A RETROSPECTIVE REVIEW OF COLORECTAL CANCER PATIENT OUTCOMES

BY

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Abstract

Colorectal cancer (CRC) occupies the second highest spot of the leading cause of cancer related mortalities in Canada. It is heterogenous in development and presentation, therefore, treatment strategies must be personalized to best treat the individual conditions of each patient. Prognostic factors are used by physicians as a guiding tool in creating treatment strategies. For that reason, it is important to understand the value that each prognostic factor provides in estimating prognosis. Prognostic molecular biomarkers have recently demonstrated their importance in predicting response to systemic therapies. In this study, a retrospective chart review was conducted at a single institution located in New Brunswick, Canada, in order to evaluate the demographic, clinical, and pathologic prognostic factors and their individual influence on patient outcome in terms of overall survival and reoccurrence free survival. This study also determined the simultaneous effect of multiple prognostic factors, lymph node yield and resection margins, on patient outcome. In addition, adherence to specific CRC guidelines at this institution were assessed for compliance in regards to lymph node yield and resection margins. This study showed that for the relatively small sample of patients, the strongest prognostic factors for overall survival was histologic grade of the tumour, while the strongest prognostic factors for reoccurrence free survival were age at diagnosis, distal resection margin and circumferential resection margin. When lymph node yield and resection margins were assessed simultaneously their influence on patient outcome was not significant in improving reoccurrence free survival even with adherence to guidelines. There was relatively high compliance to CRC guidelines for both lymph node yield and circumferential resection margins. Furthermore, this study also assessed the practicality of molecular investigating for a potential prognostic biomarker, the *RAD18* gene, in formalin fixed paraffin embedded (FFPE) tissue. This was done by evaluating a method for retrieving high quality DNA from FFPE. Gel electrophoresis indicated high DNA fragmentation and unsuccessful amplification as a result of the protocol used for FFPE tissue. Overall, these results allow for recognition of survival and reoccurrence trends. Because there is no central database of CRC patient outcome after surgery in the province of New Brunswick, our study may point to areas where further investigation is needed. In the future, we hope this will provide a foundation for a larger survival analysis that will increase knowledge on OS and RFS, allowing for more successful CRC patient outcome in New Brunswick.

Chapter One: Introduction

1.1.0 Epidemiology and etiology of colorectal cancer

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in men and the second most commonly diagnosed cancer in women worldwide, representing a major global health concern (Bray et al., 2018). In 2018, CRC-related deaths numbered approximately 862,000 in total, making it the second most common cause of cancer-related mortality in the world (Bray et al., 2018). Similarly, in Canada, CRC is the third most commonly diagnosed cancer, responsible for an estimated 9,500 deaths in 2019 alone (Canadian Cancer Statistics, 2019). As of January 2020, it was revealed that New Brunswick currently occupies the second spot for highest rate of new cancer cases in Canada with colorectal cancer ranking in the top three of the most diagnosed cancers in this province (Canadian Cancer Statistics, 2020).

Colon and rectal cancer are similar in both morphology and configuration and are therefore collectively referred to as colorectal cancer (Li et al., 2007). Colorectal cancer (CRC) is defined as a malignancy that originates in the large bowel and has the potential to metastasize to other sites on the body (Li et al., 2007). Individual susceptibility to CRC is influenced by both environmental and heritable factors (Arvelo et al., 2015). The majority of CRC cases are sporadic, or nonhereditary, originating from mutations in the somatic cells (Mundade et al., 2014). It has been hypothesized that the diet and lifestyle are associated with the development of CRC (Durko and Malecka-Panas, 2014). High consumption of processed meats, low fiber intake, obesity, and sleep deprivation are related to increased risk (Durko and Malecka-Panas, 2014). While uncommon, heredity does play a role in approximately 5% of all patients diagnosed with CRC, who have an inherited form (Jasperson et al., 2010). These cases can be further defined as one of two types of hereditary CRC: hereditary non-polyposis colorectal cancer (HNPCC), and hereditary polyposis colorectal cancer (HPCC) (Medina Pabón and Babiker, 2019). Both of these syndromes are often associated with young onset CRC, occurring in individuals under the age of 50 (Jasperson et al., 2010).

1.2.0 Molecular pathogenesis of colorectal cancer

The molecular pathogenesis of CRC consists of a multistep process with a timeline that may span years to decades. Over 90% of CRC cases are adenocarcinomas, a type of cancer that

starts in the muciparous (mucus-secreting) glands (Fleming et al., 2012). The step-wise progression of healthy colon or rectal epithelial cells to invasive cancer cells is referred to as the adenoma carcinoma sequence (Aarons et al., 2014). This progression is the result of three distinct mechanisms which all contribute to increased genomic instability (Wolpin and Mayer, 2008). These mechanisms include chromosomal instability, which is associated with the majority of CRC cases, microsatellite instability, and CpG island methylator phenotype (Wolpin and Mayer, 2008). All three mechanisms can either work separately or in tandem to initiate and progress CRC (Wolpin and Mayer, 2008).

1.3.0 Diagnosis and treatment of colorectal cancer

Colorectal cancer is diagnosed through clinical assessment, diagnostic imaging and colonoscopies (Cancer (UK), 2011). Clinical assessment includes an evaluation of symptoms and health history (Cancer (UK), 2011). Common CRC symptoms include abdominal pain, abnormal bowel habit and rectal bleeding (Thompson et al., 2007). In addition, blood tests are performed to detect biomarkers for CRC, as well as to provide a complete blood count, and chemistry profiling to identify any abnormalities (Cancer (UK), 2011). Diagnostic imaging, such as a computerized tomography (CT) scan or a magnetic resonance imaging (MRI) scan, is performed to identify areas of the colon or rectum that may be cancerous (Cancer (UK), 2011). These scans can also can be used to locate and identify metastases (Moss, 1989).

After diagnosis is confirmed, a multidisciplinary team made up of a variety of health care professionals work together to create individualized treatment plans for patients diagnosed with CRC (Dekker et al., 2019) The team typically includes a surgeon, a medical oncologist and a gastroenterologist (Dekker et al., 2019). Depending on the extent of disease, different treatment modalities will be assigned (Dekker et al., 2019). Treatment modalities include complete surgical resection, the only curative strategy for the treatment of CRC (Dekker et al., 2019). This involves the removal of the malignant tumour, a safety margin of surrounding healthy tissue, and nearby lymph nodes (Dekker et al., 2019). If found early enough, a detected polyp (projection of tissue in epithelium of colon or rectum with the potential to be malignant) may be removed during routine colonoscopy, however, early colorectal cancers are often asymptomatic (Church, 2005). Therefore detection of advanced cases are most common and demand more complex tumor resections (Church, 2005). Surgery can be paired with preoperative (neoadjuvant) systemic

therapy, post-operative (adjuvant) systemic therapy or both (Garcia-Aguilar et al., 2016). This includes chemotherapy, radiotherapy and targeted therapy which may be administered in order to downsize a tumour and to prevent reoccurrence (Garcia-Aguilar et al., 2016).

1.3.1 Surgical procedures for rectal cancer and associated resection margins

By the time of diagnoses, the majority of rectal cancers present at an advanced stage and require tumour resection by surgery (Patel et al., 2014). Currently, the most common surgical procedure performed for rectal cancer patients is low anterior resection (Friel, 2009). Low anterior resection is defined as the surgical removal of part of or the entirety of the rectum (Friel, 2009). After resection of the rectum, the colon and the anus are joined (Friel, 2009). Recent advances in surgical technique allowed for the development of sphincter-sparing operations like low anterior resection, which maintains functionality of anal sphincter muscles (Perry and Connaughton, 2007). However, before this operative technique was considered safe, a surgical procedure called abdominoperineal resection was the most frequently used for the treatment of all rectal cancers (Perry and Connaughton, 2007). Abdominoperineal resection is defined by the complete removal of the distal colon, the rectum and the anal sphincter complex (Perry and Connaughton, 2007). This surgical procedure requires the formation of a permanent colostomy (Perry and Connaughton, 2007). If possible, abdominoperineal resection and subsequent formation of colostomies are avoided, however, in some cases where the tumour is located in the distal rectum, abdominoperineal resection is the only option for safe and complete removal of the tumour (Perry and Connaughton, 2007).

There is often an increased challenge for surgery with tumours located in the rectum compared to the colon (Chand et al., 2012). Completing total tumour clearance in the rectum is more complex due to its location within the confines of the pelvis (Chand et al., 2012). In addition to this, the rectum is surrounded by a lymphovascular envelope called the mesorectum (Chand et al., 2012). The mesorectum contains blood vessels, nerves, lymphatic drainage and lymph nodes, which are all structures that can efficiently further tumour spread (Westwood and West, 2018). Both the confined operative field and the surrounding structures susceptible to cancer spread contribute to increased risk of reoccurrence after rectal cancer surgery (Westwood and West, 2018).

Resection margins, otherwise known as safety margins, greatly influence reoccurrence rate (Krishnamurty and Wise, 2016). Resection margins refer to the non-tumourous tissue surrounding the tumour that has the potential to have been infiltrated by cancerous cells, or contain cancerous lymph nodes (Krishnamurty and Wise, 2016). The surrounding tissue is resected along with the tumor and subjected to histopathology (Krishnamurty and Wise, 2016). Findings from resection margins have implications for prognosis.

A distal resection margin (DRM) refers to the margin of tissue distal to the tumour being removed . It is determined by an acceptable distance from possible cancer spread (Krishnamurty and Wise, 2016). A five centimetre DRM is considered to be necessary for all rectal cancer cases as previous studies had found spread of cancerous cells and lymph nodes up to four centimetres from the primary tumour (Grinnell, 1954). Accordingly, Cancer Care Ontario guidelines, which are the guidelines followed by surgeons in Canada, suggest a minimum of a five centimetre DRM for tumors located in rectum (Cancer Care Ontario, 2011). They also suggest that a DRM should not be compromised in order to avoid an abdominoperineal resection (Cancer Care Ontario, 2011) .

A proximal resection margin (PRM) refers to the margin of tissue proximal to the tumour being excised (Shin and Park, 2013). It is determined by the ability to obtain a sufficient distance from possible lymphatic spread while retaining measures to reconstruct after resection (Shin and Park, 2013). Nationally accepted guidelines provided by Cancer Care Ontario, suggest a PRM of at least five centimetres in rectal cancer patients (Cancer Care Ontario, 2011) .

A circumferential resection margin (CRM) is defined as the non-peritonealized surface of resected tissue created during resection (Hermanek and Junginger, 2005). In recent years, the prognostic capability of the CRM has become increasingly apparent, particularly for rectal cancer cases (DeCaria et al., 2015). A CRM has been shown to be the most critical resection margin for assessing risk of reoccurrence (Hermanek and Junginger, 2005). It also has the greatest implications in predicting overall survival (Hermanek and Junginger, 2005). Cancer Care Ontario guidelines suggest a CRM of one millimetre or greater (Cancer Care Ontario, 2011).

1.3.2 Surgical procedures for colon cancer and associated resection margins

The most frequently performed surgical procedure for colon cancer is a hemicolectomy (Sarli et al., 2006). A hemicolectomy involves the partial resection of the colon with the formation of an anastomosis (a connection) by joining the remaining colon and the anus (Sarli et al., 2006). Two types of hemicolectomies are performed which include a right hemicolectomy for cancers located in in the cecum, the ascending colon and the hepatic flexure and a left hemicolectomy for cancers located in the distal, transverse or descending colon (Sarli et al., 2006). Another frequently performed surgical procedure is a sigmoid colectomy, in cases where the tumour is located in the sigmoid colon (Sarli et al., 2006). The operative field in right hemicolectomy allows for increased visualization compared to that of a left hemicolectomy or a sigmoid colectomy (Lim et al., 2017). As such, in colon cancer cases, the removal of a tumour by left hemicolectomy and sigmoid colectomy is often more challenging (Lim et al., 2017).

A DRM for colon cancer resection is determined by the extent of surrounding tissue with the potential to contain cancerous lymph nodes or tumour deposits (Guraya, 2016). Both a DRM and PRM are also determined by the level of ligation of the inferior mesenteric artery because if anastomosis is performed, it is essential that tissue is well vascularized (Guraya, 2016). Cancer Care Ontario guidelines suggest a DRM and PRM of at least five centimetres in colon cancer cases (Cancer Care Ontario, 2011).

A CRM in colon cancer cases includes the colon's mesenteric attachment point and the non-peritonealized surface cut during resection (Amri et al., 2015). Cancer Care Ontario guidelines suggest a CRM that maintains a one millimeter space between the non-peritonealized surface and the tumor (Cancer Care Ontario, 2011).

1.3.3 Open versus laparoscopic surgery

All of the surgical procedures mentioned above can be completed through two different surgical approaches. This includes laparoscopic surgical resection and open surgical resection. The laparoscopic approach to colorectal surgery has surged in popularity over the last 20 years (Mik et al., 2016). Laparoscopic surgery is considered minimally invasive as it requires several small incisions opposed to the single large incision used during open resection (Mik et al., 2016).. These modifications have been shown to decrease blood loss, reduce postoperative pain, shorten the length of hospitalization, and accelerate both the patient recovery rate and return of gastrointestinal function (Bagshaw et al., 2012; Krishnamurty and Wise, 2016; Nussbaum et al.,

2015). While medical professionals are in agreement that laparoscopic surgery is relatively safe, whether laparoscopic surgery produces equivalent oncologic outcome compared to open resection continues to be the subject of discussion (Nussbaum et al., 2015). There is particular concern regarding laparoscopic rectal cancer resection, which inherently involves reduced visibility during surgery in the lower pelvis leading to increased incidence of cancer spread to resection margins (Nussbaum et al., 2015). Without adequate visibility there is an increased possibility of incomplete tumor resection and inadequate lymph node retrieval (Bagshaw et al., 2012; Nussbaum et al., 2015).

1.3.4 Systemic therapy for colorectal cancer

If cancer is identified at an early stage, surgical resection alone is often successful at addressing CRC (Brenner et al., 2014). However, more advanced cases require systemic treatment (Brenner et al., 2014). Neoadjuvant therapy is administered preoperatively before curative resection (Feeney et al., 2019). In cases where a tumor is inoperable, chemotherapy or radiotherapy may be used in an attempt to reduce the tumor size, increasing the possibility for resection (Feeney et al., 2019). Adjuvant therapy (as opposed to neoadjuvant therapy) is administered post-operatively after complete resection to reduce the risk of local recurrence (Milinis et al., 2015). The two main types of systemic therapy used to treat colorectal cancer includes radiotherapy and chemotherapy.

Radiotherapy (also known as radiation therapy) uses ionizing radiation (IR) to damage the DNA of cancerous cells by inducing double stranded DNA breaks. The repair of the DNA double stranded breaks is done through the DNA repair system, mostly the non-homologous end-joining (NHEJ) pathway (Ramzan et al., 2014). The NHEJ pathway can successfully repair the damage done by IR, however double stranded DNA breaks are often difficult to repair and this results in cellular death of cancer cells preventing further cell division and proliferation (Wang et al., 2018). It is thought that the cause of resistance to IR, observed in some CRC individuals, lies within the DNA repair system (Schulz et al., 2019)

Chemotherapy is the delivery of chemotherapeutic agents intravenously or orally (Liu et al., 2015). The ultimate goal of chemotherapeutic agents is to eliminate all cancer cells while avoiding extensive damage to normal tissue (Liu et al., 2015). The cytotoxic effects of chemotherapy include impairing mitosis of cancerous cells through the degradation of DNA and

the spindle apparatus necessary for cell division (Longley et al., 2003). Fast dividing malignant (cancerous) cells are most affected (Liu et al., 2015). The most frequently administered chemotherapeutic agents for colorectal cancers are 5-fluorouracil, oxaliplatin, irinotecan, raltitrexed, trifluridne and tipiracil (Valentini et al., 2006).

Targeted therapy is an alternative therapeutic cancer treatment. This therapy functions to identify target molecules required for cancer growth through the use of antibodies (Singh and Marshall, 2019). These antibodies bind to the targeted molecules on malignant cells, stopping their spread or causing their cellular death (Singh and Marshall, 2019). The target molecules include vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR), both of which encourage cancerous growth (Singh and Marshall, 2019). By inhibiting these molecules, cancer cell development will likewise be hindered (Singh and Marshall, 2019). In colorectal cancer, monoclonal antibody therapy is used, the most common being the anti-EGFR antibody therapy (Martinelli et al., 2009).

