Plasma microRNA biomarkers in rectal cancer patients undergoing neoadjuvant therapy

By

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Abstract

Colorectal cancer (CRC) is the second most common cause of cancer related death worldwide with an expected 1.8 million new cases this year (1). CRC claims approximately 1200 New Zealand lives per year and rectal cancer patients account for approximately 25% of CRC cases (2). The mainstay of treatment for locally advanced rectal cancer is neoadjuvant therapy followed by surgical excision. The purpose of neoadjuvant therapy is to reduce rates of local recurrence, increase sphincter preservation and to improve tumour resectability (3), however the risk to patients is exposure to unnecessary toxins and their effects. Most importantly, patient response to neoadjuvant therapy is varied, and complete pathological response will occur in 15-27% of patients (4). Therefore, a significant proportion of patients undergoing therapy will suffer unnecessary delays to surgery and toxic exposure. There are currently no clinically utilised biomarkers to predict response to neo-adjuvant therapy. Development of predictive markers would enable optimal treatment of patients and avoid ineffective and potentially harmful treatments. miRNA are short regulatory transcripts that are stable in biofluids. Their dysregulation has been extensively reported in CRC and their usefulness in the detection of cancer and as predictors of prognosis and treatment response is a rapidly growing area of research. Whilst the majority of work has been on tissue biomarkers, blood biomarkers would be less invasive and expose patients to less risk.

This thesis has investigated changes in circulating miRNA levels in patients who have locally advanced rectal cancer and have undergone neoadjuvant therapy. The main aim of this study was to ascertain if miR-21, miR-29a, miR-143, and miR-145 levels in plasma prior to treatment can predict response to SCR and LCCCR therapy. Plasma samples were taken prior to and after neoadjuvant therapy. RNA was extracted and miRNA levels were analysed using RT-qPCR. Relative expression was evaluated and compared to pathological specimen reports after surgery. In this pilot study we have found a significant increase in the relative expression miR-143 in plasma after SCR. There was no difference in expression of miR-21, miR-29a, miR-143 or miR-145 in patients with
stage II and III rectal cancer compared to healthy controls. There was no difference in expression of target miRNA in patients who had a response to neoadjuvant therapy compared to those who did not respond.

This is the first study to use patients’ plasma to evaluate the expression of these miRNA and examine whether this can predict TRG. This is important because finding a predictive blood biomarker is a more accessible and potentially clinically useful tool than tumour biopsy. Findings from this study will contribute to the pool of research on the use of miRNA’s in CRC for diagnostic and predictive tests. As this is a pilot study, future samples being collected from these patients may also contribute to knowledge on recurrence.
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## Abbreviations

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<th>Definition</th>
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<tbody>
<tr>
<td>5-FU</td>
<td>5’ fluorouracil</td>
</tr>
<tr>
<td>AJCC</td>
<td>American Joint Commission on Cancer</td>
</tr>
<tr>
<td>APR</td>
<td>abdominoperineal resection</td>
</tr>
<tr>
<td>AR</td>
<td>anterior resection</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CEA</td>
<td>carcinoembryonic antigen</td>
</tr>
<tr>
<td>CRC</td>
<td>colorectal cancer</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CT</td>
<td>computerised tomography</td>
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<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>IRS-1</td>
<td>insulin receptor substrate-1</td>
</tr>
<tr>
<td>LCCR</td>
<td>long course chemoradiation therapy</td>
</tr>
<tr>
<td>MDM</td>
<td>multidisciplinary meeting</td>
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<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>MMR</td>
<td>mismatch repair</td>
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<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MSI</td>
<td>microsatellite instability</td>
</tr>
<tr>
<td>NLR</td>
<td>neutrophil to lymphocyte ratio</td>
</tr>
<tr>
<td>NZ</td>
<td>New Zealand</td>
</tr>
<tr>
<td>pCR</td>
<td>complete pathological response</td>
</tr>
<tr>
<td>PNI</td>
<td>perineural invasion</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>reverse transcription quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SCR</td>
<td>short course radiation</td>
</tr>
<tr>
<td>TAMIS</td>
<td>transanal minimally invasive surgery</td>
</tr>
<tr>
<td>TEMS</td>
<td>transanal endoscopic microsurgery</td>
</tr>
<tr>
<td>TME</td>
<td>total mesorectal excision</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumour Nodes Metastasis staging classification</td>
</tr>
<tr>
<td>TRG</td>
<td>tumour regression grade</td>
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Chapter 1:

