for detecting PTEN loss (138).

Our study showed that the expression of PTEN was significantly lower in all examined tumour areas compared to normal colon mucosa, as detected by the Dako and CellSignaling antibodies. The Neomarkers antibody exhibited a similar trend, although it did not reach statistical significance. Staining for PTEN levels with all three antibodies showed a gradual decrease from the tumour edge to the main tumour mass, invasive front, and lymph node metastasis, indicating a potential role for PTEN in carcinogenesis and progression. The results we obtained are consistent with previous findings in the literature (139-142). Our study provides novel insights by assessing the comparative effectiveness of three frequently utilized antibodies, highlighting the need for caution when interpreting results obtained using various antibodies. These findings call for the establishment of standardized and validated protocols to ensure the optimal use of this potentially valuable tool in future research and clinical applications. We did not observe significant differences in staining intensity within tumour regions and matched lymph node metastasis, but we did observe a pattern of gradual decrease towards the invasive front. This contrasts with multiple studies reporting heterogeneous PTEN expression in CRC using IHC (138, 143). We observed comparable PTEN expression levels in both the primary tumour mass and liver metastasis, indicating a high degree of consistency regarding the PTEN status in primary colorectal cancer and its corresponding liver metastases, as reported previously (22, 102).

Our analysis did not reveal any significant differences in PTEN expression based on the location of the tumour in the large bowel, contrary to previous studies that reported lower PTEN expression in distal tumours compared to proximal tumours (28, 141, 144). Additionally, we did not observe any relationship between PTEN expression levels, as determined by the three antibodies, and clinicopathological parameters such as pT status, Dukes classification, and clinical stage. The literature reports variable results regarding the relationship between clinical stage, prognosis, and PTEN expression levels determined by IHC. Sawai et al. reported a significant association between the loss of PTEN expression and advanced TNM stage (22), whereas Taniyama et al. found no such connection between PTEN expression and stage or grade in sporadic CRC (145). Our findings suggest that these discrepancies could be attributed to differences in the



performance of diverse antibodies employed for detection and a lack of standardization in both the technical aspects of staining and analytical methodologies.

In the present study, we observed a correlation between *KRAS* status and PTEN expression only when staining was performed using the Neomarkers antibody. No correlation was found between *KRAS* status and PTEN expression levels when staining was performed using either the Dako or the CellSignaling antibody. However, this correlation was only detected in a small subset of cases that harboured mutations in exon 13. Interestingly, our negative findings using the Dako and CellSignaling antibodies align with previous studies that reported no statistically significant correlation between PTEN expression and mutations in *PIK3CA* or *KRAS/NRAS/BRAF* in primary CRC tissue samples or their corresponding liver metastases (102). We did not find any correlation between PTEN status and tumour grade using any of the antibodies, which is consistent with the findings of previous studies by Lin and Jin et al. (146, 147).

The occurrence of MSI in sporadic colorectal cancer is approximately 10-15% (148). A research identified the presence of frameshift mutations in the poly(A) tracts of the PTEN gene in colorectal cancers, indicating that PTEN may be a target of MSI-based colorectal carcinogenesis (149). We observed an MSI phenotype in 5.5% of our patient population. Furthermore, we observed the absence of a noteworthy correlation between the MSI phenotype and the levels of PTEN expression. However, the limited number of MSI tumours in our study may have resulted in insufficient statistical power to detect a significant association.

In our study, we examined the prognostic significance of PTEN expression by categorizing CRCs into PTEN-normal and PTEN-loss cases using any of the antibodies. We found that PTEN-loss, as determined by any of the antibodies, did not have any prognostic impact on RFS. Additionally, ROC analysis failed to identify a cut off H-score that could separate the cohort based on PTEN expression levels. Bohn et al. conducted a study that showed differences in the prognostic and predictive values of PTEN loss between colon and rectum cancers. Their separate analysis revealed a significant association between PTEN status and OS in rectal cancers only. In our study, there was an underrepresentation of rectal tumours which limited to perform similar investigations. Other studies have also reported no association between PTEN loss and prognosis, which is consistent with our findings (102, 146, 147).