1.4.0 The tumour-node-metastasis staging system

Currently, the gold standard of prognostication for colorectal cancer is the tumour-node-metastasis (TNM) staging system produced by American Joint Committee on Cancer (AJCC) (Tong et al., 2018). The tumour-node-metastasis system is used to describe the degree to which a cancerous tumour has progressed (Tong et al., 2018). Once the stage of CRC is known, physicians try to develop the best treatment strategy for optimal patient outcome. There are three elements considered when determining the stage of colorectal cancer (Tong et al., 2018). They include the tumour stage, regional lymph nodes and the presence of metastatic disease. The tumour (T) stage describes the size of the primary tumor based on invasion-depth into nearby tissues, and is given as a number from one (smaller and shallower) to four (larger and deeper) (Brenner et al., 2014). The extent of lymph node involvement is defined by the N stage, and ranges from N0 (no lymph node involvement) to N3 (multiple lymph node involvement/ larger lymph nodes) (Brenner et al., 2014). The presence or absence of metastasis is defined by the M stage, and is given as either M0 (no metastases) or M1 (metastases) (Brenner et al., 2014). The TNM categories are combined to define an overall stage for a cancer. The stages range from stage 0 (least severe) to stage IV (most severe, metastatic spread) (Brenner et al., 2014). Staging

helps to generate a prognosis, which is necessary for health care professionals to create individualized treatment plans (Tong et al., 2018).

1.5.0 Prognostic factors of colorectal cancer

The TNM staging system provides a foundation for estimating patient outcome. However, it has been widely established that CRC is not a homogeneous disease (Molinari et al., 2018). Accordingly, there is great variation of patient outcome for individuals within the same TNM stage. Therefore, there are several other prognostic factors that physicians will consider when making an estimate of post-operative outcome (Athanasakis et al., 2018). Prognostic factors can consist of demographic factors which describe socioeconomic characteristics of patients like age and sex, clinical factors which can describe details of the treatment strategy used, pathological factors which describes characteristics of the tumourous cancer and prognostic molecular biomarkers. Physicians must determine how to incorporate established prognostic factors alongside the TNM stage in the development of optimal treatment strategies for each patient (Athanasakis et al., 2018).

1.5.1 Lymph node yield as a prognostic factor

Lymph nodes are an integral component of the immune system. They act as filters of foreign particles and cancerous cells, making them an essential element in cancer diagnosis and treatment (Ji, 2016). Lymph node involvement is a major part of cancer staging which, in turn, dictates individualized treatment plans and prognosis (Brenner et al., 2014). During surgical resection, lymph nodes are removed along with the tumor (Brenner et al., 2014). In order to prevent underdiagnoses, sufficient numbers of lymph nodes must be removed and analyzed for an accurate detection of node-positive disease. Cancer Care Ontario guidelines currently suggest the removal of at least 12 lymph nodes, referred to as the lymph node yield (LNY), in CRC cases (Cancer Care Ontario, 2011). A reduced LNY has been shown in several studies to be associated with reduced survival in CRC (Bui et al., 2006; Norwood et al., 2010; Wang et al., 2019c).

1.5.2 Tumour location as a prognostic factor

It is widely recognized that colon and rectal cancer share many features because of their anatomic continuity (Hong et al., 2012) They share the same risk factors, symptoms and similar

development patterns (Hong et al., 2012). Although, rectal and colon cancer are related anatomically, they have a separate blood supply, drainage and innervation system (Dai et al., 2017), which is thought to be the reason behind differences in their overall survival and reoccurrence rates. Rectal cancer is associated with a much greater reoccurrence rate and a decreased survival rate compared to colon cancer (Hong et al., 2012). For this reason, the location of the tumour has been identified as having prognostic value (Hong et al., 2012). It is also acknowledged that right sided and left sided colon cancer differ in many aspects (Baran et al., 2018). They exhibit variability in both histological and molecular characteristics (Baran et al., 2018). Right sided colon cancers are commonly associated with the microsatellite instability pathway tumours while left sided tumours often originate by the chromosomal instability pathway (Baran et al., 2018). The difference in tumour biology is thought to stem from the different embryological origins of these tissues; the right colon arises from the mid gut while the left colon arises from the hind gut (Wang et al., 2019). This variation translates into differences in overall survival and reoccurrence free survival. Right sided tumours are easier to resect because right hemicolectomies offer an increased operative field, however, right-sided colon cancers are associated with worse prognosis (Moritani et al., 2014; Yahagi et al., 2016). It is thought that this is because left sided cancers are easier to detect at an earlier stage due to differences in polyp morphology, as right sided polyps tend to be more flat (Baran et al., 2018). Therefore, at diagnosis, right side colon cancers are more advanced (Baran et al., 2018).

1.5.3 Tumour size as a prognostic factor

Tumour size is a widely assessed pathological parameter and is calculated from the widest horizontal tumour diameter (Dai et al., 2017). Although tumour size is already evaluated as the T stage in the TNM staging system, this is based on the depth of tumour invasion into bowel tissue and is not a fixed measurement of size. There is currently no general consensus for the prognostic value of tumour size for CRC outcome (Dai et al., 2017). There have been several studies that have shown no association between tumour size and patient outcome (Crozier et al., 2007; Miller et al., 1985; Steinberg et al., 1986). On the contrary, there have also been several studies that have shown that large tumour size is associated with negative patient outcome in CRC (Balta et al., 2014; Mejri et al., 2017; Saha et al., 2015).

1.5.4 Histologic tumour grade as a prognostic factor

Histologic tumour grade is assessed by trained pathologists which provide a description of the degree of differentiation (Rakha et al., 2010). Well-differentiated tumours have high homology compared to normal epithelial colorectal tissue and poorly differentiated tumours present highly abnormal cells (Rakha et al., 2010). Histologic grade has been shown to have great prognostic value. Multiple studies have demonstrated that the prognostic importance of tumour grade is comparable to that of lymph node status (Galea et al., 1992; Sundquist et al., 1999). As expected, those with poorly differentiated tumours have a worse prognosis than those with well differentiated tumours (Manxhuka-Kerliu et al., 2009).

1.6.0. Prognostic molecular biomarkers of colorectal cancer

Demographic, clinical and pathologic prognostic factors have been used for decades in estimating prognosis and are deemed fundamental for developing individualized treatment strategies (Manxhuka-Kerliu et al., 2009). In recent years, the importance of molecular biomarkers has also become evident (Sepulveda et al., 2017). The detection of molecular biomarkers, specifically gene mutations, is important for determining how individual patients will respond to systemic therapies (Sinicrope et al., 2016). Currently, common histopathology practice includes testing for microsatellite instability (MSI), BRAF mutation and KRAS mutation (McGee et al., 2018). Testing for MSI is important in determining response to 5FU-based chemotherapy (Kawakami et al., 2015). Testing for mutations on *KRAS* and *BRAF* protooncogenes provide information on how patients might respond to anti-EGFR antibody therapy (Ducreux et al., 2019).

1.6.1 RAD18 gene expression as a prognostic molecular biomarker

In recent times, a commonly used treatment modality for rectal cancer is to use neoadjuvant radiotherapy with subsequent surgical resection and adjuvant chemotherapy (Nacion et al., 2018). While this has become the standard, research has shown that only a small number of patients benefit from neoadjuvant therapy, which requires that surgery is postponed until after therapy is administered (Yan et al., 2019). For this reason, it would be beneficial to know if patients are likely to respond to a neoadjuvant treatment beforehand, so that surgery is not delayed without benefit (Gruenberger et al., 2008). A recent investigation of *RAD18*, has shown

it to be a predictor of a patient's response to chemotherapy and radiotherapy and has indicated the potential for this gene to be an important molecular biomarker in treatment of rectal cancer (Yan et al., 2019).

The *RAD18* gene is an E3 ubiquitin-linked enzyme and is involved in the DNA damage-repair system (Xie et al., 2014). If DNA is damaged during replication, development of the replication forks is stalled until it is repaired (Xie et al., 2014). Inability to advance the replication fork results in permanently suspended replication and subsequent cellular death (Xie et al., 2014). In order to survive, cells adapt, using DNA-damage tolerance (DDT), skipping the repair, and completing the cell cycle (Xie et al., 2014). A DDT mechanism, known as translesion DNA synthesis (TLS), involves replacing stalled replicative polymerase, caused by DNA lesions, with TLS polymerases (Xie et al., 2014). Unlike replicative polymerase, TLS polymerase can insert bases opposite damaged nucleotides in order to complete replication (Xie et al., 2014). The substitute polymerase has low fidelity, meaning that it is highly error prone, which leads to genomic instability (Xie et al., 2014). Despite this, increased mutagenesis is favoured over incomplete replication or cellular death (Vaisman and Woodgate, 2017).

In order to bypass the lesion, TLS polymerases require the help of proliferating cell nuclear antigen (PCNA). In non-damaged conditions, PCNA bound to polymerase allows for normal insertion of nucleotides into a DNA sequence. In damaged DNA, PCNA, ubiquitinated by RAD6/RAD18 proteins, allows polymerase to bypass the lesion and continue the cell replication cycle (Wang, 2001). It has been hypothesized that this protein interaction decreases the fidelity of replicative polymerase, allowing it to insert nucleotides opposite damaged bases (Vaisman and Woodgate, 2017). For this reason, the level of expression of *RAD18* has been shown to be associated with resistance to IR or 5-FU chemotherapy (Yan et al., 2019), (Xie et al., 2014). In these studies, high expression of *RAD18* was associated with decreased IR- and 5FU-mediated apoptosis when compared to cells with low *RAD18* expression (Yan et al., 2019), (Xie et al., 2014).

The ability to detect the molecular biomarkers, such as the one mentioned here, has considerable prognostic implications for the management of CRC (Sinicrope et al., 2016). In order to create improved outcomes for a greater number of CRC patients, there is a need for newer and more predictive biomarkers (Sinicrope et al., 2016). Detection of *RAD18* expression, for example, would be very useful for improving rectal cancer patient prognoses. Before this can

be accomplished, however, more research is needed to create the foundation for *RAD18* gene expression analysis in clinical and pathological practice.

1.6.2 Investigating prognostic molecular biomarkers of CRC from formalin fixed paraffin embedded tissue.

In order to investigate molecular biomarkers of CRC, a reliable method for obtaining a large resource of nucleic acids from CRC patients is needed. Fresh frozen tissue has been established as the sample of choice in past studies who aimed to detect gene mutations in molecular biomarkers investigations (Kalmar et al., 2013). Fresh frozen tissue samples represent the most reliable source of quality nucleic acids (Kalmar et al., 2013). However, fresh frozen tissue is often not abundantly available in clinical practice as it is costly and requires liquid nitrogen for snap-freezing of resected tissue right after surgical removal (Gao et al., 2020). In contrast, formalin fixed paraffin embedded (FFPE) tissue is used a preservative method in routine clinical practice for storing patient samples (Gao et al., 2020). FFPE tissue offers several advantages compared to other preservative methods due to its efficient and inexpensive protocol (Rabelo-Gonçalves et al., 2014). Therefore, FFPE tissue of cancer patients is relatively abundant in hospital settings (Rabelo-Gonçalves et al., 2014).

FFPE tissue blocks maintain cellular morphological features like cytoskeletal and protein structure (Klopfleisch et al., 2011). However, harsh conditions in the fixation and embedding processes affect the quality of nucleic acids. During fixation, a reaction between the nucleic acids and the added formaldehyde results in the crosslinking of proteins and other biomolecules found in the tissue (Patel et al., 2016). Isolated DNA and RNA can be retrieved, but in a fragmented form (Patel et al., 2016). Fragmentation is increased through several other factors including the age of the FFPE section, the concentration of formaldehyde added during fixation and the duration of time between the fixation and embedding procedures (Lin et al., 2009).

Growing interest in using these tissues for such studies has led to efforts in optimizing nucleic acid extraction from FFPE tissue (Kokkat et al., 2013). Currently, several studies exist comparing commercially available DNA and RNA extraction kits. The silica membrane-based extraction method has been shown to provide higher quality DNA and RNA from FFPE tissue compared to alternative methods using magnetic beads, salt precipitation or the use of organic solvents (Gilbert et al., 2007; Hedegaard et al., 2014; Janecka et al., 2015). A reliable extraction

method of nucleic acids from FFPE tissue will allow for a greater overall understanding of CRC pathogenesis on a molecular level.

1.7.0 Goals of current research study

It has been well established that colorectal cancer is a heterogeneous disease, thus there is considerable variability in the development, presentation and outcome for CRC patients. Individualized treatment plans are established for every CRC patient in order to increase the likelihood of recovery. Prognostic factors provide guidance for the formation of treatment plans. To increase CRC patient success, it is important to understand the demographic, clinical and pathologic factors influencing patient outcome. Currently, an accessible central database of colorectal cancer patient outcomes does not exist in New Brunswick, Canada. As a result, we have no indication of how established prognostic factors are influencing overall survival or reoccurrence free survival in this province. Evaluating the prognostic value of factors that are known to have an influence on promoting survival and reducing reoccurrence would allow for greater patient success, especially because in New Brunswick CRC diagnosis rates are at an all-time high. The goal of this study, therefore, is to evaluate demographic, clinical and pathological factors that influence CRC patient outcomes in a single-institution setting through a retrospective chart review.

There are three research goals within the retrospective chart review. The first and main research goal is to determine the individual effect of each prognostic factor on patient outcome. The second was to determine the simultaneous effect of lymph node yield, distal resection margin, proximal resection margin and circumferential resection margin on patient outcome in order to determine if improved outcome is observed when CRC guidelines are followed. The third is to determine the frequency of adherence to guidelines at this institution by examining how the institution's lymph node yield, as well as their distal resection margins, circumferential resection margins, and proximal resection margins conformed to the CRC treatment-guidelines.

Finally, systemic therapy response, particularly radiotherapy, has been shown to be dependent on *RAD18* gene expression, however few studies exist on this topic to-date. The final goal of this study is to assess the ability of the silica membrane-based extraction method to extract quality nucleic acids, in this case DNA, from FFPE tissue. The results generated through this research will provide a foundation for a future project aiming to look at *RAD18*'s potential

role as a predictor for chemotherapy and radiotherapy-response in patients suffering from CRC, and especially those patients with rectal carcinomas.

1.7.1 Experimental strategy

To assess the demographic, clinical and pathologic prognostic factors that affect colorectal cancer patient outcome, a retrospective chart-review of CRC patients was conducted at the Upper River Valley hospital in New Brunswick, Canada. Patient data were manually extracted from the institution's database. Statistical analysis was then performed on the patient data of interest to associate prognostic factors with resulting-outcome, in terms of overall survival and reoccurrence free survival. An additional part of this study included assessing the steps required for investigating molecular biomarkers such as gene expression of the *RAD18* gene in FFPE tissue samples. The first step of this process included evaluating the ability of the QIAamp® DNA FFPE Tissue Kit, which uses the silica membrane-based extraction method to extract DNA from FFPE tissue.

1.7.2 Significance of study

Colorectal cancer continues to be one of the leading causes of cancer-related death worldwide. Advances in medical research have improved CRC diagnosis-rates as well as the outcomes for those diagnosed with CRC, both through more effective and streamlined screening procedures, improved surgical techniques, and highly targeted treatment methods. It is important to understand the prognostic factors that are associated with improved outcomes for those diagnosed with CRC. This study aimed to provide a foundation for the assessment of prognostic factors and their associated CRC patient outcomes, allowing for the recognition and continued study of parameters worthy of further investigation in New Brunswick.

Furthermore, CRC patient outcome can be improved through the detection of molecular biomarkers that have a role in prognosis. There is need for more molecular biomarkers that can reliably predict how patients will respond to certain treatments. As such, determination of the prognostic role of the *RAD 18* gene would be a significant addition to the current knowledge-base. The final goal of this study was to provide the preliminary steps for developing the methodology for investigating *RAD18* expression in the FFPE tissue samples of CRC patients.

