CORRELATION OF TUMOR ASSOCIATED MACROPHAGES (TAMS) DENSITY AND M2 PROPORTION WITH THE PATHOLOGICAL STAGE OF COLORECTAL CANCER

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BY

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MBBS

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Dedicated to Arsalan, my husband

For his endless support

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LIST OF ABBREVIATIONS

S. NO.	ABBREVIATIONS	STANDS FOR
1	CRC	Colorectal cancer
2	TAMs	Tumor associated macrophages
3	IGF	Insulin like growth factor
4	APC	Adenomatous polyposis coli
5	IBD	Inflammatory bowel disease
6	MSI	Microsatellite instability
7	HNPCC	Hereditary non- polyposis colorectal cancer
8	MMR-D	Mismatch repair deficient
9	FAP	Familial adenomatous polyposis
10	CHRPE	Congenital hypertrophy of retinal pigment epithelium
11	ESMO	European society of medical Oncologists
12	MAP	MutY homolog- associated polyposis
13	HMPS	Hereditary mixed polyposis syndrome
14	JPS	Juvenile polyposis syndromes
15	PJS	Peutz- Jeghers syndrome
17	CIN	Chromosomal instability
18	HP	Hyperplastic polyp
19	SSA/P	Sessile serrated adenoma or polyp
20	TSA	Traditional serrated adenoma
21	MSS	Microsatellite stable
22	MMR-P	Mismatch repair proficient
23	CIMP	CpG island methylator phenotype
24	CMS	Consensus molecular subtype
25	TME	Tumor microenvironment
26	EVs	Extracellular vesicles
27	TANs	Tumor associated neutrophils
28	CAF	Cancer-Associated Fibroblasts
29	EMT	Epithelial to mesenchymal transition
30	VEGF	Vascular endothelial growth factor

31	TEC	Tumor-derived endothelial cells
32	LPS	Lipopolysaccharides
33	IFN γ	Interferon gamma
34	TNFα	Tumor necrosis factor, alpha
35	TGF β	Transforming growth factor beta
36	pTNM	Pathological staging
37	EO-CRC	Early onset- colorectal cancer
38	IHC	Immunohistochemistry
39	IF	Immunofluorescence
40	FFPE	Formalin fixed paraffin embedded
41	DFS	Disease free survival
42	OS	Overall survival
43	NCCN	National comprehensive cancer network
44	CAP	College of American pathologist
45	USPSTF	United States preventive services task force

ABSTRACT

Colorectal cancer (CRC) is the second most common malignancy among adults in Pakistan. With increasing focus on precision medicine and personalized chemotherapy, focus has shifted to finding a finer print in the pathological assessment. Tumor Associated Macrophages (TAMs) can serve as an additional pathological marker to enable further therapeutic targets and improve cancer therapy for CRC. TAMs have two polarization states – anti-tumor M1 and protumor M2. This cross-sectional study was conducted to determine statistical correlation of M2-macrophage proportion among all TAMs and TAM density in tumor stroma and tumor front, with the pathological stage of colorectal cancer, at the PNS Shifa Hospital, Karachi, during a period of six months. Clinical and pathological records were reviewed. With anti-CD68+ determining the total TAMs infiltrate and anti-CD163+, the M2 polarized macrophages, the proportional distribution of M2 was ascertained and correlated with tumor histological stage along with other clinico-pathological parameters. Histopathology specimen of 43 patients were selected. Among the included patients, two-thirds were male patients (n=30, 69.8%) with a median age of 54 years (Range 18-80 years of age). Tumors were most commonly found in cecum, sigmoid colon and rectum (combined n= 29, 67.4,%) with overall propensity toward the left side of the colon, along with rectum (n=25, 58%). The tumor were mostly well differentiated (n=21, 48.8%) adenocarcinoma (n=36, 83.7%), staged as pT3 (n=26, 60.5%), pN1 (n=23, 53.3%) with a median size of 4.5 cm in the greatest dimension. A small proportion of tumor had identified tumor perforation (n=3, 7%) and perineural invasion (n=9, 20.9) but more than half the specimen showed lympho-vascular invasion (n=24, 55.8%). Overall TNM stage III was more prevalent (n=26, 60.5%). The tumor grade and dimensions were independently distributed in relation to the age groups. There was a higher frequency of cancer in right colon among males (n=15, 50%), compared to females. The two measures that showed significant difference between the two genders were macroscopic tumor perforation and lympho-vascular invasion. There was no significant difference in TAMs

density and M2 proportion, in the tumor stroma and tumor front, tumor size, pathological tumor grade and pathological tumor stage. Lympho-vascular infiltration showed significant statistical difference in the TAMs density, at the tumor front. Otherwise, no difference was found for macroscopic tumor perforation, perineural invasion as well as the lympho-vascular invasion in the tumor stroma. For M2 proportion also no difference was found in any of these categories. Comparison of TAMs density and M2 proportion, for early versus late-stage CRC also did not reveal any statistical difference. Based on logistic regression analysis, sensitivity was calculated to be 84.6% with a specificity of 76.5% for modified model. In conclusion, TAMs density and M2 proportion, in the tumor stroma and the tumor front of colorectal cancer specimens, did not reach statistically significant correlation in this study with the tumor stage. The predictability of advanced tumor stage (TNM stage 3 or 4) when assessed based on TAMs density and M2 proportion only, had a low sensitivity and specificity and was not statistically significant but the predictability improved remarkably for the model that also included the tumor grade and lympho-vascular invasion, reaching statistical significance.

Keywords: Colorectal cancer, TAMs, M2 macrophages, CD68+, CD163+. Immunohistochemistry, pathological stage

CHAPTER 1

INTRODUCTION

1.1 Introduction

Colorectal cancer (CRC) is the third most common malignancy worldwide (Rawla, Sunkara, & Barsouk, 2019) and the second most common among adults in Pakistan according to a referenced cancer registry (Yousaf, 2018). With increasing focus on precision medicine and related personalized chemotherapy, focus has shifted to finding a fine print in pathological assessment and reporting of cancers (Tran et al., 2015). This has direct bearing on treatment and prognosis of patients with colorectal cancer as well (Yau, 2019). During the last decade significant research has been done to establish Tumor Associated Macrophages (TAMs) as an additional pathological marker to enable further therapeutic targets and improve cancer therapy that includes CRC (Y. Chen et al., 2019; Waniczek et al., 2017). Aspects of the CRC epidemiology and related risk factors, basis of anatomical pathology and tumor pathogenesis warrant additional discussion to elaborate the theoretical basis of this treatise.

1.1.1 Epidemiology

Colorectal cancers (CRCs) collectively are the third commonly diagnosed malignancies worldwide, with colon cancer being the fourth common and rectal cancer eighth in line. It is also the second highest cause of cancer related mortality with estimated 881,000 deaths in 2018. The incidence is likely to increase by 60% by the year 2030, with estimated 2.2 million new cases and 1.2 million deaths globally (Bray et al., 2018). A global pattern of increasing incidence of CRC is also being identified in younger population aged between 20-49 years (Rawla et al., 2019) partly attributable to the sedentary lifestyle and changes in the dietary pattern (Arnold et al., 2017).

In Pakistan as well CRC represents second most common malignancy overall and the most common malignancy among men. Additionally, CRC affects a disproportionate number of younger Pakistani population, with estimated 50% affected patients found to be less than 50 years of age according to an institution- based epidemiological study (Yousaf, 2018). Younger patients present with aggressive and advanced disease due to the lack of awareness regarding the disease and non-existent screening programs (Hasan et al., 2017; Tajamal, 2019).

To address this burgeoning challenge, improved cancer therapy and patient survival can be achieved by better prognostication at diagnosis with molecular markers and more importantly finding newer therapeutic targets for better treatment.

1.1.2 Risk Factors

The development and progression of Colorectal cancer is a multistep process accompanied by the gradual involvement of various oncogenic pathways. The tumor starts as a non- neoplastic mucosal proliferation called polyp or adenoma. Over a period of 10-20 years, this benign lesion accumulates mutations involving the oncogenes, tumor suppressor genes and the mismatch repair genes (Sierra, 2016). Thus, giving rise to invasive cancer.

Majority of the colorectal cancers are sporadic. The incidence of sporadic cancer has been increasing in the countries experiencing economic transition, probably due to the adoption of lifestyle of the developed nations. This mainly includes the changing dietary and activity patterns. Both these lifestyle modifications have been shown to be associated with promotion of intestinal inflammation and changes in the gut flora, providing a fertile soil for the development of polyp and its progression to cancer. Similarly, hereditary factors facilitate hyperproliferation and carcinogenesis by conferring selective advantage to some mucosal epithelial cells. In short, CRC is potentially preventable through the identification and modification of lifestyle associated risk factors as well as genetic testing and regular screening for non- modifiable risk factors (Arnold et al., 2017; Rawla et al., 2019).

1.1.2.1 Modifiable Risk Factors

Strong evidence exists, linking sedentary lifestyle and obesity with metabolic abnormalities and persistent low- grade inflammation, leading to increased risk of colorectal cancer (Friedenreich, Ryder-Burbidge, & McNeil, 2020). Modification of these risk factors can result in 30- 40% decrease in the global incidence of CRC (Poirier et al., 2019).

Obesity and Physical Inactivity

Physical inactivity and obesity are established risk factors for various cancers including the colorectal cancer (McTiernan et al., 2019). Decreased physical activity and obesity, especially the abdominal obesity is associated with insulin resistance leading to hyperinsulinemia (Avgerinos, Spyrou, Mantzoros, & Dalamaga, 2019). Insulin resistance results in increased circulating levels of insulin and insulin like growth factor (IGF)-1, involved in angiogenesis and cell proliferation.

Furthermore, adipocytes play an important role in promoting inflammation by secreting various pro- inflammatory cytokines (Interleukin-6 and Tumor necrosis factor - alpha) and adipokines like leptin (Booth, Magnuson, Fouts, & Foster, 2015).

Incorporating variable degrees of exercise in one's daily routine lowers the risk by reducing the time that the carcinogens stay in contact with the colonic epithelium, due to increased gastrointestinal transit time (Friedenreich et al., 2020)

Diet

Association of dietary factors with CRC differ according to the gender and site of the tumor. High carbohydrate intake increases the risk of right sided colon cancers in females but rectal cancers in males. High fat intake (polyunsaturated fat, cholesterol and sucrose) has been related to an increased risk of right sided colon cancer. Red and processed meat increase the risk, owing to the formation of polycyclic hydrocarbons during cooking. Though the exact mechanism is still not clear (Kim et al., 2015). Fiber, fruits and vegetables have protective effect. The role of folic acid is contradictory. Increased levels of Vit D, iron and calcium are inversely related to the risk (Song, Garrett, & Chan, 2015). Diet rich in soy products also lead to cancer risk reduction in females, probably due to the metabolic and structural similarities to estrogen.

Tobacco

Carcinogens found in tobacco and tobacco smoke have been linked to an increase in the precursor lesions of CRC i.e., the adenomatous polyps. The heterocyclic compounds present in tobacco smoke have been described to be responsible for mutation in adenomatous polyposis coli (APC) gene in rats as well as in humans (Sierra, 2016). Smoking is also associated with tumors exhibiting KRAS and BRAF mutations, as well as CpG island methylator phenotype (Dolatkhah et al., 2018).

Alcohol

Strong evidence suggests the causal relationship of moderate to heavy alcohol consumption to colorectal cancers. Acetaldehyde, a metabolite of ethanol is the major carcinogen identified and acts as a genotoxic agent (Bagnardi et al., 2015). Folate, vitamins A, B12 and B6 deficiencies result due to impaired absorption attributable to heavy alcohol consumption. Folate is required for the synthesis, repair and methylation of DNA.

NSAIDS

Colorectal cancer and cardiovascular disease (CVD) have a number of risk factors in common, like, older age, diabetes, obesity, physical inactivity and smoking. Aspirin usage was recommended in 2016 for the primary prevention of CVD and colorectal cancer. Till to date, multiple studies have confirmed the favorable role of Aspirin and other NSAIDS in lowering the risk of adenomas progressing to CRC (Seaton et al., 2019).

Hormones

Oral contraceptives and estrogen only hormone replacement therapy have a protective role to offer in pathogenesis of CRC. Female hormones are associated with decreased production of insulin like growth factor-1 and secondary bile acids, both having a role in carcinogenesis (Sierra, 2016). Cautious use of these hormones is recommended as the risks may overshadow the benefits.

1.1.2.2 Non- Modifiable Risk Factors

About 20% of the cases of colorectal cancer are familial, defined as more than one, affected first degree relative. The risk of developing the disease is 2-4 times higher as compared to general population, particularly in population below 45 years of age (De Rosa et al., 2015). About 7-10% of CRCs are hereditary, evolving from non- polyposis or polyposis syndromes. Inflammatory bowel disease (IBD) accounts for only 1-2 % of cases (Sehgal et al., 2014).

Race and Ethnicity

Race and ethnicity dictate the differences in incidence and survival of CRC in different nations across the world. These variations further reinforce the role of genetic and environmental factors in the pathogenesis of CRC. The African American population of the

USA show increased incidence and decreased survival rates as compared to whites. Access to quality health care is a notable factor as well (Rawla et al., 2019).

Variations in the incidence of CRC among different ethnicities range from 4- 6.8% in Pakistan (Badar & Mahmood, 2017; Qureshi et al., 2016). Increased consumption of smoked meat has been attributed to the higher incidence of CRC in Pathans and Balochis (Idrees, Fatima, Abdul-Ghafar, Raheem, & Ahmad, 2018).

Gender

Males have 1.5 times increased propensity of developing CRC (Bray et al., 2018). The proximal colon cancers are common in females. These are associated with MSI and are considered more aggressive than the left sided ones. The 5year survival rate for CRC is lower in females as compared to males, especially in females over 70 years. This could be due to the lack of estrogen in older women, which is considered a defense factor against microsatellite instability (MSI) (Kim et al., 2015).

Hereditary Syndromes

Hereditary non- polyposis colorectal cancer (HNPCC) syndrome or Lynch Syndrome is an autosomal dominant syndrome, responsible for 2% - 4% of all the cases of CRC and 70% - 80% lifetime risk (Wilkins, McMechan, Talukder, & Herline, 2018). It due to a heterozygous mutation in one of the DNA mismatch repair (MMR) genes, MLH1, MSH2, MSH6 and PMS2, leading to microsatellite instability (MSI)(Schubert, Morreau, de Miranda, & van Wezel, 2020). MLH1 mutations are responsible for young- onset CRC and MSH2 mutations are notorious for increased risk of extracolonic manifestations (Byrne & Tsikitis, 2018), with endometrial cancer being the commonest. Screening colonoscopy

starting as early 25 years is recommended for first degree relatives of patients with HNPCC (Balmaña, Balaguer, Cervantes, & Arnold, 2013).

Familial adenomatous polyposis (FAP) is an autosomal dominant syndrome, which accounts for about 1% of all the cases of CRC. Resulting due to the heterozygous mutation in the Adenomatous polyposis coli (APC) gene, the diagnosis is based on the presence of at least 100 adenomas. By the time the patient reaches 40- 50 years, the risk of developing CRC nears 100%. 95% of the patients with FAP also develop duodenal adenomas. Other extraintestinal cancer sites include stomach, thyroid and biliary tree. Congenital hypertrophy of retinal pigment epithelium (CHRPE) is also diagnostic presenting as patchy discoloration of the eye's fundus (Byrne & Tsikitis, 2018). The European Society of Medical Oncologists (ESMO) advises sigmoidoscopy, every 2 years for the patient and relatives, starting age 12-14 years. Upper GI endoscopy and cervical ultrasound are part of screening too (Balmaña et al., 2013).

Attenuated FAP also results due to the germline mutation in APC gene but is characterized by the presence of less than 100 adenomas and development of CRC in the sixth or seventh decades of life. Patients may present with duodenal adenomas but CHRPE is absent. ESMO guidelines suggest colonoscopy starting at the age of 18- 20 years (Byrne & Tsikitis, 2018).

MutY homolog- associated polyposis (MAP) is an autosomal recessive syndrome, usually diagnosed in the fourth decade of life. It is characterized by the presence of less than 100 adenomas and has features including proximal location, a mucinous component and increased number of tumor infiltrating lymphocytes, that overlap lynch syndrome and sporadic MSI tumors (Valle, 2014).

Other rare polyposis syndromes include polymerase proofreading associated-polyposis and hereditary mixed polyposis syndrome (HMPS). Both have dominant pattern of inheritance. Former results due to mutations in DNA polymerase Ɛ (POLE) and δ (POLD1) .It presents as multiple large adenomas and early onset CRC. Female carriers have increased risk of endometrial cancer (Valle et al., 2014). HMPS, on other hand is characterized by the presence of mixed population of hamartomatous polyps and adenomas but lacks extraintestinal manifestation (Valle, 2014)

Juvenile polyposis syndromes (JPS), Peutz- Jeghers syndrome (PJS) and PTEN hamartoma tumor syndrome (PHTS) come under the heading of hamartomatous polyposis syndromes. Germline mutations in SMAD4 or in BMPR1A have been identified in 40% of the patients with JPS. Either a positive family history, presence of 3-10 juvenile polyps in colorectum or polyps in stomach and small intestine are required to meet the diagnostic criteria. Risk of developing gastrointestinal malignancies lies between 9%- 50%.

PJS is an autosomal dominant syndrome characterized by the presence of germline mutation in STK11, a tumor suppressor gene. The diagnosis of PJS is based on the presence of specific hamartomatous polyp comprising of a central core of smooth muscle, showing a tree like branching pattern and covered by the native mucosa (Byrne & Tsikitis, 2018). Mucocutaneous pigmentation involving the lips , nostrils, labial mucosa and perianal region is characteristic, present in 95% of the patients with PJS. The risk of colon, small bowel, pancreas, breast, ovary, uterus and testicular malignancies significantly increases in patients with PJS.

PHTS comprises of multiple rare syndromes, including the Cowden syndrome with PTEN mutations. The lifetime risk of developing CRC is 9% and 85% for breast cancer (Tan et al., 2012). Other associated anomalies include autism and microcephaly.

Although , all the discussed syndromes are rare but in order to devise a patient specific treatment and surveillance plan, it is necessary to know their lifetime cancer risks.

Inflammatory Bowel Disease (IBD)

IBD is responsible for 1% - 5% of the cases of CRC, depending on the duration of disease. The risk of CRC is 2- 6 times higher in IBD patients than the general population. Patients $\leq$ 15 years of age comprise the highest risk group probably because of the longer duration of disease and the more aggressive phenotype (Dulai, Sandborn, & Gupta, 2016). Both the variants of IBD, namely ulcerative colitis and Crohn’s disease are considered autoimmune with a hereditary component (Rawla et al., 2019).

Crohn's disease involves the perianal region, colon and terminal ileum in a discontinuous fashion with transmural inflammation, granulomas, fissuring and ulceration. Strictures are common. Ulcerative colitis on other hand, can involve the entire colon, including the rectum in a continuous manner. The inflammation is limited to mucosa and submucosa. Crypt abscesses are common. Chronic inflammation plays a key role in IBD-CRC through the release of cytokines and chemokines and their effect on the epithelial cells growth and survival (Guan, 2019).

CRC in IBD is believed to develop from multifocal dysplasia in contrast to sporadic CRC, which arise from one or two foci of dysplasia. Chromosomal instability (CIN) and microsatellite instability (MSI) follow the same frequency as in sporadic CRC but the mutations can be identified long before the definite diagnosis of dysplasia can be made. Thus, the IBD related CRC follows an "inflammation- dysplasia- carcinoma" pattern, rather than the classical "adenoma- carcinoma" sequence (Keller, Windsor, Cohen, & Chand, 2019).

The aim of surveillance is the early detection of dysplasia through regular colonoscopies and colectomy if dysplasia is detected, so as to reduce the risk of progression to CRC (Dulai et al., 2016).

In short, lifestyle modifications and surveillance hold the key to the reduction in risk and mortality associated with CRC.

1.1.3 Pathological basis of colorectal cancer

Developing embryologically from the midgut, hindgut and the proctodeum, large intestine or colon extends from the cecum to the anal canal. It measures between 1100-2108mm in adults (Wozniak, Pytrus, Kobierzycki, Grabowski, & Paulsen, 2019) and consists of cecum, appendix, ascending colon, transverse colon, descending colon , rectum and anal canal. The large intestine receives its blood supply mainly from the branches of the superior and inferior mesenteric arteries and their corresponding veins. Sympathetic, parasympathetic and sensory nerve supply is via superior and inferior mesenteric plexus (Kahai, Mandiga, & Lobo, 2019)**.**

Histologically, it is composed of mucosa, submucosa, muscularis externa and serosa. The mucosa is devoid of villi but contains deep crypts to perform the prime function of reabsorption of water, essential nutrients and minerals.

The finer print of colon epithelial cell regeneration holds the key to understanding the aberrant transition that leads to colon cancer. Located at the base of the crypts, the pluripotent colon stem cells continually regenerate for renewal of epithelium. Differentiation follows during migration out of the crypt into enterocytes and specialized epithelial cells including goblet, Paneth and endocrine cells. In about two weeks, these cells experience programmed cell death or apoptosis followed by shedding and eventual disposal with feces (Rawla et al., 2019). An array of signaling proteins meticulously choreograph this highly regulated transition, commoner examples being TGF-β and WNT (Medema & Vermeulen, 2011). It is during this continual process that cells acquire mutations. When an inciter like the localized inflammatory process or increased epithelial breakdown or alternatively a food-derived toxin induces heightened epithelial proliferation, the process of mutation also accelerates. A typical example of this would be Inflammatory Bowel Disease (IBD). The genetic and epigenetic mutation would be directed to impart selective survivability advantage to these hyper-proliferating cells by delaying cell death and immortalizing these eventually (Ewing, Hurley, Josephides, & Millar, 2014). This simplified representation of adenoma-carcinoma sequence is thus imbedded into the histological milieu of the living colonic epithelium.

Thus, tumorigenesis of colorectal cancer is a multistep process, resulting secondary to the accumulation of genetic and epigenetic mutations, through the involvement of a number of molecular pathways. In an era of personalized treatment, it is important to understand these pathological processes for devising novel treatment plans for the patients.

1.1.3.1 Neoplastic Precursor Lesions

Neoplastic precursor lesions are largely divided into two main types based on the histological features: Conventional adenomas and serrated polyps.

Adenomas are the established precursors of CRC and account for two third of all the colonic polyps. Adenomatous polyps are more common in males and are also responsible for the development of CRC in patients with hereditary polyposis syndromes like FAP and MAP. Patients of African- American descent may present with large right sided adenomas and early onset CRC (< 50 years). Only, 5% or less adenomas progress to cancer over a period of 7- 10 years, with the risk being greatest for advanced adenomas i-e high grade dysplasia, a villous component or size > 10 mm.

Grossly, adenomas may be sessile, pedunculated, depressed or flat. Histologically, the adenomas can be classified as tubular, villous and tubule-villous.

- Tubular adenomas are the commonest, accounting for 80% of the colonic adenomas and comprising a network of branching epithelium. A tubular component of at least 75% is required to be classified as tubular.

- A villous component of at least 75% is necessary for the diagnosis of villous adenomas. The long glands extend right from the surface to the center of the polyp. Villous adenomas account for 5%- 15% of all the adenomas.

- Tubulo-villous adenomas have 25%- 75% villous component and account for 5%- 15% of all the adenomas.

All adenomas have some degree of dysplasia, and should be resected completely (Macrae, 2020).

Named so because of the characteristic saw tooth (serration) growth pattern, serrated polyps are grouped into, hyperplastic polyps (HP), sessile serrated adenoma or polyp (SSA/P) and traditional serrated adenoma (TSA).

- HP are the commonest and are usually smaller than 5mm. These polyps are common in left colon and rectum with a debatable risk of malignant potential.

- SSA/P make up 2%- 15% of all the colon polyps, range in size between 5-10mm and are common in right colon. The role of these polyps has now been confirmed as the precursor lesion for MSI-H cancers evolving through the serrated pathway.

- TSA is a rare variant, common in left colon and rectum. Some studies hold it responsible for an aggressive type of CRC (Gibson & Odze, 2016).

1.1.3.2 The Molecular Basis of Colorectal Cancer

The two major molecular pathways responsible for the pathogenesis of CRC are, the chromosomal instability pathway (CIN) and the serrated pathway. Both follow the "adenoma- carcinoma sequence" along with the step-by-step accumulation of mutations, thus resulting in genetic instability.

- CIN pathway is responsible for almost 80% of the cases of CRC and is the most well understood pathway. Conventional adenomas are the precursor lesions. Mutations in this pathway involve the APC, KRAS, SMAD4 and p53 genes. Carcinomas developing through this pathway are microsatellite stable (MSS) and mismatch repair proficient (MMR- P).

- 15% of the CRC develop through the serrated pathway. Sessile serrated adenoma/polyp (SSA/P) are the precursor lesions. BRAF gene is mutated along with the epigenetic silencing of MLH1 gene. These tumors have high levels of microsatellite instability (MSI-H) and are mismatch repair deficient (MMR-D) (Gibson & Odze, 2016).

Chromosomal Instability

Chromosomal instability results due to loss of function of APC and other tumor suppressor genes. This in turn leads to the changes in the chromosome copy number. Under normal conditions APC gene detects the misaligned chromosomes during mitosis along with the regulation of spindle microtubules.

APC is also a part of WNT signaling pathway, where its role is to control the β-catenin activity. Under resting conditions when the colonic epithelial cells are not stimulated by WNT molecules, APC gene binds β- catenin, thus leading to its destruction and keeping the levels under control. In the proliferating colonic epithelial cells, under the influence of WNT signaling, the APC- β catenin complex dissociates. β catenin cannot be degraded and its levels in the cytoplasm increase. It then translocates to the nucleus and after binding TCF leads to the proliferation of colonic epithelial cells (Kumar, 2015).

Loss of APC results in stabilization of β catenin and the resulting activation of the Myc and Cyclin D1 leads to uncontrolled cell proliferation- a seminal event for tumorigenesis (Al-Sohaily, Biankin, Leong, Kohonen-Corish, & Warusavitarne, 2012).

Microsatellite instability

Microsatellites are short repetitive segments of mono and di nucleotides scattered throughout the genome. This abundance makes them prone to mutations during replication.

MSI results due to the hypermethylation or somatic and germline mutations in the DNA mismatch repair genes (MMR). The loss of proofreading results in accumulation of mutations throughout the genome. The resultant increase in the size of microsatellite, especially if its located in the codon region of the gene may result in altered gene function in terms of protein product. Somatic mutations are responsible for 15% of all the cases of CRC, with a greater predilection for female gender, older age, a proximal colon location and a mucinous histology. While germline mutations result in Lynch syndrome (Battaglin, Naseem, Lenz, & Salem, 2018).

Aberrant DNA Methylation

DNA methylation, leading to gene silencing is necessary for regulating gene expression. This methylation is carried out by DNA methyltransferases and usually involve the cytosine bases. CpG islands are 300- 3000 bp regions of DNA with a GC content of > 50%, located within and close to the promoter region. These islands are often unmethylated in normal colonic epithelial cells (Wang et al., 2018). In case of CRC, there is hypermethylation of these islands located in the promoter region, leading to the silencing of tumor suppressor genes (El Bairi et al., 2018). This results in CpG island methylator phenotype (CIMP), which accounts for 15% of the cases of CRC. There is associated MSI due to the loss of expression of MLH1(Ewing et al., 2014).