Introduction
1.1 Colorectal cancer

Colorectal cancer (CRC) is a significant problem with a huge impact on health both globally and in Australasia. Worldwide, CRC is the third most common cancer and the second leading cause of cancer related mortality (1). New Zealand and Australia have the second highest rates in the developed world (1). In 2013, CRC was the second most commonly diagnosed cancer in both men and women in New Zealand with approximately 3,200 new cases registered and 1,200 deaths (2).

CRC places a high demand on the health system and is costly. Whilst the rates of CRC are declining, the actual number of registered cases are on the rise due to the increase in the New Zealand population and the increasing average age (5). Therefore, the number of CRC diagnoses is expected to increase by 26% by the year 2026 which will lead to an increased need for follow up endoscopy, surgery, chemotherapy and clinical follow up/surveillance (6). The forecasted annual costs for CRC is projected to increase from $83.6 million in 2014 to $100 million in 2026 (6).

Whilst the difference between colon and rectal cancer are somewhat anatomical, the therapeutic approaches of surgery and chemoradiation are different. For this reason, we must consider them separately. The PIPER Project (Jackson 2015) is the largest national CRC retrospective cohort study and reports that in New Zealand rectal cancer accounts for around 25% of all CRC cases. 62% of patients in this cohort were male and the majority were in the age bracket of 60-80 years old. Māori and Pacific patients presented with higher rates of metastatic rectal cancer than non-Māori, non-Pacific patients. Of concern, there was a high rate of Emergency Department presentations (14%), and a high proportion (19%) of patients presented with late stage, metastatic disease (7).

Despite major advances in detection and treatments for patients with rectal cancer, there is still a lack of understanding as to which patients will benefit the most from
which treatment. Further research is required to investigate how we can tailor treatment more appropriately for individual patients.

1.2 Presentation and management of patients with rectal cancer

Patients with rectal cancer may be asymptomatic or may present with change in bowel habit, loss of weight, tenesmus, and bleeding per rectum (8). In some cases patients may only have iron deficiency anaemia. These signs and symptoms can be nonspecific but should prompt early investigation of the colon and rectum. Patients in New Zealand have access to endoscopy via population screening through the Ministry of Health Bowel Cancer Screening program, through surveillance if at an increased risk of CRC, or if presenting with symptoms (9). Once rectal cancer has been diagnosed, prompt assessment of stage should occur (9). Preoperative staging should consist of; complete history and physical exam, complete blood count, liver and renal function tests, carcinoembryonic antigen (CEA), digital rectal examination and rigid sigmoidoscopy, colonoscopy, chest abdominal and pelvic computerised tomography (CT), and magnetic resonance imaging (MRI) of the pelvis or endorectal ultrasound (9).

The stage of cancer defines the local and distant extend of disease and is required for guidance of treatment options (10). The most widely used classification of CRC carcinomas is the tumour, nodes and metastases (TNM) classification (11) (Table 1). This is based on the local depth of tumour invasion (T), presence and number of lymph nodes effected (N), and presence of distant metastatic disease (M) (10). Lower T stage (T0-2) leads to statistically significant decreased rates of local recurrence, metastatic disease free survival and overall survival (12).
<table>
<thead>
<tr>
<th>Stage</th>
<th>T</th>
<th>N</th>
<th>M</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>$T_{is}$</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>I</td>
<td>T1/T2</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>II</td>
<td>T3/T4a/T4b</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>III</td>
<td>T1/T2/T3/T4</td>
<td>N1a/b/c/N2a/b/c</td>
<td>M0</td>
</tr>
<tr>
<td>IV</td>
<td>Any T</td>
<td>Any N</td>
<td>M1a/M1b</td>
</tr>
</tbody>
</table>