Chapter two: Materials & Methods

2.1.0 Ethical considerations for retrospective chart review

Permission for secondary use of colorectal cancer (CRC) patient data from the Upper River Valley Hospital, located in Waterville, New Brunswick, Canada was granted by the Research ethics board of the Horizon Health Network on August 1, 2019 **(**ROMEO File #: 100350 Event 6158). A waiver of consent and fees was approved for this study on June 10, 2019 by Horizon Health Research ethics board (ROMEO File #: 100350 Event 6158). All chart reviews were completed by the student research assistant of the chief of surgery at the Upper River Valley Hospital.

2.2.0 Study population and setting

The study population included patients that received treatment for CRC by surgical resection between the years of January 2016 and December 2019 at the Upper River Valley Hospital. All patients were either diagnosed at the Upper River Valley Hospital or referred to this institution by their family physician. The Upper River Valley Hospital provides services to the rural communities of western New Brunswick, notably the towns of Woodstock and Hartland.

2.3.0 Study design

In order to obtain information on prognostic factors at this institution, a retrospective chart review was conducted. All chart reviews took place at the surgical department of the Upper River Valley Hospital between September 2019 and January 2020. Patient records were reviewed from patients that received treatment for colorectal cancer at the Upper River Valley Hospital between January 2016 to December 2019. Exclusion criteria included patients who did not undergo surgical resection, those who died before undergoing surgical resection and those that did not wish to undergo resection. Patient records were obtained from office notes and operating room records, as well as electronic-files and reports from the on-site electronic medical record (EMR) system, MEDITECH ERH. Patient records were analyzed for preselected prognostic factors. This included two demographic factors including age and sex; six clinical factors including treatment modalities, type of surgical procedure performed, surgical approach and the length of stay in the hospital after resection and eight pathologic factors including

tumour stage, tumour location, tumour size, histologic grade of tumour, lymph node yield, distal resection margin, proximal resection margin and circumferential resection margin. Data, with identifiers redacted, was inputted into an excel spread sheet and stored at Mount Allison University in Sackville, NB (data available upon request). Survival analysis was carried out with the primary endpoints being overall survival and reoccurrence free survival.

2.3.1 Prognostic factors of interest

The prognostic factors used in this study included demographic, clinical and pathologic factors. All of the factors were assessed as a categorical variable and none were assessed as a continuous variable. Preselected prognostic factors and their associated categories that were assessed in this study. Age at diagnosis was categorized into two groups which included those that were 50 years or younger and those over the age of 50 years old. Treatment modality was categorized according to four different treatment regimens. Categories included those who received neoadjuvant and adjuvant therapy in the addition to surgery, those who received neoadjuvant therapy in addition to surgery, those who received adjuvant therapy in addition to surgery and those who underwent surgery without the addition of either type of systemic therapy. Surgical procedure described five different procedures. This included right hemicolectomy, left hemicolectomy, sigmoid colectomy, abdominoperineal resection and low anterior resection. Surgical approach included the two different methods of surgical excision. This was assessed as laparoscopic resection or open resection. Length of stay in hospital was categorized in two groups which includes those who stayed in hospital admittance less than 7 days after surgery and those who were in hospital admittance for 7 days or more after surgery.

The tumour stage was categorized as stage I, stage II, stage III and stage IV based on the Tumour Node Metastasis (TNM) staging system. Tumour location was categorized as left colon, right colon and rectum. Tumour size was calculated from widest horizontal tumour diameter and was categorized by 4.5 centimeters (cm) or less or greater than 4.5 cm. Histologic grade of the tumour was classified as poorly differentiated and well differentiated. Lymph node yield was categorized according to the guideline recommended lymph node yield. This included a lymph node yield less than 12 lymph nodes and a lymph node yield of 12 lymph nodes or greater. All three resection margins reviewed were also categorized based on guideline recommendations. The distal resection margin was grouped into a margin less than 5 cm and a margin of 5 cm or

greater. The proximal resection margin was also grouped into a margin less than 5 cm and a margin of 5 cm or greater. The circumferential resection margin was grouped into a margin less than 0.1 cm and a margin of 0.1 cm or greater.

2.3.2 Patient outcome

For the purposes of this research, patient outcome was defined as overall survival (OS) and reoccurrence free survival (RFS). Overall survival was measured from the date of surgery to the date of documented death. This point was censored by the last date of follow up. Reoccurrence free survival was defined from the date of surgery to the date of confirmed cancer reoccurrence. This was also censored at the date of the last documented follow up.

2.4.0 Statistical analysis for retrospective chart review

In order to address the first research goal, which was to determine the individual effect of each prognostic factor on patient outcome, a univariate analysis was performed. Probability curves were constructed according to the Kalan Meier method (Efron, 1988) to show OS and RFS for each of the patient groups within each of the demographic, clinical and pathologic prognostic factors. The log rank test (Bland and Altman, 2004) was used to compare differences in time-to-event distribution for each patient group within the prognostic factor, with the event being either death or reoccurrence. Time was measured as the number days after surgery. A multivariate Cox proportional hazards model (Lin and Wei, 1989) was used to evaluate the simultaneous effect of multiple prognostic factors (lymph node yield, distal resection margin, proximal resection margin and circumferential resection margin) on patient outcome (OS and RFS), which was the second research goal. Statistical analyses did not need to be performed for the third research goal, as it was presented as a frequency of guideline adherence. All tests had the α level set at 0.05 for statistical significance. All statistical analyses were performed using R software (Version 3.6.2, “Dark and Stormy Night”).

2.5.0 Formalin–fixed paraffin embedded tissue and fresh tissue samples

To assess the ability of the silica membrane-based extraction method to extract quality DNA from FFPE tissue, the QIAamp® DNA FFPE Tissue Kit (QIAGEN, Cat # 56404), which uses the silica membrane extraction method, was used. Permission to use FFPE tissue samples was given by the Mount Allison University Research Ethics Board. Two FFPE tissue samples were used. They consisted of samples of most convenience from the Lloyd bio bank and the Aiken Lab (Mount Allison University). The samples included *Homo sapien* (human) skeletal muscle FFPE tissue and *Semibalanus balanoidea* (barnacle) FFPE tissue. A fresh tissue sample, *Ixodes scapularis* (tick) tissue, was used as a control for non-formalin fixed tissue in this study. This was also a sample of most convenience provided by the Lloyd bio bank (Mount Allison University).

2.6.0 DNA extraction from FFPE tissue samples

Extraction of DNA from FFPE tissue samples and the fresh tissue control sample were performed using the QIAamp® DNA FFPE Tissue Kit (QIAGEN, Cat # 56404), according to the manufacturer's instructions. Using a sterile razor blade, excess paraffin was cut off each of the FFPE tissue samples and discarded. Sections of FFPE tissue were cut 10 µm thick and placed into 1.5 microcentrifuge tubes. The *Ixodes scapularis* (tick) tissue was minced and placed into a 1.5 microcentrifuge tube. In a fume hood, xylene was added to each of the tubes and mixed by vortexing for ten seconds. In order to further break down the tissue, all samples were grinded with sterile plastic pestles. The samples were then centrifuged at 13, 300 RPM for 2 minutes. The supernatant was removed from each microcentrifuge tube using a fine pipet tip. The pellet was washed with 1 ml of 95% ethanol and mixed by vortexing for 15 seconds. The samples were then centrifuged again at 13, 300 RPM for 2 minutes.

Supernatant was discarded and the pellet was dried at room temperature (20-25°C) in an open tube for 15 minutes until all residual liquid had evaporated. The samples were then resuspended in 180 µl of Buffer ATL (200 ml tissue lysis buffer, cat# 19076) and 20 µl of proteinase K, then vortexed for 15 seconds. All samples were incubated in a 90 °C water bath for 1 hour. Once removed, samples were allowed to cool and then centrifuged for 15 seconds at 13, 300 RPM to remove excess liquid from inside the lid. Next, 200 µl of Buffer AL (Lysis Buffer AL, cat# 19075) was added to each solution and mixed by vortexing for 10 seconds. This was

followed by the addition of 200 µl of 95% ethanol and mixed again by vortexing for 10 seconds. The entire lysate was then carefully transferred to a QIAamp MiniElute column (in a 2 ml collection tube, cat# 19201) and centrifuged at 8 000 RPM for 1 minute.

The QIAamp MiniElute column was then placed into a new 2ml collection tube and washed with 500 µl of Buffer AW2 (Wash Buffer (1) concentrate, c# 19081). Collection tubes containing the flow through were discarded and replaced with a new collection tube. The samples were then washed with 500 µl of Buffer AW2 (Wash Buffer (2) concentrate, c#19082) and then collection tube with flow through was discarded and replaced. The membrane was dried by centrifugation at 13, 300 at 3 minutes to rid it of residual ethanol. The QIAamp MiniElute columns were placed in 1.5 ml microcentrifuge tubes and 100 ul of Buffer ATE was applied to the center of membranes. Samples were then incubated at room temperature (20-25°C) for 5 minutes followed by centrifugation at 13, 300 RPM for 1 minute. All DNA samples were stored at -20°C until PCR amplification was performed.

2.2 PCR amplification

In this study, DNA quality was assessed by success of PCR amplification of the DNA extracted from the FFPE samples. PCR amplifications were completed for each of the FFPE tissue samples and the fresh tissue sample with three different amplifying different sized amplicons for detection of the conserved regions of mitochondrial *Cytochrome C Oxidase subunit 1* gene (Table 1; Sigma-Aldrich Custom Oligos). In order to assess the PCR amplifiability of a much less abundant gene found in the human FFPE sample assessed, a primer for the detection of the rRNA intragenic region of the bacterial species, *Borrelia burgdorferi* (*sensu stricto*) was also used (Table 1; Sigma-Aldrich Custom Oligos).

In a PCR workstation, a master mix was made with each of the primers in sufficient amounts for all of the samples and the negative controls. The mixes (per sample/control) contained 8.5 µl nuclease free water, 12.5 µl GoTaq®, 1µl forward primer and 1µl reverse primer. In each of the PCR tubes, 23 µl of master mix was added. The negative controls were placed in the first and last PCR tube to DNA contamination. In the negative control tubes, 2 µl of nuclease free water was added. While in the rest of the PCR tubes, 2 µl of DNA sample was added. Samples were then placed in a thermocycler (Eppendorf Mastercycler ep Gradient S). The thermocycler program was different for all four primers used.

The thermocycler program for PCR using HCO2198 and LCO1490 primers started with an initial 5 minute denaturation period. This was followed by a series of 3 steps, repeated for a total of 35 cycles. This included a denaturation period at 95 °C for 15 seconds, an annealing period at 60°C for 15 seconds and an elongation period of 30 seconds at 72°C. This was followed by a final extension period at 72°C for 5 minutes. For the mlCO313 primers, the thermocycler program described by Leray et al. was used (Leray et al., 2013). This started with a 5 minute denaturation step at 95°C. After this, a series of 3 steps were carried out for 16 cycles. This included a 10 second denaturation period at 95°C, a 30 second annealing period at 65°C and a 1 minute extension period at 72°C. This was followed by 25 cycles of 46°C for 30 seconds.

The thermocycler program for PCR using the Uni-Minibar130 primers, described by Meusiner et al., started with a 2 minute denaturation period at 95°C (Meusnier et al., 2008). This was followed by a series of 3 steps that were repeated in 5 cycles. This included a 1 minute denaturation period at 95°C, followed by a 1 minute annealing period at 55°C and a 30 second elongation period at 72°C. This was then followed by a series of 3 steps repeated for 35 cycles. This included a 1 minute denaturation period at 95°C, a 1 minute annealing period a 53°C and a 30 second elongation period at 72°C. This program finished with a final extension period at 72°C for 5 minutes. For the NML Bbss qpcr primers, the thermocycler program started with an initial 5 minutes denaturation period. This was followed by a series of 3 steps, repeated for 35 cycles which included a denaturation period at 95°C for 15 seconds, an annealing period at 53°C for 30 seconds and an elongation period at 72°C for 30 seconds. This was again, followed by a final extension period at 72°C for 5 minutes. All samples were kept at 4 °C until they were removed from the thermocycler and placed into storage at -20 °C until the second round of PCR was performed.

Two rounds of PCR were completed for each of the samples with all four of the primers. An identical procedure was performed for the second round however, instead of adding template DNA to the 23 µl of master mix in a PCR tube, 2 µl of PCR product from the first PCR round was added. The samples were placed again in the thermocycler. The same thermocycler programs, as mentioned above, for each primer were performed. All samples were kept at 4 °C until they were removed from the thermocycler and placed into storage at -20 °C until gel electrophoresis could be performed.

Table 2.1: Primers used for detection of *Cytochrome C Oxidase Subunit* 1 gene and the rRNA intragenic region of *Borellia burgdorferi.*

Primer label	Target gene	Sequence (5'-3')	Amplicon length (bp)	Melting temperature (°C)	Annealing temperature (°C)
HCO2198	*Cytochrome C Oxidase Subunit I*	TAAACTTCAGGGTGACCAAAAAATCA	710	63	60
LCO1490		GGTCAACAAATCATAAAGATATTGG		58	
mlCO313 F		GGWACWGGWTGAACWGTWTAYCCYCC	313	70.5	65
mlCO313 R		GGRGGRTASACSGTTCASCCSGTSCC		68	
Uni-Minibar130 CO1 F		CAAAATCATAATGAAGGCATGAGC	130	65.2	55
Uni-Minibar130 CO1 R		TCCACTAATCACAARGATATTGGTAC		58.5	
NML Bbss qpcr F	rRNA Intragenic region of *Borrelia burgdorferi*	CGAGTCTTAAAAGGGCGATTTAGT	200	64.3	53
NML Bbss qpcr R		GCTTCAGCCTGGCCATAAATAG		65.6	

2.6.2 Gel electrophoresis

A 100mL 1.2% (w/v) agarose gel was made using 1X, 0.05M sodium borate (SB) buffer. The SB buffer contained 1L double distilled water with 0.8% w/v NaOH and 4.5% w/v boric acid. Liquified gel was stained with 5µl SYBR® Safe DNA Gel Stain (Thermo Fisher Scientific, cat # S33102) and set to shape. Gels were submerged in 1X SB running buffer and samples were loaded into the wells of the gel. Alongside the samples, a 1kB DNA ladder (GeneDireX, cat #DM010-R500) was loaded into the wells of the gel. Samples were run at 108V for 50 minutes. The gel was then placed on a UV transilluminator (Labnet DNA Light) and a photo was taken with a cell phone. Successful PCR amplification was defined as a fluorescing, definitive band at the location of the expected amplicon, as indicated by the target amplicon of each primer.

Chapter three: Results

The goal of this study was to assess the demographic, clinical, and pathologic prognostic factors that affect patient outcome in terms of overall survival and the reoccurrence-free survival of patients diagnosed with colorectal cancer at a single institution setting. In order to do this, a retrospective chart review was conducted and data was statistically analyzed to answer three research goals. The research goals included determining the individual effect of each prognostic factor of patient outcome, determining the simultaneous effect of multiple prognostic factors (lymph node yield, distal resection margin, and circumferential resection margin) on patient outcome and finally to determine adherence to guidelines at this institution. An additional goal of this study included assessing the steps required for molecular investigation of gene expression in formalin fixed paraffin embedded (FFPE) patient tissue samples. The first step in this process is extracting nucleic acids from FFPE samples and I report here an assessment of the ability of the QIAamp® DNA FFPE Tissue Kit to extract PCR-amplifiable DNA from FFPE tissue.

3.1.0 Clinical, demographic, and pathological factors of study population

Data from 60 cases of CRC that were treated by surgery at the Upper River Valley Hospital were included in the final analysis. The clinical features of patients are shown in Table 3.1. The mean age of the study participants was 69.8 ± 11.0 and ranged from 49 years to 88 years of age. Of the study population, 60% were males and 40% females (Table 3.1).

Treatment modalities consisted of either a combination of preoperative radiotherapy (neoadjuvant therapy), surgery and postoperative (adjuvant therapy), a combination of preoperative therapy and surgery, a combination of surgery and postoperative therapy and lastly, surgery alone. All of the CRC patients reviewed in this study received chemotherapy and/or radiotherapy and none were administered monoclonal antibody therapy as a systemic therapeutic agent.