1.1.3.3 Consensus Molecular Subtypes

Keeping in view the heterogenous nature of CRC, in the recent times more attention has been paid to the underlying molecular mechanisms. For this purpose, the Colorectal Cancer Subtyping Consortium introduced a novel classification system in 2015 based on transcriptional profiling. This classification system divides CRC into four molecular subtypes for better prognostication and targeted therapy.

- **CMS1** accounts for 14% of all the CRC, of which 12% are sporadic and the rest are inherited (LS). Precursor lesion for this subtype is serrated adenoma. Adjuvant therapy is not recommended for stage II tumors while patients with stage III tumors do not benefit from fluorouracil therapy alone. The 5 year survival rate is 73%.

- **CMS2 (canonical)** accounts for 39% of the cases of CRC. Precursor lesions are conventional adenomas. This sub type is characterized by the loss of APC and Tp53, tumor suppressor genes, along with the activating mutations in KRAS. For patients with stage III disease standard adjuvant chemotherapy is recommended. The 5 year survival for this subtype is 77%.

- **CMS3 (metabolic)** accounts for 13% of the cases. This subtype exhibit CIN but the somatic copy number alterations are low as compared to CMS 2 and CMS4. KRAS mutations are the most frequent in this subtype. Enhancement in glutamine, fatty acid and lipo-phospholipid metabolism was observed, when studied with messenger RNA. Treatment options are limited due to a high percentage of KRAS mutations and the absence of an identifiable gene target. The 5 year survival rate is 75%.

- **CMS4 (mesenchymal)** tumors show MSS, CIN and a very high somatic copy number alteration. The tumor microenvironment is inflammatory owing to high TGFβ signaling as compared to CMS1. Mutated gene include APC, KRAS, TP53 and PIK3CA. These tumors are often diagnosed late thus, responsible for the worst 5 year survival of 62% (Thanki et al., 2017).

In short, awareness about these molecular subtypes is important in order to tailor the treatment according to the individuals mutations and the pathways involved.

1.1.4 Adenocarcinoma and it's Histologic Variants

Adenocarcinomas with intestinal differentiation account for more than 90% of the colorectal malignancies. Carcinomas with mucinous, serrated, and signet ring cell differentiation are less common but warrant a little discussion. Other uncommon variants described by WHO include, neuroendocrine, squamous cell, adenosquamous, and undifferentiated carcinomas.

The terms adenocarcinoma NOS (not other wise specified), adenocarcinoma with intestinal differentiation and conventional adenocarcinoma are synonymous (Sternberg, 2015) (Fleming, Ravula, Tatishchev, & Wang, 2012). Conventional adenocarcinomas arise from the epithelial lining of the colorectal mucosa and are composed of glands with cells showing dysplastic features. This glandular differentiation forms the basis of tumor grading that correlates with the survival. 15%- 20% of the colorectal adenocarcinomas are grade I or well differentiated (> 95% of gland formation), 60%- 70% are grade II or moderately differentiated (between 50%- 95% of gland formation) and 15%- 20% are grade III or poorly differentiated (< 50% of gland formation) (Sternberg, 2015). The grading criteria discussed above are only applicable to the conventional adenocarcinomas.

Recently a two tiered grading has been proposed, which classifies tumors into low and high grade, based on glandular differentiation of less or greater than 50% respectively. WHO recommends assigning grades based on the least differentiated areas. Tumor buds and poorly differentiated cell clusters are considered high grade because of non-existent glands (M. Johncilla & Yantiss, 2020).

1.1.4.1 Mucinous Adenocarcinoma

Mucinous adenocarcinoma accounts for 10%- 20% cases of CRC and is more common in younger patients. These tumors occur commonly in the promixal colon and

have already metastasized to the lymph nodes and the distant structures at the time of diagnosis. Poor response to chemotherapy further complicates the clinical picture (J. J. Yahaya, Msokwa, & Mremi, 2019). For a tumor to be called mucinous, it should have an extracellular mucinous component of > 50% of the tumor volume (Luo, Cen, Ding, & Wu, 2019). Those with less than 50% of the mucinous component are called adenocarcinoma with mucinous differentiation. Mucinous adenocarcinomas show expansile growth pattern with single or clusters of tumor cells floating in mucin pools. Initially, these tumors were termed high grade owing to their aggressive behavior. But studies have shown that 50% of the tumors which are MMR deficient, as in lynch syndrome, are associated with a better prognosis. Such tumors behave in a low grade fashion as compared to MMR proficient tumors (Liddell et al., 2017).

1.1.4.2 Serrated Adenocarcinoma

8% of the CRCs are histologically classified as serrated adenocarcinomas. Prominent features include, eosinophilic cytoplasm with vesicular nuclei and prominent nucleoli. Foci of mucinous differentiation may be present but the tumors lack the luminal necrotic debris, as in conventional adenocarcinoma (Daiki Hirano et al., 2017). BRAF mutations are present in 20% of cases along with CpG island hypermethylation and MMR deficiency. Here again, MMR deficiency correlates with favorable prognosis as compared to MMR proficient tumors (D. Hirano et al., 2019).

1.1.4.3 Signet Ring Cell Carcinoma

This histologic variant accounts for less than 1% of colorectal cancers and has a higher predilection for patients younger than 40 years of age. It is defined as a tumor

having more than 50% cells with abundant intracytoplasmic mucin with eccentric and hyperchromatic nuclei. Tumors with less than 50% of the signet ring cell component are called adenocarcinoma with signet ring cell differentiation. Right colon is commonly involved and advanced stage is usually encountered at the time of diagnosis (Barresi & Pedrazzani, 2020).

In case of signet ring cell tumors the MMR status does not affect the biologic behavior. Both the MMR deficient and proficient carcinomas are associated with poor prognosis and behave as high grade carcinoma (Melanie Johncilla, Chen, Sweeney, & Yantiss, 2018).

1.1.5 Pathologic Stage Classification (pTNM)

The pathological staging of resected CRC specimen correlates with the prognosis (Fleming et al., 2012). This classification relies on the information provided by clinical staging, operative findings and the histopathological assessment of the resected surgical specimen and is valid only if there is a negative history of any treatment before surgery. It is symbolized by a lowercase “p” (Gress et al., 2017).

1.1.5.1 Pathologic Stage Classification (pTNM, AJCC 8th Edition)

Primary Tumor (pT)

pTX: Primary tumor cannot be assessed

pT0: No evidence of primary tumor

pTis: Carcinoma in situ, intramucosal carcinoma (involvement of lamina propria with no extension through muscularis mucosae

pT1: Tumor invades the submucosa (through the muscularis mucosa but nit into the muscularis propria)

pT2: Tumor invades the muscularis propria

pT3: Tumor invades through the muscularis propria into peri-colorectal tissue

pT4: Tumor invades the visceral peritoneum or invades or adheres to adjacent organ or structure

pT4a: Tumor invades through the visceral peritoneum (including gross perforation of the bowel through tumor and continuous invasion of tumor areas of inflammation to the surface of the visceral peritoneum)

pT4b: Tumor directly invades or adhere to adjacent organs or structures

Regional Lymph Nodes (pN)

pNX: Regional lymph nodes cannot be assessed

pN0: No regional lymph node metastasis

pN1: One to three regional lymph nodes are positive (tumor in lymph nodes measuring ≥ 0.2 mm), or any number of tumor deposits are present and all identifiable lymph nodes are negative

pN1a: One regional lymph node is positive

pN1b: Two or three regional lymph nodes are positive

pN1c: No regional lymph nodes are positive, but there are tumor deposits in the subserosa, mesentry or, non-peritonealized pericolic, or perirectal/mesorectal tissues

pN2: Four or more regional lymph nodes are positive

pN2a: Four to six regional lymph nodes are positive

pN2b: Seven or more regional lymph nodes are positive

Distant Metastasis (pm) (required only if confirmed pathologically in this case)

pMX: Metastasis cannot be assessed

pM1: Metastasis to one or more distant sites or organs or peritoneal metastasis is identified

pM1a: Metastasis to one site or organ is identified without peritoneal metastasis

pM1b: Metastasis to two or more sites or organ is identified without peritoneal metastasis

pM1c: Metastasis to the peritoneal surface is identified alone or with other site or organ metastases

(Pathologists, 2020)

In contrast to the other parts of gastrointestinal tract, like esophagus, stomach and small bowel, where the term “invasive carcinoma” denotes mucosal invasion (pT1), submucosal invasion of the colorectum is necessary for the diagnosis of invasive carcinoma (pT1). One reason could be the scarcity of lymphatics in the mucosa, which prevents its spread to the lymph nodes and distant organs. Therefore, in case of CRC, the mucosal invasion is labelled high grade dysplasia by the pathologist and is classified as Tis (Fleming et al., 2012).

1.1.6 Tumor Microenvironment (TME)

A formative event in the initiation and progression of cancers is the aberrant cellular differentiation that bypasses immunological defenses by modifying the molecular signals and receptors. As discussed earlier, the tumor cells are a product of multiple genetic mutations and epigenetic influences like hypermethylation of CpG islands. The mutation into oncogenes and inactivation of tumor suppressor genes, for example, kick-starts the

evolution into undifferentiated cancer cells. This would be rapidly checked by the multi-layered defense mechanisms working diligently through a complex interplay of cellular elements like pericytes, dendritic cells, macrophages, fibroblast and specialized lymphocytes (Labani-Motlagh, Ashja-Mahdavi, & Loskog, 2020), duly alerted by the stromal chemokines and cytokines (Y. Yuan, Jiang Y., Sun, C., Chen, Q, 2016). The extracellular matrix (ECM) is composed of structural proteins like collagens and elastin, along with a complex meshwork of glycoproteins like fibronectin and laminins, along with the proteoglycans (Wei, Liu, Zhang, Min, & Zhu, 2020) that influence cellular adhesion, as well as modulate cell proliferation, and intercellular communication (Arneth, 2019). Moreover, it harbors humoral elements like TGF-β, TNF-α and interleukins (IL-6) (Baghban et al., 2020) that would be released on stromal disruption and activate tissue immune cells to attack the abnormal tumor cell . All these cellular, structural and humoral elements, that surround the tumor cells constitute the Tumor Microenvironment (TME). So successful tumor progression can only occur if multiple elements of the tumor micro-environment, are abnormally altered.

Extracellular vesicles (EVs), including the exosomes and microvesicles are released by all the cells of the body, including the tumor cells. Their uptake is selective and thus serve as a mean of communication between specific cells. Exosomes are endosomal in origin and can transport lipids, proteins as well as RNAs. In TME, the exosomes secreted by the tumor and immune cells help mediating their effects on each other, thus resulting in the modulation of tumor microenvironment. Tumor cell derived exosomes cause inhibition of natural killer and T cells, promote angiogenesis, metastasis and polarization of macrophages and neutrophils to TAMs and tumor associated neutrophils (TANs). The transferring of RNA species can reprogram the recipient cell as well. On the other hand, exosomes released by the immune cells can lead to tumor cell apoptosis. Figure 1.1 depicts the effects of tumor and immune cells related exosomes on tumor microenvironment.

The integral role of TME has been extensively studied for colorectal cancers (CRC). For example, it has been shown that the evident stiffness of tumor relates to the fibrosis generated by Cancer-Associated Fibroblasts (CAF). The biophysical forces generated by tumor cell proliferation in this stiff environment, interacting with CAFs, lead to release of

Activin A, which is a member of TGF-β family. Among the downstream effects of activin A includes contribution to epithelial to mesenchymal transition (EMT). Study shows that Activin A achieves this via the ligand-dependent CRC epithelial cell migration (Bauer et al., 2020). Fibrosis and stiffness of colorectal cancers has been shown to increase with tumor progression (Kawano et al., 2015).

With the burgeoning evidence of TME's essential role in cancer progression and increasing knowledge about related cellular and molecular signaling pathways, there has been an increasing focus on targeting these cellular and molecular elements in the treatment of cancers. A whole new class of cancer therapeutics is based on immune-modulation. These immunotherapies, like the immune checkpoint inhibitor Pembrolizumab (PD-1 inhibitor), and Atezolizumab (PDL-1 inhibitor), specifically target the immune cells in the TME. These drugs restore the T cell inhibition induced by the cancer cell generated humoral binders (Peng et al., 2020). This re-activates the cytotoxic function of CD8+ cells, for example, against chemotherapy resistant melanomas (Brochez et al., 2018), renal cell carcinomas, small-cell lung cancers and head and neck squamous cell cancers (Hirata-Nozaki et al., 2019). A myriad of other pathways provides avenues to manipulate TMEs cellular, structural and humoral elements for therapeutic advantage. An established approach in metastatic CRC targets the vascular endothelial growth factor signaling (VEGF) that is instrumental in tumor angiogenesis. The new vasculature ensures adequate tumor nutrition, potentiate metastasis and the Tumor-derived endothelial cells (TEC) induces resistance to chemotherapeutic agents (Baghban et al., 2020). Bevacizumab is humanized monoclonal antibody that inhibits VEGF-A and blocks angiogenesis. Over the last decade several studies have shown survival benefit to patient with addition of Bevacizumab to chemotherapy and surgical resection in patients with metastatic colorectal cancers (Hopirtean & Nagy, 2018). Tumor associated macrophages (TAM) provide another potential target for this approach, that will be explored in the following section.

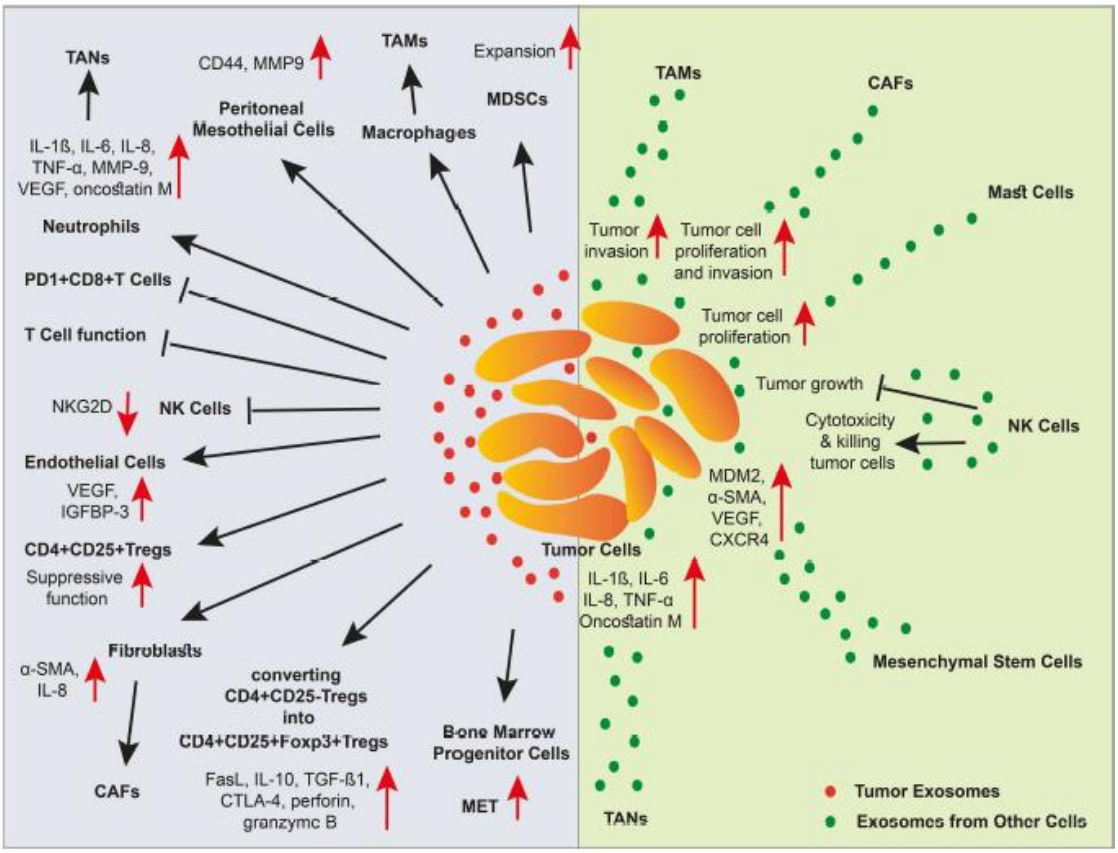


Figure 1.1: Effect of tumor and immune cells derived exosomes on tumorMicroenvironment. Adapted from "The Tumor Microenvironment: A Milieu Hindering and Obstructing Antitumor Immune Responses", from Labani-Motlagh, A., Ashja-Mahdavi, M., & Loskog, A, (2020), *Front Immunol, 11*, 940. Copyright © 2020 Labani-Motlagh, Ashja-Mahdavi and Loskog. Adapted with permission.

1.1.7 Tumor Associated Macrophages (TAMs)

Macrophages are white blood cells, derived from the peripheral blood monocytes. Their phagocytic properties, responsible for clearing the cellular debris and tumor cells along with the other harmful foreign agents render them a vital component of mononuclear phagocyte immune system (Yona & Gordon, 2015).

Depending on the internal environment of the body, as dictated by the immune and tumor cells when these macrophages are employed to the tumor microenvironment, they are translated to tumor associated macrophages (Zhou et al., 2020).

The role of Tumor Associated Macrophages (TAMs) in cancer tissues is central to understanding the approach of improving cancer's pathological assessment and subsequent care. It is established that the tumor microenvironment (TME) is a necessary player for the initiation and progression of solid tumors. TAMs comprise the main bulk of the infiltrating immune cells in TME in comparison to the dendritic cells, T cells and the other types of antigen presenting cells (Y. Chen et al., 2019).

Plasticity and adaptability are the two hallmarks of macrophages (Guttman & C. Lewis, 2016). In general, the macrophages can be classified into two major types depending on their polarization states, i.e., classically activated M1 and alternatively activated M2 macrophages (Zhou et al., 2020). M1 and M2 macrophages are the two extremes of the polarization spectrum with a number of unaccounted subtypes in between (Y. Chen et al., 2019; Jayasingam et al., 2019).

M1 macrophages are considered pro inflammatory and bactericidal. This polarization state is induced by the factors such as, interferon (IFN) γ and lipopolysaccharides (LPS) . M1 macrophages secrete a number of Th1 inducing cytokines like, tumor necrosis factor (TNF) α , interleukin 12 (IL-12), IL-6 and IL-18. These cells have high antigen presenting capacity and also produce reactive oxygen species (ROS), thus are responsible for directly killing the tumor cells (anti tumor) (Pinto et al., 2019) (Guttman & C. Lewis, 2016).

M2 macrophages are immunosuppressive, anti inflammatory and pro tumor. Their polarization is choreographed by IL-4, IL-13 and IL-10. These cells in turn secrete transforming growth factor (TGF)- β and IL-10, which are responsible for the immunosuppressive nature of tumor microenvironment (Y. Chen et al., 2019) (Pinto et al., 2019).

In the case of tumors, TAMs highly resemble M2 macrophages , as both are activated in response to the similar cytokines and secrete some common factors, while exhibiting few differences as well (Y. Chen et al., 2019). A number of growth factors, cytokines, chemokines and enzymes produced by TAMS play an important role in tumor growth and progression. IL-6 increases the chemoresistance by activating the STAT3 pathway and indirectly increasing the anti- apoptotic protein Bcl2 in colorectal and other solid tumors (Xu, Ye, Huang, Yan, & Li, 2019) (Y. Yin et al., 2017). TAMs promote neovascularization by increased secretion of vascular endothelial growth factor (VEGF), platelet derived growth factor and TGF β (Tamura, Tanaka, Yamamoto, Akasaki, & Sasaki, 2018). Matrix metalloproteinases (MMPs) promote invasion and metastasis. TGFβ induces EMT in colorectal cancers through smad/snail signaling pathway (Cai et al., 2019b). Various chemokines like CCL2, CCL5, including other cytokines and enzymes already mentioned can hinder the CD4+ and CD8+ functions and also result in the recruitment of natural Tregs (nTregs), thus resulting in unsuccessful immunosuppressive therapy (Y. Chen et al., 2019).

1.1.7.1 Tumor Associated Macrophages and Prognosis in CRC

In relation to colorectal cancers, TAMs have been evaluated for prognosis in several studies and contrary to the trend in most other cancers, a higher density of TAMs in CRC appears to correlate with better prognosis. Decreased survival associated with TAMs have been found, for example, among breast, melanoma and kidney malignancy patients (Edin et al., 2012). This is explained by the tumor friendly functions of TAMs including the secretion of various growth factors for the neoplastic cells and blood vessels, like vascular

endothelial growth factors (VEGF), transforming growth factor -B (TGF- B) and platelet derived growth factor (PDGF) (Gulubova et al., 2013). In patients with CRC, several studies have shown better survival in cases where a higher infiltration of TAMs was found. This was especially true if instances of higher concentration of TAMs were localized to tumor front as opposed to tumor stroma (Edin et al., 2012). Possible explanation of this paradox in colorectal cancers was theorized to be a higher concentration of anti-tumoral M1 polarized macrophages compared to pro-tumoral M2 macrophages, in these tumors (Pinto et al., 2019). Some studies have demonstrated this difference in relative density, or higher M1:M2 ratio, correlated with better prognosis (C. Yang et al., 2019). Alternatively, this may relate to the secretion of TGF-β by TAMs, that plays a tumor suppressor role in early stages of cancer (Itatani, Kawada, & Sakai, 2019). This effect is lost and paradoxically turned to tumor-progressor function of TGB-β, with the loss or mutation of SMAD-4 in the nucleus of tumor cell (Cai et al., 2019a). This stage has also demonstrated a higher propensity for metastases. In summary, for colorectal cancers, higher density of TAMs represents a good prognostic factor but when the proportion of M2-like TAMs predominate, likely under the influence of altered tumor microenvironment, the prognosis worsens.

1.1.7.2 Tumor Associated Macrophages and Targeted Therapies

Being an important component of TME, tumor associated macrophages are a desirable target for cancer treatment. The therapeutic strategies mainly aim at clearing and inhibiting the activation of TAMS by targeting CSF-1/CSF1R signaling to suppress the tumor growth, promoting the phagocytic activity of macrophages by blocking CD47-SIRPα signaling, limiting monocyte recruitment by targeting CCL2R and inhibition of TAMs by PD-L1 antibody to promote phagocytic activity. Monoclonal antibodies directed against the LILRB1 component of the LILRB1/ MHC class 1 identification mechanism and genetically engineered TAMs lacking the SIRPα and LILRB1 receptors, are few other under trial

targets, directed at increasing the phagocytic activity of TAMs (Barkal et al., 2018). As promising these treatment options might seem, there is still a long way before they could become a part of regular treatment for solid tumors, as discussed in the following chapter.

1.1.7.3 Immunohistochemistry

Histologically we can identify TAMs and M2 phenotype with immune-histochemistry by using antibodies directed against CD68+ and CD163+ cells. A number of previous studies have confirmed the role of CD68 and CD163 as pan macrophage and a marker for M2 macrophages respectively (Barbosa, Sá, Pinto, & Freitas, 2015) (Jamiyan, Kuroda, Yamaguchi, Abe, & Hayashi, 2020) (Hu et al., 2017).

CD68 is a glycoprotein, which is heavily glycosylated and mainly associated with the endosomal/lysosomal compartment (Chistiakov, Killingsworth, Myasoedova, Orekhov, & Bobryshev, 2017). It is expressed by a number of tissue macrophages, as well as the Kupffer cells, alveolar, splenic, germinal center and lamina propria macrophages (Minami, Hiwatashi, Ueno, et al., 2018). The function of CD 68 is not well understood but the major localization in late endosomes point towards an important role in antigen processing and the protection of lysosomal membranes against their own hydrolytic enzymes. CD 68 also acts as a receptor for oxidized LDL (low density lipoprotein).

CD163 is a transmembrane protein and functions to mediate the endocytosis of haptoglobin- hemoglobin complexes. This scavenger receptor is exclusively and highly expressed by macrophages (Skytthe, Graversen, & Moestrup, 2020)

Immunohistochemical staining for CD163 shows diffuse staining of M2 macrophages membrane and cytoplasm (Hu et al., 2017), while for CD68 , the staining is mainly cytoplasmic. Morphologically, CD68+ cells are rounded or dendritic, whereas CD163+ cells are usually dendritic (Salmi et al., 2019)

1.2 Hypothesis

1.2.1 Null Hypothesis

1- There is no correlation of M2-like TAMs proportion with pathological stage of CRC.
2- There is no correlation of TAM density in tumor stroma with pathological stage in CRC.
3- There is no correlation of TAM density in tumor-front with the pathological stage of CRC.

1.2.2 Alternate Hypothesis

1- There is correlation of M2-like TAMs proportion with pathological stage of CRC.
2- There is correlation of TAM density in tumor stroma with pathological stage in CRC.
3- There is correlation of TAM density in tumor-front with the pathological stage of CRC.

1.3 Objectives of Study

1- To determine statistical correlation of M2-macrophage proportion among TAMs with pathological stage of colorectal cancer.

2- To determine statistical correlation of TAM density in tumor stroma and tumor front with pathological stage of colorectal cancer.

1.4 Statement of the Problem

Colorectal Cancer is responsible for substantial morbidity and mortality in adults, especially in Pakistan. Earlier diagnosis and improved staging, utilizing better molecular and cellular markers, with new immunohistochemical targets, may potentially provide novel therapeutic targets and help in improving the overall treatment and prognosis of the disease.

1.5 Significance of Study

Colorectal cancers are among the commonest malignancies, and lead to significant proportion of cancer related deaths, worldwide. Pakistani population also shows similar epidemiological characteristics with added adversity of affecting increasingly younger individuals(Yousaf, 2018). Conventionally, cancer treatment was largely determined by tumor stage based on its size, nodal involvement, and documented metastasis. Over the last decade, focus of cancer therapy has shifted from cytotoxic chemotherapy alone to a complex mix of targeted therapies that are directed against a wide range of molecular targets. These molecular targets enable immunomodulation and apoptosis leading to much improved rate of cure and patient survival. This molecular signature thus, gives much more meaningful information about tumor biology and its prognosis.

This study aims to contribute by better defining TAM character of tumor microenvironment in colorectal cancers. This will be done by enumerating the total number of TAMs in tumor stroma and tumor front, following immune-histochemical (IHC) staining with anti-CD68+ as a pan-macrophage marker. Additionally, the proportion of M2 among all TAMs will be calculated based on counts of macrophages with IHC staining positive for anti-CD163+. This will again be separately enumerated for tumor stroma and front. A correlation of this proportion with the histological stage and other clinicopathological parameters would then be performed (Sanjay Kakar, 2017).

The rationale of this assessment is that this differentiation to M2 may represent an early marker for pre-clinical metastatic disease, followed by decreased TAM density as an additional marker representing poor prognosis. Establishing M2 proportion as a prognostic marker will improve molecular staging of colorectal cancer (Guinney et al., 2015). Additionally, it would provide new potential therapeutic target for treatment in colorectal cancer patients.