Another objective of our investigation was to evaluate the infiltration of immune cells in colorectal cancer within systematically selected tumour regions spatiotemporally in 137 patients. We examined the full spectrum of tumour progression, from the primary tumour location to the invasive front, through lymph node metastases, and eventually to the liver. We analysed a range of leukocytes, including T cells, helper T cells, cytotoxic T cells, B cells, mature B cells/activated macrophages, and natural killer cells, as well as the immune checkpoint markers CTLA-4, PD-L1, and PD-1, in each location. Recent data indicates that the infiltration pattern of immune cells is predictive and prognostic of treatment outcomes (150). Current evidence suggests that it is important to evaluate immune cells in the primary tumours and liver metastases of colorectal cancers separately (151).

Our findings revealed an increased number of natural killer (NK) cells in the primary tumour area compared to metastatic regions, in agreement with the notion that NK cells play a crucial role in guarding against gut carcinogenesis but become exhausted with tumour progression (152). We also observed a higher occurrence of mature B cells and PD-1<sup>+</sup> expressing cells in the primary tumour mass, while no difference was detected in T cells and their subpopulations, or in the levels of CTLA-4<sup>+</sup> and PD-L1<sup>+</sup> cells between primary and metastatic samples. According to our research, B cells initially respond to the formation of the primary tumour and its effects in the local area. However, in the case of metastasis, their involvement in the interaction is less noticeable. This aligns with the understanding that B cells are part of the surveillance system that monitors the gut microbiome and the development of cancer, and their role is likely indirect through the recruitment of T cells to the tumour and the production of immunoglobulins (153). Furthermore, a higher density of B cells is associated with a more favourable prognosis in right-sided colorectal cancer (154). A greater number of metastatic lymph nodes was found to be linked to decreased B cell counts. As the lymph node metastatic status became more advanced, higher numbers of leukocytes, particularly T cells, were observed. A recent publication reported that an increased presence of T<sub>reg</sub> cells was associated with greater lymph node involvement, suggesting a potential role for this subpopulation in facilitating tumour progression (155, 156).

In the context of liver metastases we noted disparities in natural killer and leukocyte counts: fewer NK cells were identified in patients with advanced-stage disease

at diagnosis (Stage I-II vs. IV), while the leukocyte count was slightly higher in Stage IV CRC (Stage III vs. IV). Infiltration of M2-like tumour-associated macrophages has been associated with an elevated occurrence of liver metastasis in colorectal cancer, along with the facilitation of disease progression within the liver. Additionally, TGF- $\beta$ -induced epithelial-mesenchymal transition in cancer stem cells serves as a mechanism for liver metastasis formation in CRC (157). Helper T cell and PD-L1<sup>+</sup> cell counts did not show any significant differences in the selectively investigated primary tumour regions. Both our study and others found that NK cells were more prominently located peritumorally (158). Leukocytes, including T cells, killer cells, B cells, and mature B cells, as well as CTLA-4 and PD-1 expressing cells, have been found to exhibit higher expression in regions of lymph node metastasis. This finding is not unexpected, as lymph nodes serve as both communication and physiological spaces for immune cells and immune functions.

In the field of oncology, there have been various approaches to understanding this delicate interaction between the host system and the tumour. In CRC, the number of surgically removed and pathologically analysed lymph nodes is a quality measure that improves the outcome of the disease (159). Inflammatory infiltration, including lymphocytes, in the primary tumour has been found to be not only prognostic but also to yield the number of harvested lymph nodes (160, 161). These findings have been validated through multiomic annotated datasets, offering supplementary evidence that a substantial lymph node count in resections of colon cancer stems from an adaptive immune response between the host and the tumour. Therefore, efforts to maximize the number of examined lymph nodes may be misleading. Our findings offer further understanding of the immune cell composition of CRC, but additional research is necessary to obtain more precise information on their specific role in tumour initiation and progression, as well as their potential involvement in therapeutic intervention.