Surgery without neoadjuvant or adjuvant therapy was the most common treatment modality (58.3%) used in this study followed by surgery with adjuvant therapy (30.0%) and surgery with neoadjuvant therapy (6.7%) (Table 3.1). Overall, 5.0% of patients received both neoadjuvant and adjuvant therapy in addition to surgery (Table 3.1).

The majority of tumours (51.7%) were located in the right colon, while 20% were located in the left colon and 15% were located in the rectum (Table 3.1). Information on location was

missing for 3.33% of cases reviewed. Accordingly, a right hemicolectomy was performed most frequently (56.7%), followed by low anterior resection (26.7%), sigmoid colectomy (8.3%), left hemicolectomy (6.7%) and finally abdominoperineal resection (1.7%) (Table 3.1). The surgical approach used most often was laparoscopic resection (53.3%) followed closely by open resection (46.7%). The length of stay in hospital, in days, was relatively consistent with these treatments. Approximately half of the patients (46.7%) stayed in hospital for less than one week after their surgical procedure (Table 3.1), while in 53.3%, of patients stayed in hospital for over one week after surgery (Table 3.1).

Overall, most (41.7%) of the patients observed in this study had tumours classified as stage II tumours (Table 3.1), with 33.3% of cases classified as stage III tumours and 16.7% of cases as stage I tumours (Table 3.1). Information on stage for 8.3% of cases was not recorded (Table 3.1). It was found that 35% of tumours exceeded 4.5 cm in diameter while 48.3% of tumours were less than 4.5 cm in diameter; the information was missing for 16.7% of cases (Table 3.1). Results from data of the histologic grade of the tumours observed in this study revealed that 73% were designated as well differentiated and 18% of cases showed tumours designated as poorly differentiated while information from 8.3% of cases was not recorded (Table 3.1).

In the majority of CRC cases (81.7%), 12 or more lymph nodes were resected while in 18.3% of cases under 12 lymph nodes were resected (Table 3.1). With regard to distal resection margins (DRM), 41.6% of cases showed a DRM of 5 centimetres (cm) or more, while 35% of cases presented a margin that was less than 5 cm (Table 3.1). Information on DRM was missing for 14% of cases. Proximal resection margin (PRM) measurements showed similar proportions in each group. In 21.7% of cases the PRMs were found to be 5 cm of more, while in 35% of cases the PRMs were under 5 cm and 43.4% of cases had missing information (Table 3.1). In the majority (76.7%) of cases, circumferential resection margins (CRM) were above 0.1 cm, while in one (1.7%) case the CRM was under 0.1 cm and 21.7% of cases had missing information (Table 3.1).

Table 3.1: Demographic, clinical, and pathological factors of colorectal cancer assessed in this study

Factor	Frequency n (%)	Variable	Frequency n (%)
Age		**Tumour Location**	
≤ 50	2(3.3)	Right colon	31(51.7)
> 50	58(96.7)	Left colon	12(20.0)
Sex		Rectum	15(25.0)
Male	36(60.0)	Not recorded	2(3.33%)
Female	24(40.0)	**Tumour Size**	
Treatment Modalities		≤ 4.5 cm	29(48.3)
Surgery+Neoadjuvant+Adjuvant	3(5.0)	> 4.5cm	21(35.0)
Surgery+Neoadjuvant	4(6.7)	Not recorded	10(16.7)
Surgery+Adjuvant	18(30.0)	**Histologic grade**	
Surgery	35(58.3)	Well differentiated	44(73.0)
Type of surgical procedure		Poorly differentiated	11(18.0)
Right Hemi Colectomy	34 (56.7)	Not recorded	5(8.3)
Left Hemi Colectomy	4(6.7)	**Lymph node yield**	
Sigmoid Colectomy	5(8.3)	< 12	11(18.3)
Low anterior Resection	16(26.7)	≥ 12	49(81.7)
Abdominoperineal resection	1(1.7)	**DRM**	
Surgical approach		< 5 cm	21(35.0)
Open	28(46.7)	≥ 5 cm	25(41.6)
Laparoscopic	32(53.3)	Not recorded	14(23.3)
Length of stay in hospital		**PRM**	
< 7 days	28(46.7)	< 5cm	21(35.0)
≥ 7 days	32(53.3)	≥ 5 cm	13(21.7)
Tumour Stage		Not recorded	26(43.3)
Stage I	10(16.7)	**CRM**	
Stage II	25(41.7)	< 0.1 cm	1(1.7)
Stage III	20(33.3)	≥ 0.1 cm	46(76.7)
Not recorded	5(8.3)	Not recorded	13(21.7)

3.2.0 Overall survival and reoccurrence-free survival after surgical resection

The overall survival (OS) analysis was based on 5 deaths among the 60 patients (8.3%) that were analyzed as of February 2020. The OS time showed a plateau at approximately 80%, starting at around 650 days (1.7 years) after surgery (Figure 3.1). The reoccurrence-free survival (RFS) was based on 10 events among 60 patients (16.7%). Reoccurrence events in this study began at around 585 days (1.6 years) after surgery (Figure 3.1). The RFS time plateaued at approximately 62%, beginning at around 992 days (2.7 years) after surgery (Figure 3.1).

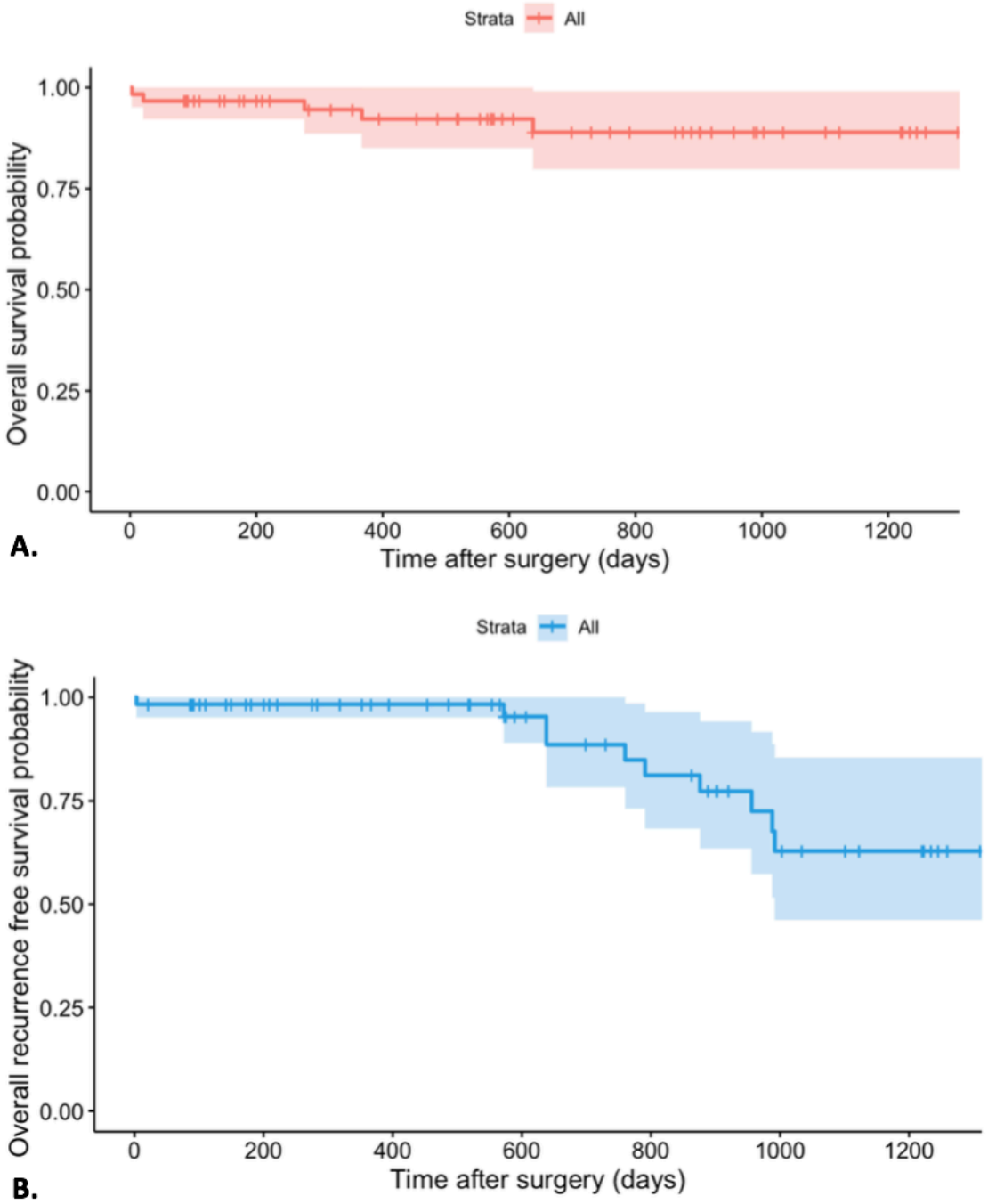

Figure 3.1: A. Kaplan Meier overall survival (OS) curve displaying survival probability over time in days after surgery (n=60). Downward steps represent an event (death). Vertical ticks represent a censored observation (patient who did not experience death by last follow up). Pale red area indicates 95% confidence interval of the survival curve B. Kaplan Meier overall reoccurrence-free survival (RFS) curve displaying reoccurrence-free survival probability over time in days after surgery (n=60). Downward steps represent an event (reoccurrence of cancer). Vertical ticks represent a censored observation (patient who did not experience reoccurrence by last follow up). Pale blue area indicates 95% confidence interval of the survival curve.

3.2.1 Univariate analysis of overall survival and reoccurrence free survival

In order to assess OS and RFS differences among the demographic, clinical, and pathological variables assessed in this study, a univariate analysis was used. This included the Kaplan Meier method to create a survival curve for every subgroup within each prognostic variable and the log rank test to determine if there was a statistically significant difference between each of the survival curves.

Demographic and clinical variables included age, sex, treatment modality, type of surgical procedure performed, surgical approach and length of stay in hospital after surgery (Table 3.2). In spite of higher incidence of CRC in patients over 50 years old, there was no statistically significant difference in OS between patients that were older than 50 years or younger than 50 years of age (P= 0.67) (Table 3.2). However, as visualized on the Kaplan Meier survival curve, those over the age of 50 had an increased mortality rate (Figure 3.2). A similar trend was observed for RFS, as those over the age of 50 had an increased reoccurrence rate (Figure 3.3). In this case, a statistically significant difference in RFS was found between the two age groups (P=0.044) (Table 3.2). There was no statistically significant difference in OS or RFS was found between patient sex (P=0.84; P=0.5) (Table 3.2).

For all four treatment modalities used in this study, the Kaplan Meier survival curves revealed that OS and RFS favoured patients whose treatment modality included surgery with the addition of neoadjuvant therapy and those who received surgery with the addition of both neoadjuvant and adjuvant therapies (Figure 3.2, Figure 3.3), however, there was no statistically significant difference found between any of the treatment modality groups (P=0.91; P=0.18) (Table 3.2). For the five different surgical procedures performed; right hemicolectomy, left hemicolectomy, sigmoid colectomy, low anterior resection and abdominoperineal resection, a trend of increased OS and RFS was shown for those that received a right hemicolectomy (Figure 3.2; Figure 3.3). Once again there was no statistically significant difference of OS or RFS was found (P=0.83; P=0.6) (Table 3.2).

The OS or RFS of patients slightly favoured those that received laparoscopic surgery compared to those that received open resection (Figure 3.2; Figure 3.3). However, there was not a statistically significant difference detected between the two surgical approaches (P= 0.72; P= 0.94) (Table 3.2). Patients that stayed in hospital admittance for more than 7 days after surgery and patients that stayed less than 7 days after surgery did not show a statistically significant

difference in OS or RFS probability (P= 0.93; P=0.28) (Table 3.2). It was concluded that none of the demographic of clinical prognostic factors assessed in this study showed statistically different OS probabilities, but a statistically significant difference in RFS was detected for age (Table 3.2).

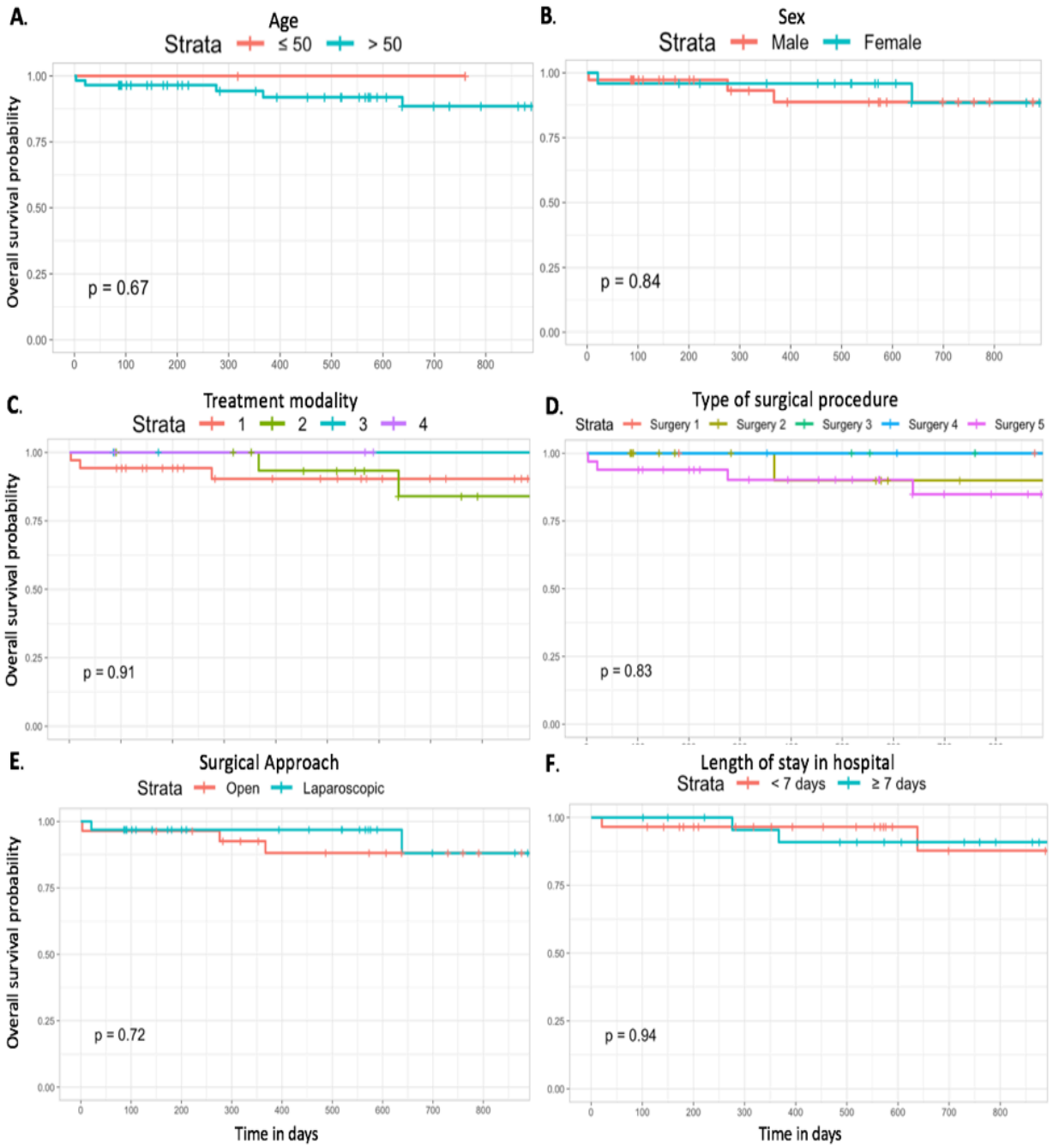

Figure 3.2: Kaplan Meier overall survival curves of clinical prognostic factors displaying survival probability over time in days after surgery (n=60). Downward steps represent an event (death). Vertical ticks represent a censored observation (patient who did not experience death by last follow up). A. Age survival plot. B. Sex survival plot. C. Treatment modality survival plot indicating 1: Surgery; 2: Surgery+Adjuvant therapy; 3: Surgery+Neoadjuvant therapy; 4: Surgery+Neoadjuvant+Adjuvant therapy. D. Surgical procedure survival plot indication 1: Abdominoperineal resection; 2: Low anterior resection 3: Sigmoid colectomy; 4: Left hemicolectomy; 5: Right hemicolectomy. E. Surgical approach survival plot. F. Length of stay in hospital survival plot. P values displayed in each of the survival plots indicate result from log rank test.