A simplified protocol would ensure easier adaptability for clinical application. Few earlier studies have applied it in a similar manner (C. Yang et al., 2019). No such studies have till now been published in Pakistan.

1.6 Operational Definitions

1.6.1 Tumor Stroma and Tumor Microenvironment (TME)

The stromal component of the tumor, responsible for the initiation and progression of the cancer is regarded as the tumor microenvironment. TME consists mainly of fibroblasts, endothelial cells and inflammatory cells related to innate and adaptive immune responses, including Tumor Associated Macrophages (TAM). Tumor stroma, in general is the phenotypic representation of TME in anatomical pathology.(Whiteside, 2008; Y. Yuan, Jiang Y., Sun, C., Chen, Q, 2016)

In this study, for colorectal cancers, TAM density and proportion of M2 are being proposed to represent protumor or antitumor status of the TME, with implication for cancer prognosis.

1.6.2 Tumor Associated Macrophages (TAMs)

TAMs comprise the main bulk of the infiltrating immune cells in TME. Anti-CD68+ IHC marker will be used to identify TAMs phenotype in colorectal specimens for this study. Further characterization will be based on 5 “hotspots” analysis, i.e., fields with most intensive CD 68+ recruitment, in the tumor stroma.(Morita et al., 2017; Salmi et al., 2019)

1.6.3 TAMs Density

TAMs density was assessed based on IHC anti-CD68+ staining. Actual count of CD68+ macrophages was determined in the observed field at x400 magnification. The mean number of CD68+ macrophages, per 5 HPF (5 spots), for each slide (tumor front and stroma), were labeled as TAMs density (Salmi et al., 2019).

TAMs density was further divided into high and low after calculating the median value for both tumor front and stroma as described by (Hu et al., 2017).

1.6.4 M2 Subtype Proportion

The proportion of M2 subtype proportion was calculated for each tissue specimen using the formula:

$$\frac{Mean\ number\ of\ CD163\ cell}{Mean\ number\ of CD68\ cells}\ \text{X}\ 100 = M2\ subtype\ proportion$$

1.6.5 M2 Macrophages

Macrophages expressing anti-CD163+ IHC marker were considered M2 phenotype or alternatively activated macrophages, for this study. Actual count of CD163+ macrophages was determined in the observed field at x400 magnification, within 5 hotspots in the tumor stroma and along the tumor front. Mean number of CD163+ M2 cells was then calculated for the tissue specimen.

M1 macrophages*: M1 phenotype was directly stained or counted in this study. Indirect determination was made by subtracting the proportion of M2 phenotype from all TAMs (i.e., M1= TAM - M2). This allowed a simplified assessment overall with improved clinical applicability if found to be significant*.

1.6.6 Pathological Stage of Colorectal Cancer

Pathological staging(pTNM) is based on pathological evaluation of the surgically resected specimens.(Gress et al., 2017) For colorectal cancers this is based on the site of resection, tumor location, tumor size and extension, nodal involvement and possible metastatic tumor deposits submitted for anatomic, gross and microscopic pathological review.

1.6.7 Invasive Tumor Front (IT)

Invasive tumor front or the tumor – host interface is defined as the most advanced, progressing edge of tumor, comprising between three to six tumor cell layers or detached tumor cells with marked cellular atypia (Yang et al., 2019),(Patil, Augustine, & Rao, 2016).

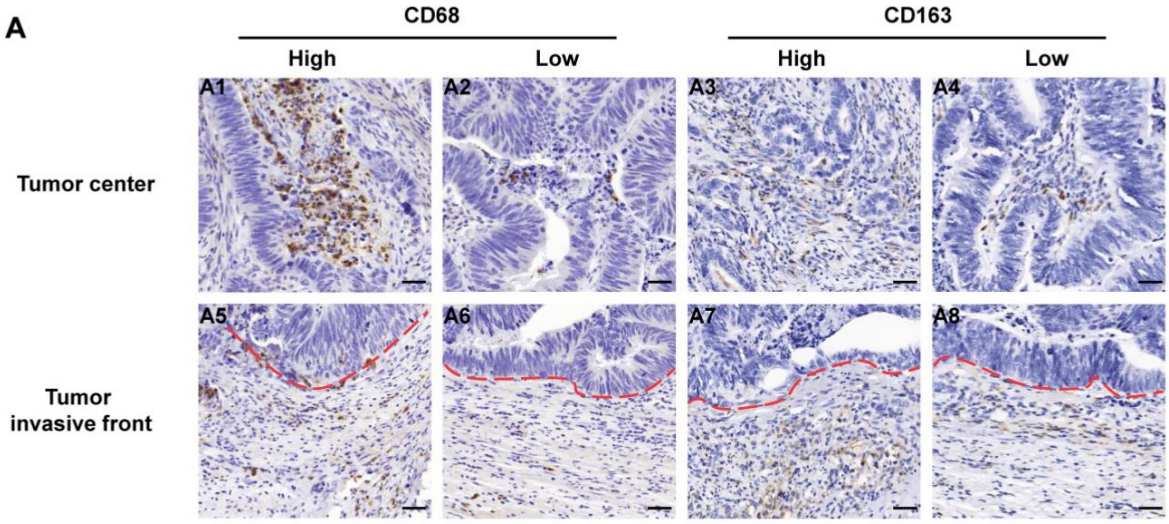


Figure 1.2: Immunohistochemical analysis of macrophages in colorectal samples. The interrupted red line denotes the tumor front or the host tumor interface. Adapted from "Elevated CD163(+)/CD68(+) Ratio at Tumor Invasive Front is Closely Associated with Aggressive Phenotype and Poor Prognosis in Colorectal Cancer", from Yang, C., Wei, C., Wang, S., Shi, D., Zhang, C., Lin, X., . . . Xiong, B. (2019) .*Int J Biol Sci, 15*(5), 984-998. © Ivyspring International Publisher. Adapted with permission.

CHAPTER 2

LITERATURE REVIEW

2.1 The Changing Epidemiological Trends in CRC

Colorectal cancer has been evolving epidemiologically, and more so over the last two decades. The demographics, for example is increasingly representing a younger age group i.e., less than 50 years of age (H. Huang et al., 2020), presenting with more advanced disease and graver prognosis (Abdelsattar et al., 2016). Left sided / rectal tumors with poorer differentiation and Signet ring cell histology are common in this EO- CRC (Early onset- colorectal cancer) group. Signet ring CRCs are responsible for 3% -13% of cases especially in patients younger than 30 years of age (Mauri et al., 2019). This demographic trend is also evident in Pakistan (Hasan et al., 2017; Tajamal, 2019). Paradoxically, during this time, there has been a declining incidence in those older than 50 years, largely attributable to routine colonoscopic screening, weeding out the adenomatous polyps prior to transition to full blown carcinoma (Siegel et al., 2020). Additionally, adenocarcinomas are being diagnosed earlier and treated sooner leading to a better prognosis. Finally, the treatment repertoire has expanded significantly. This has been, in no small measure, the result of improving understanding of tumor biology, along with the identification of newer molecular and cellular diagnostic markers and targets for therapy. A whole array of immunotherapies, for instance, modify tumor response to patient's immune cells and chemotherapeutic agents. This treatise aims to analyze the existing body of information on

Tumor Associated Macrophages (TAM), as an additional prognostic tool and a potential therapeutic target for colorectal cancers.

2.2 The Pathology Report: influential determinant of prognosis and treatment

The pathology report of a resected colorectal cancer specimen, is the most influential determinant of the patients prognosis as well as subsequent cancer treatment, in today's paradigm of Personalized Medicine (Fleming et al., 2012). The elements of microscopic depth of tumor invasion, tumor free margins, lympho-vascular and perineural invasion, tumor deposits, the total number of lymph nodes involved define the tumor stage. Additionally, presence or absence of tumor budding, and micro-satellite instability (MSI), along with the finer molecular elements like *KRAS, BRAF and PIK3CA* mutations all dictate the treatment for each patient (Fleming et al., 2012; Tran et al., 2015). This reliance is based on the increasing understanding of how the histological phenotype represents tumor biology, of a particular patient, along with the conventionally understood cancer stage. The molecular signatures add to the phenotype and helps in recognizing the milieu even better. This has allowed clinicians to identify specific targets and tailor cancer therapy for each affected patient, setting the paradigm of personalized medicine.

2.3 Epithelial-to-Mesenchymal Transition: driving the metastatic transition

A seminal event in cancer progression, for example, is conversion from localized disease to metastatic disease. For several cancers, this is known to start with epithelial-to-mesenchymal transition (EMT) (Rankovic, Zidar, Zlajpah, & Bostjancic, 2019; Savagner, 2010), further assisted by neo-vascularization and increasing survivability of tumor cells in the blood. Studies on breast (Su et al., 2014), hepatocellular carcinoma (Chen, Gingold, & Su, 2019; Minami, Hiwatashi, Sakoda, et al., 2018), and colorectal cancers (Cai et al., 2019a; Idrees et al., 2018; Rankovic et al., 2019), among several others, have demonstrated this progression. In general, EMT is characterized by loss of cell-cell junction as well as apical-basal polarity among tumor cells. This results primarily due to downregulation of E-cadherin along with upregulation of N-cadherin and Snail that signify a mesenchymal transition (Lin, Xu, & Lan, 2019). Experimental studies have demonstrated that Tumor Associated Macrophages (TAMs) can induce the EMT in breast cancer. In turn, through a feedback loop, the mesenchymal-like tumor cells can activate TAMs – induced to secrete an array of pro-tumor cytokines (Su et al., 2014). Similar experimental results were obtained with co-cultures of TAMs and hepatic cell lines, with increased clusters of HCC cancer-stem-cells (CSC), resulting in EMT and higher invasive capability of tumors (Fan et al., 2014; Wan et al., 2014). For colorectal cancers as well, experimental studies have shown TAMs inducement of EMT (Cai et al., 2019a; Edin et al., 2012). The histological phenotype for this TAMs associated tumor progression and resulting poor prognosis has also been established through several studies for breast, (Jeong, Hwang, Kang, Shin, & Kwon, 2019; Morita et al., 2017; Xixi Zhao1 &) hepatocellular,(Minami, Hiwatashi, Sakoda, et al., 2018; Minami, Hiwatashi, Ueno, et al., 2018) pancreatic, gastric,(Y. K. Huang et al., 2019; Zhang et al., 2016) as well as colorectal cancers. In general, a higher infiltration of TAMs in resected tumor specimens correlates with worse prognosis and reduced patient survival.

2.4 TAM Subtypes: opposing roles of M1 and M2

Over the last decade, there has been significant focus on defining role of TAMs in cancer biology and its effect on patients' survival. Additionally, there has been further focus on TAMs subtypes, i.e., M1 and M2, their varied distribution, and relative densities correlate with cancer prognosis and patients' survival. In general, tumor microenvironment determines the change in character, for example, from anti-tumor M1 predominant to pro-tumor M2 dominant macrophage polarization. M2 in turn has been shown to promote all the aspects of TME leading to tumor spread, mentioned earlier (Y. Chen et al., 2019; Lin et al., 2019). This pathological construct has been shown to be valid by several studies showing poor prognosis with change in TAM density and increasing M2 proportion in different cancers, including CRC (Edin et al., 2012; Jeong et al., 2019; Pinto et al., 2019).

2.5 Differential Distribution and Density Affects Prognosis: Other Cancers

Jeong et al.,(Jeong et al., 2019) in their study on differential distribution of TAM subtypes in tumor nests versus tumor stroma – based on tissue microarrays of 367 invasive breast cancer patients – concluded that, the type of TAMs and whether they are distributed in tumor nests or stroma correlates with cancer prognosis and patients survival. Overall higher number of TAMs were associated with poor prognostic markers, like higher histological grade and hormone receptors negativity. Interestingly higher M1 subtype in tumor stroma resulted in better patient survival as opposed to higher distribution of M2 subtype in tumor nests.

Similarly, Yang et al.,(M. Yang et al., 2018) studied 200 cases of basal- like breast carcinoma. They used CD68 and CD163 as pan macrophage and M2 immunohistochemical

markers, respectively to assess the prognostic significance of TAMs infiltration in tumor nests and stroma. Increased stromal infiltration by CD68 and CD163 macrophages correlated with higher recurrence and mortality rate, as well as higher tumor grade and larger tumor size. Additionally, CD163 macrophages alone emerged as the independent predictors of overall and recurrence free survival after multivariate analysis.

Hu et al.,(Hu et al., 2017), utilizing IHC, looked at the role of M2 macrophages in tumor progression, in 100 samples of esophageal squamous cell carcinoma from their Kazakh population. They observed that the density of CD163 positive macrophages was higher in tumor stroma as compared to tumor islets. Moreover, this higher density in tumor stroma was positively associated with lymph node metastasis and advanced clinical stage.

A metanalysis of 16 studies on association of TAMs with breast cancer prognosis by Zhao et al.,(X. Zhao et al., 2017) that included data from 4,541 patients, demonstrated similar association of high TAMs infiltration with markers of poor prognosis and worse patient survival. Distinctively the metanalysis showed better correlation with non-selective macrophage biomarker (viz, CD68) instead of markers that were selective for M1 or M2 subtypes.

For hepatocellular carcinoma, Minami et al.,(Minami, Hiwatashi, Sakoda, et al., 2018) evaluated resected specimens from 105 patients, for expression of TAMs immunohistochemical markers. They observed correlation of higher expression of TAMs with higher disease stage and worse prognosis.

Evaluating TAMs density with prognosis in gastric cancer, Yin et al.,(S. Yin et al., 2017) in metanalysis of 19 studies and 2242 patients found significant association of generalized high TAMs density with poor survival. They found correlation specific with M2 subtype.

2.6 Is colorectal cancer different?

Interestingly, similar studies for colorectal cancers have yielded mixed results – some studies correlating worse prognosis and others showing improved survival (Pinto et al., 2019). The likely explanation lies in the fine print of the dominant subtype and differential distribution in tumor stroma, nests or front. In general. the studies that looked at intensity of pan-TAMs, tended to show improved overall survival or no correlation with prognosis, while other studies that took account of subtypes, especially the M2 predominance or differential distribution of subtypes, demonstrated a poor overall survival or prognosis. Simply stated, the prognosis depended on which polarization was predominant (i.e., M1 or M2) and where were these present (i.e., tumor stroma or tumor nests or tumor front).

Edin et al., (Edin et al., 2012) for example, concluded that there is no difference in density of M1 and M2 in tumor specimens. Moreover, overall increased infiltration of TAMs correlated with better prognosis, in their study of 485 consecutive CRC patients.

Contrarywise, Waniczek et al.,(Waniczek et al., 2017) from data of 89 retrospectively enrolled patients, found that TAMs infiltration intensity, positively correlated with worse patient survival. They were also able to distinguish that the relative risk was twice with infiltration in tumor stroma as compared to tumor front.

Gullabova et al.,(Gulubova et al., 2013) looking at 210 primary colorectal cancer specimens, found that low levels of (non-fractionated) TAMs were associated with unfavorable prognosis, among CRC patients.

With a different approach, Yang et al., (C. Yang et al., 2019) evaluating immunohistochemistry in 81 colorectal cancer patients, determined M2:TAMs ratio in tumor stroma (center) and tumor front and concluded that this ratio in tumor front was better at prognosticating than either of these cell types alone. Moreover increased CD163/CD68 ratio at the tumor front positively correlated with lympho-vascular invasion and tumor stage.

In 201 Greek patients with CRC, increased density of stromal CD68 macrophages showed association with prolonged overall survival , less tumor budding and no lymph node metastasis. Interestingly, increased CD163 macrophage infiltration was also found to be associated with good prognostic markers, like less lymph node metastasis (LNM) and lower tumor grade, in this study by Koelzer et al (Koelzer et al., 2016)

In 78 patients with CRC, Krijgsman et al.,(Krijgsman et al., 2020) measured the serum levels of CD163 using ELISA. Stromal and intraepithelial distribution of TAMs was assessed by immunofluorescence. They found above median levels of serum CD163 to be associated with shorter overall and disease free survival. Whereas, no association was identified for the clinical outcome and the density of tams in stroma or epithelium.

A meta-analysis of 17 studies including 3749 patients with CRC, by Zhao et al (Y. Zhao et al., 2019), looking at the prognostic value of TAMs confirmed the association of shorter overall and disease free survival with increased CD163 macrophage infiltration. This meta-analysis also revealed the association between high density of TAMs and no distant and lymph node metastasis, non-mucinous histology, MSI-H status and increased CD8+ T cell infiltration.

2.7 Connecting the Dots: Summating TAMs in Colorectal Cancer

Working with these diverse conclusions, regarding association of TAMs and prognosis in colorectal cancer, there is a need to clarify possible pathogenesis that may connect these together. The key here is understanding the temporal sequence of events, starting from early stage disease to later advanced stages. The tumor microenvironment is continually changing under the influence of tumor cells. These cells tend to accumulate genetic mutation with the rapid growth cycle. These mutations lead to change in the cytokines secreted, by tumor cells. These cytokines induce a change in polarization of macrophages. The macrophages change character from anti-tumor M1 to protumor M2

subtype. These M2 like TAMs, then start secreting various growth factors, like vascular endothelial growth factors (VEGF), transforming growth factor -β (TGF- β) and platelet derived growth factor (PDGF) promoting angiogenesis, tumor growth, invasion, and migration. This feedback loop, in turn induces epithelial to mesenchymal transition, initiating tumor invasion and metastasis. This may explain the findings discussed earlier from different studies. In the initial stages of cancer, the tumor provokes an inflammatory, anti-tumor response from immune cells including tissue macrophages. These macrophages are predominantly M1 type. These tend to restrict tumor progression. So, these specimens from earlier stage disease, reveal M1 predominance, and good prognosis. But as the tumor cells go through multiple cycles, newer generation of more mutated cells become predominant in the tumor tissue and start changing the character of tumor microenvironment. This leads to evolution of tumor associated macrophages to M2 type. Under the pro-tumor influence of M2 subtype, the tumor center or core increases in size, its phenotype reflects increasingly undifferentiated grading, representing higher stage of cancer. Hence the studies find a poorer prognosis for these tumor specimens with higher density of M2 macrophages. Similarly, the studies that looked in to the M1:M2 ratio also reflect worse outcome with higher M2 ratio.

2.8 TAMs as Novel Therapeutic Target for Cancer Immunotherapy

From a therapeutic standpoint, TAMs provide multiple potential routes to augment their anti-cancer activity (L. Yang & Zhang, 2017). Reducing or blocking monocyte recruitment into tissues, inducing apoptosis of TAMs already present in the tissue, blocking specific TAM activity like promoting angiogenesis through receptor binding, re-educating or repolarizing TAMs from pro-tumor M2 to anti-tumor M1 type, for example, are some pathways . Each of these would translate to either direct cellular phagocytosis or cytotoxicity of tumor cells, enable better therapeutic response to chemotherapeutic agents

by unblocking the cell-death function in tumor cells, blocking the tumor promoting functions like angiogenesis among several other possibilities (Anfray, Ummarino, Andón, & Allavena, 2019). In effect this would result in delaying tumor progression or actual tumor regression resulting in improved patient survival. A wholesome volume of research has been directed to these potential targets and its beginning to provide evidence of clinical efficacy.

2.8.1 Blocking TAMs Recruitment

Blocking TAMs recruitment would theoretically reduce the effect of TAM induced modulation of TME that, although beneficial in initial stages of cancer, are deleterious as tumor progresses. A potential target that triggered significant interest was chemokine CCL2 and its receptor CCR2 (Pinto et al., 2019). Monoclonal antibody against CCL2, carlumab, has been tested in phase I and phase II trials. Although showed encouraging results in the mouse model (Sanford et al., 2013), and good tolerance in humans but unfortunately did not translate into therapeutic efficacy as TAM recruitment was not affected among a cohort of prostate cancer patients (Pienta et al., 2013) and a diverse set of solid tumors (Brana et al., 2015). Interestingly in combination with FOLFIRINOX (fluorouracil irinotecan plus leucovorin and oxaliplatin) alone, or in combination with CCR2 antagonist as chemotherapy for pancreatic adenocarcinoma (Nywening et al., 2016), it did lead to TAM depletion in tumors and contributed to partial response in half the patients by hampering tumor growth and metastasis. Among the CRC patients a subset of patients with advanced disease showed encouraging response when Maraviroc, an antagonist to CCR5 receptor of CCL5 chemokine, was used in a preclinical study (Halama et al., 2016).

2.8.2 Inducing TAMs depletion

TAMs depletion, already recruited to the tumor, would also potentiate anti-tumor activity in principle, similar to the recruitment blockage paradigm. A wide array of targets and molecules have been employed in basic and clinical research for TAMs depletion (Zhou et al., 2020). Two of these, namely Bisphosphonates and Trabectidin, are already in clinical use as anti-cancer agents for specific indications. Bisphosphonates are mostly used in patients with bony metastases from solid tumors e.g., breast (Diel et al., 1998; Powles et al., 2002) and prostate cancers (Macherey et al., 2017) or against myeloid element in hematological malignancy. As inhibitors of the farnesyldiphosphonate synthase, these tend to accumulate in bone hydroxyapatite where they are taken up by the bone macrophages (osteoclasts), leading to their apoptosis (Junankar et al., 2015). This macrophage apoptosis is also witnessed non-selectively in non-bony tissues, for example in the liposomal formulation of clodronate, and has found efficacy in reducing visceral as well as bony metastasis in patients with breast cancer. Trabectidin is an alkylating chemotherapeutic drug that is approved for treatment of advanced ovarian cancer (Ventriglia et al., 2018) , liposarcoma, leiomyosarcoma and other soft tissue sarcomas (Gordon, Sankhala, Chawla, & Chawla, 2016). Along with its direct cytotoxic effect on neoplastic cells, it has been shown to markedly reduce tissue concentration of TAMs by 30-70% through the TRAIL dependent pathway of apoptosis (Germano et al., 2013).

Another enticing target to induce TAMs apoptosis has been through the Colony Stimulating Factor-1 (CSF-1) and its receptor CSF1R pathway (Mantovani, Marchesi, Malesci, Laghi, & Allavena, 2017). This pathway has major role in maturation and differentiation of macrophages and monocytes. Antibodies directed against the CSF1R receptor has been shown in murine models to significantly reduce the number of TAMs in tumor tissue that appears to be more selective for M2 macrophages. Emactuzumab, is the more widely used agent that has been utilized in clinical trials of solid tumors (Gomez-Roca et al., 2019; Machiels et al., 2020) like breast, prostate and ovarian cancers. The anti-neoplastic agents commonly used in these tumors tend to upregulate CSF1/CSF1R complex leading to

increasing recruitment, activation and differentiation of macrophages to TAMs, and blocking this pathway has resulted in significant reduction of TAMs population in these tumors even in clinical studies. An alternative to antibody approach has been to utilize tyrosine kinase inhibitor like pexidartinib to block the CSF1R receptor. This has found clinical efficacy and approval in enhancing the response in advanced prostate in combination with hormonal therapy. In general, because of the nonspecific response against macrophages throughout the body, these present a lot of side effects and further work is being directed toward agents that would provide selectivity for M2 macrophages in tumor tissues by targeting for example CD-163 receptors for cell selection.

2.8.3 Reeducating TAMs: M2 to M1

Utilizing TAMs plasticity i.e., ability of converting to M2 from M1 phenotype and vice versa provide other potential means to influence TME. Reeducating M2 to M1 would revert the antitumor potential and may potentially improve patient survival (van Dalen, van Stevendaal, Fennemann, Verdoes, & Ilina, 2018; M. A. F. Yahaya, Lila, Ismail, Zainol, & Afizan, 2019). Alternatively, preventing M1 conversion to M2 may also achieve this goal by preventing pro-tumoral effects of M2. This construct is still in the realm of experimental or preclinical studies. There are a host of theoretical pathways to achieve that and an increasing number of candidate drugs to modulate these pathways. An example is Zoledronic acid and its effects on TAMs repolarization from M2 to M1 (Giuseppina Comito1 & 2016). An indirect clinical correlation was provided by Comito et al., through their clinical trial with addition of Zoledronic Acid to endocrine therapies among 1803 premenopausal breast cancer patients, as part of ABCSG-12 randomized trial, was shown to achieve improved survival (Gnant et al., 2011). More commonly experimental models focus on STAT3 and NK-κB pathways disruption, using for example antibodies like anti-CD 40 antibody, or designer molecules, e.g., FLLL32, a diketone analogue of curcumin to achieve repolarization (M. A. F. Yahaya et al., 2019).

2.8.4 TAMs Manipulation: other alternatives

Alternative strategies, mentioned earlier, that promote the phagocytic activity of macrophages by blocking CD47-SIRPα signaling, inhibition of TAMs by PD-L1 antibody to promote phagocytic activity, monoclonal antibodies directed against the LILRB1 component of the LILRB1/ MHC class 1 identification mechanism and genetically engineered TAMs lacking the SIRPα and LILRB1 receptors, are few other strategies for TAMs manipulation (Barkal et al., 2018). Most of these are still a long way from clinical utility though and mostly experimental constructs at this time.

2.9 Enumerating TAMs: the technical fine print

All the theoretical basis of TAMs as tools to prognosticate CRCs or other cancers are meaningless without conversion to laboratory-based practice paradigm. The basic steps of outlining TAMs population in the tumor's pathological specimen, distinguishing polarized subtypes, enumerating and defining their densities in different zones (e.g., tumor stroma and tumor front), and comparing ratios, need to be standardized and reproducible in the clinical lab setting. Only then this tool will translate to practice.

2.9.1 Evaluating TAMs and polarization states: the preferred method

Advancements in immunological methods provide multiple methods for accessing TAMs in pathological specimen (Wu et al., 2020). Flow cytometry (Krijgsman et al., 2020), gene expression profiling (Orecchioni, Ghosheh, Pramod, & Ley, 2019; Oshi et al., 2020), and immunohistochemistry are the more commonly used in research lab setting with the high-throughput single cell sequencing (Wu et al., 2020) providing the cutting edge technology for the purpose. There is significant limitation to all of these methods except IHC in the clinical lab though. The requirement for destruction of tissue architecture along with cost and time feasibility is an example. IHC is thus the preferred method in this regard, especially as it enables assessment of TAMs and its subtypes in relation to histological phenotype of the whole tissue specimen akin to TME.

Among the two commonly used IHC methods i.e., immunofluorescence and the chromogenic method, the preference, in general, is for conventional IHC instead of immunofluorescence (IF). This again owes to the practical considerations. IF, for instance needs special handling as it commonly requires fresh frozen tissue and fluorescence microscope. In contrast, IHC is performed on paraffin-embedded sections and light microscopes (Jayasingam et al., 2019).

IHC requires determination of best antibody markers to differentiate TAMs from other cells in the specimen as well as outlining the M1 and M2 polar types. There is no consensual practice in this regard and studies have used different markers or marker combinations for distinguishing TAMs and subtypes. Broadly speaking, anti-CD68 is most commonly utilized for marking TAMs, without differentiation. For M1 macrophages, usually applied antibodies are HLA DR, iNOS or inducible nitric oxide synthase, and pSAT1. In contrast, anti-CD204, CD206, and CD163 are frequently used for M2 subtype (Wu et al., 2020). Some have used two markers for staining each subtype to theoretically improve specificity in assessment (Greening, Bess, & Muldoon, 2018). Mostly the combination is of CD68 with any one antibody for the specific subtype, for example CD68/CD163 for M2. This approach obviously increases the cost and processing needs.