$T_{is} = \text{carcinoma in situ}, T1 = \text{tumour invades submucosa}, T2 = \text{tumour invades muscularis propria}, T3 = \text{tumour invades into pericolorectal tissues}, T4 = \text{tumour penetrates to the surface of the (a) visceral peritoneum or (b) directly invades, or is adherent to other organs or structures}, N0 = \text{No regional lymph node metastasis}, N1 = \text{Metastasis in 1-3 lymph nodes (a=1, b=2-3, c = no lymph nodes but deposits in subserosa, mesentery or non-peritonialised perirectal/mesorectal tissues}}, N2 = \text{metastasis in 4 or more regional lymph nodes (a = 4-6, b = 7+), M0 = no distant metastasis, M1 = Distant metastasis (a = 1 organ site, b = 2+ organ sites, c = peritoneal metastasis).}

The 5 year relative survival rate for all stages of rectal cancer has improved from the mid 1970s until the period 2006-2012 from 48% to 68%, reflecting improvements in detection and treatment (13). Five year survival rates are approximately 90% for localised disease, 70% for disease with regional spread and 12% for disease with distant spread (13). In New Zealand, 76% of rectal cancer patients present with stage I-III disease (non-metastatic) (7).

Histological features other than TNM stage have been investigated as to their impact on prognosis; such as histopathological variants of adenocarcinoma, and tumour growth characteristics. For example, perineural, lymphatic and vascular invasion have all been widely investigated regarding their prognostic value in rectal cancer. Patients with tumours without perineural invasion (PNI) have approximately four times the rate of survival at 5 years compared to patients with PNI (14). The presence of PNI is also associated with poorly differentiated tumours, lymphatic and blood vessel invasion (14).

Radiological features, notably on MRI, are able to predict prognosis in terms of risk of local and distant recurrence (15). MRI is used to evaluate the following to identify high

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Table 1 Colorectal Cancer TNM Stage (AJCC 7th Edition)
risk patients who will likely require neoadjuvant therapy; circumferential resection margin, the depth of tumour spread beyond the muscularis propria, extramural vascular spread, and lymph node status (15). For example, extramural vascular invasion is associated with more locally advanced tumours and with the development of liver metastasis (15). The rate of relapse-free survival at 3 years in patients with positive findings of extramural vascular invasion is less than half that of those with negative findings (15).

The management of rectal cancer is guided by both patient factors and tumour factors. First, it is recommended that all patients with rectal cancer are discussed in a colorectal multidisciplinary meeting (MDM) (9). Local excision of a T1 rectal cancer may be used in selected patients if the tumour is mobile and less than 3cm, and not poorly differentiated on biopsy (9). The standard management of locally advanced rectal cancer (Stage II and III disease) is neoadjuvant radiation or chemoradiation followed by total mesorectal excision (TME) surgery. This current multimodal approach has led to significant improvement in patient outcomes over the recent years. For example when TME is performed with an R₀ resection (microscopically margin negative resection), short course radiotherapy (SCR) of the pelvis (5 Gy/day for 5 days) has been demonstrated to further reduce the 2-year risk of local failure after surgery in comparison with TME alone (2.4% vs 8.2%) (3).

1.3.1 Surgery

The TME concept was a revolutionary change in the surgical management for rectal cancer in that it greatly improved local recurrence rates. A complete TME is described as “complete removal of the lymph node bearing mesorectum along with its intact enveloping fascia” (16). Current mainstay surgical options for locally advanced rectal cancer include; laparoscopically assisted or open high/low anterior resection (AR), abdomino-perineal resection (APR), and Hartmann’s procedure. These all utilise the TME technique and are considered to be major abdominal operations. Minimally invasive options are sometimes used for early T1 disease and include transanal excisions
such as Transanal Minimally Invasive Surgery (TAMIS) and Transanal Endoscopic Microsurgery (TEMS), but these do not utilize the TME dissection plane.