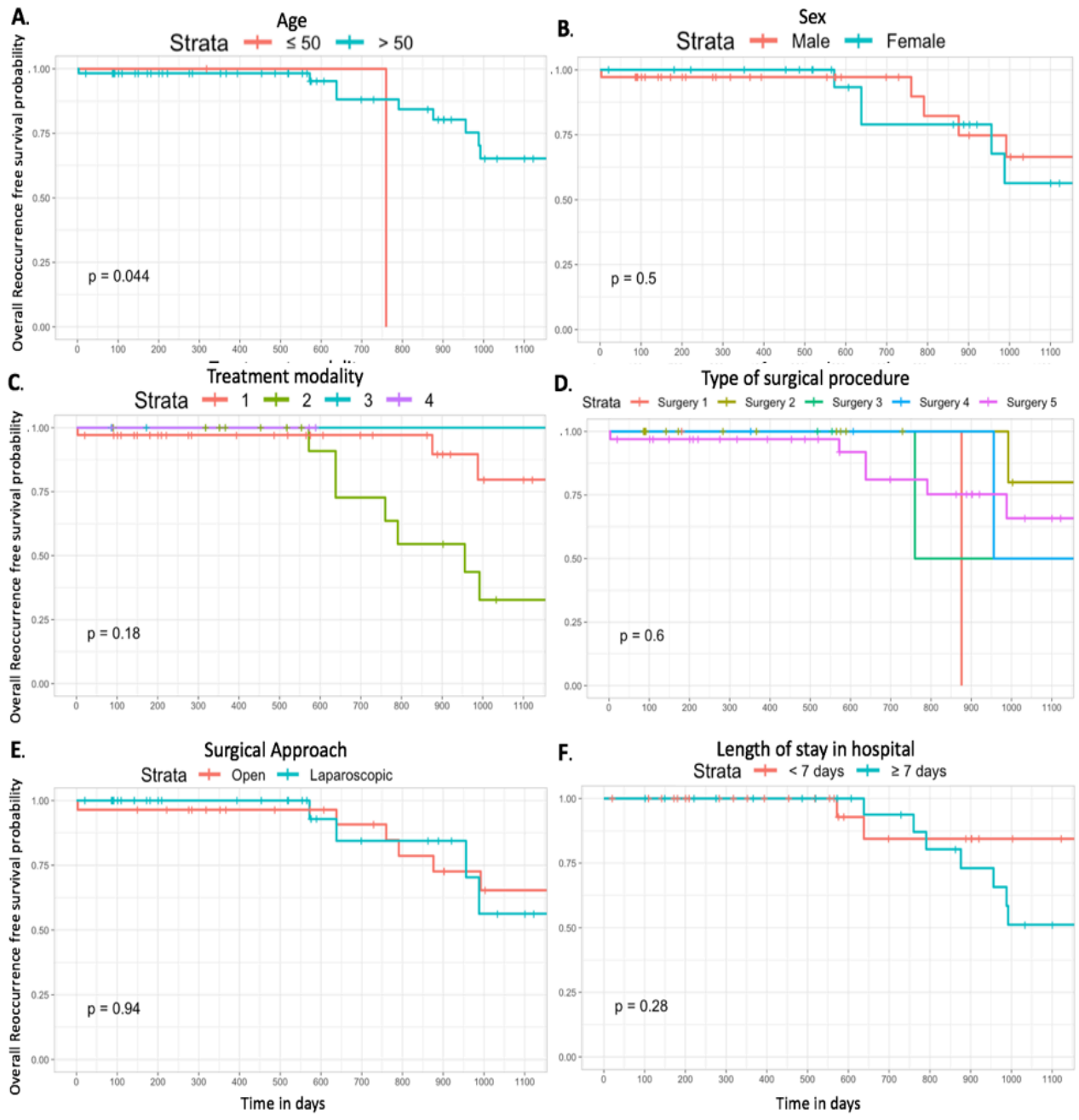

Figure 3.3: Kaplan Meier overall survival curves of pathological prognostic factors displaying reoccurrence free survival probability over time in days after surgery (n=60). Downward steps represent an event (reoccurrence). Vertical ticks represent a censored observation (patient who did not experience reoccurrence by last follow up). A. Age survival plot. B. Sex survival plot. C. Treatment modality survival plot indicating 1: Surgery; 2: Surgery+Adjuvant therapy; 3: Surgery+Neoadjuvant therapy; 4: Surgery+Neoadjuvant+Adjuvant therapy. D. Surgical procedure survival plot indication 1: Abdominoperineal resection; 2: Low anterior resection 3: Sigmoid colectomy; 4: Left hemicolectomy; 5: Right hemicolectomy. E. Surgical approach survival plot. F. Length of stay in hospital survival plot. P values displayed in each of the survival plots indicate result from log rank test.

Table 3.2: Association of demographic and clinical factors with overall survival (OS) and reoccurrence free survival using the log rank test

Factor	*P* value[1] (OS)	*P* value[1] (RFS)
Age	0.67	0.044
Sex	0.84	0.5
Treatment Modalities	0.91	0.18
Type of surgical procedure	0.83	0.6
Surgical approach	0.72	0.94
Length of stay in hospital	0.94	0.28

Pathological factors included tumour stage, tumour location, tumour size, histologic grade, lymph node yield and all three resection margins (DRM, PRM and CRM) (Table 3.3). In this study, no CRC cases classified as stage IV tumours were reviewed. There was a trend of higher overall mortality for patients who had tumours classified as stage III, followed by those with tumours classified as stage II and the stage I (Figure 3.4). However, there was no statistically significant difference in OS probability (P=0.13) (Table 3.3). There was also a trend of increased reoccurrence for patients that had tumours classified as stage II (Figure 3.5). Once again, however, there was no statistically significant difference in RFS probability (P=0.66) (Table 3.3).

Despite high incidence of right colon tumour location opposed to the left colon and the rectum tumour location, no statistically significant difference in OS or RFS between all three tumour locations was seen (P=0.41; P=0.67) (Table 3.3). There was however, a trend of reduced mortality and reoccurrence for those with a tumour located in the rectum (Figure 3.4; Figure 3.5). There was also a trend of increased mortality for those with a tumour greater than 4.5 cm and a trend for reduced reoccurrence for those with tumours under 4.5 cm (Figure 3.4; Figure 3.5). However, the size of the tumour showed no statistically significant difference in OS or RFS between tumours in the two size groups (P=0.95; P=0.62) (Table 3.3).

Histologic grade of the tumour was grouped into well-differentiated or poorly differentiated. It was discovered that there was a statistically significant difference in OS between the two groups (P=0.0035) with a trend for increased survival for patients with tumours classified as well differentiated as indicated by the Kaplan Meier survival curve (Figure 3.4). However, difference in histological grade failed to be a statistically significant variable when looking at RFS (P=0.42) (Table 3.3).

Even though, lymph node yield was over 12 lymph nodes in the majority of cases, no statistically significant difference in both OS and RFS was seen for patients that had 12 or more lymph nodes resected and patients that had fewer than 12 lymph nodes resected (P=0.29; P=0.85) (Table 3.3). There was a trend, however, of increased OS and RFS for those that had fewer than 12 lymph nodes resected (Figure 3.4; Figure 3.5). The difference in OS between patients with a DRM over 5 cm and under 5 cm was not statistically significant (P= 0.14) (Table 3.3). However, the difference in RFS was statistically significant for the two groups of DRM (P=0.012) (Table 3.3). Similarly the difference in OS between a CRM over 0.1 cm and under 0.1 cm did not differ significantly (P=0.69), however the difference in RFS did differ significantly (P=0.03) (Table 3.3). The difference in OS between a PRM over 5 cm and under 5 cm did not differ significantly for either OS or RFS (P=0.71; P=0.71) (Table 3.3).

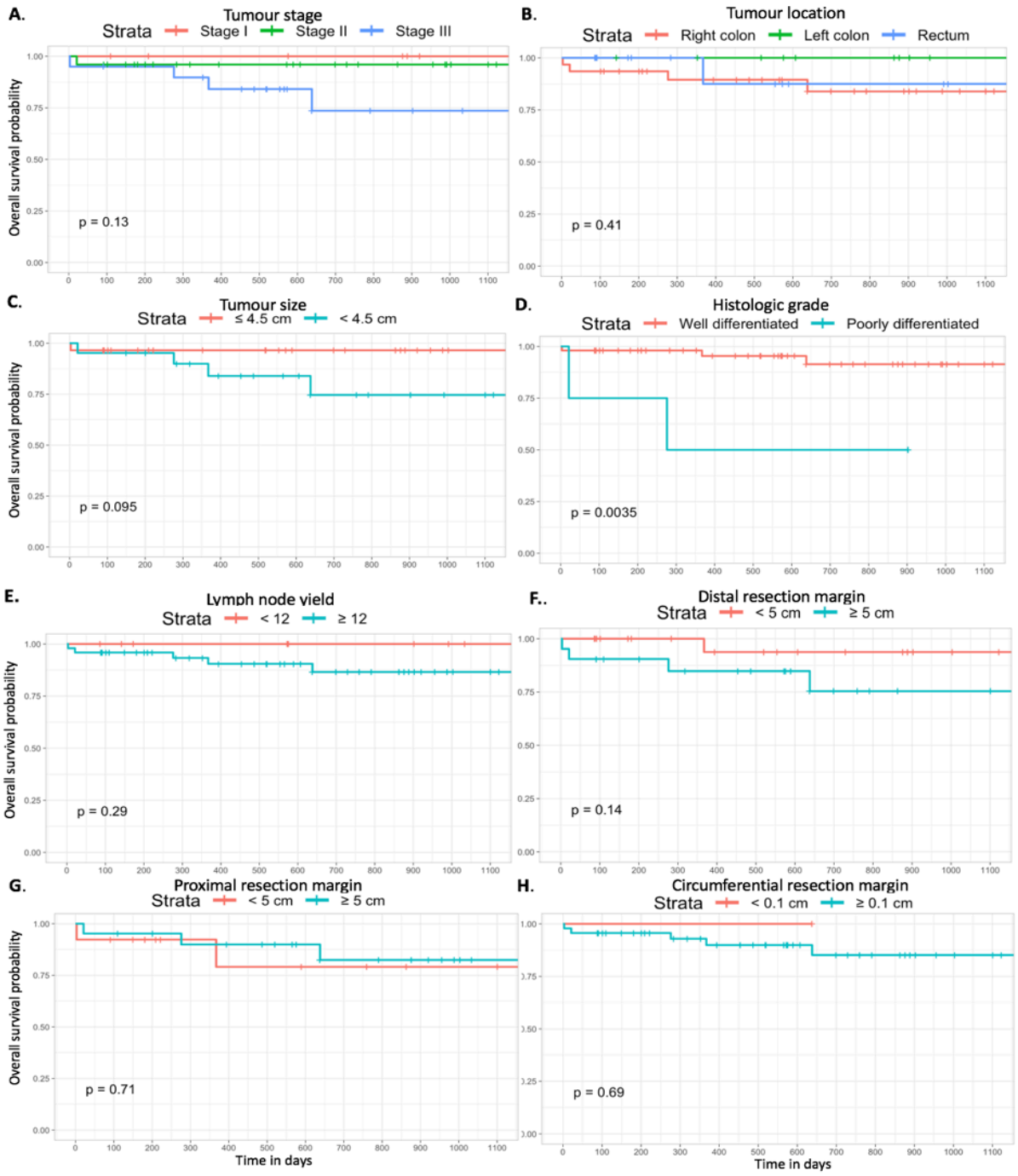

Figure 3.4: Kaplan Meier overall survival curves of pathological prognostic factors displaying survival probability over time in days after surgery (n=60). Downward steps represent an event (death). Vertical ticks represent a censored observation (patient who did not experience death by last follow up). A. Tumour stage survival plot. B. Tumour location survival plot. C. Tumour size survival plot. D. Histologic grade survival plot. E. Lymph node yield survival plot. F. Distal resection margin survival plot. G. Proximal resection margin survival plot. H. Circumferential resection margin survival plot. P values displayed in each of the survival plots indicate result from log rank test.

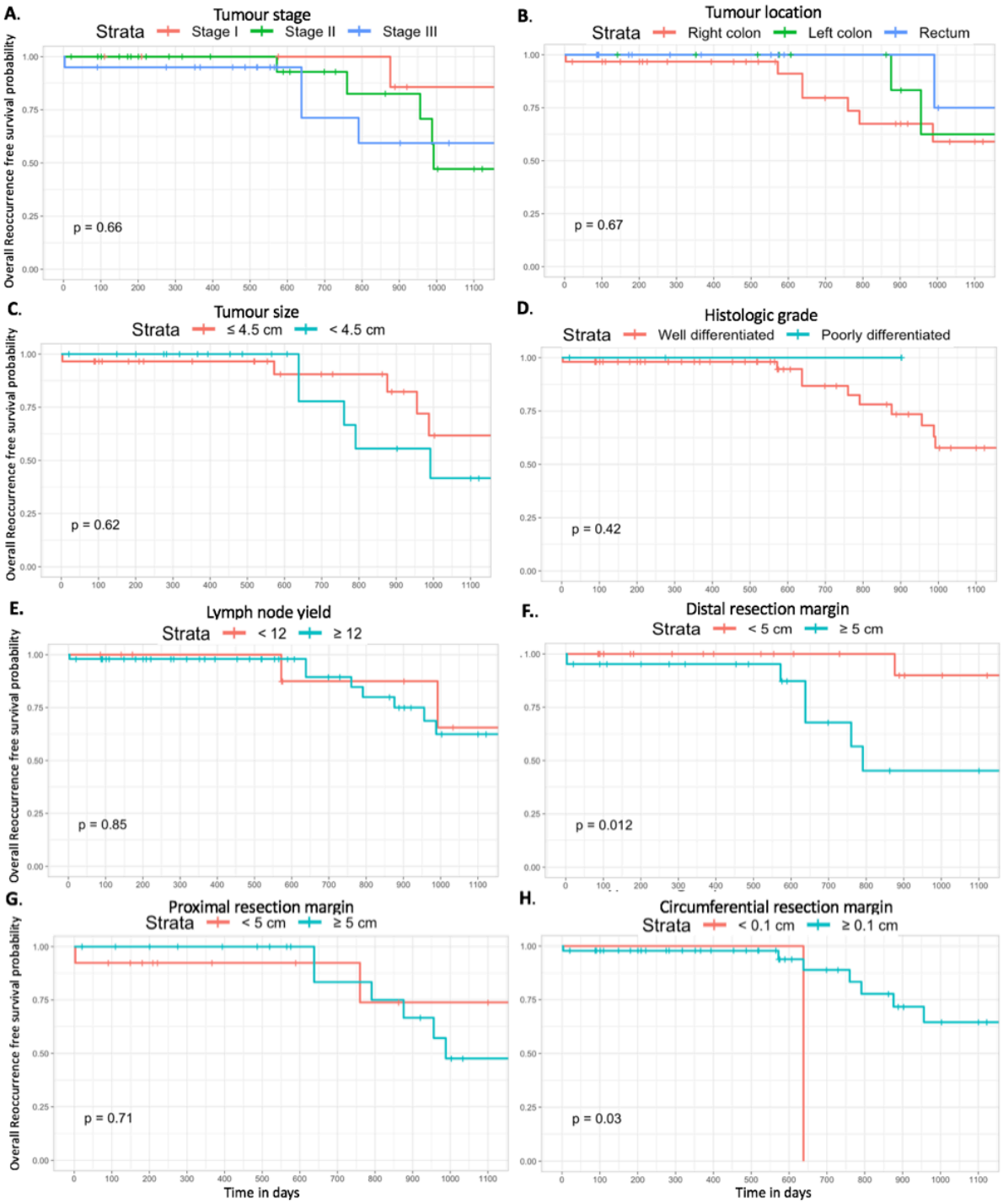

Figure 3.5: Kaplan Meier overall survival curves of pathological prognostic factors displaying reoccurrence free survival probability over time in days after surgery (n=60). Downward steps represent an event (reoccurrence). Vertical ticks represent a censored observation (patient who did not experience reoccurrence by last follow up). A. Tumour stage survival plot. B. Tumour location survival plot. C. Tumour size survival plot. D. Histologic grade survival plot. E. Lymph node yield survival plot. F. Distal resection margin survival plot. G. Proximal resection margin survival plot. H. Circumferential resection margin survival plot. P values displayed in each of the survival plots indicate result from log rank test.