2.9.2 Enumerating TAMs: quantification methods and standardization issue

Commonly an averaging method of either 3, 5 or 10 high density or “hot” fields is performed to quantify TAMs and subtypes. In study setting, many investigators employed two pathologists for countering inter-observer variance. Others used automated image-assessment software.

Simplistic in theory but in practice a number of processing variations can introduce discrepancy in enumerating TAMs. These include tissue collection, fixation, thickness, staining and assessment. Till these issues are sorted, inter-rater differences will hamper standardization (Jayasingam et al., 2019).

CHAPTER 3

METHODOLOGY

3.1 Study Design

Cross-sectional study

3.2 Subjects

Paraffin embedded colorectal tissue blocks

3.3 Setting

PNS Shifa Hospital, Karachi, Pakistan

3.4 Inclusion Criteria

- Both the genders
- Patients > 18 years of age
- Primary colorectal cancer specimens, with the adenocarcinoma morphological type

3.5 Exclusion Criteria

Following specimens will be excluded:

- Specimens obtained from patients < 18 years of age
- Poorly fixed tissues
- Endoscopic biopsy specimen
- Metastatic tumors

3.6 Duration of Study

Individual study period: 2-3 days

Total period of study: 6 months

3.7 Sample Size

A total of 43 specimen were evaluated, based on average of population incidence estimate of colorectal cancer in Pakistan at 6.9% (Idrees et al., 2018), *see the calculation below*), with a 5% confidence level and 95% confidence interval.

3.8 Sample Size Estimation

Based on average of population incidence estimate of 6.9% (based on data from seven studies from Pakistan, ranging from 5% to 7.5%.(Idrees et al., 2018)

Population size (for finite population correction factor or fpc)(*N*):	1000000
Hypothesized % frequency of outcome factor in the population (*p*):	5.9%+/-5
Confidence limits as % of 100(absolute +/- %)(*d*):	5%
Design effect (for cluster surveys-*DEFF*):	0.5

Sample Size(*n*) for Various Confidence Levels

Confidence Level (%)	Sample Size
95%	**43**
80%	19

90%	31
97%	53
99%	74
99.9%	121
99.99%	169

Equation

Sample size n = $[DEFF*Np(1-p)]/ [(d^2/Z^2_{1-\alpha/2}*(N-1)+p*(1-p)]$

Results from OpenEpi, Version 3, open source calculator.

3.9 Sampling Technique

Convenient sampling (non-probability)

3.10 Human Subjects and Consent

No direct involvement of human subjects. Paraffin embedded colorectal cancer tissue blocks will be used.

3.11 Materials Used

- Formalin fixed paraffin embedded tissue blocks of colorectal cancer
- Surgical pathology records
- Hematoxylin and Eosin stained slides of all samples
- Positive tissue controls: Formalin fixed paraffin embedded tissue blocks of liver and lymph node
- Primary antibodies, CD163 (clone ZM29), prediluted monospecific Mouse monoclonal and CD68 (clone KP1) prediluted Mouse monoclonal supplied by Zeta corporation
- Zeta max HRP polymer detection kit containing the amplifier and HRP polymer
- Chromogen substrate
- Hematoxylin for counter stain
- Poly-lysine coated slides and cover slips
- Xylene
- Ethanol
- Distilled water
- Wash buffer
- Peroxide block
- Heating utensil for antigen retrieval

- Mounting medium
- Timer
- Light microscope

3.12 Parameters of Study

3.12.1 Clinical Parameters

Clinical parameters include:

- Gender
- Age
- Site of tumor
- Size of tumor
- Presence or absence of macroscopic tumor perforation

3.12.2 Pathological parameters

Pathological parameters include:

- Histologic type of tumor
- Histologic grade of tumor
- pTNM stage
- Presence or absence of perineural invasion
- Presence or absence of lympho-vascular invasion
- CD68+ cells in tumor front and stroma
- CD163+ cells in tumor front and stroma

3.13 Protocol of Study

3.13.1 Hematoxylin and Eosin (H&E) staining

Sections were proceeded through numerous solutions as follows:

- Xylene I – 10 minutes
- Xylene II – 10 minutes
- Absolute alcohol – 10 minutes
- 95% alcohol – 5 minutes
- 80% alcohol – 5 minutes
- 70% alcohol – 5 minutes
- Tap water rinse – 2 minutes
- Harris hematoxylin – 5- 10 minutes
- Acid alcohol 1%, 3-5 dips, then washed with tap water
- Ammonia water, 3-5 dips, then rinsed with tap water for 10 minutes
- Eosin – 2 minutes
- 70% alcohol – 5 quick dips
- 80% alcohol – 5 quick dips
- 95% alcohol – 5 quick dips
- Absolute alcohol – 2 changes, 5 minutes each
- Xylene – 5 minutes
- Mounted in Dako toluene free mounting media

Resulting in blue nuclei and cytoplasm displaying varying shades of pink

1. *Kiernan, J. A. (2008). Histological and histochemical methods: theory and practice. 4th ed. Bloxham, UK; Scion. Retrieved from https://www.protocolsonline.com/histology/dyes-and-stains/haematoxylin-eosin-he-staining/*

3.13.2 Immunohistochemistry

- Clinical data, biopsy reports and all relevant information were reviewed and recorded.
- H&E stained slides of CRC were retrieved and reviewed by a senior histopathologist, and the required tissue blocks retrieved.
- Formalin fixed paraffin embedded (FFPE) blocks were then cut into 4 µm thick sections using microtome and placed on poly L-lysine coated slides.
- Slides were fixed in oven at 80^{o}C for 20- 25 minutes.
- De-paraffinization and rehydration performed using Xylene and ethanol.
- After de-paraffinization, endogenous peroxidase activity was blocked by treating the sections with H_2O_2 for 5 minutes.
- Antigen retrieval was then performed using a pressure cooker, by boiling in Citrate buffer (PH 6.0) for 7 minutes at 98^{o}C.
- Following that, sections were allowed to cool down for 25 minutes.
- Washing with phosphate buffer saline (PBS) solution (PH 7.2) was then performed.
- Next, sections were incubated with prediluted mouse monoclonal CD68 (KP1) and monospecific mouse monoclonal CD163 (ZM29) antibodies for 1 hour at room temperature.
- After thorough washing with PBS, primary antibody was visualized using Zeta max HRP polymer detection kit.
- Finally, the sections were counterstained with hematoxylin and mounted.
- Positive tissue control for CD68 was lymph node and liver for CD163.
- These controls were run with the initial few slides and were treated in the similar fashion as the sample specimens.

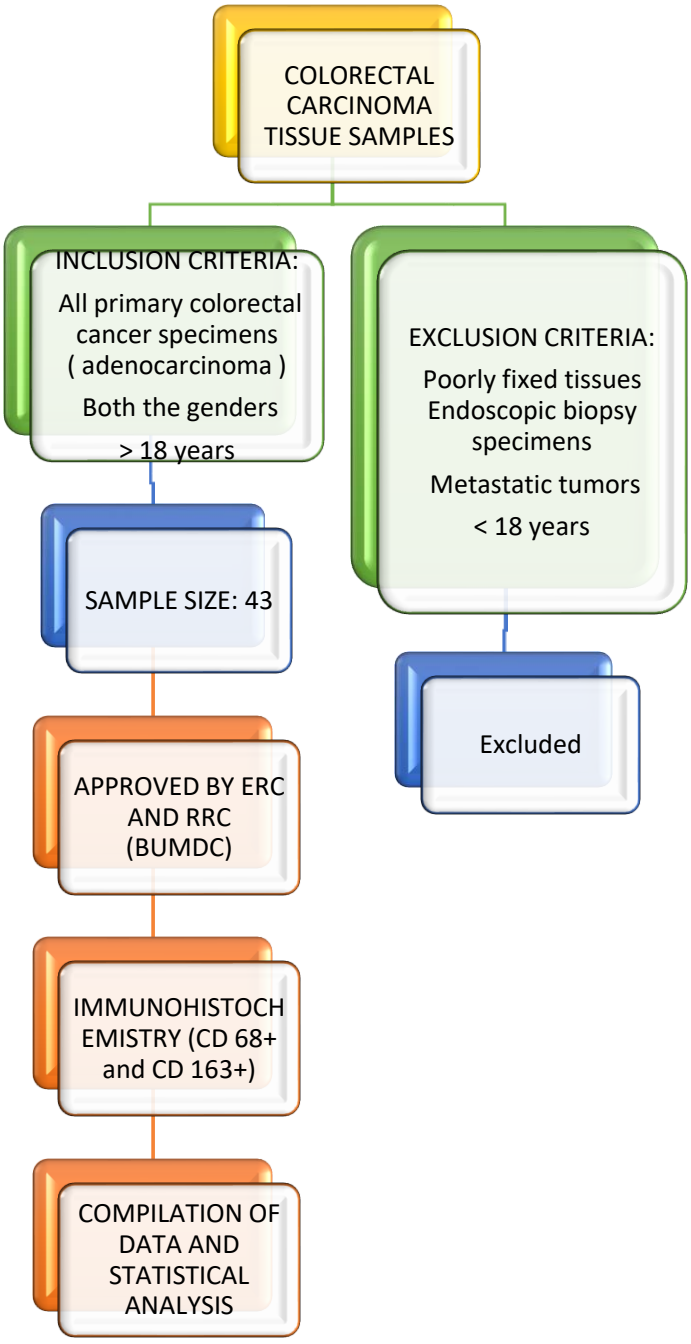
1. *Pinto, M. L., Rios, E., Durães, C., Ribeiro, R., Machado, J. C., Mantovani, A., ... & Oliveira, M. J. (2019). The two faces of tumor-associated macrophages and their clinical significance in colorectal cancer. Frontiers in immunology, 10, 1875.*

3.13.3 TAMs enumeration

- After completing the IHC, slides were studied by light microscopy at low- power fields (LPFs, 40x- 100x), using Leica DM 1000 microscope.
- Tumor stroma and tumor front were defined.
- Cells immunoreactive to CD68/ CD163 antibodies and exhibiting macrophage like morphology were considered as M1 and M2 macrophages respectively.
- The total number of CD68+ and CD163+ macrophages were counted in a continuous line , from left to right, in 5 high- power fields (HPFs, 400x).
- The counting was done along the tumor front and in the tumor stroma for both the antibodies along with a senior pathologist.
- The mean number of CD68+ and CD163+ macrophages, per HPF (5 spots), for each slide (tumor front and stroma) was defined as TAMs and M2 density.
- TAMs density was further divided into high and low after calculating the median value for both tumor front and stroma.
- M2 proportion was calculated as discussed earlier and was further divided into high and low after calculating the median value for both the tumor front and stroma.
- Results were analyzed statistically using SPSS version 23.

1. *Salmi, S., Siiskonen, H., Sironen, R., Tyynelä-Korhonen, K., Hirschovits-Gerz, B., Valkonen, M., ... & Pasonen-Seppänen, S. (2019). The number and localization of CD68+ and CD163+ macrophages in different stages of cutaneous melanoma. Melanoma research, 29(3), 237.*
2. *Morita, Y., Zhang, R., Leslie, M., Adhikari, S., Hasan, N., Chervoneva, I., ... & Tanaka, T. (2017). Pathologic evaluation of tumor-associated macrophage density and vessel inflammation in invasive breast carcinomas. Oncology letters, 14(2), 2111-2118.*
3. *Hu, J. M., Liu, K., Liu, J. H., Jiang, X. L., Wang, X. L., Chen, Y. Z., ... & Li, F. (2017). CD163 as a marker of M2 macrophage, contribute to predict aggressiveness and prognosis of Kazakh esophageal squamous cell carcinoma. Oncotarget, 8(13), 21526.*

3.14 Algorithm of Study



3.15 Statistical Analysis

All the data collected was entered into specifically designed database in Statistical Package for the Social Sciences (SPSS version 23). Descriptive statistics were generated for the baseline characteristics including patients' demographics, tumor's pathological characteristics, differential counts of TAMs densities and M2 proportions. Measure of central tendencies and dispersion were calculated with mean and standard deviation for continuous variables and for the categorical data frequencies and percentages were determined. We also assessed medians in cases where the distribution plots demonstrated significant outliers and skewed tails.

Further analysis was performed in two steps. First a cross-tabulation of main variables was performed to determine significant differentiation of subgroups utilizing Pearson's chi square across all variables. In cases where quantitative variables were in symmetrical dichotomous distribution across the cross-tabulating groups and a 2X2 table was generated, a McNemar t-test was also applied. Only the statistically significant differentiation was reported in such cases. Where appropriate p value < 0.05 will be considered statistically significant.

In the next step we performed a logistic regression analysis to determine significant correlation of TAMs densities and M2 proportion in the tumor stroma and the tumor front with the main outcome variable, viz, pathological tumor stage.

Chapter 4

Results

Histopathology specimen of 43 patients were selected according to the selection criteria outlined earlier and the sample size calculation. Their related clinical information was also obtained and recorded on the study proforma and entered in the electronic database. Following data analysis, baseline characteristics of the patients and tissue specimen were outlined and subsequently compared in relation to the age, gender, histological characteristics, and pathological stage as detailed in the following sections.

4.1 Baseline Characteristics

Among the included patients, two-thirds were male patients (n=30, 69.8%) with a median age of 54 years (Range 18-80 years of age). Most of the samples represented colonic resection specimens as opposed to the remaining third that were obtained after rectal or rectosigmoid resection (n=12, 27.9%). Tumors were most commonly found in cecum, sigmoid colon and rectum (combined n= 29, 67.4,%) with overall propensity

toward the left side of the colon, along with rectum (n=25, 58%), in terms of distribution. Further details are summarized in Table 4.1.1.

The tumor characteristics demonstrated well differentiated tumors (n=21, 48.8%) and adenocarcinoma (n=36, 83.7%) as the predominant histology, staged as pT3 (n=26, 60.5%), pN1 (n=23, 53.3%) with a median size of 4.5 cm in the greatest dimension. No specimen was associated with histologically proven tumor metastasis. A small proportion of tumor had identified tumor perforation (n=3, 7%) and perineural invasion (n=9, 20.9) but more than half the specimen showed lympho-vascular invasion (n=24, 55.8%). Overall TNM stage III was more prevalent (n=26, 60.5%). Further details are included in table 4.1.2.

	A. Demographics		
1	Age	mean(±SD)	51.02 (16.9)
2	Gender	n (%)	
	2.1 Male		30 (69.8)
	2.2 Female		13 (30.2)
	B. Specimen details		
3	Procedure:	n (%)	
	3.1 Right Hemicolectomy		18 (41.9)
	3.2 Left Hemicolectomy		7 (16.3)
	3.2 Sigmoidectomy		7 (16.3)
	3.3 Rectal Resection (APR or LAR)		11 (25.5)
4	Tumor Site:	n (%)	
	4.1 Cecum (including ileocecal valve)		11 (25.5)
	4.2 Right Colon (including hepatic flexure)		6 (13.9)
	4.3 Transverse Colon		1 (2.3)
	4.4 Left Colon (including splenic flexure)		3 (7.0)
	4.5 Sigmoid Colon		10 (23.3)
	4.6 Rectosigmoid		4 (9.3)
	4.7 Rectum		8 (18.6)
5	Tumor Size:	mean (±SD)	
	5.1 Greatest Dimension (cm)		6.18 (4.8)
	5.2 Additional Dimension (cm)		3.9 (2.1)

Table 4.1.1 Baseline Characteristics

	A. Histology	n (%)
1	Histologic Type	
	1.1 Adenocarcinoma	36 (83.7)
	1.2 Mucinous adenocarcinoma	6 (14.0)
	1.3 Signet-ring cell adenocarcinoma	1 (2.3)
2	Histologic Grade	
	2.1 G1: Well differentiated	21 (48.8)
	2.2 G2: Moderately differentiated	14 (32.5)
	2.3 G3: Poorly differentiated	8 (18.6)
	B. TNM	
3	Primary Tumor	
	3.1 pT1	3 (7.0)
	3.2 pT2	8 (18.6)
	3.3 pT3	26 (60.5)
	3.4 pT4	6 (14.0)
4	Regional Lymph Node	
	4.1 pN0	17 (39.5)
	4.2 pN1	23 (53.3)
	4.3 pN2	3 (7.0)
5	Distant Metastasis	
	5.1 pMX	43 (100)
	5.2 pM1	0 (0)
	C. Additional Features	
6	Perforation	
	6.1 Not identified	40 (90.3)
	6.1 Present	3 (7.0)
7	Lympho-vascular invasion	
	7.1 Not identified	19 (44.2)
	7.2 Present	24 (55.8)
8	Perineural invasion	
	8.1 Not identified	34 (79.1)
	8.2 Present	9 (20.9)
	D. Pathological Tumor Stage	
9	9.1 Stage I	5 (11.6)
	9.2 Stage II	12 (27.9)
	9.3 Stage III	26 (60.5)
	9.4 Stage IV	0 (0)

Table 4.1.2: Tumor Characteristics

4.2 Age

In order to study the relationship of age with the demographic and tumor characteristics, we employed two different stratification criteria. The primary criteria was the clinically defined, early-onset versus late-onset or standard-onset colorectal cancer. This arbitrary definition is used regularly in clinical studies whereby 50 years of age sets the cutoff between the two groups. As traditionally, CRC occurs commonly in patients more than 50 years of age, being the rationale for the related screening age, all patients at or above 50 years were grouped in to late or standard-onset group. Contrariwise all patient at or under 49 years of age were included in the early-onset CRC group. The other criteria we used to distinguish age groups was similarly arbitrary with less than 25 years, 25-50 years and more than 50 years of age representing the three age stratifications.
For the later stratification, expectedly, most patient were more than 50 years of age (n=24, 56%, as shown in figure 4.2.1.). Crosstabulation to explore significant association of these age strata with demographic and tumor characteristics, based on Pearson's Chi-square showed, that the tumor grade and dimensions were not independently distributed in relation to these age groups as detailed in table 4.2.1. and table 4.2.2. These are further presented graphically in figures 4.2.2 to figure 4.2.6.

Similar analysis for early onset versus late-onset CRC demonstrated a sizable proportion of patients with early-onset CRC (n=17, 40%, as shown n figure 4.2.7). This is somewhat unexpected, and we will explore this further in the discussion section. As regards to association of age of onset with demographic and tumor factors again demonstrated that tumor grade was significantly related to this age distribution. No other factor including tumor dimension showed similar significant association, as detailed in table 4.2.3, table 4.2.4 and table 4.2.5. These are graphically represented in figures from 4.2.8 to 4.2.12.

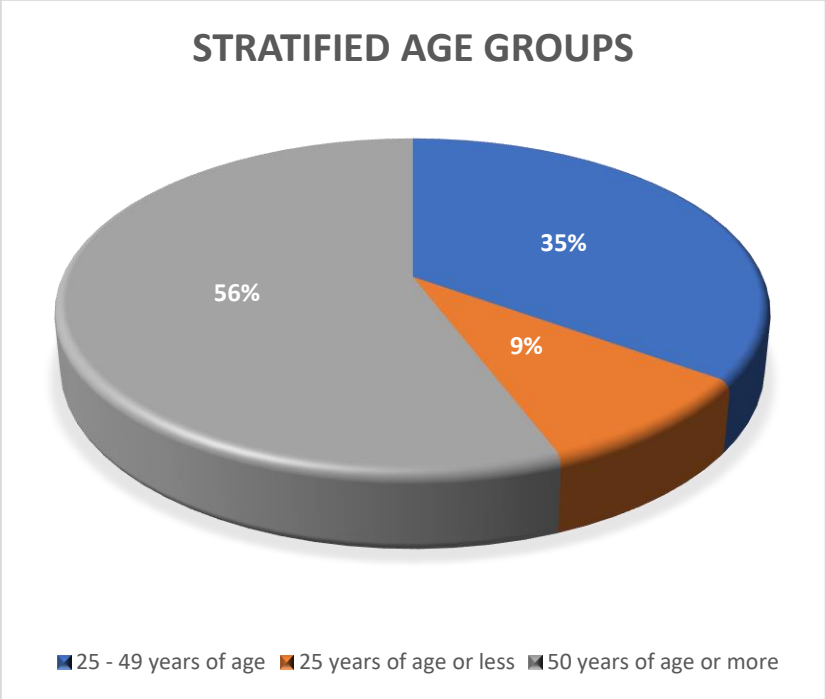


Figure 4.2.1: Stratified Age Groups

		Age Stratified				
		25 years of age or less	25 - 49 years of age	50 years of age or more	Total	*p*-value
Gender	Male	1	11	18	30	
	Female	3	4	6	13	
Total		4	15	24	43	*0.20*
Tumor site	Left colon	2	6	8	16	
	Rectum	1	4	3	8	
	Trans.Colon*	0	1	2	3	
	Right colon	1	4	11	16	
Total		4	15	24	43	*0.34*
Histology	Adenocarcinoma	2	13	21	36	
	Mucinous adenoca.	1	2	3	6	
	Signet- ring cell ca.	1	0	0	1	
Total		4	15	24	43	*0.09*
Tumor grade	G1	1	5	15	21	
	G2	0	7	7	14	
	G3	2	2	0	4	
	Not applicable	1	1	2	4	
Total		4	15	24	43	***0.05***
Stage	I	0	3	2	5	
	II	0	4	8	12	
	III	4	8	14	26	
Total		4	15	24	43	*0.52*

*Transverse colon, including the hepatic and splenic flexures

Table 4.2.1: Cross-tabulation of Stratified Age with Demographic and Tumor Characteristics

	Age Stratified			
	Age 25 years or less	Age 25 - 49 years	Age 50 years or more	Chi-square
	Mean (± s.d.)	Mean (± s.d.)	Mean (± s.d.)	*p*-value
Tumor dimension 1	6.55 (4.88)	6.01 (2.56)	6.42 (5.88)	***0.001***
Tumor dimension 2	2.25 (0.65)	4.54 (2.03)	4.00 (2.11)	***0.019***

Table 4.2.2: Cross-tabulation of Stratified Age with Tumor Dimensions (Based on Pearson's Chi-square)

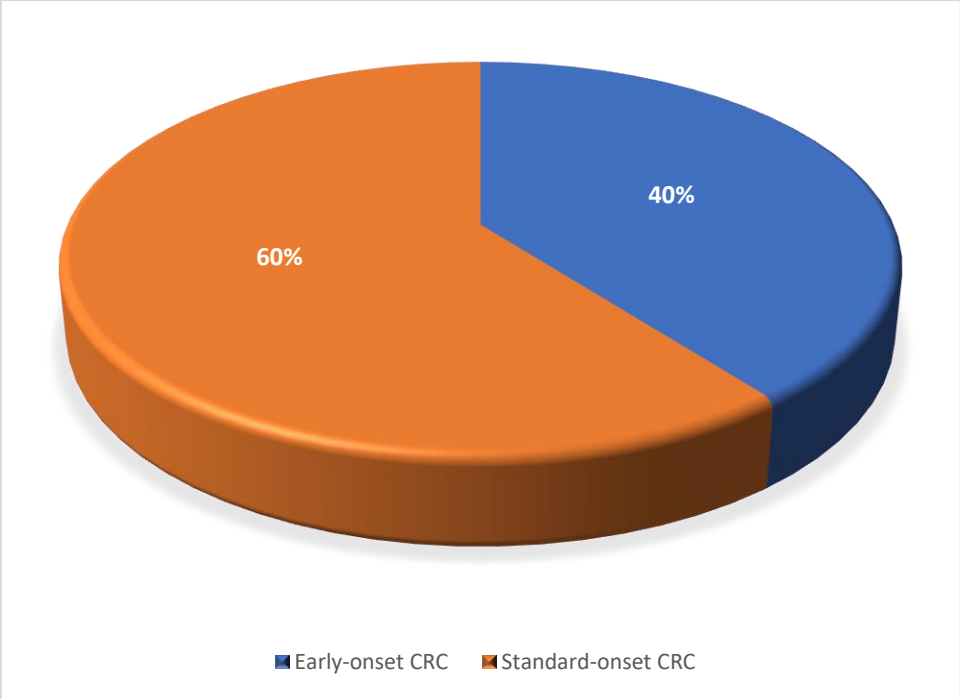


Figure 4.2.2: Early- onset CRC vs Late-onset CRC

		Age Group: Early Onset Versus Late Onset CRC			
		Eary-Onset CRC	Late-Onset CRC	Total	*p*-value
Gender	Male	11	19	30	
	Female	6	7	13	
Total		17	26	43	*0.55*
Tumor site	Left colon	7	9	16	
	Rectum	5	3	8	
	Trans. Colon*	1	2	3	
	Right colon	4	12	16	
Total		17	26	43	*0.34*
Histology	Adenocarcinoma	13	23	36	
	Mucinous adenoca.	3	3	6	
	Signet- ring cell ca.	1	0	1	
Total		17	26	43	*0.37*
Tumor grade	G1	5	16	21	
	G2	6	8	14	
	G3	4	0	4	
	Not applicable	2	2	4	
Total		17	26	43	***0.03***
Stage	I	2	3	5	
	II	3	9	12	
	III	12	14	26	
Total		17	26	43	*0.46*

*Transverse colon, including the hepatic and splenic flexures

Table 4.2.3: Cross-tabulation of Age of Onset with Demographic and Tumor Characteristics

	Size Distribution		
	Tumor Dimension 1	Tumor Dimension 2	Chi-square Test
	Mean (± s.d.)	Mean (± s.d.)	*p*-value
Early-onset	5.88 (3.26)	3.74 (2.11)	*0.18*
Standard-onset	6.38 (5.67)	4.06 (2.13)	*0.90*

Table 4.2.4: Cross-tabulation of Age of Onset with size distribution (*Based on Pearson's chi-square)*

	Macroscopic tumor perforation		Lympho-vascular invasion		Perineural invasion	
	Not identified (n)	Present (n)	Not identified (n)	Present (n)	Not identified (n)	Present (n)
Early-onset CRC	15	2	7	10	15	2
Standard-onset CRC	25	1	12	14	19	7
p value	0.31		*0.74*		*0.23*	

Table 4.2.5: Cross-tabulation of Age of Onset with : tumor perforation, lympho-vascular invasion, perineural invasion. (*Based on Pearson's chi-square)*

4.3 Gender

For analysis of gender-related differences in the patient population and their tumor characteristics, similar cross-tabulation was utilized as defined in the age section (Section 4.2). In general male to female ratio in the study sample was 2.3:1 (figure 4.3.1).

The trends identified included a higher frequency of cancer in right colon among males (n=15, 50%), compared to females, who showed more predilection for the left colon (n=8, 61%), both groups having well-differentiated tumors (males 47%, females 54%) and adenocarcinoma (males 87%, females 77%) as the most common histology, and a higher frequency of stage III tumors (males 63%, females 54%). These are detailed in table 4.3.1 and graphically represented in figures from 4.3.2 to 4.3.8

The median tumor size was bigger in females in both dimensions (see table 4.3.2) without reaching significance. Nearly 80% of either gender had perineural invasion. The two measures that showed significant difference between the two genders, based on McNemar T-test, were macroscopic tumor perforation and lympho-vascular invasion. The details of later assessment are presented in table 4.3.3.