In rectal cancer the plane of dissection is an important prognostic factor for local recurrence. The hazard ratio (HR) for the risk of local recurrence is decreased when the plane of dissection removes the mesorectum (HR = 0.32 (95% CI 0.16-0.64)) compared with the intramesorectum (HR = 0.48 (95% CI 0.25-0.93)), as is risk of local recurrence at 3 years 4% (3-6%) for mesorectal and 13% (8-21%) for muscularis propria dissection (17).

1.3.2 Neoadjuvant therapy

The New Zealand National Guidelines for the Management of Early Rectal cancer state that patients with rectal cancer who are at risk of local recurrence should undergo either preoperative SCR or preoperative LCCR (9). There is no current guideline in New Zealand regarding time delay after therapy to surgery and there is some variation across the country as to how this is carried out. At Wellington Hospital, neoadjuvant therapy consists of either long course radiation (50.4 Gy in 25 fractions) combined with oral chemotherapy generally over 6 weeks, or short course radiation of 25 Gy over 5 fractions, typically over one week. Surgery occurs after the neoadjuvant therapy, and at Wellington Hospital this is typically 6 weeks after long course therapy and 3-5 days after short course therapy.

The chemotherapy agent used most commonly is Capecitabine, a 5-Fluorouracil (5-FU) pro-drug (18). This agent has nearly 100% bioavailability in the oral form. It is metabolized to 5-FU via three metabolic steps and disrupts DNA and RNA synthesis and repair, leading to cell death (18). It is taken as adjuvant therapy for advanced or metastatic colon cancer – either as a monotherapy or with combination drugs, and also in rectal cancer with concurrent radiotherapy with a recommended dose of 850 mg/m² twice daily. Known side effects specific to Capecitabine are diarrhoea, leukopenia, stomatitis, hand and foot syndrome, vomiting and neuropathy (18).
The purpose of neoadjuvant therapy is to decrease local recurrence, increase the likelihood of sphincter preservation and to improve tumour resectability (3). An important concern regarding neoadjuvant therapy is the exposure of patients to unnecessary toxins and their effects. Side effects of neoadjuvant chemoradiation include proctitis, sexual and urinary dysfunction, fatigue, nausea and vomiting, and diarrhoea (19). In addition to this, the proportion of patients who undergo neoadjuvant therapy that will not have a favorable response will suffer unnecessary delays to surgery. In New Zealand patients may need to relocate from rural centers to tertiary hospital centers for the duration of their therapy, which can lead to additional social isolation and stress to patients and their families.

However, patient tumour response to neoadjuvant therapy is varied, and a complete pathological response (pCR) will occur in 15%-27% of patients undergoing preoperative treatment followed by TME surgery (4). Patients with pCR after chemoradiation have better long-term outcomes, and it is thought that pCR might be indicative of a prognostically favourable biological tumour profile (20). There is therefore great interest in finding biomarkers that could indicate the likelihood of response prior to administering this treatment.

1.4 Assessing response to neoadjuvant therapy

Response to neoadjuvant therapy is an important prognostic indicator for patients and is measured after surgery using both Tumour Regression Grade (TRG), and pathological stage by TNM stage.

1.4.1 Tumour Regression Grading

Tumour Regression Grade (TRG) is now part of the standard synoptic reporting of a pathological specimen, particularly for patients who have undergone LCCR. Tumour regression grading systems are predictive of both disease free survival (21), local recurrence, and local node involvement (12).
There are a number of grades utilised for tumour regression reporting including Dworak (22), Mandard, Dworak/Rodel, American Joint Commission on Cancer (AJCC), (23), and the Ryan classification (24, 25). No absolute standard method is used worldwide but all of these systems are based on the on the degree of fibrosis and percentage of viable cancer cells as assessed by a trained pathologist. Grades vary from absence of regression for non-responders, to decreasing amounts of tumour cells within fibrotic tissue, to no tumour cells, termed complete regression (pCR) (24). For research purposes, patients are typically grouped into complete responders, intermediate, or poor responders.