Table 3.3: Association of pathological variables with overall survival (OS) and reoccurrence free survival using the log rank test

Variable	*P* value[1] (OS)	*P* value[1] (RFS)
Tumour Stage	0.13	0.66
Tumour Location	0.41	0.67
Tumour Size	0.95	0.62
Histologic grade	0.0035	0.42
Lymph node yield	0.29	0.85
DRM	0.14	0.012
PRM	0.71	0.71
CRM	0.69	0.03

3.2.2 Multivariate analysis of overall survival and reoccurrence free survival

After completion of the univariate analysis, a multivariate analysis (Cox proportional hazard model) was used to answer the second research goal addressing the simultaneous effects of lymph node yield, distal resection margin and circumferential resection margin on OS or RFS. This analysis was done with these prognostic factors in order to determine if increased patient outcome is observed when lymph node yield and resection margin guidelines are followed.

I was unable to analyze the effects of lymph node yield and the three resection margins, DRM, PRM and CRM with OS, as the variables have infinite values and therefore, the Cox function is not usable for the data. However, I was able to analyze the effects of covariates, lymph node yield and two of resection margins, DRM and CRM with RFS. The resection margin, PRM was excluded from the analysis as it had infinite values from too many unrecorded values and so was not usable in the Cox function. A Cox proportional hazards model was constructed for determining hazard ratios (HR). Hazard ratios represent the probability that an individual will experience an event, in this case reoccurrence of cancer, at a particular point in time after surgery.

The hazard ratio of 0.29 for lymph node yield indicates that patients who had less than 12 lymph nodes resected have a reduced risk of reoccurrence compared to patients who had 12 or more lymph nodes resected (Table 3.4). Therefore, having less than 12 lymph nodes resected reduces the hazard by 71% after adjusting for patient's DRM and CRM. However, as indicated by the p-value, this result is not statistically significant (P=0.324) (Table 3.4).

A fairly large hazard ratio of 11.2 was seen for DRM (Table 3.4). This indicates that at any particular time, patients who had a DRM of less than 5 cm was 11.2 times more likely to experience reoccurrence compared to those that had a DRM of 5 cm of more. The p-value of 0.05 indicates that it is right on conventional significance borderline (P=0.05) (Table 3.4). The hazard ratio of 0.19 for CRM indicated that the patients that had a CRM of more than 0.1 cm have a reduced risk of reoccurrence compared to those that had a CRM of less than 0.1 cm. Patients with a CRM of 0.1 cm or greater reduces the hazard by 81%. However, The p-value of CRM is greater than 0.05 (P=0.159), therefore the hazard ratio fails to be statistically significant (Table 3.4).

Table 3.4: Association of three pathological variables with overall survival (OS) using Cox regression analysis

Variable	OS HR (95 % CI)	OS *P* value[1]
Lymph node yield	0.29 (0.024 - 3.4)	0.324
DRM	11.2 (0.995 - 16.0)	0.05
CRM	0.19 (0.018 - 1.9)	0.159

3.3.0 PCR amplification of DNA from formalin fixed paraffin embedded tissue samples using primers amplifying different sized amplicons

The first steps for investigating the potential molecular biomarkers, such as *RAD18*, is to find a reliable method for extracting nucleic acids from formalin fixed paraffin embedded tissue (FFPE). In this study, DNA was extracted rather than RNA as it is often endures formalin fixation better than RNA and remains more intact. In order to assess the ability of the QIAamp® DNA FFPE Tissue Kit (QIAGEN, Cat # 56404) to extract PCR-amplifiable DNA from FFPE tissue, I performed PCR amplifications with primers for detection of the conserved regions of mitochondrial *Cytochrome C Oxidase subunit 1* gene with DNA from samples of convenience, which included *Homo sapiens* skeletal muscle FFPE tissue, *Semibalanus balanoidea* (barnacle) FFPE tissue and *Ixodes scapularis* (tick) fresh tissue as a control sample. Primers for detection of the conserved regions of mitochondrial *Cytochrome C Oxidase subunit 1* gene with DNA were used as it was known, without uncertainty, that all samples assessed for contain this gene.

PCR products from the first round were re-amplified and then electrophoresed on 1.2% agarose gel and visualized under UV illumination. PCR amplification with primers

HCO2198 and LCO1490 (Folmer et al., 1994), primers which amplify 710-bp amplicons revealed no amplification, resulting in only smears on the gel (lanes EO5, EO6 and EO7; Figure 3.6 A.). The absence of clear bands indicates unsuccessful DNA amplification and suggests fragmentation of DNA in both the FFPE tissue samples and the fresh tissue sample.

To determine if an amplicon could be produced with primers targeting smaller regions, I completed two rounds of PCR amplification of the samples with a new primer pair with a shorter target amplicon. PCR amplification with mlCO313 primers (Leray et al., 2013) which amplifies 313 bp amplicons revealed very low intensity DNA smears. Lanes with both the FFPE tissue samples and the fresh tissue sample showed no amplification (Figure 3.6 B.). Two rounds of PCR amplification were then performed with a Uni-Minibar130 CO1 primers (Meusnier et al., 2008) whose target amplicon is even shorter, 130 bp. Agarose gel electrophoresis again revealed larger and higher intensity DNA smears (Figure 3.6 C.). Lane EO6, containing the barnacle DNA displayed a faint band at approximately 142.3 bp (Figure 3.6 C.). Lane EO6 also had the greatest range of smearing starting at the approximately 501.2 bp and finishing at around 102 bp (Figure 3.6 C.).

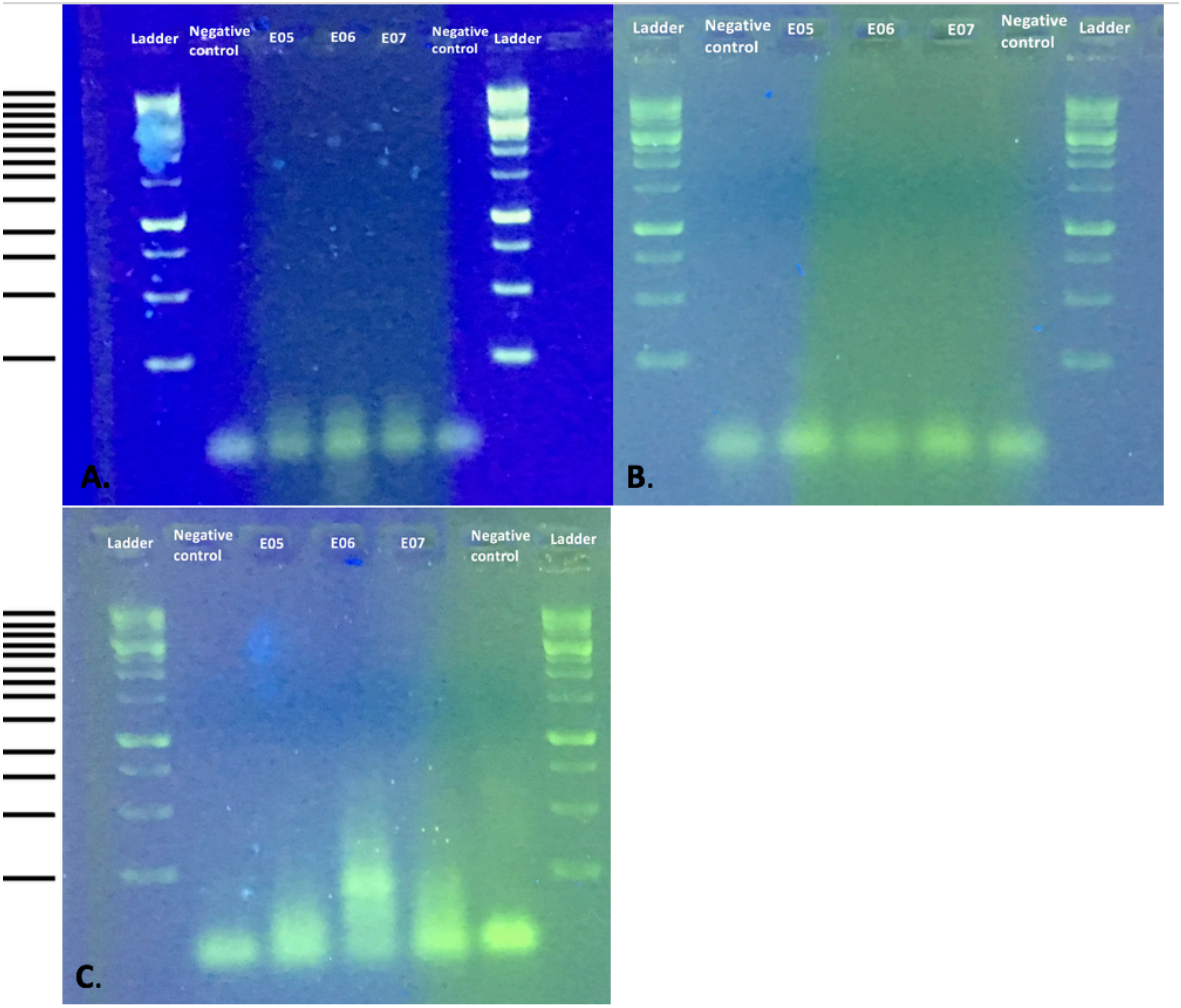

Figure 3.6: Agarose gel (1.2%) on UV transilluminator displaying results of two rounds of PCR on extracted DNA from FFPE tissue samples and fresh tissue samples. A. PCR with HCO2198 and LCO1490 primers (predicted amplicon size 710 bps). B. PCR with mlCO313 primers (predicted amplicon size 313 bps) C. PCR with Uni-Minibar130 CO1 primers (predicted amplicon size 130 bps). DNA was extracted from FFPE skeletal muscle tissue (EO5), FFPE barnacle tissue (EO6) and fresh tick tissue (EO7). Both negative controls bordering the samples contain nuclease free water instead of template DNA. Neighboring the negative samples are 1 kb GeneDirect ladders (10000 bp-250bp). Smears on the bottom of the gel in all lanes, including the negative controls, are residual primers. Base pair length was estimated using the linear regression equation produced by the standard curve of known base pair sizes from the 1 Kb GeneDirect ladder (Appendix 1).

3.3.1 PCR amplification of DNA from FFPE tissue samples using specialized primer sets

In order to assess the potential to amplify a gene segment that is much less abundant than the mitochondrial *Cytochrome C Oxidase subunit 1 gene*, I performed PCR amplifications with primers for detection of a rarer gene; that for the rRNA intragenic region of the bacterial species, *Borrelia burgdorferi* (*sensu stricto*). The detection of this gene was chosen as the human sample was known to be infected with this bacteria. Once again, this was done as a matter of convenience as the primers were available. Two rounds of PCR amplification were performed with National Microbiology Laboratory (NML) Bbss qpcr primers whose target amplicon is 200 bp. Agarose gel electrophoresis revealed no amplification, no bands or smears were seen, indicating that the DNA template was not successfully amplified. The positive control that contains *Borrelia burgdorferi*-infected tick DNA displayed a band at approximately 199.2 bp, indicating that the PCR was successful, but indicating that DNA suitable for amplification was obtained from the FFPE blocks.

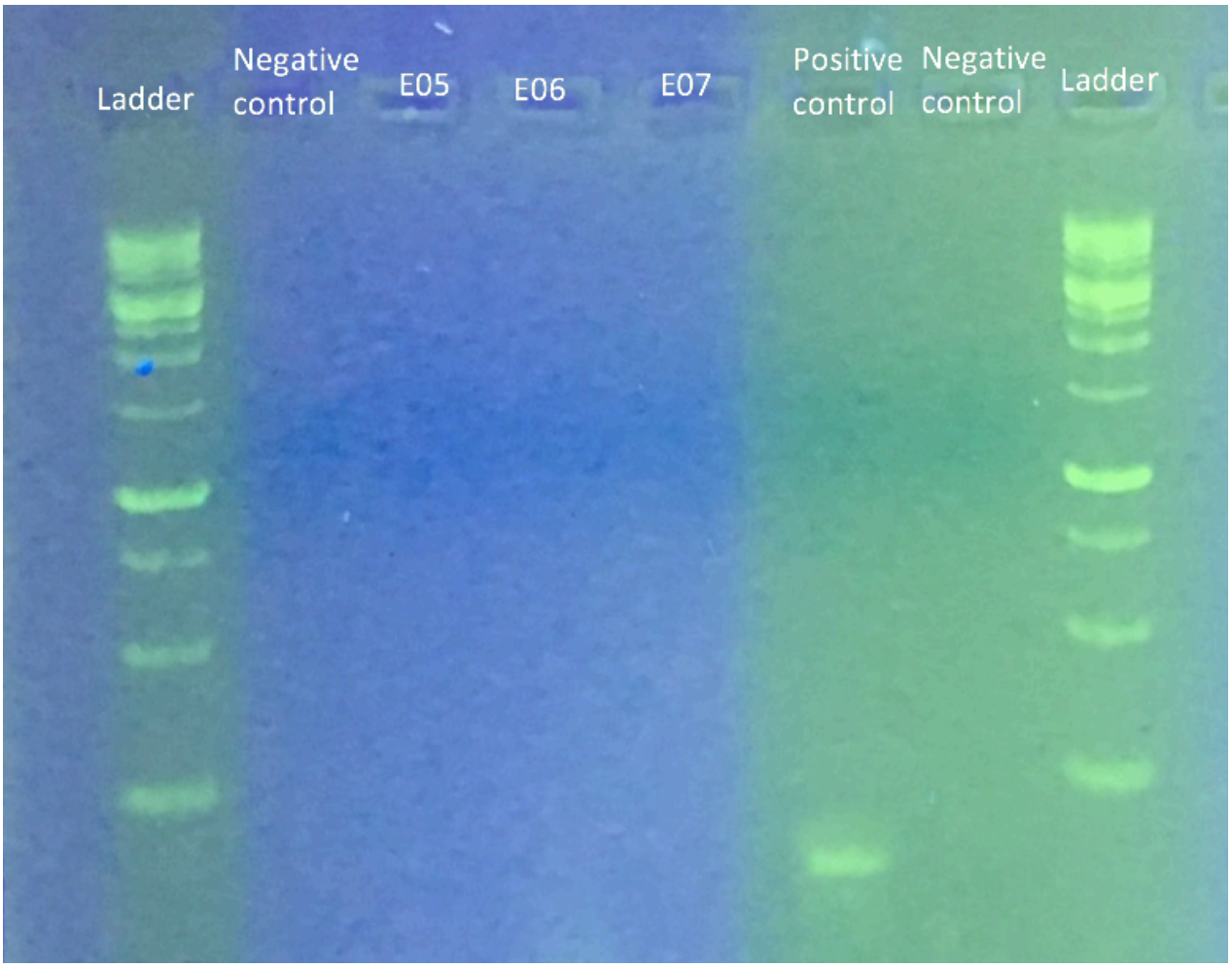

Figure 3.7: Agarose gel (1.2%) on UV transilluminator displaying results of two rounds of PCR on extracted DNA from FFPE tissue samples and fresh tissue samples with primers. Lanes are labeled as followed: FFPE skeletal muscle tissue (EO5), FFPE barnacle tissue (EO6) and fresh tick tissue (EO7). The positive control contains positively infected tick with DNA with *Borrelia burgdorferi* (ID# 968). Both negative controls bordering the samples contain nuclease free water instead of template DNA. Neighboring the negative samples are 1 kb GeneDirect ladders.

Chapter four: Discussion

This study aimed to evaluate demographic, clinical, and pathological prognostic factors that affect colorectal cancer (CRC) patient outcome. The patient cohort was CRC patients treated by surgical resection at the Upper Valley Hospital in New Brunswick, Canada from January 2016 to December 2019. Patient outcome was defined, for the purposes of this research, as overall survival probability (OS) and reoccurrence-free survival probability (RFS). In order to assess patient outcomes, patient charts were reviewed and analyzed for data on preselected prognostic factors as described in section *2.2.1* of Materials & Methods. The data was then statistically analyzed to answer the three main research goals: the individual effect of each prognostic factor on patient outcome, the simultaneous effect of multiple prognostic factors: lymph node yield (LYN), distal resection margin (DRM), circumferential resection margin (CRM) on patient outcome and how the institution's lymph node yield and resection margins adhere to the CRC treatment-guidelines.