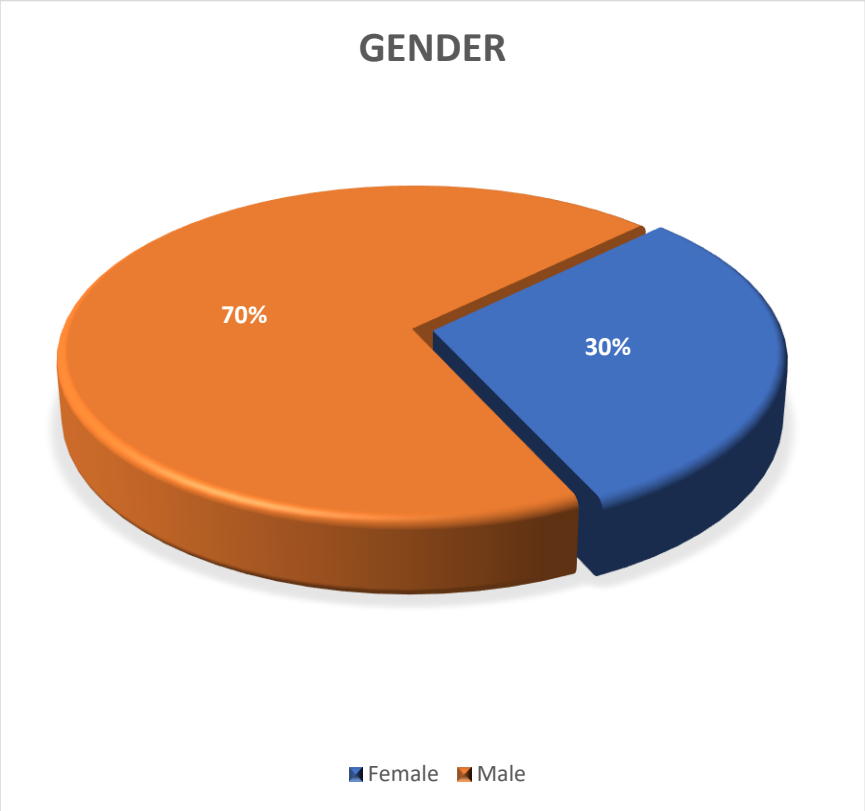


Figure 4.3.1: Gender distribution of CRC patients

		Gender			
		Male	Female	Total	*p*-value
Tumor site	Left colon	8	8	16	
	Rectum	7	1	8	
	Trans. Colon*	3	0	3	
	Right colon	12	4	16	
Total		30	13	43	*0.12*
Histology	Adenocarcinoma	26	10	36	
	Mucinous adenoca.	4	2	6	
	Signet- ring cell ca.	0	1	1	
Total		30	13	43	*0.29*
Tumor grade	G1	14	7	21	
	G2	11	3	14	
	G3	3	1	4	
	Not applicable	2	2	4	
Total		30	13	43	*0.70*
Stage	I	4	1	5	
	II	7	5	12	
	III	19	7	26	
Total		30	13	43	*0.56*

*Transverse colon, including the hepatic and splenic flexures

Table 4.3.1: Cross-tabulation of Gender with Demographic and Tumor Characteristics

	Size Distribution	
	Tumor Dimension 1	Tumor Dimension 2
	Mean (± s.d.)	Mean (± s.d.)
Male	6.12 (5.36)	3.77
Female	6.32 (3.45)	4.31
p-value[1]	*0.53*	*0.75*

Table 4.3.2: Cross-tabulation of Gender with tumor size distribution *Based on Pearson's chi-square*

	Macroscopic tumor perforation		Lympho-vascular invasion		Perineural invasion	
	Not identified (n)	Present (n)	Not identified (n)	Present (n)	Not identified (n)	Present (n)
Male	29	1	11	19	24	6
Female	11	2	8	5	10	3
p value[1]	***0.006***		***0.05***		0.45	

Table 4.3.3: Cross-tabulation of Gender with tumor perforation, lympho-vascular invasion, perineural invasion, *Based on McNemar T Test*

4.4 Tumor Site and Sidedness:

College of American Pathologists reporting criteria divides colon and rectum in to ten zones for defining tumor site. Based on these criteria, as demonstrated in figure 4.4.1, cecum, sigmoid colon and rectum altogether represented two thirds all the CRC specimens in this series (n=29). Sidedness into a simple left and right-sided CRC is a more clinically relevant criterion that as shown prognostic value. The term right sided colon cancers includes all the CRCs up till the splenic flexure, whereas left sided colon cancers include the malignancies of the descending colon , sigmoid colon and the rectosigmoid (Gowarty, Durham, Wong, & Chen, 2019). This criterion was thus utilized for further analytical assessment.

Generally, more than half the tumors were left-sided (including rectum) (n=24, 56% as shown in figure 4.4.2). Cross-tabulation demonstrated a fairly even distribution of gender, histology, grade and stage between left and right-sided CRCs, as detailed in table 4.4.1. These are graphically presented in figures from 4.4.3 to figure 4.4.6. There were similarly no evident differences for macroscopic tumor perforation and perineural invasion among the two sides. The mean size of right-sided tumor was relatively larger and there was reversal of distribution as regards to lympho-vascular infiltration for the two sides but even these like all other criteria did not show statistical significance, as exhibited in table 4.4.2 and 4.4.3.

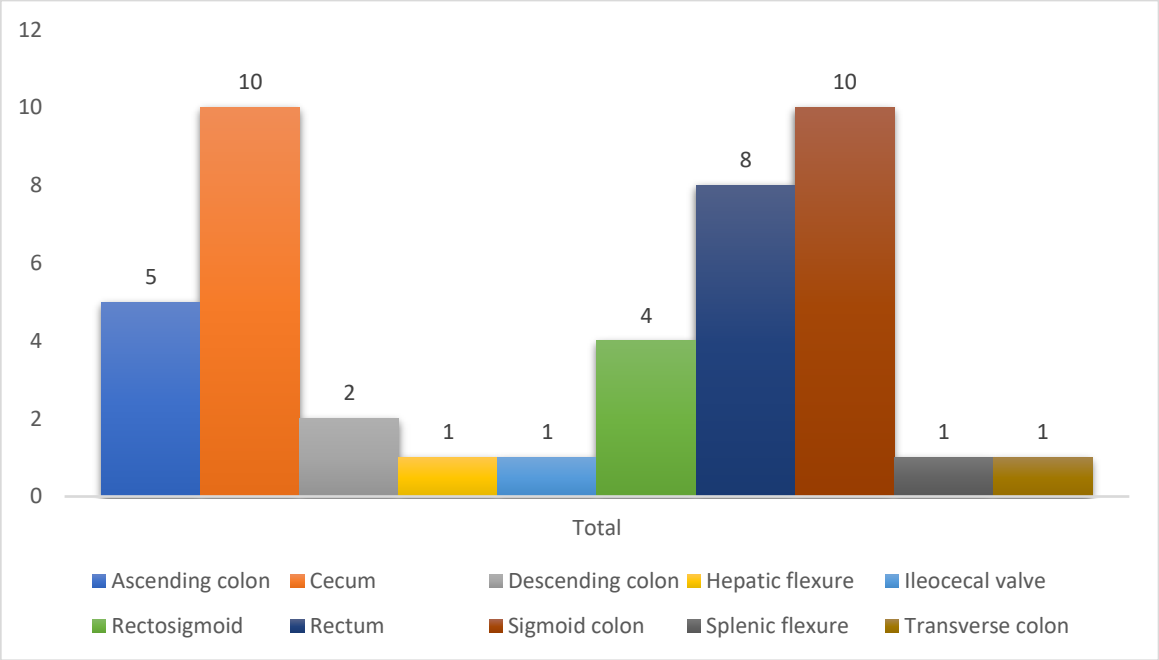
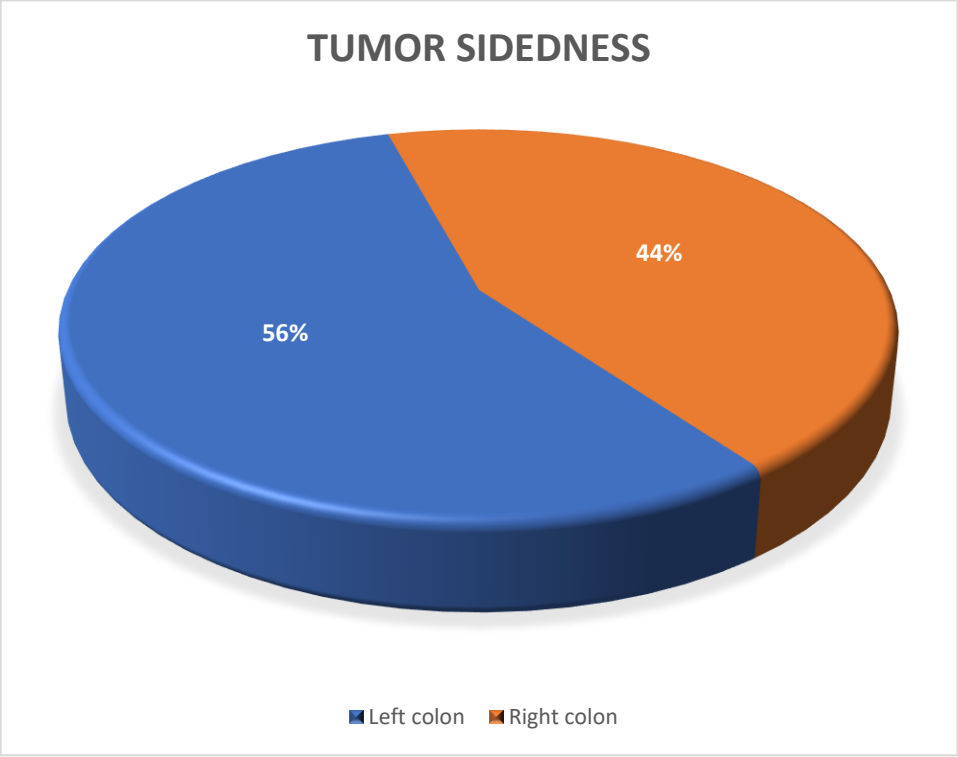


Figure 4.4.1: Tumor Site: ***based on (College of American Pathologists) CRC pathology reporting criteria***



4.4.2 Tumor Site: *based on sidedness criterion*

		Tumor Sidedness			
		Left Colon	Right Colon	Total	*p*-value
Gender	Male	15	15	30	
	Female	9	4	13	
Total		24	19	43	*0.24*
Histology	Adenocarcinoma	21	15	36	
	Mucinous adenoca.	2	4	6	
	Signet- ring cell ca.	1	0	1	
Total		24	19	43	*0.34*
Tumor grade	G1	11	10	21	
	G2	8	6	14	
	G3	4	0	4	
	Not applicable	1	3	4	
Total		24	19	43	*0.18*
Stage	I	2	3	5	
	II	7	5	12	
	III	15	11	26	
Total		24	19	43	*0.75*

Table 4.4.1: Cross-tabulation of Tumor side with Demographic and Tumor Characteristics

	Size Distribution	
	Tumor Dimension 1	Tumor Dimension 2
	Mean (± s.d.)	Mean (± s.d.)
Right Colon	5.93 (3.06)	4.39 (2.29)
Left Colon	6.38 (5.92)	2.29 (1.91)
Chi-square Test (*p*-value)	0.83	0.39

Table 4.4.2: Cross-tabulation of Tumor Side with tumor size distribution, *(Based on Pearson's Chi-Square Test)*

	Macroscopic tumor perforation		Lympho-vascular invasion		Perineural invasion	
	Not identified (n)	Present (n)	Not identified (n)	Present (n)	Not identified (n)	Present (n)
Right Colon	18	1	8	11	17	2
Left Colon	22	2	11	13	17	7
p value[1]	*0.69*		*0.80*		*0.13*	

Table 4.4.3: Cross-tabulation of Tumor Side with tumor perforation, lympho-vascular invasion, perineural invasion, (*Based on Pearson's Chi-Square Test)*

4.5 Tumor associated macrophages (TAMs) and M2: *densities, proportions and ratios*

The primary objective of this study was to analyze the distribution of TAMs in relation to the tumor stage. Let us look at the distribution of TAMs and subtypes first. Overall, the mean number of TAMs was relatively higher in the tumor front as compared to tumor stoma, but only reached significance for M1 subtype. This is shown in table 4.5.1. For further analyses of TAMs density we stratified the densities into high density and low density based on the median values (see table 4.5.2). Similarly, the M2 proportion in the stroma and the tumor front was also categorized.

Crosstabulation with the age of onset of CRC showed a very homogenous distribution of high and low density of TAMs and M2 proportion in the tumor stroma and at the tumor front irrespective if it was early-onset or late-onset CRC. Comparative densities were higher overall for late-onset CRC as compared to early onset but none of the comparisons showed significant difference based on Pearson's chi-square, as detailed in Table 4.5.3.

Regarding gender, one significant difference was found for the distribution of M2 proportion, whereby tumor front had lower percentage of TAMs among females as opposed to higher proportion in males. This trend of M2 was also evident in the stroma but did not reach statistical significance. The related data is presented in table 4.5.4.

Subsequent evaluation showed no significant difference in TAMs density and M2 proportion, in the tumor stroma and tumor front for the tumor site (table 4.5.5), tumor size (table 4.5.6), pathological tumor type (table 4.5.7), pathological tumor grade (table 4.5.8), and pathological tumor stage (table 4.5.9).

Lympho-vascular infiltration showed significant statistical difference in the TAMs density at the tumor front. Otherwise, no difference was found for macroscopic tumor perforation, perineural invasion in the tumor stroma or front, for the TAMS density as well

as the lympho-vascular invasion in the tumor stroma. For M2 proportion also no difference was found in any of these categories. The details are collated in table 4.5.10.

	Population	**Tumor Stroma** mean (± s.d.)	**Tumor Front** mean (± s.d.)	***p*-value**[1]
	Densities			
1.	TAMs (anti-CD68+)	31.7 (14.05)	40.1 (18.37)	*0.14*
2.	M2 (anti-CD163+)	22.5 (9.70)	30.0 (14.86)	*0.49*
3.	M1 (calculated)	9.2 (8.98)	10.9 (9.36)	***0.02***
	Proportions			
4.	M2 (%)	72.9 (14.89)	75.3 (14.29)	*0.12*
	Ratios			
5.	M1:M2	0.48 (0.69)	0.42 (0.42)	*0.28*

Table 4.5.1: Densities, proportions and ratios of TAMs with subsets in tumor stroma and tumor front. (*Based on Pearson's chi-square)*

Cell population	Tumor Area	Median	High	Low
TAMs Density	Stroma	32	≥ 32	≤ 31
	Front	40	≥ 40	≤ 39
M2 Proportion	Stroma	77	≥ 77	≤ 76
	Front	78	≥ 78	≤ 77

Table 4.5.2: TAMs: Definition of high and low TAMS densities and M2 proportions

	Population	Tumor Area	Density/ Proportion	Early-onset CRC n	Late-onset CRC n	*p*-value[1]
A	TAMs (anti-CD68 +) Density	Front	High	9	13	*0.85*
			Low	8	13	
		Stroma	High	9	13	*0.85*
			Low	8	13	
B	M2 (anti-CD163 +) Proportion	Front	High	9	13	*0.85*
			Low	8	13	
		Stroma	High	8	14	*0.66*
			Low	9	12	

Table 4.5.3: TAMs density and M2 proportion: Age group (early-onset, late-onset). (*Based on Pearson's chi-square)*

	Population	Tumor Area	Density/ Proportion	Male n	Female n	*p*-value[1]
A	TAMs (anti-CD68 +) Density	Front	High	16	6	*0.66*
			Low	14	7	
		Stroma	High	15	7	*0.81*
			Low	15	6	
B	M2 (anti-CD163 +) Proportion	Front	High	19	3	***0.01***
			Low	11	10	
		Stroma	High	18	4	*0.07*
			Low	12	9	

Table 4.5.4: TAMs density and M2 proportion: Gender. (*Based on Pearson's chi-square)*

	Population	Tumor Area	Density/ Proportion	Right Colon n	Left Colon n	*p*-value[1]
A	TAMs (anti-CD68 +) Density	Front	High	13	9	*0.65*
			Low	11	10	
		Stroma	High	12	10	*0.86*
			Low	12	9	
B	M2 (anti-CD163 +)	Front	High	11	11	*0.43*
			Low	13	8	
		Stroma	High	10	12	*0.16*
			Low	14	7	

Table 4.5.5: TAMs density and M2 proportion: Side (right vs left colon).

(*Based on Pearson's chi-square)*

	Population	**Tumor Area**	**Density/ Proportion**	**Tumor Dimension 1** Mean (± s.d.)	**Tumor Dimension 2** Mean (± s.d.)
A	TAMs (anti-CD68 +) Density	Front	High	6.47	4.07
			Low	5.88	3.79
			***p*-value**[1]	0.83	0.86
		Stroma	High	5.52	4.07
			Low	6.88	3.79
			***p*-value**[1]	0.68	0.62
B	M2 (anti-CD163 +) Proportion	Front	High	6.30	3.91
			Low	6.07	3.95
			***p*-value**[1]	0.37	0.55
		Stroma	High	6.61	4.30
			Low	5.73	3.55
			***p*-value**[1]	0.47	0.50

Table 4.5.6: TAMs density and M2 proportion: ***tumor size. (Based on Pearson's chi-square)***

	Population	Tumor Area	Density/ Proportion	Adeno carcinoma n	Mucinous n	Signet-ring cell n	*p*-value[1]
A	TAMs (anti-CD68 +) Density	Front	High	19	3	0	0.58
			Low	17	3	1	
		Stroma	High	17	5	0	0.15
			Low	19	1	1	
B	M2 (anti-CD163 +) Proportion	Front	High	18	4	0	0.43
			Low	18	2	1	
		Stroma	High	17	5	0	0.15
			Low	19	1	1	

Table 4.5.7: TAMs density and M2 proportion: ***pathological tumor type. (Based on Pearson's chi-square)***

	Population	Tumor Area	Density/ Proportion	Grade I n	Grade II n	Grade III n	Not applicable n	*p*-value[1]
A	TAMs (anti-CD68 +) Density	Front	High	10	8	1	3	0.51
			Low	11	6	3	1	
		Stroma	High	10	6	2	4	0.23
			Low	11	8	2	0	
B	M2 (anti-CD163 +) Proportion	Front	High	9	8	3	2	0.63
			Low	12	6	1	2	
		Stroma	High	9	8	2	3	0.63
			Low	12	6	2	1	

Table 4.5.8: TAMs density and M2 proportion: *pathological tumor grade. (Based on Pearson's chi-square)*

	Population	Tumor Area	Density/ Proportion	Stage I n	Stage II n	Stage III n	*p*-value
A	TAMs (anti-CD68 +) Density	Front	High	2	5	15	0.56
			Low	3	7	11	
		Stroma	High	3	6	13	0.91
			Low	2	6	13	
B	M2 (anti-CD163 +)	Front	High	2	5	15	0.56
			Low	3	7	11	
		Stroma	High	2	5	15	0.56
			Low	3	7	11	

Table 4.5.9: TAMs density and M2 proportion: ***pathological tumor stage. (Based on Pearson's chi-square)***

	Population	**Tumor Area**	**Density/ Proportion**	**Tumor perforation** (present) n	**Lympho-vascular invasion** (present) n	**perineural invasion** (present) n
A	TAMs (anti-CD68 +) Density	Front	High	2	16	5
			Low	1	8	4
			***p*-value**[1]	0.57	**0.02**	0.76
		Stroma	High	3	12	3
			Low	0	12	6
			***p*-value**[1]	0.79	0.86	0.22
B	M2 (anti-CD163 +) Proportion	Front	High	1	13	5
			Low	2	11	4
			***p*-value**[1]	0.52	0.65	0.76
		Stroma	High	2	12	5
			Low	1	12	4
			***p*-value**[1]	0.57	0.86	0.76

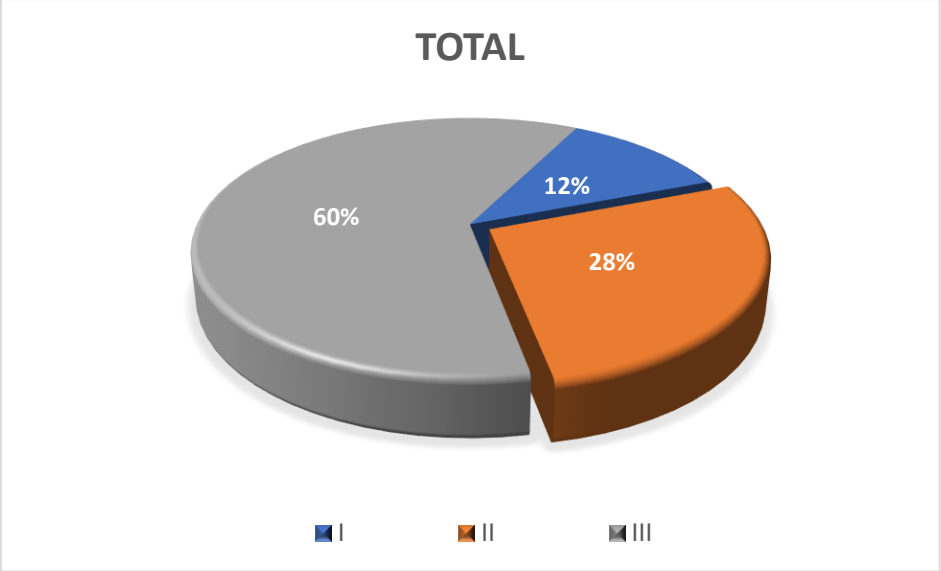
Table 4.5.10: TAMs density and M2 proportion: *tumor perforation, lympho-vascular invasion, perineural invasion. (Based on Pearson's chi-square)*

4.6 Pathological Tumor Stage

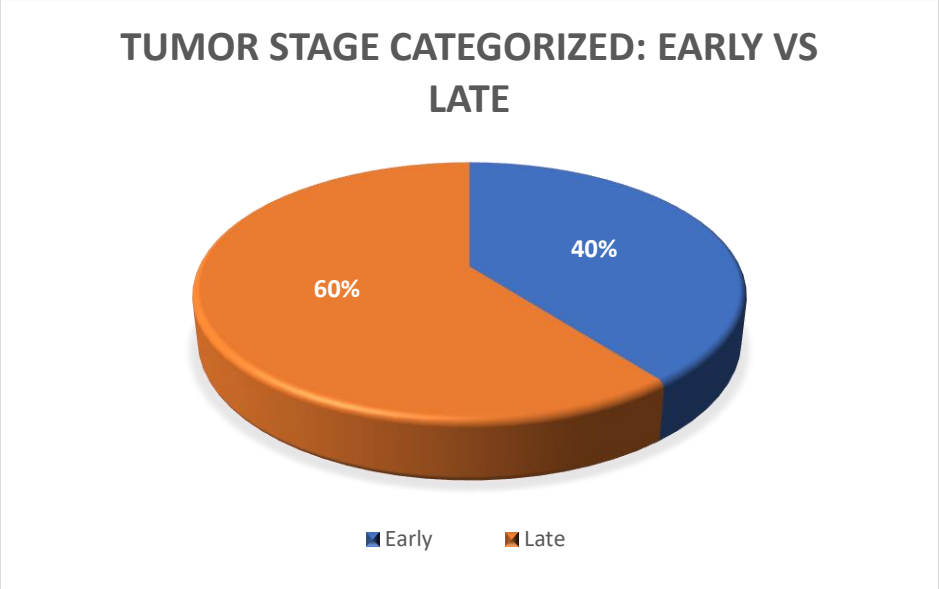
The study population included more than half of Stage III tumors (n=26) and a smaller proportion of Stage II (n=12) and Stage I disease (n=5) in the decreasing order of frequency (see figure 4.6.1). As the stage-wise comparison has already been discussed in each prior section, the analysis presented in this section relates to stage categorized as early vs advanced disease presentation. Early stage represented by Stage I and Stage II disease collectively, and Stage III and Stage IV disease characterized as late disease. As practically we did not find any specimen with pathological stage IV disease, late stage essentially represented Stage III disease in this series.

Comparing between early and late-stage CRC, no statistical difference was found for age of onset, gender, tumor sidedness, pathological tissue type or grade, macroscopic tumor perforation or peri-neural invasion. Lympho-vascular invasion, remarkably again demonstrated significant difference (p = 0.005) between the two groups. These are detailed in table 4.6.1.

Comparison of TAMs density and M2 proportion, for early versus late stage CRC also did not reveal any statistical difference, based on Pearson's chi-square, for either tumor front or stroma, as presented in table 4.6.2.



4.6.1 Pathological Tumor Stage: *proportional distribution*



4.6.2 Categorized Pathological Tumor Stage: *proportion of early vs late stage CRC*

		Tumor Stage Categorized: Early vs Late			
		Early	Late	Total	*p*-value
Onset-age	Early-onset CRC	5	12	17	
Group	Late-onset CRC	12	14	26	
Total		17	26	43	*0.27*
Gender	Male	11	19	30	
	Female	6	7	13	
Total		17	26	43	*0.55*
Tumor site:	Left colon	9	15	24	
Left vs Right	Right colon	8	11	19	
Total		17	26	43	*0.75*
Histology	Adenocarcinoma	15	21	36	
	Mucinous adenoca.	2	4	6	
	Signet- ring cell ca.	0	1	1	
Total		17	26	43	*0.66*
Tumor grade	G1	9	12	21	
	G2	6	8	14	
	G3	0	4	4	
	Not applicable	2	2	4	
Total		17	26	43	*0.39*
Tumor	Not identified	16	24	40	
Perforation	Present	1	2	3	
Total		17	26	43	*0.82*
Lympho-vas	Not identified	12	7	19	
Invasion	Present	5	19	24	
Total		17	26	43	***0.005***
Perineural	Not identified	15	19	34	
Invasion	Present	2	7	9	
Total		17	26	43	*0.23*

Table 4.6.1: Comparison of Traits for Early vs Late Stage CRC

		Tumor Stage Categorized: Early vs Late			
		Early	Late	Total	*p*-value
TAMs (CD-68)	High	7	15	22	
Density: Front	Low	10	11	21	
Total		17	26	43	*0.28*
TAMs (CD-68)	High	9	13	22	
Density: Stroma	Low	8	13	21	
Total		17	26	43	*0.85*
M2 (CD-163)	High	7	15	22	
Proportion: Front	Low	10	11	21	
Total		17	26	43	*0.28*
M2 (CD-163)	High	7	15	22	
Proportion: Stroma	Low	10	11	21	
Total		17	26	43	*0.28*

Table 4.6.2: Early vs Late Stage CRC: Comparison of TAMs Density and M2 Proportion

4.7 Logistic Regression Analysis

The logistic regression was conducted to determine whether TAMs Density with M2 proportion in tumor stroma and front, could predict the likelihood of an advanced pathological stage of CRC. This was in line with the primary objectives of this study and represented our basic prediction model, referred to as model 1 in further discussion. The results of the logistic regression are detailed in Table 4.7.1, 4.7.2 and 4.7.3. This model was not statistically significant with a P value of 0.49 (Table 4.7.2), and the R-square showed improved predictability between 7.7% to 10.4% (Table 4.7.3) only. It only correctly classified 60.5% of the cases, similar to what could be predicted without using this model (Table 4.7.1, see table footnote *e*). Sensitivity was 76.9% and specificity was 35.3 (Table 4.7.1, see table footnote *c* and *d*).