The NanoString method was used to investigate the gene expression of selected immune-related genes in available liver metastases with paired primary colorectal cancers. In total, 11 genes exhibited a differential expression that was statistically significant, with 7 of them being downregulated and 4 upregulated. Additionally, 29 genes displayed a differential expression pattern that was of a more subdued or marginal nature. Previous research has identified the Wnt-beta-catenin and TGF-beta pathways, as well as downstream activators of PI3K/AKT signalling, as the primary mechanisms in

the formation of CRC liver metastasis (162). However, we focused our analysis on 770 genes previously associated with immune function. Our findings indicate no discernible difference in the expression of immunological genes between right-sided and left-sided tumours, although our sample size was small. Gene set enrichment analysis revealed a reduction in the expression of genes related to innate immune responses, and we observed marginal increases in the expression of *LTB* and *TNFRSF8* genes, which are part of the TNF superfamily and their receptors. A limited number of studies investigated signalling associated with liver metastasis formation in CRC; it was however documented that genes during tumour progression were strongly associated with either the cell adhesion/focal adhesion/chemokine signalling pathway/PI3K-AKT signalling pathway or innate immune response/complement activation/acute-phase response (163-165).

Upon analysing the prognosis prediction grounded in the expression of genes within primary colorectal cancer, we found that a decreased DSS was associated with a lower *TNFRSF8* (CD30) expression, and a decreased PFS was seen with higher *DEFB1* counts. In liver metastases, shorter PFS was associated with a lower *C4B*, *CFI*, and *IL1RAP* expression and higher *CARD9* counts. Only a few studies have investigated the signalling pathways involved in liver metastasis formation in CRC. However, previous research has shown that genes associated with tumour progression are strongly linked to either the cell adhesion/focal adhesion/chemokine signalling pathway/PI3K-AKT signalling pathway or the innate immune response/complement activation/acute-phase response. In our analysis of gene expression in primary CRC, we found that a shorter DSS was associated with decreased expression of *TNFRSF8* (CD30), and a shorter PFS was associated with increased expression of *DEFB1*. In liver metastases, a shorter PFS was linked to decreased expression of *C4B*, *CFI*, and *IL1RAP*, while increased expression of *CARD9* was associated with shorter PFS. In CRC progression, the genes *C4B*, *MBL2*, *CARD9*, and *TNFRSF8* were found to impact DSS, while *IL27* and *LTB* were prognostic for PFS, and *DEFB1* had a significant effect on both DSS and PFS (166). These markers have been documented across various cancer types with varying patterns of expression, and some have implications for therapy in oncology or other specialties(167). Of particular importance in the pro- and anti-tumorigenic inflammation in CRC are *CARD9* and leukotriene signalling, which require further research to better understand the

resistance mechanisms against immunotherapy in CRC and other solid tumours (168, 169).

Limitations of our studies include the followings. To gain a more comprehensive understanding of potential predictive factors for treatment response in MSI CRC patients, it would have been advantageous to augment our study by analysing additional pathobiological factors, such as *BRAF*, and expanding the cohort size. To determine PTEN-loss, more standardized studies should be carried out, with a focus on larger and more homogeneous patient populations, utilising established validation studies and standardized immunohistochemistry protocols. The use of digital evaluation methods may also would have been beneficial. The immune study did not conduct a comprehensive assessment of immune-based biomarkers and immune checkpoint markers across all tumour regions for the entire study population. Additionally, gene analysis via NanoString was limited to only a subset of patients. Another constraint of the study was that only the NanoString subpopulation could be analysed for patient survival, as different treatment options were available for older and later patient enrolments, leading to potential bias in survival data analysis. The NanoString subpopulation was homogeneous with respect to the time of diagnosis, surgery, and other relevant factors.