The time delay from the end of neoadjuvant therapy to surgery is now known to impact on tumour regression (26). Prolonging the time to surgery after SCR from the conventional 5 days to 4 weeks markedly increases the rate of complete pathological response and tumour downstaging and also lowers the rate of post operative complications. However, there is no difference in overall survival, sphincter preservation, or R₀ resection rates (26).

1.4.2 Pathological staging

The AJCC TNM staging system is universally accepted for pathological specimen reporting for rectal cancer and allows for national and international comparisons. The pathological stage allows for decisions to be made about whether the patient may benefit from adjuvant chemotherapy and to give some indication of prognosis. These decisions are made on a case by case basis by the Colorectal MDM. Currently, TNM staging is considered the best prognostic classification tool to help guide therapeutic decisions for CRC (27). Whilst the initial TNM stage is made from imaging completed prior to surgery, following surgery, the stage is re-reported by pathology. Differences in preoperative stage and pathological stage can be used as evidence of downstaging of the tumour in response to neoadjuvant treatment, however TRG is considered more accurate as it could be that the differences were due to the comparison of two different modalities.
1.5 Predictive biomarkers for response to neoadjuvant therapy

Given that currently we cannot predict with confidence which patients will benefit from neoadjuvant therapy, the individual patient requirement for this treatment is uncertain. The previously mentioned factors for predicting local recurrence risk, and overall survival are all based on post-operative specimen analysis. There is considerable interest and investigation into robust biomarkers that can be used prior to surgery to aid in risk stratification and to personalise treatment. Patients that respond well to neoadjuvant therapy may not need surgery or adjuvant treatment, and patients that will not respond may proceed straight to surgery and avoid the need for any ineffective toxic therapy.

In recent years there have been considerable advances in developing the use of molecular biomarkers for the prognosis and prediction of treatment response in CRC. In terms of prognosis, examples of clinically utilised tissue biomarkers are Microsatellite Instability (MSI) and the KRAS gene. MSI is a phenotype of a DNA mismatch repair (MMR) system defect and is observed in 10-15% of spontaneous CRC cases (28). Patients can be classified into MSI-H (high), MSI-L (low) or microsatellite stable tumours. Patients with MSI-H or MMR deficient tumours have a favourable prognosis, and do not derive any benefit from adjuvant 5-FU based chemotherapy (29). KRAS is a commonly mutated oncogene in CRC. In patients with advanced CRC, mutations of KRAS predict a failure to respond to Cetuximab; a monoclonal antibody against epidermal growth factor receptor, compared to supportive care alone. Patients with a wild type KRAS gene have a doubling of the median overall survival (median 9.5 months vs 4.8 months $p<0.001$) and progression free survival (3.7 vs 1.9 months, $p<0.001$)(30). These are examples of how molecular biomarkers can be used for predicting response to therapies in CRC patients.

Extensive research has been conducted on tissue based biomarkers to determine if there is correlation with pCR. Hur et al. used tissue microarrays and immunohistochemistry to look at 12 biomarkers including Ki-67, p53, p21, and Bax (31). Of these 12 markers, the expression levels of four (p53, VEGF, p21, and Ki67) differed
significantly between patients who achieved pCR and those that did not. When these four markers were combined into a scoring system, there was good sensitivity (96.3%) but not specificity (46.3%) for pCR (31). It is noted however, that this was a retrospective study looking at 81 samples. The results from this study were potentially subject to selection bias with only those with enough tissue being able to be examined (31). The appropriateness of this scoring system would still need to be validated with an independent cohort.