We then added the factors determined to be significantly different among comparison groups from prior discussion, viz, lympho-vascular infiltration and tumor grade. This is referred to as model 2 in subsequent discussion. Interestingly, adding these factors improved test's predictive ability remarkably. Chi-square test for all these factors (or coefficients) showed that model was statistically significant with a p-value of 0.024 (Table 4.7.2) and improved predictability between 33.6% to 45.5% (table 4.7.3). It correctly classified 81.4% of the cases (Table 4.7.1, see table footnote *e*). The test sensitivity was calculated to be 84.6% with a specificity of 76.5%.

Model 1 (TAMS density + M2 Proportion)											
Observed: Without independent predictors			Predicted			Observed: With the independent predictors			Predicted		
			Stage Type: Early or Late		Percentage Correct				Stage Type: Early or Late		Percentage Correct
			Early	Late					Early	Late	
Step 0	Stage Type: Early or Late	Early	0	17	.0	Step 1	Stage Type: Early or Late	Early	6	11	35.3[c]
		Late	0	26	100.0			Late	6	20	76.9[d]
	Overall Percentage				60.5		Overall Percentage				60.5[e]
Model 2 (TAMS density + M2 Proportion + Tumor Grade + Lympho-vasc. Infiltration)											
Observed: Without independent predictors			Predicted			Observed: With the independent predictors			Predicted		
			Stage Type: Early or Late		Percentage Correct				Stage Type: Early or Late		Percentage Correct
			Early	Late					Early	Late	
Step 0	Stage Type: Early or Late	Early	0	17	.0	Step 1	Stage Type: Early or Late	Early	13	4	76.5[c]
		Late	0	26	100.0			Late	4	22	84.6[d]
	Overall Percentage				60.5		Overall Percentage				81.4[e]

a. Constant is included in the model.

b. The cut value is .500

c. Represents specificity.

d. Represents sensitivity

e. Represents overall predictability

Table 4.7.1. Logistic Regression Analysis: Comparison of Model 1 and Model 2

	Chi-square	df	Sig.		Chi-square	df	Sig.
Model 1: TAMS density + M2 Proportion	3.424	4	.490	Model 2: TAMS density + M2 Proportion+ Tumor Grade + Lympho-vasc. Infiltration	17.638	8	.024

Table 4.7.2. Omnibus Tests of Model Coefficients

Model	-2 Log likelihood	Cox & Snell R Square	Nagelkerke R Square	Model	-2 Log likelihood	Cox & Snell R Square	Nagelkerke R Square
1	54.289[a]	.077	.104	2	40.075[a]	.336	.455

Table 4.7.3. R-square Model Summary

a. Estimation terminated at iteration number 20 because maximum iterations has been reached. Final solution cannot be found.

PHOTOMICROGRAPHS

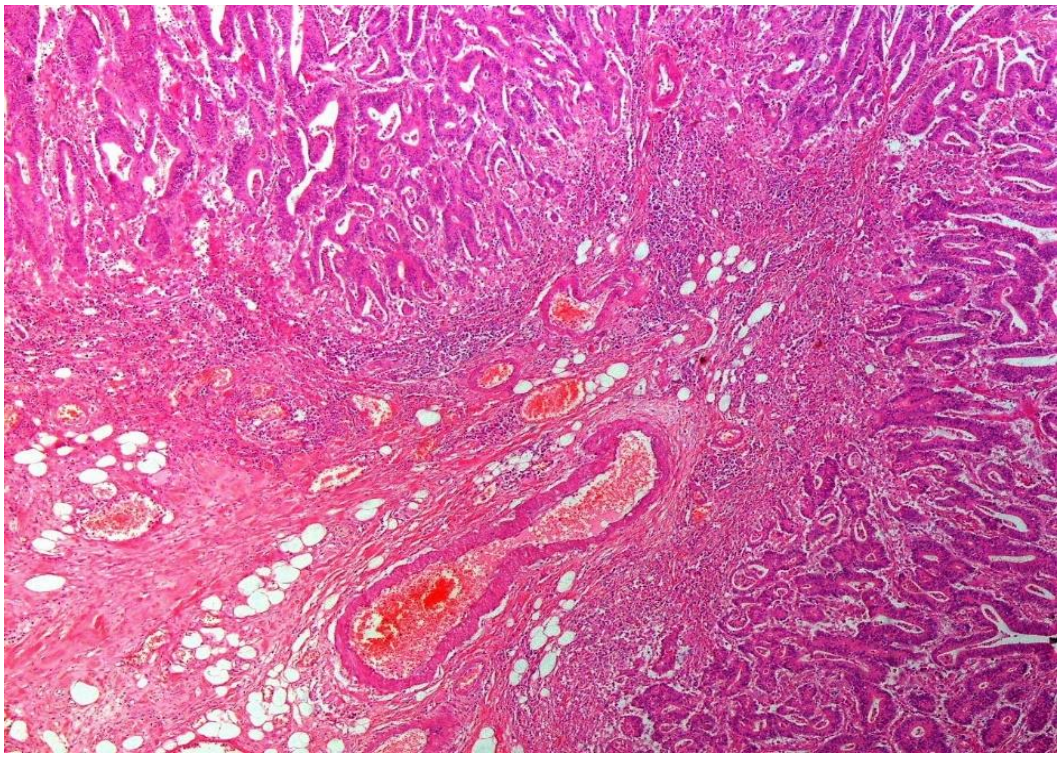


Image no. 1: Invasive front: Colorectal carcinoma (Hematoxylin & Eosin x40 magnification)

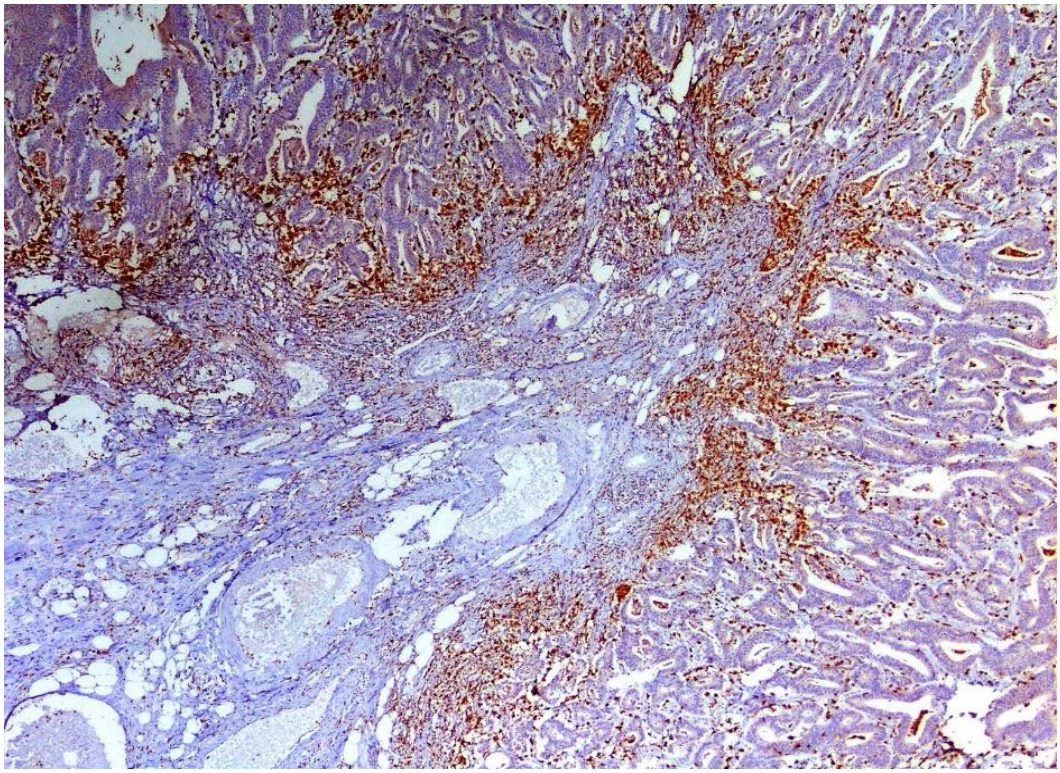


Image no. 2: Invasive front: Colorectal carcinoma, CD 163 macrophages outlining the invasive front (Immunohistochemistry x40 magnification)

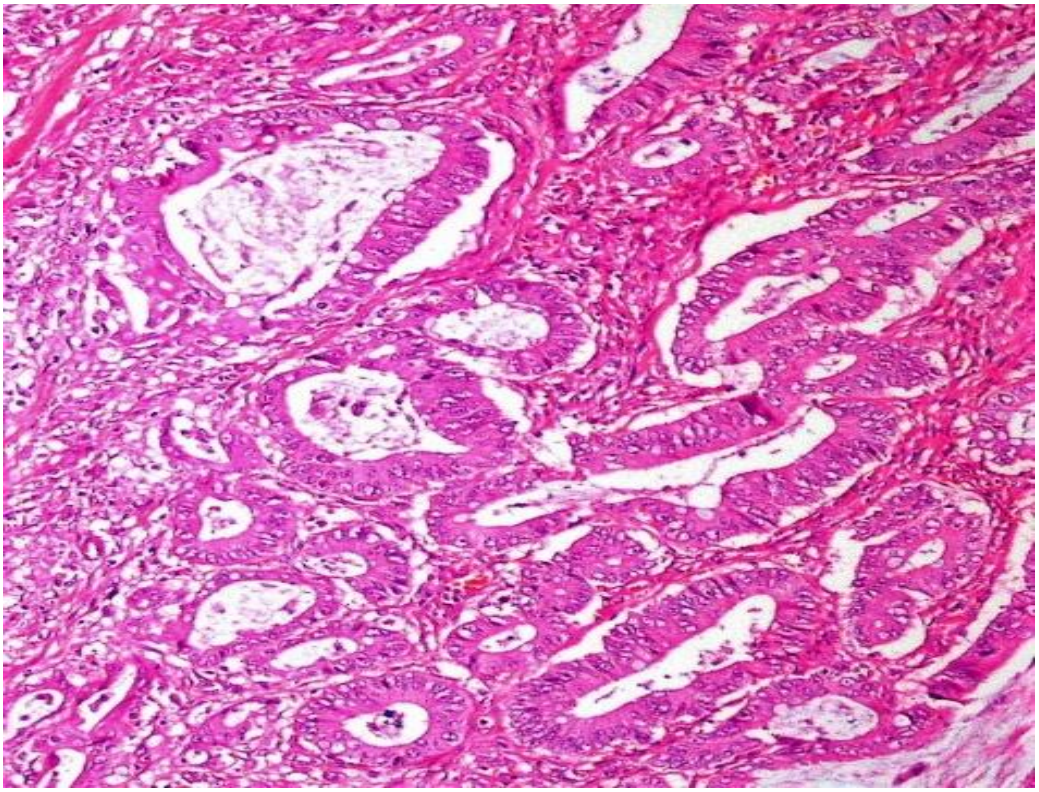


Image no. 3: Tumor stroma: Colorectal carcinoma (Hematoxylin & Eosin X100 magnification)

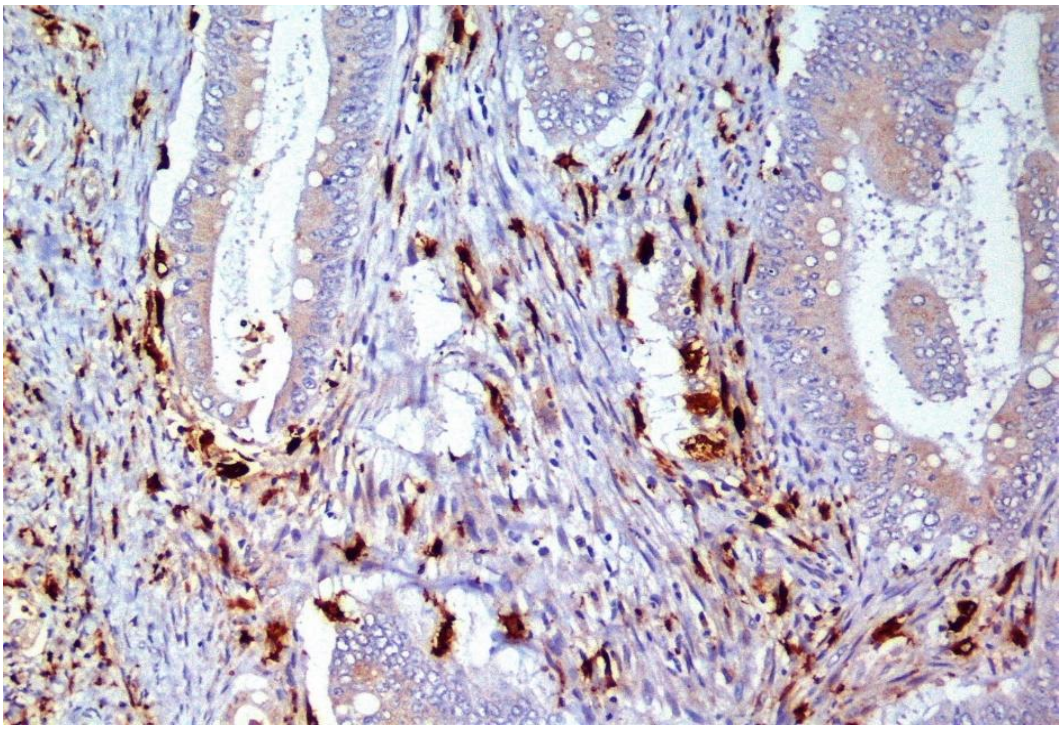


Image no. 4: Tumor stroma: Infiltrated by CD 68+ macrophages, exhibiting cytoplasmic staining (Immunohistochemistry x200 magnification)

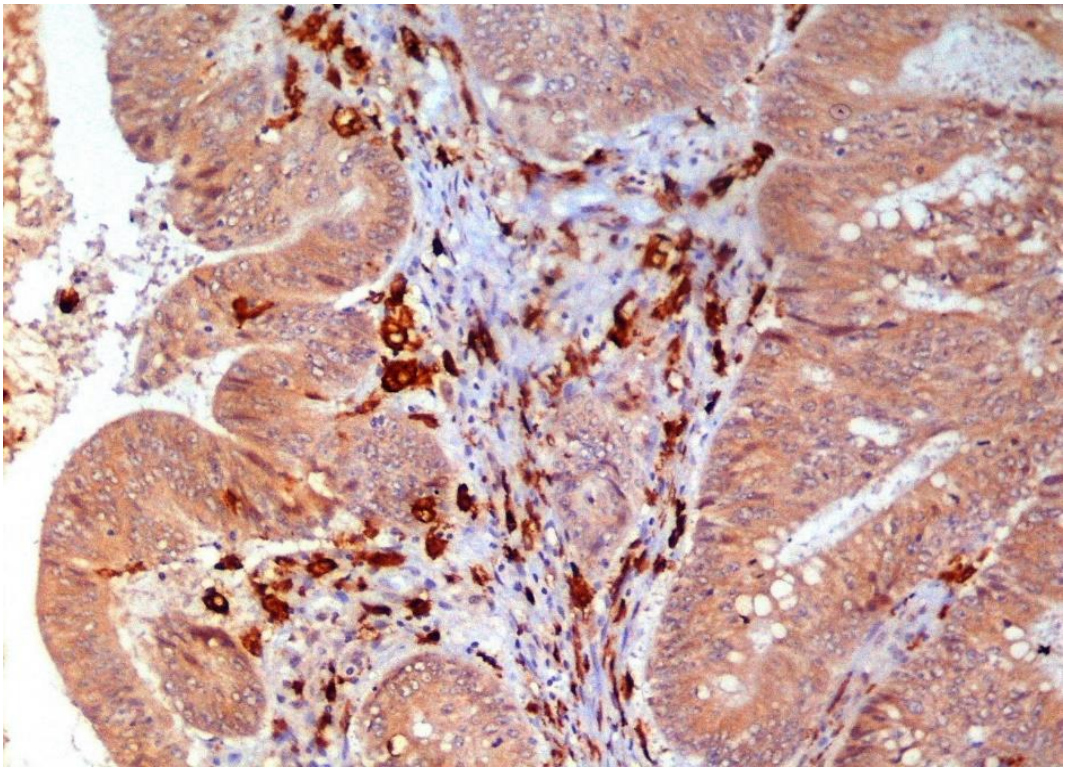


Image no. 5: Tumor stroma: infiltrated by CD 163+ macrophages, exhibiting membranous and cytoplasmic staining (Immunohistochemistry x200 magnification)

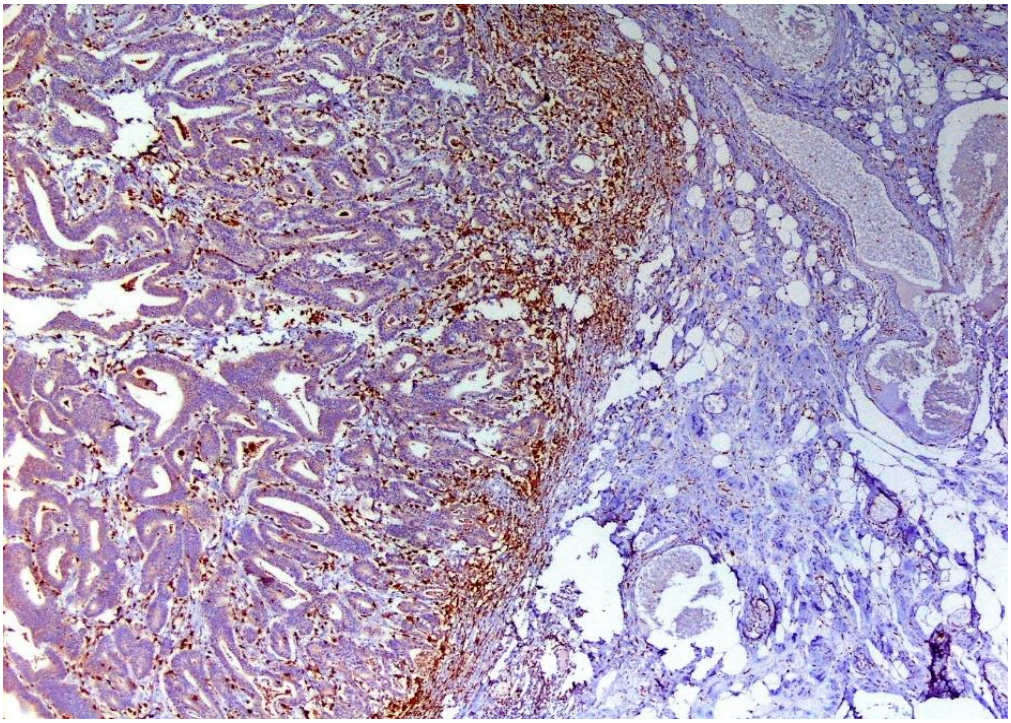


Image no. 6: Invasive front, outlined by CD 68+ macrophages (Immunohistochemistry x40 magnification)

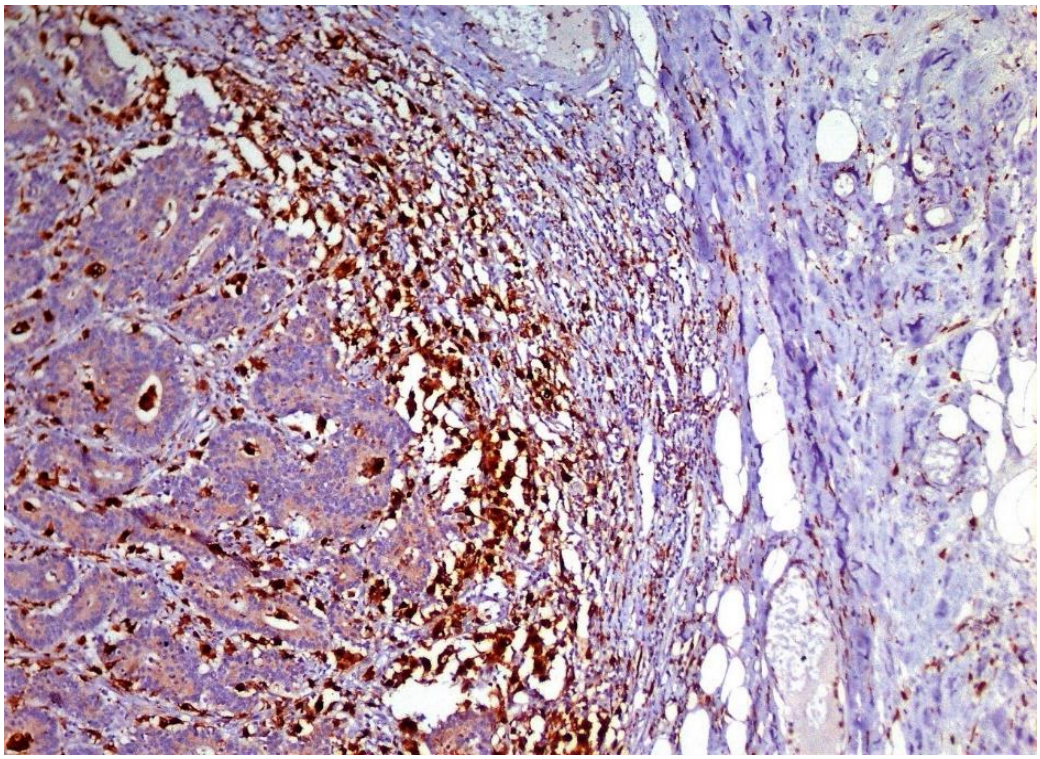


Image no. 7: Invasive front (same as in image 6), outlined by CD 68+ macrophages (Immunohistochemistry x100 magnification)

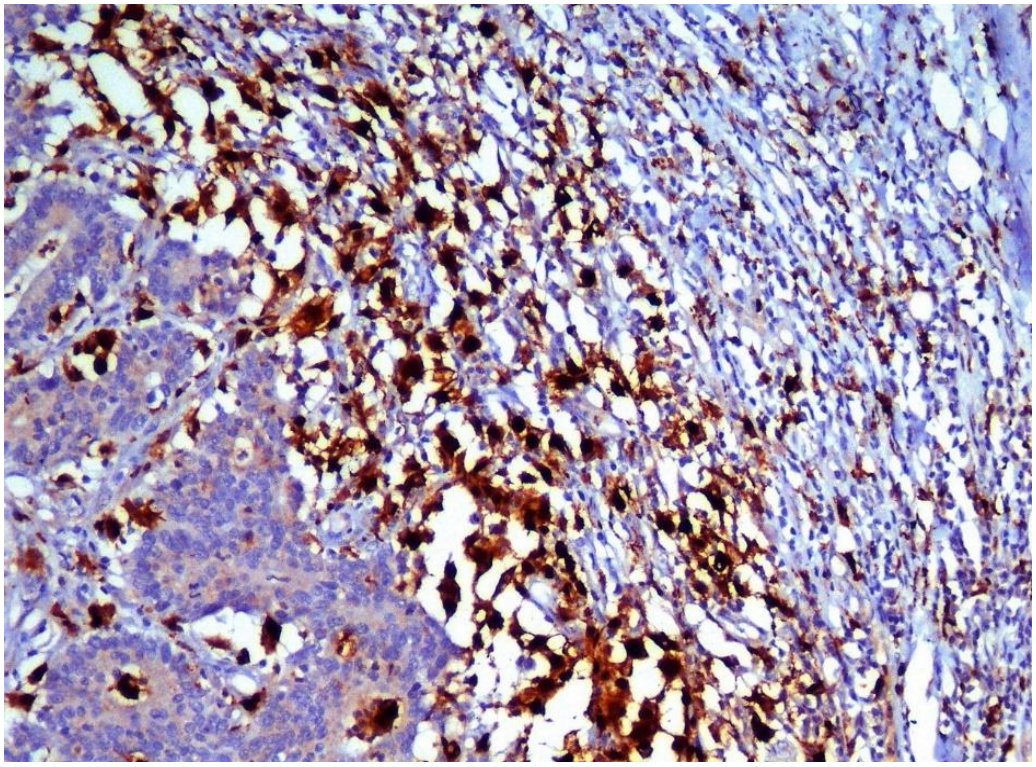


Image no. 8: Invasive front (same as in image 6), outlined by CD 68+ macrophages (Immunohistochemistry x200 magnification)

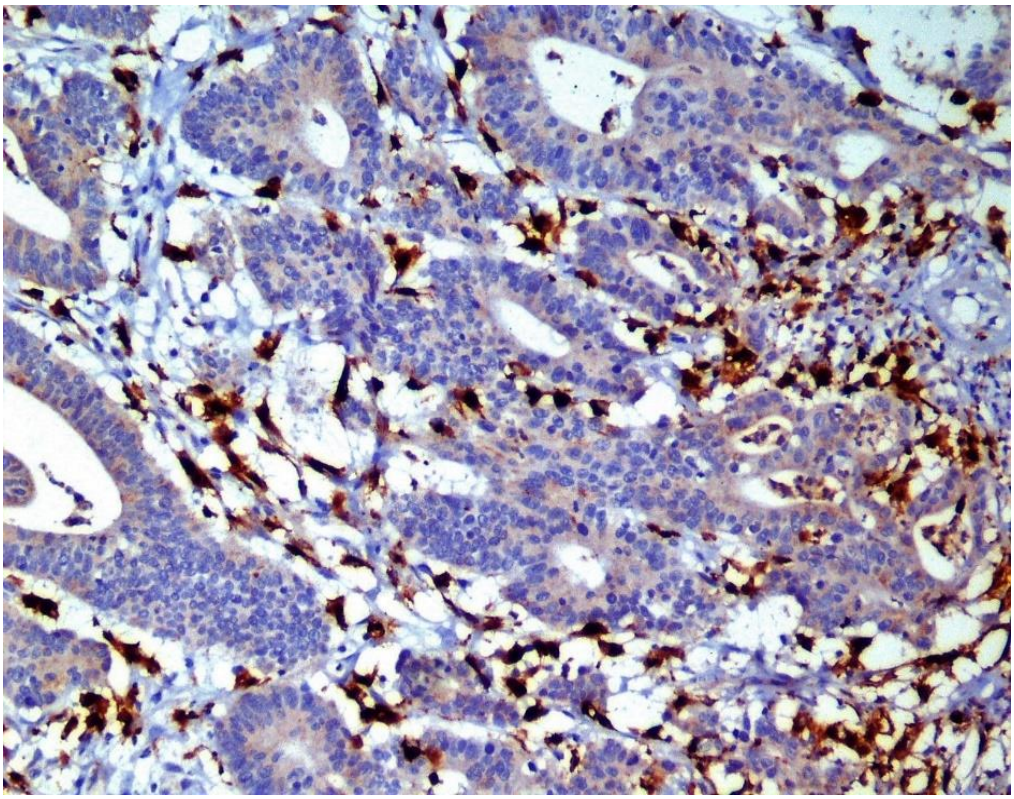


Image no.9: Tumor stroma, infiltrated by CD 68+ macrophages (Immunohistochemistry x200 magnification)