It was shown that the strongest prognostic factor for determining OS was histologic grade, with worse outcome for those with tumours classified as poorly differentiated. None of the demographic or pathological factors assessed showed a statistically significant difference between patient groups within each prognostic factor in regard to OS. The strongest prognostic factors for determining RFS was patient age at diagnosis. While DRM and the CRM were shown to be strong prognostic factors when assessed individually, when assessed simultaneously with lymph node yield these prognostic factors did not significantly affect RFS. Compliance to guidelines was shown to be the highest for LNY and CRM.

This study also aimed to assess the feasibility of molecular investigating of predisposition gene variants, for example the *RAD18* gene, in formalin fixed paraffin embedded (FFPE) tissue. In initiating such a study, I started by assessing the ability of the QIAamp® DNA FFPE Tissue Kit to extract nucleic acid, in this case DNA, from FFPE tissue in a condition and length compatible with PCR amplification. Results from this work indicated considerable DNA fragmentation as a result of this protocol in both FFPE and fresh tissues.

4.1.0 Overall survival and overall reoccurrence free survival rates after surgical resection

Surgical resection is currently the only curative method for treating CRC (Walker et al., 2014). It has proved to be highly effective in both increasing overall survival and preventing reoccurrence (Walker et al., 2014). However, even with intervention by surgery, around 30 – 40% of CRC patients will develop reoccurrence in the first few years (Walker et al., 2014). This study revealed that patients who underwent surgical resection for CRC at this institution had a relatively high overall survival incidence (91.7%) compared to recent studies conducted elsewhere in Canada (Lefresne et al., 2018; Vergara-Fernandez et al.; Yu et al., 2019), although the small sample size means that these results must be interpreted with caution. The overall reoccurrence rate (16.3%) observed in this study was similar to those in previous studies (Kobayashi et al., 2007; Primrose et al., 2014; Yamano et al., 2018). In order to prevent reoccurrence and promote overall survival, increased knowledge on the predictive value of each prognostic factor is important.

4.2.0 Demographic prognostic factors associated with colorectal cancer incidence and outcome

In this retrospective study, we first reviewed the demographic prognostic factors of patients that underwent CRC treatment by surgical resection which included age and sex of the study population to determine trends of CRC incidence and outcome. The mean age of CRC patients reviewed (69.8 ± 11.0 years) was similar to findings in other studies conducted in Canada, which reported a mean age of 72 years in a study of Manitoba patients (McKay et al., 2014) and a mean age of 72 years for a cohort of Ontario patients (Karim et al., 2017). This is consistent with the well-established finding that the probability of being diagnosed with CRC progressively rises after the age of 50 years old (Haggar and Boushey, 2009). In this study, age was examined in two sub groups; one that included those over the age of 50 and one that included those under the age of 50. As expected, incidence of CRC of those under 50 years old was just 3.3% (n=2). This is consistent with the age–dependent incidence of CRC found in previous studies. For example, a recent large cohort study conducted in 2019 using Canadian national cancer registries saw a significantly higher proportion of CRC incidence of those over the age of 50 (Brenner et al., 2019).

There was decreased mortality rate for those under the age of 50, however the OS difference between age groups was not significant (P=0.67). This reflects the fact that older

adults typically have more comorbidities and therefore have an increased risk of dying. Age was, however, determined to be a strong prognostic factor for RFS as there was a significant difference found between the different age groups (P=0.044). It was determined that RFS favoured those under the age of 50, with fewer relapses in this age group. These results were partially supported by a previous study that found that although small, there was a significant increased risk of mortality for those over the age of 50 (McKay et al., 2014). This study also found that RFS was better for those over the age of 50 years, with fewer relapses for this age group (McKay et al., 2014), which differs from what was found in the present study. They suggested that the trend could be explained by the finding that younger individuals are more likely to experience more aggressive cancers compared to older adults (McKay et al., 2014). A power analysis, revealed that in order to obtain a statistically significant value of this prognostic factor from the number of deaths and events of reoccurrence recorded in this study, the number of CRC patients reviewed must be of at least 178 patients. The present study reviewed many fewer patients (n=60) compared to the study conducted by McKay et al. (n= 2086). Therefore, such a trend would not have been observable in a cohort of the size used here.

CRC incidence in males is generally higher than in females (White et al., 2018). This was also seen in our study with 60% of the study population being male (Table 3.1). It is controversial whether sex has a role in predicting prognosis. Some studies have determined that females have a better prognosis versus males (Hendifar et al., 2009; Majek et al., 2013; Yang et al., 2017) while other studies showed no sex-specific difference in OS or RFS (Wang et al., 2000; White et al., 2018). This study did not find a significant difference in either OS or RFS between male and female patients (P=0.84; P=0.5). Therefore, age was the only demographic factor with a significant prognostic role in determining RFS; neither of the demographic factors assessed were found to have a significant prognostic role in determining OS in this study, however, with a larger sample size, small differences might become evident.

4.2.1 Clinical prognostic factors associated with colorectal cancer treatment outcomes

The clinical factors assessed in this study described the treatment strategy used for each patient reviewed. This included the treatment modality, type of surgical procedure, surgical approach and length of stay in the hospital after surgery. Treatment modality, which describes if chemotherapy or radiotherapy (or both) were used preoperatively (neoadjuvant), post-operatively

(adjuvant), both, or neither in addition to surgery, was the first clinical prognostic factor analyzed. For the treatment of colon cancer, surgery in addition to adjuvant therapy is most commonly used (Milinis et al., 2015), while neoadjuvant therapy with subsequent surgery followed by adjuvant therapy has become common practice for treatment of rectal cancer in recent years (Feeney et al., 2019). In this study, location of the tumour was most frequently seen in the colon compared to the rectum. Accordingly, the treatment modality used most often was surgery alone followed in frequency by surgery with the addition of adjuvant therapy.

Previous studies have shown clinical benefits of neoadjuvant and adjuvant therapies with respect to prolonging overall survival and reducing reoccurrence rates (Lee and Chu, 2018; Sargent et al., 2001; Sargent et al., 2009). In this study, OS and RFS was better for patients whose treatment modality included surgery with the addition of neoadjuvant therapy and those who received surgery with the addition of both neoadjuvant and adjuvant therapies. Results also indicated that while there was higher reoccurrence events for those that received surgery with the addition of adjuvant therapy, the difference was not significant and no significant difference in OS and RFS was found between each of the treatment modalities (P= 0.91; P=0.18). Once again, sample size was small. Therefore, no inferences can be drawn from these observational trends. A power analysis revealed that in order to obtain a statistically significant value for this prognostic factor from the number of deaths and events of reoccurrence recorded in this study, the number of CRC patients reviewed must be of at least 431 patients.

Although small, it is unclear why this trend was observed as the main purpose of adjuvant therapy is to eliminate micro-metastatic disease and thereby, reduce the risk or reoccurrence (Derks and van de Velde, 2018). Some studies, however, have observed similar results particularly with adjuvant radiotherapy (Sofia Vala et al., 2010; Vilalta et al., 2016). The effect may be explained by enhanced angiogenesis caused by radiotherapy which has the potential to promote tumour growth (Marques et al., 2020; Sofia Vala et al., 2010). It also could be explained by overexpression of certain genes involved in the DNA damage repair system, like the *RAD18* gene, that can hinder the functionality of radiotherapy causing radiotherapy resistance for some individuals (Wu et al., 2019). The results found in this study (although not significant) and the previously mentioned studies highlight the need for increased research on the predictive biomarkers that improve CRC patient outcome.

Five different surgical procedures were reviewed in this study: right hemicolectomy, left hemicolectomy, sigmoid colectomy, low anterior resection and abdominoperineal resection. Sphincter-saving surgery was most frequently performed and only one individual among those reviewed underwent abdominoperineal resection (the complete removal of the anus, rectum and sigmoid). Sphincter-saving surgery is defined as a surgical procedure that maintains intestinal continuity without the need to create an end stoma (Johnson and Heise, 2010) and this class includes all of the other surgical procedures that were reviewed in this study. Once again, there was no significant difference for OS or RFS between the five different surgical procedures that were performed (P=0.83: P=0.5). However, there was a trend of reduced OS and RFS for patients that underwent a right hemicolectomy surgical procedure. This makes sense, as right sided tumour are associated with more aggressive cancer types. However, again, the samples size was small so interpretation may not be reliable. A similar study that looked at the difference between patient outcome of those that received right colectomies compared to those that received left colectomies saw no significant difference in mortality which is consistent with the findings in the present study (Bagshaw et al., 2012; Nussbaum et al., 2015).

Surgical approach describes if surgical resection was completed laparoscopically or through open resection. While some studies argue that there is reduced visibility in the lower pelvis during laparoscopic surgery (Bagshaw et al., 2012; Nussbaum et al., 2015), other studies have found that laparoscopy provides a brighter image allowing surgeons to achieve a more precise resection (Chiu et al., 2019). In this study, there was a fairly equal proportion of patients reviewed that received laparoscopic (53.3%) and open resection (46.7%) (Table 3.1). The results indicated that OS and RFS was slightly better for patients who received laparoscopic surgery, however, this difference was not statistically significant (P=0.71; P=0.94). The largest randomized controlled trial that has been conducted to date which analyzed OS and RFS of patients that received laparoscopic versus open resection also found no statistically significant difference in survival or reoccurrence (n=872) (Group, 2009).

The overall median length of stay in hospital after surgery (8.4 ± 5.1) was comparable to that found in other studies. For example, the median post-operative length of stay after CRC surgery in a similar study conducted by Seow-En *et al.* in 2019 was reported to be 8 days (Seow-En et al., 2019) and a study conducted by Aravai et al, in 2016 showed a median post-operative length of stay to be 10 days (Aravani et al., 2016). The OS and RFS analysis indicated that there

was no significant difference in patient outcome between those that stayed in hospital for more than 7 days after surgery versus those that stayed fewer than 7 days (P=0.94; P=0.28). These results differ from a similar study done by Shin et al. in 2012 in which identified length of hospital stay to be a statistically significant prognostic factor of overall survival (Shin et al., 2017). Many factors can influence postoperative length of stay in hospital including age, site and stage of the tumour and whether the operation was performed laparoscopically or through open resection (Shin et al., 2017). In the 2012 study, they investigated survival of patients of 70 years or older (Shin et al., 2017). The greater overall age of patients assessed may have enhanced the prognostic value of length of stay in hospital compared to the prognostic role that it had in the present study. Also, sample size was much smaller compared to the study conducted by Shin et al (2017). A power analysis revealed that in order to obtain a statistically significant value for this prognostic factor from the number of deaths and events of reoccurrence recorded in this study, the number of CRC patients reviewed for this prognostic factor must be of at least 432 patients.

Overall, none of the clinical factors assessed were found to have a significant prognostic role for OS or RFS of patients who underwent curative resection.

4.2.2 Pathological prognostic factors associated with colorectal cancer outcome

The pathological factors assessed describe the characteristics of CRC tumours. This included tumour stage, location, size, histologic grade as well as lymph node yield and the three resection margins; distal resection margin (DRM), proximal resection margin (PRM) and circumferential resection margin (CRM).

The Tumour Node Metastasis (TNM) staging classification system provides information on the size, lymph node spread and metastatic status that are combined to define the stage of each individual cancer. It has been established that the stage of tumour is the most important factor for predicting overall survival probability of patients with CRC (Storli et al., 2011). As such, physicians are trained to use it as a prognostication tool in the development of treatment strategies for individual CRC patients. Results from the present study indicated that OS was best for patients with tumours classified as stage I, followed by stage II and stage III tumours. However, there was no significant difference in OS probability between the three groups (P=0.13; P=0.66). It was also indicated that patients with stage II tumours were most likely to

experience reoccurrence compared to patients with stage I or III classified tumours, however, there was also no significant difference in RFS probability between the three groups. Again, sample size was small, so interpretation of trends must be taken with discretion. Although small, this trend could be due to the fact that stage III-diagnosed patients are more likely to be treated by more aggressive measures compared to stage I or stage II cancers leading to decreased reoccurrence. It is also possible that the true stage for those diagnosed with stage II cancer was underestimated leading to reoccurrence after curative resection. A power analysis revealed that in order to obtain a statistically significant value for this prognostic factor from the number of deaths and events of reoccurrence recorded in this study, the number of CRC patients reviewed must be of at least 351 patients.

It has been established that different clinical and biologic characteristics are associated with CRC tumours located in different anatomical subsites (Wang et al., 2019a). Literature reports have shown that tumour location (colon and rectum) and sidedness (left and right) are associated with different patient outcomes (Wang et al., 2017; Warschkow et al., 2016). Several previous studies have determined that patients with right-sided tumours have a poorer overall prognosis with respect to OS and RFS compared with patients with left sided tumours (Moritani et al., 2014; Petrelli et al., 2017; Wang et al., 2019b). It has also been shown that rectal cancers have a worse prognosis compared to colon cancers (Lee et al., 2013). In the present study, there was a trend towards increased mortalities and reoccurrence in patients with right-side colonic tumours. There was also a trend for reduced mortalities and reoccurrences for patients with tumours located in the rectum. However, there was not a significant difference in OS and RFS between each of the three tumour sites assessed. A power analysis revealed that in order to obtain a statistically significant value for this prognostic factor from the number of deaths and events of reoccurrence recorded in this study, the number of CRC patients reviewed must be of at least 428 patients.

Although tumour size is a widely assessed parameter in designing cancer treatment, the prognostic value of tumour size is still undecided amongst medical professionals (Dai et al., 2017). In this study, the results indicated that OS favoured those with a tumour less than 4.5 cm, while RFS survival favored tumours over 4.5 cm. However, neither finding was statistically different for either OS or RFS between the two groups (P=0.095; P=0.62). In this study, the cut off value of 4.5 cm was chosen as it was the median tumour size. A study conducted in 2014

saw a median tumour size identical to the one found in the present study at 4.5 cm, in which was used as a cut off value to assess tumour size as a categorical variable (Balta et al., 2014). This study likewise found no significant difference between tumour size groups in OS or RFS (Balta et al., 2014).

Histologic grade was categorized as either poorly differentiated or well-differentiated. The results from this study indicated that there was a statistically significant difference in OS for the two categories (P=0.0035). Those with tumours classified as well differentiated had an increased OS compared to those with tumours classified as poorly differentiated. Tumours classified as well differentiated had a reduced reoccurrence rate, although the difference was not significant (P=0.42). This finding, which is to be expected, is consistent with a previous study that concluded poorly differentiated tumours were associated with decreased OS (Schneider and Langner, 2014).

It is widely accepted that 12 or more lymph nodes is the recommended lymph node yield (LNY) during the surgical removal of a CRC tumour. LNY is used in the staging process as it can indicate if cancer has spread and low lymph node yield has been associated with poorer OS and RFS in multiple studies (Backes et al., 2017; Chan and Tan, 2018; Chan et al., 2018; Foo et al., 2020; Kim et al., 2009). However, in this study, both OS and RFS favoured patients with fewer than 12 lymph nodes resected, although it was not statistically significant (P=0.29; P=0.85). Sample size is most likely the explanation behind this trend observation. However, though small, this trend could be explained by the fact that patients in this group were underrepresented in this study, the proportion of those with over 12 lymph nodes resected (81.7%) was much high than those that had a LNY of less than 12 (18.3%). It also could be explained by the fact that further advanced tumours are more likely to induce a stronger immunologic response compared to earlier tumours, allowing easier detection of lymph nodes (Foo et al., 2020). If so, those with LNY of fewer than 12 may have had a less advanced tumour, and therefore increased OS and RFS. A power analysis revealed that in order to obtain a statistically significant value for this prognostic factor from the number of deaths and events of reoccurrence recorded in this study, the number of CRC patients reviewed for must be of at least 407 patients.