Despite this progress, tissue biomarkers have some significant limitations and have not been implemented clinically. First, harvesting tumour biopsies can expose patients to some risk of perforation and bleeding. Statistics from the New Zealand bowel cancer screening program show a rate of bleeding in patients with tissue removed during colonoscopy to be 7.9/1000 colonoscopies, and perforation rate to be 1.2/1000 (32). It is important to note however, that these rates refer to full colonoscopies including polypectomies, not just biopsies. Also, endoscopic biopsies can be unreliable due to sampling error. CRC tumours are heterogenous in nature (33) and a biopsy may not be representative as to what is happening in other parts of the tumour. In addition to this, in most cases endoscopy is performed once per patient usually prior to surgery due to its relatively invasive nature.

1.5.1 Liquid biopsies

Liquid biopsies, or blood-based biomarkers present as an attractive alternative to tissue biopsy as they are easily accessible and safe to acquire. If taken prior to neoadjuvant therapy starting, they would have a higher clinical utility to help with decision making regarding treatment, all prior to obtaining a surgical specimen. It would also be possible to take multiple or consecutive samples during treatment to monitor response at no further risk to the patient.

One example of a clinically used blood biomarker in CRC is CEA, which is used as a marker for disease recurrence. CEA is more sensitive at detecting CRC recurrence than
chest X-ray, ultrasound of the liver, liver function studies, or colonoscopy and is comparable to CT scan (34). There have been multiple studies exploring the predictive role of CEA in response to neoadjuvant therapy. The majority of studies have demonstrated that a lower CEA is associated with higher rates of pCR however these studies are retrospective in their design and all have different CEA cut off values (35). CEA is not currently used clinically for predicting response to neoadjuvant therapy as data is still requires validation in larger prospective trials (35).

Part of the routine preoperative work up for CRC patients includes measuring C Reactive Protein (CRP), white blood cells, neutrophils, lymphocytes. It has been shown that CRP is a sensitive but non-specific marker of inflammation. Despite known links between inflammation and cancer, there is a very weak association between elevated CRP and increased risk of CRC as demonstrated in a meta-analysis by Tsilidis et al. They found that there was an increased relative risk for colon cancer with each increase in unit of CRP, with a RR 1.13, (95% CI; 1.00-1.27) but not for rectal cancer, (RR 1.06 (95% CI 0.86-1.30)) (36). The Neutrophil to Lymphocyte Ratio (NLR) has also been explored for its predictive use. A meta-analysis by Li et al. looking at solid tumours (including breast, bladder, rectal and gastric) demonstrated a lower NRL was associated with better overall survival, disease free survival and recurrence free survival (37). Specifically, when looking at neoadjuvant therapy in rectal cancer patients, patients with lower NLR had an increased rate of pCR (OR 2.01, 95% CI = 1.14-3.55).

Despite this work, there are no blood biomarkers used in a clinical setting to predict response to neoadjuvant therapy in rectal cancer. There are several new classes of biomarkers including microRNAs (miRNA) which are particularly attractive because they are stable in blood. Emerging research suggests that miRNA may be potentially useful in the early detection, prognosis and as therapeutic targets for CRC (38).
Table 2 Biomarkers in tissue and plasma investigated for pCR predictive capability

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Biomarker</th>
<th>pCR Predictive Capability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour tissue DNA mutation/DNA methylation</td>
<td>KRAS</td>
<td>Unlikely to be able to predict response to treatment (35).</td>
</tr>
<tr>
<td></td>
<td>TP53</td>
<td>Mutation possibly associated with resistance to LCCR (39), but majority of studies reveal no correlation between p53 and treatment outcome (40)</td>
</tr>
<tr>
<td>Gene expression</td>
<td>NPTX2</td>
<td>Decreased levels associated with increased response to therapy (41).</td>
</tr>
<tr>
<td>Proteins</td>
<td>NF-KB</td>
<td>No significant association (42).</td>
</tr>
<tr>
<td>Tumour microenvironment</td>
<td>PD-L1</td>
<td>Inconsistent findings (42).</td>
</tr>
<tr>
<td>miRNA</td>
<td>miR-135b</td>
<td>Expression levels significantly correlated with pCR .</td>
</tr>
<tr>
<td></td>
<td>miR-21</td>
<td>Increased in pCR (43).</td>
</tr>
<tr>
<td></td>
<td>miR-145</td>
<td>Significant correlation - patients with a low intratumoral post-therapeutic expression had a worse response to neoadjuvant therapy AUC of ROC curve 0.696 ($p = 0.031$) (44).</td>
</tr>
</tbody>
</table>
### Patient plasma