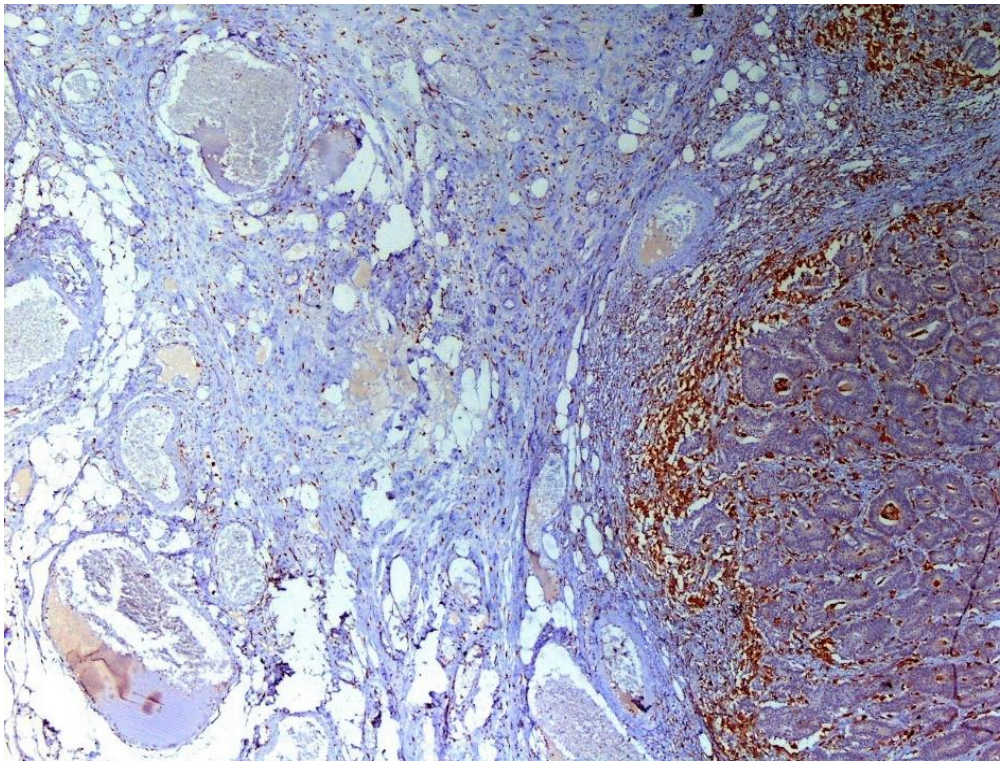


Image no. 10: Invasive front, outlined by CD 163+ macrophages (Immunohistochemistry x40 magnification)

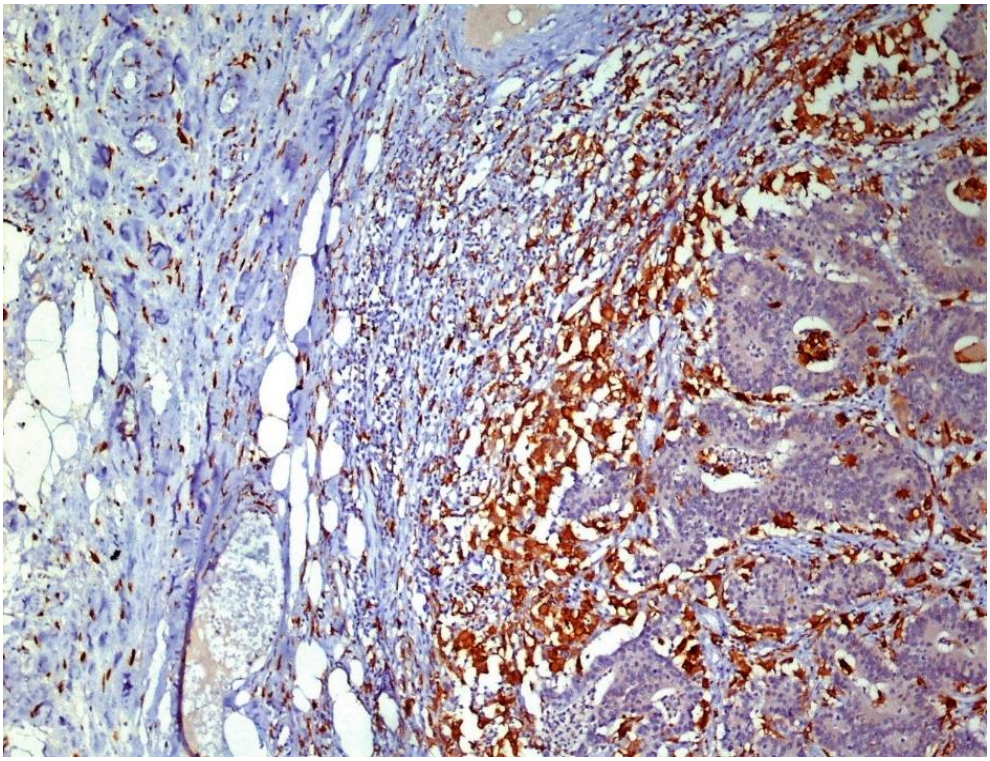


Image no. 11: Invasive front (same as in image 10), outlined by CD 163+ macrophages (Immunohistochemistry x100 magnification)

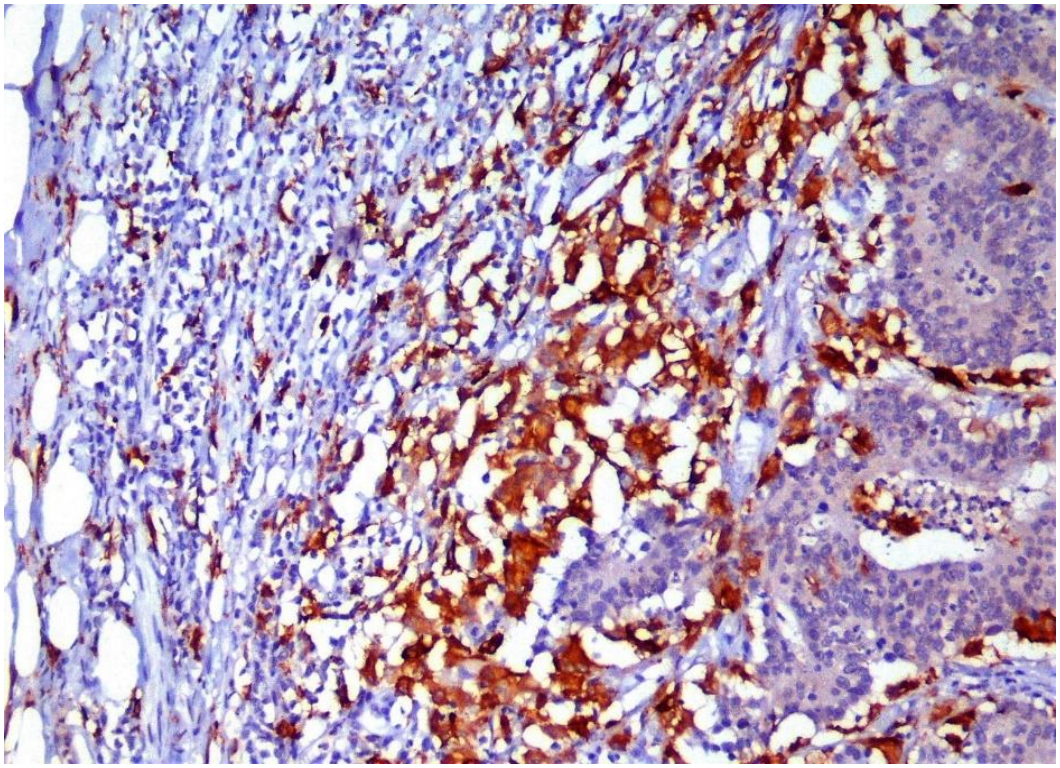


Image no. 12: Invasive front (same as in image 10), outlined by CD 163+ macrophages (Immunohistochemistry x200 magnification)

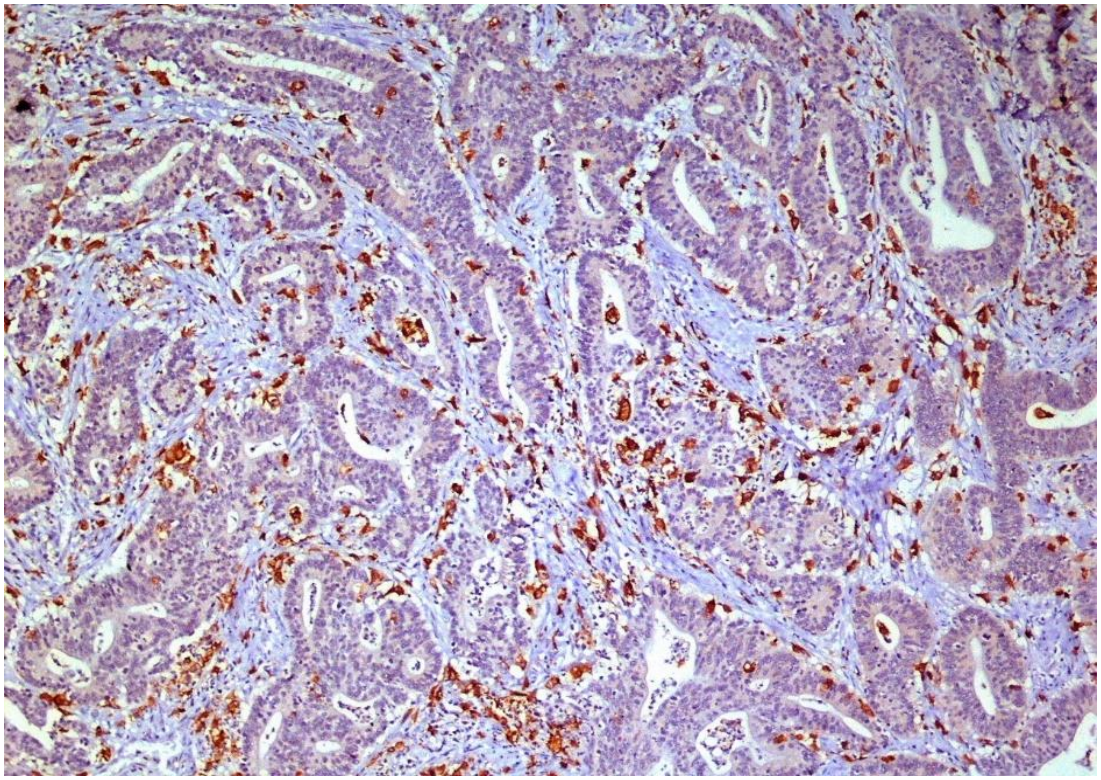


Image no. 13: Tumor stroma, infiltrated by CD 163+ macrophages (Immunohistochemistry x100 magnification)

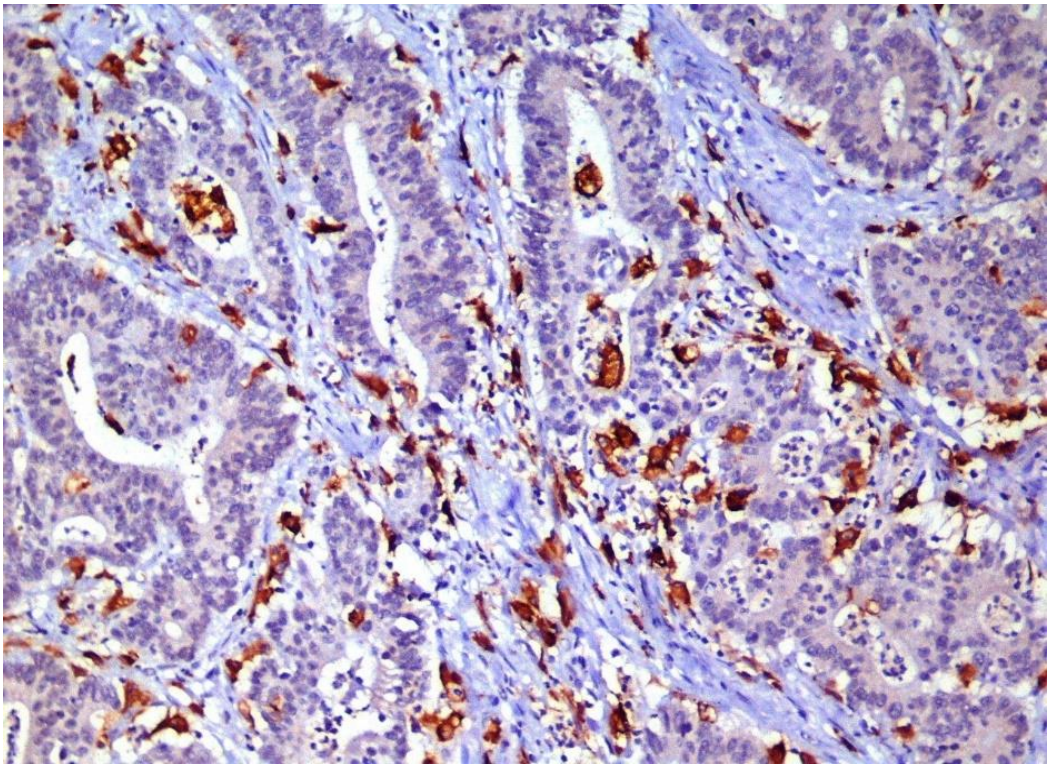


Image no.14: Tumor stroma (same as in image 13), infiltrated by CD 163+ macrophages (Immunohistochemistry x200 magnification)

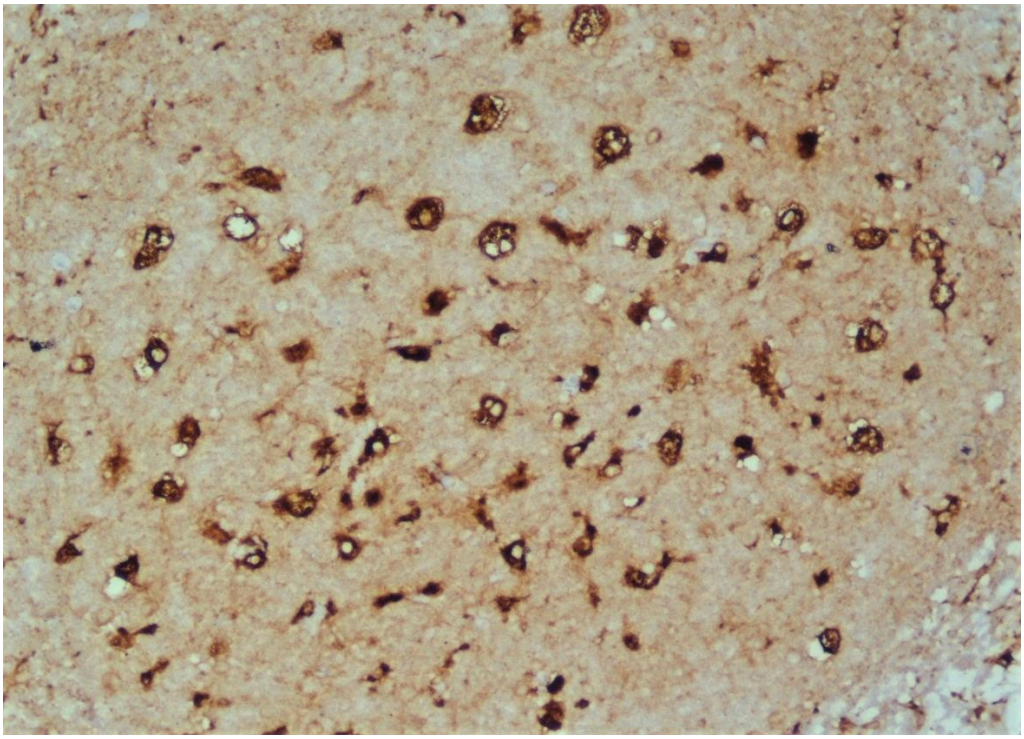


Image no. 15: Lymph node, positive control for CD 68 antibody (Immunohistochemistry x 200 magnification)

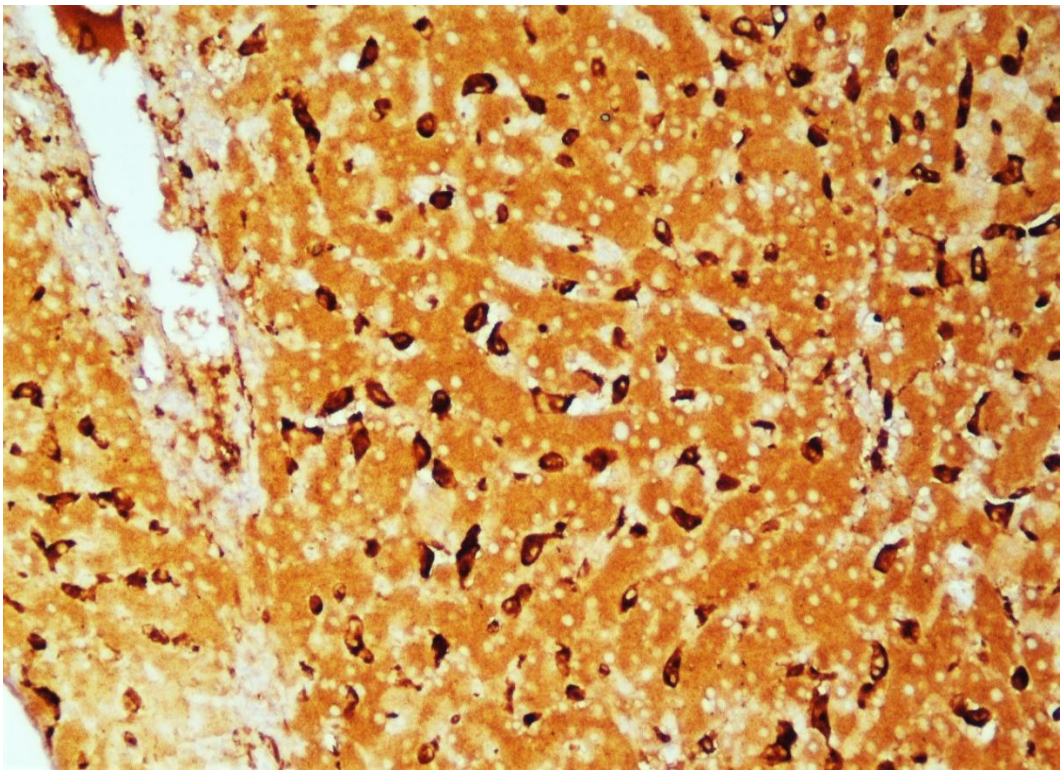


Image no. 16: Liver, positive control for CD163 antibody

(Immunohistochemistry x 200 magnification)

Chapter 5

Discussion

Colorectal cancers being among the commonest malignancies worldwide, has been in the fore of medical research. With precision medicine and targeted therapy, the paradigm needs higher definition for optimizing outcome for every patient. Our focus in this study was to explore whether the density of tumor associated macrophages and the proportion of its M2 subset in the tumor stroma or tumor front or by extension their relative ratios are related to the disease stage. The rationale of this discourse, as discussed earlier is the possibility of altering the tumor microenvironment to improve cancer prognosis by downstaging the disease through the pharmacological or immunological manipulation of TAMs. Establishing this association thus serves as the first step to progress on this potential therapeutic strategy. In the following discussion, first the main results and their implication for this objective will be examined, followed by comparison to other similar studies done prior, then the strengths and weakness of the study will be highlighted and finally the recommendations for future research will be made.

The main result was the determination, based on logistic regression analysis, regarding statistically significant association between TAMs and M2 proportion with the tumor stage. We did this in two iterations. The first analysis was based on TAMs and M2 proportion as independent predictors of tumor stage as a combined metric. For the second iteration, we also added lympho-vascular invasion and tumor grade to the model, as these were found to have significant association in the initial exploratory analysis of this study.

Interestingly we found that the former model was not statistically significant while the later was. To place more context before explaining this finding, lympho-vascular invasion, was found to be significantly differentiated with most other factors during the exploratory analysis. Prior studies have also established the prognostic significance of lympho-vascular invasion (LVI) (Gao et al., 2021; Jiang et al., 2019) and tumor grade (Valeria, Luca Reggiani, Antonio, Rosario Alberto, & Giovanni, 2015) for colorectal cancers as independent risk factors. There are thus two possible mechanistic explanations of these findings. The first logical conclusion is that TAMs and M2 subset did not carry any significant prediction ability and adding LVI and tumor grade raised a false association, derived from the predictive power of latter factors. To check that, we retested the model, removing TAMs and M2 while leaving LVI and tumor grade as independent predictors. Although the significance remained but magnitude of predictability decreased as evident by reduced sensitivity (80.8% from 84.6%) and specificity (70.6% from 76.5%) for the model. So, despite a lower contribution, TAMs and M2 did improve test's predictability. The second possible explanation also relates to this assertion. The TAMs density and M2 proportion may carry statistical significance on their own but did not reach significance in our study due to a small sample size. We will explore this further in the later part of the discussion.

As discussed earlier, several prior studies have investigated similar association between tumor associated macrophages and its subsets in relation to the prognosis and outcome of colorectal cancers. For example, Forssell et al (Forssell et al., 2007) in a retrospective review, working with 446 resected tumor specimens, investigated for the TAMs density in all the tumor micro-areas, solely with the pan-macrophage marker CD-68. They assessed CD-68 semi-quantitatively into graded densities (from 1 to 4) and correlated with cancer-specific survival through crosstabulation, univariate followed by multivariate analysis and plotting Kaplan-Meir survival curves. They demonstrated highly significant association with the survival improving incrementally along the CD-68 infiltration density, across most of the patient subsets. Exploring for correlation with the associated clinicopathologic parameters, the study showed no correlation to gender, age, but a very strong correlation to tumor stage, grade, localization, type, and LVI at the tumor front. This older publication is of importance for being among the foundational studies that initially

explored the prognostic association of TAMs with colorectal cancers. The findings are largely in line with the current study. The primary difference is related to comparing with patient's cancer-specific survival in the Forssell et al study, while our comparison was to the pathological tumor stage. Our objective was to establish TAMs density and M2 proportion's correlation with the tumor stage, and we did not obtain survival data from patient files. So, the primary analysis between the two studies does not automatically relate. But considering that the outcome of CRC does relate to tumor stage at presentation, we may extrapolate pathological stage in this study to the survival observed in the Forssell study. One possible explanation for the difference thus observed may be that while we recorded and analyzed CD-68 cell counts, the older study used semi-quantitative grading instead. So, their analysis relying on four categorical grades reached statistical significance while out discrete counts did not. Additionally, Forssell study showed clinicopathological association with the tumor grade, and LVI similar to this study. Both of these represent progressing tumor dedifferentiation and so relate to the poorer outcome in both the studies.

Among the more recent studies that are similar to ours are those published by Waniczek et al (Waniczek et al., 2017), Yang et al (C. Yang et al., 2019) and Pinto et al (Pinto et al., 2019). The similarity between these studies is the correlation of TAMS and/or subsets with patient survival, either disease-specific (disease free survival or DFS) or overall survival (OS). This, as discussed earlier, will be considered in lieu of tumor pathological stage in our study. Also, all of these studies analyzed association with the clinicopathological factors, similar to this study. Beyond these similarities, these three studies each had distinctions among them as well as this study.

The Waniczek et al, studied only the infiltration by M2 component of TAMs indirectly, using CD-68 + iNOS immunohistochemical antibodies for their delineation. Additionally, they explored the disease-related outcome for regulatory T cell (T-regs) infiltration of tumor front and stroma. The sample included 89 tumor resection specimens of colorectal cancer patients, with case files reviewed retrospectively for clinicopathological variables and survival statistics. The main finding was a positive correlation between M2 TAMs and Tregs as regards to densities in tumor stroma and front, as well as the poor patient survival related to higher tumor infiltration by either of these

cellular components. The relative risk of death and recurrence observed was more than twice with higher M2 infiltration and a massive 12 times higher for higher Tregs infiltration of tumor tissue. In our study, we did not find direct correlation of M2 proportion with the tumor stage, even though as already discussed our refined model, that included M2 proportion along with TAMs density, LVI and tumor pathological grade as a single test, showed high sensitivity and specificity for predicting the tumor stage. Among other clinicopathological variables studied, Waniczek et al did not find any correlation with the patients' age, gender, tumor location or histological grade. In our study, gender distribution was significantly different for M2 proportion with higher M2 proportion at the tumor front in males compared to a low M2 proportion among females. Other studies have looked into gender-specific outcomes of CRC (Kim et al., 2015). We will allude to this later in this chapter.

Yang et al focused on CD163/CD68 ratio instead of pan-TAMs or subtypes for correlation with recurrence-free (RFS) and overall (OS) survival in a sample of 81 colorectal patients' specimen studied retrospectively. This assessment was based on immunohistochemical study of tumor specimens. Additionally, the study included evaluation of circulating tumor cells (CTCs) in peripheral blood, as well as E-cadherin and Vimentin as EMT marker in tumor specimens through IHC technique. The analysis showed that CD163/CD68 at the tumor front was associated with both recurrence-free survival (RFS) and overall survival (OS), based on the Kaplan-Meier curve, but not with this ratio in the tumor stroma, among patients with CRC. This was further verified through multivariate Cox regression analyses. They also demonstrated that at the tumor front the level of CD163+/CD68+ ratio was significant higher compared to the tumor stroma. Furthermore, exploratory analysis included correlation with clinicopathological markers. The ratio also closely correlated with LVI, the pathological stage, EMT markers, and CTCs counts. In our study, for comparison, instead of CD163/CD68 ratio, we tested correlation of M2 proportion at the tumor front and stroma to the tumor pathological stage and other clinicopathological variables. The rationale was that since M2 represents a subset of TAMS and not an exclusive sub-type, mathematically, these represent a proportion rather than a ratio, that by definition should be an independent group. Unfortunately, our assessment did not show correlation with the tumor stage. There were two problems identified. First was

that the multitude of percentages in a small sample rendered data dispersed in very small clusters, making determination of correlation virtually unachievable. To overcome this limitation, we simplified M2 proportion in to a high and low proportions group based on value higher or lower than the median percentage at the tumor front and stroma. Here again the median cutoffs were too high, and nearly indiscriminate among tumor micro-areas. One potential mechanistic explanation would be that since the proportion of M1 was so low in our samples that M2 proportion was nearly identical to the pan-TAMs and thus discrimination was not underscored on further analysis.