Along with lymph nodes, resection margins are also an important part of staging as they can indicate if tumour cells have migrated. They are also strongly associated with increased

reoccurrence rates as inadequate resection margins can lead to residual cancer cells remaining in the colon or rectum (Gleisner et al., 2013). As indicated by international guidelines, the DRM and the PRM should be as least 5 cm and the CRM should be as least 0.1 cm. In this study, there was no significant difference in OS for patients with a DRM, PRM or CRM that met guideline recommendations versus those that did not (P=0.14; P= 0.71; P=0.69). There was a significant difference in RFS associated with those that received a DRM and CRM that met guidelines recommendation versus those that did not (P=0.012; P=0.03). There was not a significant difference in RFS associated with the PRM (P= 0.71). A single-institution study conducted by Rocha *et al*., found similar results in that OS or RFS were higher for those with adequate resection margins, however, in this case the difference was not significant (Rocha et al., 2016). The trend seen in both the present study and the study by Rochas et al. is mostly likely due to the small sample size used. A power analysis revealed that in order to obtain a statistically significant value for this prognostic factor from the number of deaths and events of reoccurrence recorded in this study, the number of CRC patients reviewed for must be of at least 270 patients.

It is also important to note that the resection margins measurements were performed after formalin fixation. Tissue shrinkage after fixation has implications in studies such as this one. Formalin alters fresh tissue by cell dehydration in order to preserve cellular integrity. A previous study by Clarke *et al.* demonstrated that immediately after excision, the bowel shrinks 28.3% of its original size and then shrinks and additional 27.3% of its original size after 24 hours of formalin fixation (Clarke et al., 2014). Another study conducted by Goldstien *et al.* recorded a 70% shrinkage with in the first 10-20 minutes of excision and then an additional 30% after formalin fixation (Goldstein et al., 1999). Therefore, it is important to recognize that shrinkage may have affected the resection margin measurements recorded in this study. Variations in the concentration of formaldehyde used during formalin fixation in pathology labs contribute to the different amounts of shrinkage observed. In order to determine how much resection margins should be adjusted for to account for shrinkage, fixation conditions must be known.

To further investigate interactions between these conditions, a multivariate (cox proportional hazard model) analysis was conducted in order to address the second research goal of determining the simultaneous effects of both lymph node yield and resection margins on patient outcome. These specific prognostic factors were assessed to determine if RFS is improved when all three guidelines are adhered to. After controlling for the resection margins,

the hazard ratio for lymph node yield indicated that those with a LNY of 12 or less had a 71% reduced risk of reoccurrence. The hazard ratio of the DRM indicated those with a resection margin greater than 5 cm were 11.2 times more likely to experience reoccurrence. The hazard ratio of the CRM indicated that those with a resection margin less than 0.1 had reduced risk of reoccurrence by 81% of after adjusting for lymph node yield. These results reflect the results found when determining the individual effect of LNY, DRM and CRM on RFS. However, while there was a statistically significant difference found for DRM and CRM in RFS when these prognostic were assessed individually, there was no statistically significant difference found for RFS when all of these prognostic factor were assessed simultaneously. Therefore, this indicated that when all three guidelines were followed, there was not a significantly improved outcome for CRC patients. It would be expected that a lymph node yield that adhered to guidelines with the addition of adequate resection margins would increase RFS so these results were surprising. But again, it is likely variability of resection margins introduced by tissue shrinkage and small sample size explain these trends.

4.3.0 Adherence to guidelines at the Upper River Valley Hospital

The final research goal of the retrospective chart review was to examine how the institution's lymph node yield, as well as their distal resection margins, circumferential resection margins and proximal resection margin conformed to the CRC treatment-guidelines. It was determined that LNY guidelines were adhered to for 81.7% of CRC patients reviewed. For the resection margins, it was determined that DRM guidelines were adhered to for 41.6%, PRM guidelines for 21.7% of cases and CRM guidelines were adhered to for 76.7% of cases. These results indicate that this institution has an adherence to guidelines that is relatively high for LNY and CRM. Low adherence for the PRM is likely due to a large number of missing values in the pathology reports that were reviewed. Also, as mentioned previously, it is likely that measurements of resection margins was underestimated due to margin shrinkage after excision and formalin fixation. It would be worthwhile to determine the fixation conditions used at the institution in order to determine how much shrinkage could be accounted for when assessing resection margins.

4.4.0 The ability of QIAamp® DNA FFPE Tissue Kit to extract good quality nucleic acid from formalin fixed paraffin embedded tissue (FFPE)

There is a need for research into potential molecular biomarkers of CRC that could improve CRC patient outcome. For example, investigation of *RAD 18* gene expression as a molecular biomarker for determining if CRC patients will exhibit resistance to systemic therapies so its assessment could provide for a more successful patient outcome.

The first step for investigating gene expression biomarkers is to find a method of reliably retrieving nucleic acids from CRC biopsy specimens. Formalin fixed paraffin embedded tissue (FFPE) is a universally-used method for archiving patient samples in pathology so it represents an extensive resource of CRC tissue. There is no general consensus on which extraction method from FFPE tissue provides the highest quality of nucleic acid, either DNA or RNA. Although RNA would be needed in order to look at gene expression, determining the quality of DNA from these tissues was a technically more accessible first step to this process. If high quality DNA can be extracted from FFPE tissue RNA extraction could then be attempted, as it is often even more difficult to isolate intact RNA (Kofanova et al., 2020). DNA analyses also allows for the investigation of new insights through the detection of mutations, polymorphisms or epigenetic changes that may alter gene expression. Therefore, it is important to assess the quality of DNA produced by an extraction method. Accordingly, one of the aims of this study was to assess the ability of the QIAamp® DNA FFPE Tissue Kit to extract good quality DNA from FFPE tissue. This kit was chosen as it used the silica membrane extraction method which has been reported to have enhanced success compared to alternative DNA extraction methods (Sengüven et al., 2014).

Quality, measured at the length of DNA that could be PCR-amplified, was assessed using suites of primers targeting different sized regions. After DNA was extracted from the FFPE tissue samples and the fresh tissue control sample, two rounds of polymerase chain reaction (PCR) were performed with three different primers for the *Cytochrome C Oxidase subunit I*. gene, all of which had a different sized target amplicon. The *Cytochrome C Oxidase subunit I* gene was used as, being a mitochondrial gene, it provides an abundant DNA target and all samples would contain this gene. Gel electrophoresis revealed DNA smears for amplifications with the three sets of primers, however, the primer with the shortest target amplicon (130 bp) produced the largest and highest intensity DNA smears. Previous studies have shown that PCR's with shorter amplicons have greater success for amplification of DNA from FFPE tissue

(Dietrich et al., 2013; Huijsmans et al., 2010; Lin et al., 2009). Nevertheless, for all three samples tested, no amplicons were generated and the DNA smears suggested that the DNA had been extensively fragmented and damaged during preservation and extraction.

The fixation process critically affects the integrity of DNA and extraction of PCR-amplifiable DNA from FFPE tissue remains a challenge (Huijsmans et al., 2010; Panchal et al., 2019). A previous study that also saw DNA fragmentation suggested that DNA polymerase inhibition caused by fragmented DNA may explain the poor PCR performance that was observed (Dietrich et al., 2013). There are, however, some studies that have demonstrated DNA extraction with successful subsequent amplification (Lin et al., 2009; Ludyga et al.). In an attempt to reduce polymerase inhibition Dietrich et al., increased the polymerase concentration, dNTP concentration and PCR elongation time. With these changes, they saw a significant improvement in PCR amplification and were able to generate larger amplicons (Dietrich et al., 2013). Applying these adaptions in future investigations for this project could result in improved PCR performance.

It is also important to note that DNA smears were not only observed for DNA extracted from the FFPE samples but they were also observed for DNA extracted from the fresh tissue control sample. This indicates that the kit may have played a role in degrading the DNA as fresh tissue was expected to exhibit less degradation compared to FFPE tissue. This kit, and many other like it, has a step to first deparaffinize the tissue with xylene in order to remove the paraffin in the FFPE tissue samples. The procedure is essential for dissolving paraffin in the FFPE samples, however xylene is associated with reduced protein yield and integrity (Mansour et al., 2014). For the fresh tissue samples that did not contain paraffin, it is possible that the xylene may have had an effect on the overall quality of DNA that was extracted or residual xylene inhibited the polymerase.

Because the *RAD18* gene is much less abundant than the mitochondrial *Cytochrome C Oxidase subunit 1 gene*, this study also investigated the amplification of a gene segment that is less abundant. In order to do this, primers for the detection of the rRNA intragenic region of the bacterial species, *Borrelia burgdorferi* (*sensu stricto*) in which was present in the human, but not barnacle, FFPE tissue were used. It was revealed by gel electrophoresis that no amplicons were generated. This indicated that *Borrelia burgdorferi*-specific DNA was not detected. An amplicon was generated for the positive control that contained *Borrelia burgdorferi*-infected tick DNA

indicating that the primers did work. A similar study in which compared the same DNA extraction kit with other kits also used amplification of a bacterial rRNA intragenic region (from *B. pseudomallei)* and obtained similar results *(Obersteller et al., 2016)*. The researchers suggested that age of the samples may have played a role in this failure to isolate good quality DNA, as increased DNA degradation is associated with longer storage of FFPE blocks *(Obersteller et al., 2016)*. They also suggested that PCR inhibition could have been a contributing factor to decreased PCR amplification (Obersteller et al., 2016). In order to advance further with this part of the study, a reliable DNA extraction method must be established. After that, a method to extract integral RNA from FFPE tissue must be established in order to develop the methodology for visualizing *RAD 18* and other RNA from FFPE tissue to assess gene expression. The end goal of this study would be to determine if there is an association of high expression of the *RAD 18* gene with resistance to systemic therapies and patient outcomes.

4.5.0 Future directions of research

This study indicates parameters that would be of value for future research of colorectal cancer characteristics and patient treatment outcome studies in New Brunswick and Canada. Although a few of these prognostic factors showed a statistically significant difference in OS and RFS between different patient groups, considering the range and heterogeneity of colorectal cancer it is difficult to draw conclusions with the small number of patients reviewed. The main limitation to this study was the limited number of patients within the study time frame the study was conducted on patients at a single institution. Only a small number of CRC-related mortalities and cancer reoccurrences were observed in this study, which while an excellent patient outcome, limited the power of the study when conducting the OS and RFS analysis between patient groups for each prognostic factor assessed. This study did however allow for an estimation of a target sample size through a power analysis, that could be used in subsequent studies to further analyze these demographic, clinical and pathological factors. The power analysis done for the prognostic factors in this study revealed that in order to attain power for this study (and all associated prognostic factors) approximately 400-500 patients must be reviewed in total to better assess the influence of each prognostic factor on patient outcome. In order to increase the sample size for greater reliability of the results and for recognition of rare outcomes, conducting a similar study

with multicenter data from several small hospitals in New Brunswick would be valuable for future CRC research across the province.

An additional limitation encountered in this study was that resection margins were measured after formalin fixation, a process that introduces unknown variability due to tissue shrinkage, so confounds attempts to measuring adherence to guidelines. Better understanding of the specific fixation conditions and the time between sample fixation and margin measurements, and its impact on tissue in this health district would help resolve this question.

Finally, it may be worthwhile to further investigate the trend of increased reoccurrence for those who received adjuvant therapy that was shown in this study. The discovery that radiotherapy could be increasing reoccurrence in some individuals is a fairly recent finding (Marques et al., 2020). Pairing an OS and RFS analysis of those that received systemic therapy with a molecular biomarker research, such as the *RAD 18* gene expression study, would be potentially useful in explaining this phenomenon.

4.6.0 Conclusions and significance of the research project

Surgical resection remains the backbone of treatment for colorectal cancer. It has proven to enhance OS and RFS of individuals who are diagnosed with CRC. However, as demonstrated in this study, the risk of reoccurrence and death after surgery is still present. The wide range of characteristics displayed by CRC patients increases the complexity of prognostication. This study highlighted trends in OS and RFS for each prognostic factor that would be useful to further analyze in future studies. I showed that for the relatively small sample of patients reviewed in this study, the strongest prognostic factors for both OS and RFS after surgery were age of the patient and histologic grade of the tumour. The surgical distal resection margin and the circumferential resection margin were found to be strong prognostic factors when assessed individually for their effect on RFS. However, when assessed simultaneously with LNY these prognostic factors did not significant difference affect RFS. This study revealed that adherence to guidelines for the 60 patients reviewed at this institution was relatively high for LNY and CRM, while compliance to DRM and PRM guidelines was lower.

The DNA extraction method evaluated in this study was associated with highly fragmented DNA that was not suitable for PCR amplification. In order to determine a reliable method for extracting DNA from FFPE tissue, PCR modifications and different extraction

protocols need to be further assessed. After a method for extracting high quality DNA is established, further advances can be made in the molecular analysis aspect of CRC tissues.

These results lay the groundwork for future work on a larger patient cohort to support a better understanding of CRC survival. Because there is no central database of CRC patient outcome after surgery in the province of New Brunswick, our study may be helpful in the recognition of survival and reoccurrence trends. In the future, we hope this and similar studies will promote increased understanding of OS and RFS to allow for more successful CRC patient outcome in New Brunswick. Advances in biomarker testing may provide insights into the role that genomics plays in predicting the efficacy of systemic therapy.

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6.0 Appendices

6.1.0: The standard curve used for estimation of base pair length of electrophoresed gels

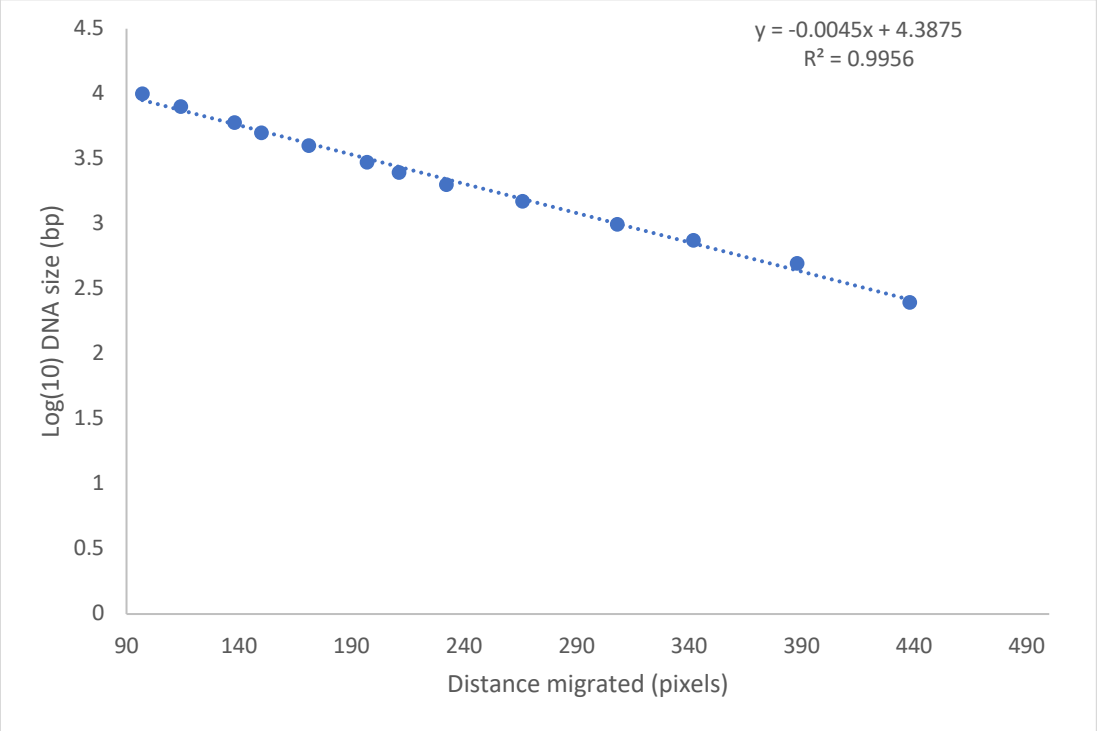

Figure 6.0: Migration distances of bands in pixels *versus* the log10 of known base pair sizes of the 1 Kbp GenedireX ladder on agarose. ImageJ software was used to measure migration distance in pixels. Linear regression equation $\log_{10}(bp) = -0.005x + 4.7671$. $R^2 = 0.97153$.