<table>
<thead>
<tr>
<th>Protein/metabolites</th>
<th>CEA</th>
<th>Conflicting results, seems to be dependent on cut off level of CEA used. Better for predicting prognosis but not TRG (42).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td></td>
<td>Elevated levels associated with pCR (45). Poor sensitivity.</td>
</tr>
</tbody>
</table>

### miRNA

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-125b</td>
<td>Significantly elevated in non-responders (46).</td>
</tr>
<tr>
<td>miR-143</td>
<td>Serum miR-143 significant association with pCR (47).</td>
</tr>
</tbody>
</table>

### Host immune response

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 and TNF-α</td>
<td>Levels after therapy higher in non-responders (48).</td>
</tr>
<tr>
<td>NLR</td>
<td>Higher NLR associated with poor response to therapy (37).</td>
</tr>
</tbody>
</table>

### Abbreviations:

- KRAS, Kirsten rat sarcoma viral oncogene homolog
- NPTX2, neuronal pentraxin II
- EGFR, epidermal growth factor receptor
- PD-L1, programmed cell death ligand 1
- MDSCs, Myeloid-Derived Suppressor Cells
- CEA, carcinoembryonic antigen
- IL, interleukin
- NLR, Neutrophil-to-lymphocyte ratio
- TNF, Tumour Necrosis Factor
- NFKB, Nuclear factor kappa-light-chain-enhancer of activated B cells

### 1.6 MicroRNA as biomarkers

MicroRNA (miRNA) are short non-coding RNA’s that are expressed in all cell types. They work by regulating translation and stability of specific target messenger RNA (38). Changes in miRNA expression are frequently associated with abnormal cell function, notably certain cancers. They are thought to target oncogenes, tumor suppressor genes, and mRNA in the cell. Therefore, miRNA have been classified as being either oncogenic or having tumour suppressor function in relation to whether their overall effect is pro or anti tumour. Their expression in cancer has now been extensively
reported and their clinical utility as both biomarkers and therapeutic targets for disease is a rapidly growing area of research (38). Importantly, miRNA are stable in biofluids due to a number of reasons (49). First, they are transported in small double lipid layer vesicles called exosomes, a form of extracellular vesicle. Secondly, a portion of miRNA that are not transported in these vesicles are co-transported with protein Argonaute 2, or they can be carried by lipoproteins (50). miRNA are stable for long periods of time when stored properly and can endure repeated freeze-thaw cycles (51).

miRNA also have a functional role in cancer, and immunology is an example of this as miRNA are key modulators of tumour immune response. miRNA serve as crucial regulators, specifically by controlling the development and functions of tumour associated immune cells and the tumour microenvironment (52).

1.7 MicroRNA in colorectal cancer

There is currently considerable interest into how the expression of miRNA can be used for CRC diagnostic, prognostic and predictive tests (53). Multiple miRNAs have been found to be over and under expressed specifically in CRC patients (54). miRNA were first shown to be dysregulated in CRC tumour tissue when compared to normal adjacent mucosa (55). Since then, they have also be found to be dysregulated in the systemic circulation of CRC patients when compared to normal controls (55). MiRNA relative expression levels have been associated with CRC stage, prognosis, tumour bulk, and response to treatment (38, 56).

We have focused on four miRNAs that have previously been associated with CRC but have not been investigated for their relationship between plasma levels and response to neoadjuvant therapy.

1.7.1 miR-21

miR-21 is an oncogenic miRNA with elevated expression levels in CRC tissue (38), (55), (57). Multiple studies have evaluated the diagnostic ability of circulating miR-21 in CRC
patients. The majority have shown significant associations with elevated miR-21 and CRC. One study has shown the ability of miR-21 in serum to differentiate between adenomatous polyps and healthy control patients (56). A few studies have found no difference in miR-21 relative expression in plasma between CRC and control patients (58).