The third study selected to cross-reference our study is by Pinto et al. For this study, researchers retrospectively reviewed a series of 150 colorectal cancer (CRC) cases and then included their tumor specimen for further evaluation by immunohistochemistry, using CD68 as pan-TAMs marker, CD80 as a marker for the M1 subtype, and CD163 as an M2 subtype marker. They utilized digital image-analysis software for the quantification. In addition to the tumor front and the tumor stroma, they also analyzed adjacent normal mucosa (ANM) for the IHC markers. Main findings were that CD163+ M2 constituted the major subtype at the tumor front, whereas CD80+ M1 were almost entirely located in the adjacent normal mucosal area. Additional tumor stage stratified analysis demonstrated that M2 subtype, was more rampant in stage II tumors, whereas M1 subtype was widespread in the T1 tumors. A higher density on pan-TAMs CD68+ cells with a lower CD80/CD163 ratio was found to be associated with decreased overall survival in patients with stage III CRC. Interestingly, their multivariate logistic regression analysis showed that the M1 subset extended a protective role regarding the risk for relapse among the patients with CRC, despite the low overall counts in the tumor areas. Another interesting observation was related to tumor localization whereby infiltration of all cell types was higher in the ANM of tumors in the right colon as compared to the left-sided colon. This amplified permeation was also evident for pan-TAMs and the M2 subtype in the tumor stroma, but the association was not seen at the tumor front for any of these cell types. The last observation has implication for tumor laterality and related gender-associated distribution of CRC, that will be explored in more detail later. In our observation, during this study, a similar finding was a very low overall proportion of M1 subtype in tumor stroma and tumor front. We did not apply specific differentiating marker for the M1 subtype, instead relying

on mathematical deduction of M1 count from total TAMs by subtracting the M2 count. Additionally, the adjacent normal mucosa (ANM) was not evaluated during current study. With a median M2 proportion exceeding 77%, the low residual count of M1 is self-evident. Despite the low calculated counts, we did find a significant difference in the distribution of M1 subtype with a relatively higher proportion noted at the tumor front compared to the tumor stroma. Although not directly comparable, would this portend a better prognosis like the reduced risk of recurrence observed in the Pinto et al study, were a formal survival analysis included in our study, is largely conjectural but a prospective possibility. Pinto et al noted that 38.7% of the specimen showed LVI but evidently did not analyze association with TAMs and subtype infiltration or patient survival statistics.

Lympho-vascular Invasion (LVI) by definition is considered to be present when on hematoxylin and eosin-stained slide, examined under light microscopy, tumor cells are seen within an endothelium-lined space or alternatively when a destruction of an endothelial-lined wall by tumor cells is observed. Other prior studies (Gao et al., 2021; Jiang et al., 2019; Lim et al., 2010) and by extension meta-analysis (H. Yuan et al., 2017) have seen association of LVI with tumor stage and patients' survival. In this study as well, we have seen LVI correlating with the tumor stage as well as other clinicopathologic features like patient's gender and TAMs density at the tumor front. Among the prior research papers, Lim et al, for example, in a study of 2417 patients, prospectively evaluated for disease-related survival in patients with colorectal cancer, detected 610 (25.2%) cases with a lympho-vascular invasion–positive tumor. On comparison with patients having LVI negative tumors, the earlier group were significantly older, with more poorly differentiated tumors, and at a more advanced TNM stage. These were also more likely to have a significantly higher preoperative serum carcinoembryonic antigen (CEA) level. On resection the specimen with LVI were more likely to have lymph-node metastases and were also more likely to recur at systemic lymph nodes following resection with a curative intent. LVI positive status was also found to be independent unfavorable prognostic factor for the 5-year overall (OS) and disease-free (DFS) survival. With evidence like this, specialty oncological guidelines (like National Comprehensive Cancer Network or NCCN) and pathological reporting standards (like College of American Pathologist or CAP), consistently recommend its inclusion in the resected colorectal specimen to aid

prognostication and guide further treatment. Moreover, when we added it to our diagnostic model, it resulted in markedly improved sensitivity and specificity, as noted earlier. Prior evidence, specialty recommendation and our observation provide ample justification for this post-hoc adjustment in our study.

Among the cross-referenced studies discussed earlier, one observation was the difference between the right-sided and left-sided CRCs, whereby a higher density of all TAMs and subtypes was observed especially in the associated normal mucosal (ANM) areas. There is evidence from a number of prior studies suggesting that tumor biology and pathogenesis may be different between the two sides. In a review of prior literature, Baran et al (Baran et al., 2018) highlighted a number of differences between the right and left side colorectal including a higher proportion of mucinous adenocarcinomas, sessile serrated adenomas, MSI-high and mismatch repair deficient tumors, and peritoneal metastases among right-sided tumor. Also, right colon cancer tends to occur more often among older patients, and females while yielding a higher immunogenicity with a high T cell infiltration, according to their review. The basis of this difference is hypothesized to be the embryological origin of the right-sided colon from the midgut and that of the left-side from the hind gut. In current study the only statistically significant difference we found as regards to the laterality of the CRC was between the early-onset CRC group compared to the late-onset group. A significantly higher proportion of right-sided CRC was observed in the late-onset cohort compared to the reverse trend in the early onset group. This is in keeping with the prior studies. Likely explanation lies in senescence and associated genetic and epigenetic aberrations accumulated over time, that have been found to be proportionately more likely to occur in the right sided colon. A study by Mukund et al (Mukund, Syulyukina, Ramamoorthy, & Subramaniam, 2020) based on comparative analysis of 411 sample of the right and left colon, obtained from The Cancer Genome Atlas-COAD cohort, highlighted suppression of certain enzymes involved in carcinogen prevention in the right colon. That may possibly explain this propensity.

Earlier we alluded to the finding in this study of a higher M2 proportion at the tumor front in males compared to a low M2 proportion among females, that brings us to the discussion of gender related differences among colorectal cancer patients. In most part the

evidence available in this regard can best be characterized as mixed with some demonstrating significant differences in the pattern of disease and outcomes between the females and the males while others showing subtle if any differences not translating in to varied outcomes of the disease. For example, Yang et al (Y. Yang et al., 2017) in a metanalysis that included 14 studies (13 retrospective cohorts and 1 RCT) , selected from initial 37, spanning from 1960 – 2017, looked into the difference in overall (OS) and cancer specific (CSS) survival between male and female groups. The pooled data demonstrated a significantly better OS for women compared to men, with some heterogeneity (HR = 0.87, 95% CI: 0.85–0.89, $p < 0.00001$; $I^2 = 26\%$, $p = 0.22$) as well as CSS with moderate heterogeneity (HR = 0.92, 95% CI: 0.89–0.95, $p < 0.00001$; $I^2 = 56\%$, $p = 0.03$) among the studies. Contrariwise, White et al (White et al., 2018) in their cross-sectional review of national data from the United Kingdom, demonstrated that there were some differences in the presentation of the disease among men and women, but no significant difference in the 5 year survival, standardized to age. The differences in presentation included earlier presentation of males, with more of them getting a diagnosis through screening colonoscopies as compared to more females presenting as emergency cases. Women also had more right-sided cancers compared to more rectal and sigmoid located cancers among men. Men had overall higher incidence with highest ratio difference of 1.7:1 for ages 70-74 years. Biologically, the propensity for right-sided tumor location explains the presentation differences for most cases. Women carried higher frequency of KRAS and BRAF mutations, micro-satellite instability and CpG island methylator phenotype (CIMP)-high. As discussed earlier about tumor laterality being related to genetic and epigenetic mutations accumulating over time related to an aberrant repair process, similarly here again, a similar propensity for abnormalities is the likely driver. These mutation result in a higher frequency for sessile type polyps that are more likely to be missed during screening endoscopies. This results in delayed diagnosis and presentation at a later stage, with obstruction presenting in the emergency room, for example. In our study, as mentioned earlier, the only gender associated significant difference was found in a higher frequency of lympho-vascular invasion among men compared to women. This may be explainable with higher frequency of stage III disease, grade 2 and 3 tumors, and early-onset CRCs seen among men compared to women in our sample. A possible explanation of this is delayed

overall presentation in our patient groups as compared to western population due to lack of routine colonoscopic screening. So, while in the White et al study, men received earlier diagnosis on screening, men in our sample did not, negating the advantage seen in their study.

In our model, logistic regression analysis showed improved sensitivity and specificity when LVI and pathological tumor grade were added. We have already discussed the role of LVI. For understanding the role of tumor's pathological grade, there has been consistent evidence over last two to three decades regarding its prognostic significance in relation to CRC. Recently Zlobec et al (Zlobec et al., 2020) compared tumor budding and tumor grade for their equivalence in prognosticating the colorectal cancer, in a sample of 771 patients. They found that tumor grade was associated with a larger tumor (pT), higher frequency of LVI, lymph node, and distant metastasis in concordance with tumor budding. Moreover, tumor grade showed significant association with the right-sided laterality and mucinous histology, but not tumor budding. Whereas tumor budding and not grading was associated with a worse overall (OS) and disease-free (DFS) survival. In the current study, we found significant association of pathological tumor grade with the age of onset of CRC, with higher grade seen in early-onset CRC and lower grades in the late-onset group. This association is also in keeping with other studies, for example, by Khan et al (Khan et al., 2016). It was demonstrated in this retrospective review of 396 patients (including 94 with early-onset CRC) that early onset disease was associated with higher pathological grade.

Early onset colorectal cancer is an entity increasingly recognized worldwide, and as already discussed in the chapter on literature review, represents the changing demography whereby younger people are developing colorectal cancers worldwide, and the trend also permeates the Pakistani population. In our patient sample nearly 40% of the subset included those aged younger than 50 years. Even though hospital samples do not represent population demographics, especially the university hospitals, being considered referral centers and thus generally represent a more complex disease pool, this trend is remarkable. We had specifically analyzed the comparative differences of early onset versus late onset groups in terms of clinic-pathological association in our study sample. The only statistically significant difference we found, as mentioned earlier, was with the tumor grade. Khan et al

(Khan et al., 2016) found a number of other statistically significant differences between early onset and late onset group including a higher TNM stage, a higher propensity for signet-ring cell histology, a poorer grade differentiation, twice the prevalence of MSI tumors and a worse five years disease specific survival in the early onset group. The reason that we do not see all these differences in our sample, can in part be explained by the lack of population screening regularly practiced in Pakistan. In most western countries, for instance, we see a trend toward earlier diagnosis and improving outcomes of CRC over last few decades, attributable in large measure to active population screening programs among their population more than 50 years of age. This very fact led to a recent downward revision of screening age to 45 years by the United States Preventive Services Task Force (USPSTF) (Stewart, 2021).

A repeated explanation for some differences in finding in current study in comparison to the prior studies is a small sample size. The effect of small sample size is that even a few outliers may skew the results in either direction, so a positive correlation or a significant association may appear uncorrelated or insignificant and vice-versa. So basically, the margin of error is limited, and any error may lead to incorrect representation of the outcome. An opposite effect is seen in studies with very large samples where even a minor difference among group may be amplified to depict statistical significance whereas such a difference would not be seen in real life situations. We followed standard protocol and a formal sample size calculation was performed with the help of a statistician but in view of the resource constraints, we chose to adopt the lower bound of the recommendation. There were also limitations related to overall logistics or obtaining and processing sample in the year of the global pandemic. It is possible that a larger sample may have reduced biases induced by possible outliers in the sample and resulted in more robust correlation among factors and outcome variables.

A valid question at the conclusion of this treatise would be – what is the global outlook for TAMs and its subsets, serving as an additional pathological test in defining the fine print, for the patient with colorectal cancers, in the era of precision targeted therapies? For now, based on more than a decade of international research on the subject and our study, it looks promising as a prognostic marker and a therapeutic target, but we are still

lacking in definitive evidence that would determine with certainty if, how and when TAMs and its subsets should be utilized in the diagnostic, prognostic and therapeutic paradigm of colorectal cancer management.

Chapter 6

Conclusion

6.1 Conclusion of the study

The following conclusion may be drawn based on the study results:

- Tumor associated macrophage (TAM) density in the tumor stroma and the tumor front of colorectal cancer specimens, did not show correlation with the tumor stage, in this study.
- Similarly, the proportion of M2 macrophages in the tumor front and the tumor stroma did not correlate with the pTNM stage and thus M2 alone is not predictive of the tumor stage .
- Moreover, the tumor grade and lympho-vascular invasion were important predictors of tumor prognosis and when added together with M2 proportion into a composite score, correlated with the pTNM stage.

6.2 Recommendations

Led by the experience during this study the following recommendation can be made for the future research on the subject:

- We recommend that TAMs density assessment should be performed semi-quantitatively as graded densities in any subsequent study. This simplifies and improves assessment. Subsequent clinical application would also be streamlined.
- We would also strongly recommend a study design based on prospective cohort or a longitudinal study, with a significantly larger sample size to optimize power, for any future study on TAMs and subtypes.
- One limitation to performing study on a larger sample is the high cost of immunohistochemical studies. We need to explore options to indigenize the process for reducing cost and improving availability.

We would also like to make the following recommendation for clinical and epidemiological practice based on our findings augmented by burgeoning globally verified trend:

- In Pakistan, a wide scale population awareness program regarding colorectal cancers should be planned, nationwide.
- Early onset colorectal cancer population is increasing in incidence. It is encouraging that recommended screening age has been lowered internationally. Same should be done in Pakistan.
- Screening protocols should be designed specifically for our target population.
- A cancer database at national level should be established.

6.3 Strengths of the study

In our opinion this study carried following strong points:

- The immunohistochemistry, for all the specimens, was performed in an identical manner by a single operator utilizing freshly acquired antibodies. As this was the first study, in our knowledge on TAMs and subtypes in Pakistan, this was done to ensure standardization through concentration of experience.

- The concept of M2 proportion instead of M1:M2 ratio is also distinctive to this study. No other study, to the best of our knowledge, utilized it earlier. It simplifies IHC, by allowing formal staining and counting of just two cell types, namely pan-TAMs and M2 subtype, instead of requiring assessment of all three (i.e., including M1 subtype). It does need formal validation in a larger study. We assessed the proportion as percentage of TAMS, as well as semi-quantitative graded assessment into high or low proportion, with arbitrary cut-off based on median percentages in each tumor area. A better assessment would be to formally evaluate cutoffs based on receiver operating characteristic curve (ROC) curve method followed by validation on a trainer subset and then a test subset.

6.4 Limitations of the study

We have identified the following weaknesses in this study:

- The sample size, although based on formal calculation, was small and likely resulted in magnified effect of outliers. A larger sample size would ensure that such a bias is countered effectively.

- It a single center, retrospective review. That introduces a selection bias, compounded by a small sample size. Additionally, it carries the inherent weaknesses of filed data obtained retrospectively. To minimize that we confined ourselves to data mostly obtained during initial assessment and the post-operative histopathology. This limited us to adopt staging instead of survival as our outcome variable. Even the staging was pathology-based rather than relying on additional imaging data. This ensured that we were able to capture complete data in nearly every instance. While that was our goal to begin with, it did restrict our final analysis and may explain, at least partly, the reason for not finding more significant correlations in current study.

CHAPTER 7

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CHAPTER 8

APPENDIX -A

Bahria University
Discovering Knowledge
Medical and Dental College, Karach

Ref no: FRC/BUMDC -13/2020-Path-115 **MS-11**

Approval of Research Proposal

Mr/Miss/Ms/Mrs/ **Dr. Fouzia Fazal**

Registration No:

Dear MS/MPhil Student,

I am pleased to inform you that your research proposal on **"Correlation of Tumor Associated Macrophages (TAMS) Density and M2 Proportion with the Pathological Stage of Colorectal Cancer"** has been approved. You may, therefore, continue your research on this theme and produce a quality thesis, as per the HEC requirements.

I take this opportunity to remind you that you must complete your thesis, and defend it successfully, by **SPRING 2021**; this is the date which marks the end of the Extended Duration of your programme. However, to remain eligible for honours and awards, you must complete the thesis, and successfully defend it, by the end of 10 week of final semester.

I wish you every success.

Dated: 29/09/20

CHAIRPERSON FRC, BUMDC

Distribution:

- DG
- Principal
- Student's File (with the HOD/PGP Coordinator)
- Student

APPENDIX – B

BAHRIA UNIVERSITY MEDICAL AND DENTAL COLLEGE

Defence phase II, Sailor Street, adjacent to PNS Shifa, Karachi. Tel: 021-35319491-9

ETHICAL REVIEW COMMITTEE

LETTER OF APPROVAL

Date: 15-Jan-21

FRC Reference:
FRC-BUMDC-13/2020-Path-115

PATRON
Prof. Ambreen Usmani
Principal & Dean
Health Sciences(BU)

CHAIRPERSON
Dr. Quratulain Javaid

SECRETARY
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Surg Cdre Amir Ejaz
Prof Reza H Syed
Ms Shabina Arif
Mr M Amir Sultan
Surg Lt Cdr Farah
Surg Lt Cdr Sadia

Dr. Fouzia Fazal

Subject: Institutional Approval of research study

Title of Study: "Correlation of Tumor Associated Macrophages (TAMs) Density and M2 Proportion with the Pathological Stage of Colorectal Cancer."

Principal Investigator: Dr. Fouzia Fazal

Reference No: ERC 21/2021

Dear Dr. Fouzia Fazal ,

Thank you for submitting the above mentioned study proposal. ERC Bahria University Medical and Dental College has reviewed this project in the meeting held on 14-Jan-2021 and gives approval. Kindly notify us when the research is complete.

Regards,

Ambreen 15/1/2021

DR. AMBREEN SURTI
Secretary, ERC
BUMDC

Quratulain 15/1/21

DR. QURATULAIN JAVAID
Chairperson, ERC
BUMDC

Cc:
DG-BUMDC
Principal BUMDC
Chairperson ERC

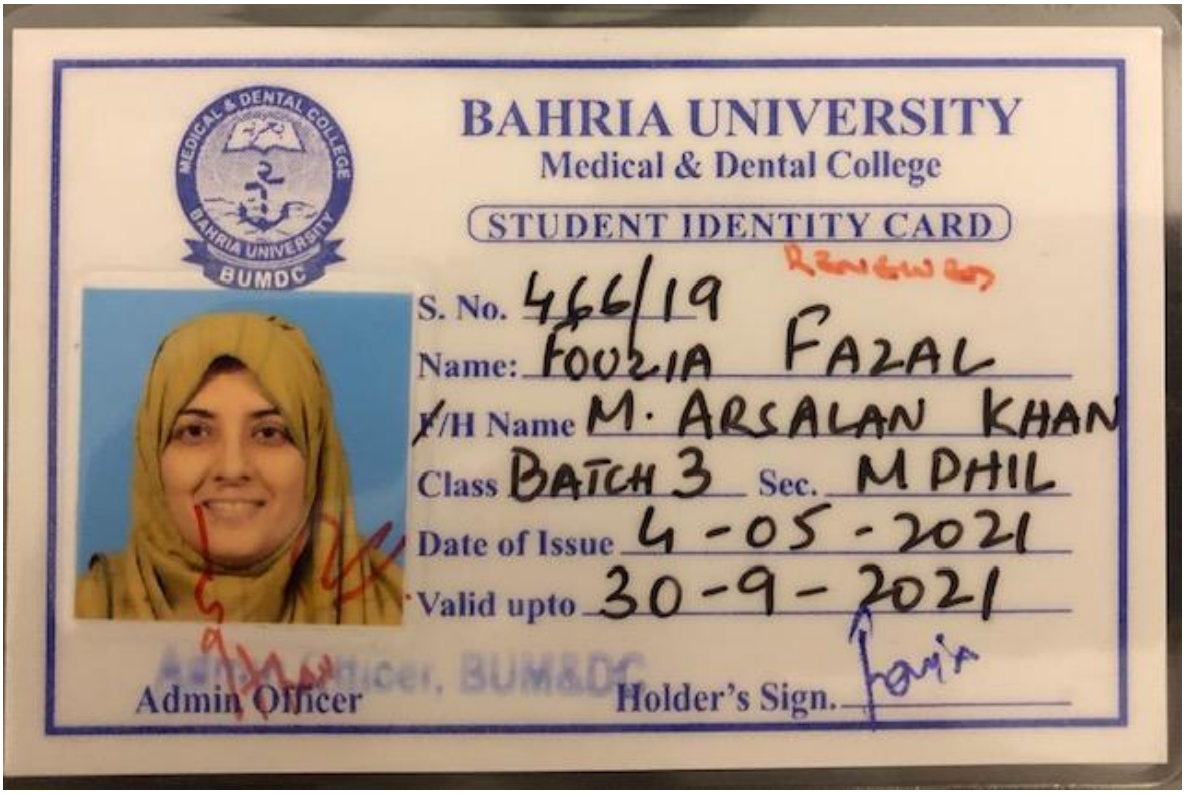
APPENDIX – C

DATA COLLECTION FORM

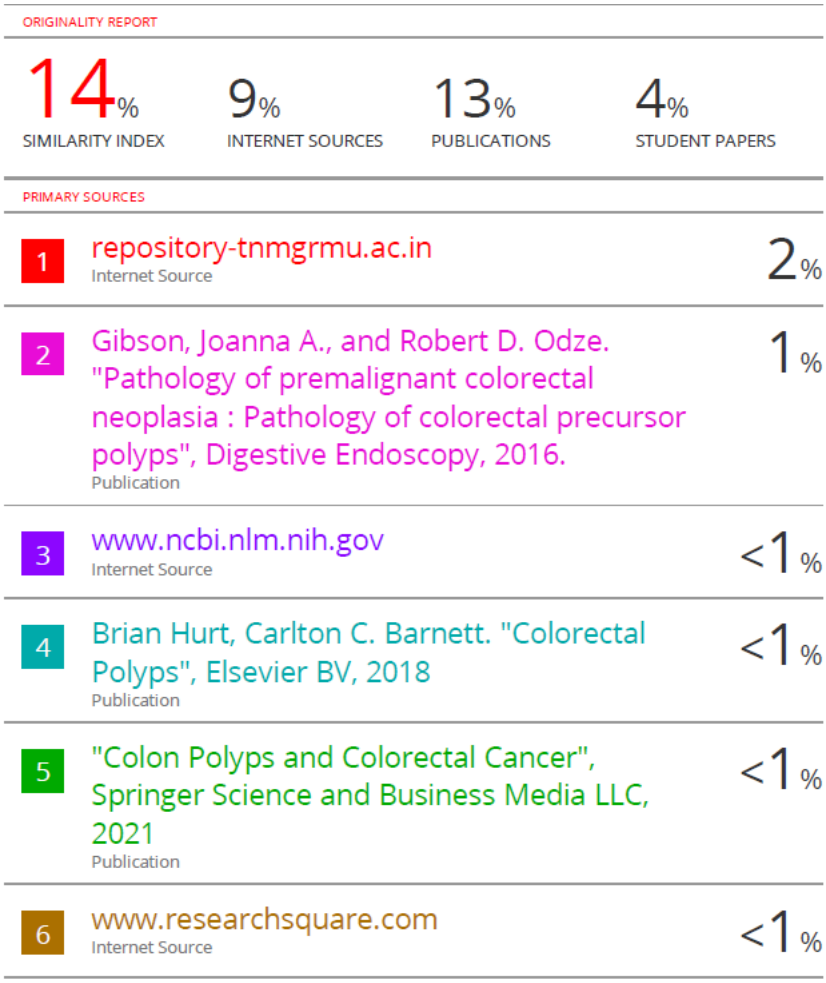
SECTION A: PATIENT IDENTIFYING INFORMATION			
1. Name			
2. Father/Husband Name			
3. Medical ID #			
4. Age			
5. Sex			
SECTION B: SPECIMEN DETAILS			
6. Specimen ID #			
7. Reporting Date			
8. Procedure	☐ Right colectomy	☐ Left colectomy	☐ Trans. colectomy
	☐ Sigmoidectomy	☐ Rectal Resection	☐ Total colectomy
9. Tumor Site	Cecum ___ Ileocecal valve ___ Right (ascending) colon ___ Hepatic flexure ___ Transverse colon ___ Splenic flexure ___ Left (descending) colon ___ Sigmoid colon ___ Rectosigmoid ___ Rectum		
10. Tumor Size	Greatest dimension (centimeters): ___ cm + Additional dimensions (centimeters): ___ x ___ cm		
11. Macroscopic Tumor Perforation	___ Not identified ___ Present ___ Cannot be determined		
12. Histologic Type	___ Adenocarcinoma ___ Mucinous adenocarcinoma ___ Signet-ring cell carcinoma ___ Medullary carcinoma ___ Micropapillary carcinoma ___ Serrated adenocarcinoma ___ Large cell neuroendocrine carcinoma ___ Small cell neuroendocrine carcinoma ___ Neuroendocrine carcinoma (poorly differentiated)# ___ Squamous cell carcinoma ___ Adenosquamous carcinoma ___ Undifferentiated carcinoma		

13. Histologic Grade	___ G1: Well differentiated ___ G2: Moderately differentiated ___ G3: Poorly differentiated ___ G4: Undifferentiated ___ GX: Cannot be assessed
14. TNM Staging	
15. Margins	***Proximal Margin*** ___ Cannot be assessed ___ Uninvolved by invasive carcinoma + Distance of tumor from margin: ___ mm or ___ cm ___ Involved by invasive carcinoma ***Distal Margin*** ___ Cannot be assessed ___ Uninvolved by invasive carcinoma + Distance of tumor from margin (millimeters or centimeters): ___ mm or ___ cm ___ Involved by invasive carcinoma **Radial or Mesenteric Margin** ___ Not applicable ___ Cannot be assessed ___ Uninvolved by invasive carcinoma ***Distance of tumor from margin*** (required only for rectal tumors) (millimeters or centimeters): ___ mm or ___ cm ___ Involved by invasive carcinoma (tumor present 0-1 mm from margin)
16. Additional Findings	***Lymphovascular Invasion*** (select all that apply) ___ Not identified ___ Present + ___ Small vessel lymphovascular invasion + ___ Large vessel (venous) invasion) + ___ Intramural + ___ Extramural ___ Cannot be determined ***Perineural Invasion*** ___ Not identified ___ Present ___ Cannot be determined ***Tumor Budding*** ___ Number of tumor buds in 1 "hotspot" field (specify total number in area=0.785 mm2): _____ ___ Low score (0-4) ___ Intermediate score (5-9) ___ High score (10 or more) ___ Cannot be determined
SECTION C: STUDY CHARACTERISTICS	
17. TAMs Density	(Observed field at x400 magnification, within 5 hotspots in the tumor stroma and along the tumor front) __+__+__+__+__=__ / 5 = __
18. M2 Subtype Proportion	- Mean number of CD163 cells (Observed field at x400 magnification, within 5 hotspots in the tumor stroma and along the tumor front) __+__+__+__+__=__ / 5 = __ - $\frac{Mean\ number\ of\ number\ CD163\ cell}{Mean\ number\ of CD68\ cells}$ X 100 = $M2\ subtype\ proportion$ ___ %

APPENDIX – D


BAHRIA UNIVERSITY
Medical & Dental College
STUDENT IDENTITY CARD
S. No. 466/19
Name: FOUZIA FAZAL
F/H Name M. ARSALAN KHAN
Class BATCH 3 Sec. M PHIL
Date of Issue 4-05-2021
Valid upto 30-9-2021
Admin Officer
Holder's Sign.

APPENDIX – E



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