## 6 Conclusion

Despite numerous studies, personalized therapy for CRC continues to pose a significant challenge. The heterogeneity of CRC has been well-recognized and must be considered in clinical decision-making at multiple levels, including epigenetic, genetic, transcriptomic, proteomic, and microenvironmental levels (170). Based on the outcomes of our investigations, we deduce the subsequent conclusions:

- Our cohort MMR status did not have a prognostic nor predictive impact on RFS and OS with any of the provided oncological treatments.
- There was no significant difference in the therapeutic response observed between MMS and MSI tumours in stage II and III CRC when treated with 5-FU.
- No significant difference was identified among the three scoring methods we employed concurrently to evaluate PTEN expression, considering three principal factors: intracellular localization, intensity, and frequency.
- PTEN expression did not show statistically significant variation in staining among tumour regions, lymph nodes, or localisations in the colon. Our observations were limited to a gradual decrease trend observed towards the invasive front.
- Neither of the administered PTEN antibodies demonstrated prognostic significance in any of the conditions tested.
- In our immune study we observed a higher quantity of NK cells and more mature B cells expressing PD-1 in the primary tumour region compared to metastatic lymph nodes, where B cell counts were significantly lower and leukocyte counts were higher.
- 11 differentially expressed immune-related genes were identified between primary tumour and liver metastasis samples, highlighting significant alterations in the innate immune response and the TNF superfamily pathways. These changes in gene expression were linked to shorter survival times in CRC patients.

## 7 Summary

**Objectives:** This study aimed to perform a detailed analysis of selected CRC samples and consecutive metastases to evaluate the frequency, heterogeneity, prognostic, and predictive potential of various biomarkers.

**Methods:** Selected regions of CRC specimens and corresponding lymph nodes, and/or liver metastases were evaluated using tissue microarrays and immunohistochemistry. Different biomarkers such as MSI, PTEN, and tumour infiltrating immune cells and immune checkpoint markers of colorectal cancer patients were investigated. The study also performed an immune panel gene expression assay on 12 primary tumours and 12 liver metastases.

**Results:** There was no significant difference in MMR status between tumour regions and lymph nodes, however in 14 cases, there was a difference between the primary tumour and liver metastases. MMR status was not found to have prognostic or predictive values. For stage II and III CRC treated with 5-FU, there was no significant difference in MMR status between MMS and MSI tumours. The main tumour region showed an elevated count of natural killer cells, mature B cells, and PD-1+ expressing cells in comparison to metastases. A significant decrease in B cell counts was noted with an increase in the number of metastatic lymph nodes. Advanced lymph node metastasis was correlated with higher leukocyte counts, particularly T cells. A set of eleven immune-related genes exhibited differential expression between primary tumours and liver metastases. Additionally, modifications in the innate immune response and the tumour necrosis factor superfamily pathways were observed. The Dako and CellSignaling PTEN antibodies stained the cytoplasm, while the Neomarkers PTEN antibody stained the cell nuclei. PTEN expression was significantly lower in patients with mKRAS (exon13). Although PTEN expression decreased in colorectal cancer, neither of the three PTEN antibodies used demonstrated a significant correlation with clinicopathological data, nor did they have any prognostic value.

**Conclusions:** Although our findings offer a deeper understanding of the role of certain biomarkers in CRC, further investigations are necessary to obtain more accurate information regarding their precise involvement in tumour initiation and progression, as well as their potential role in therapeutic interventions.

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## 9 Bibliography of the candidate's publications

### 9.1 Publications related to the phd thesis

Ágoston EI, Baranyai Z, Dede K, Bodoky G, Kulka J, Bursics A, Harsányi L, Szász AM. **Occurrence, intratumoural heterogeneity, prognostic and predictive potential of microsatellite instability following surgical resection of primary colorectal carcinomas and corresponding liver metastases.** Orv Hetil. 2015 Sep 6;156(36):1460-71. Hungarian. doi: 10.1556/650.2015.30218. PMID: 26320600. Impact factor: 0.291

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### 9.2 Publications not related to the phd thesis

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