As well as overexpression in CRC, miR-21 has been investigated as a marker for pCR. Lopes-Ramos et al. (57) found that in tumour biopsies taken prior to neoadjuvant therapy, there was markedly increased expression of miR-21-5p, miR-1290-3p and miR-1246 in patients with complete response, and increased expression of miR-205-5p in patients with incomplete response. The authors then evaluated which miRNA could predict response to chemoradiation with highest accuracy and found that miR-21 had an area under the curve (AUC) 0.94 on receiver operating curve (ROC) with sensitivity of 100% and specificity of 85% (57). They did not find on validation studies that the other miRNA were differentially expressed. Of note the “complete responders” group was comprised of both those with pathological specimen evidence of pCR and also those with clinical evidence of complete response (assessed clinically endoscopically and radiologically). Those that were considered to be clinically pCR were enrolled in a Watch and Wait program. Therefore, not all of the patients have pathological specimens to validate pCR response. The authors had a group of patients who they initially termed “complete responders” but then went on to develop early local recurrence. Interestingly, these patients had significantly lower miR-21 expression to the pCR group and expression levels were lower, similar to the incomplete responders group. This could demonstrate that miR-21 could be useful to select patients who are suitable for Watch and Wait management strategies. Regarding mechanisms for how miR-21 may effect neoadjuvant response, the authors also found that in vitro studies using colorectal tumour cell lines (HCT116 and SW480), SATB1, a miR-21 target gene which is associated with multidrug resistance may be directly involved with poor response to neoadjuvant therapy.

In a retrospective study by Carames et al. (43), the authors found miR-21 overexpression in rectal tumour tissue samples taken post neoadjuvant treatment and
post-surgery in 77% of patients. The authors state that miR-21 overexpression correlated with postoperative tumour grade and pathological response. Patients were considered to have “low” expression of miR-21 when expression levels (ΔC_T) were below 2.8. Of the 10 patients that had pCR, 6 had “low” and four had “high” miR-21. For those with incomplete response, 8 had “low” miR-21 and 52 had “high” miR-21 (p =0.0130). This is a notably small number of patients with pCR, more of which had a “low” miR-21. The main issue with this retrospective strategy is with using samples taken after neoadjuvant therapy and surgery. We cannot extrapolate that these changes in expression would be the same in patients prior to treatment and therefore useful clinically to answer predictive questioning.

Currently it is not clear as to how useful circulating miR-21 is in a predictive context. While the abovementioned studies have looked at tissue miR-21, plasma miR-21 has not been analysed for their role in predicting response to neoadjuvant therapy.

1.7.2 miR-29a

miR-29a has been reported to be dysregulated in a number of cancers including prostate cancer, myeloid leukemia, lung cancer, and glioblastoma, cholangiocarcinoma and CRC (59). Specifically miR-29a has been shown to be upregulated 2.3 fold in rectal cancer tissue (55). MiR-29a has been associated with tumour aggressiveness and with later stage cancer. Tang et al. have reported that an increased expression of miR-29a leads to a decreased expression of KLF4 gene, which is a well-established tumour suppressor gene in CRC and has been implicated in cell proliferation, migration and invasion (60). The authors also analysed the expression levels in 85 cases of CRC tissues (surgical specimens) using RT-qPCR and found a high expression of miR-29a was significantly correlated with metastatic disease (p = 0.28). Serum levels of miR-29a have been reported to be significantly higher in stage III CRC patients compared to controls (61). We have selected this as a target miRNA because circulating levels could potentially give some biological information on the rectal tumour that would be useful for deciding if neoadjuvant therapy is necessary.
1.7.3 miR-143 and miR-145

MiR-143 and miR-145 have both been reported to have decreased expression in CRC tissue. miR-143 expression has also been shown to decrease specifically in colon cancer tissue, but not in rectal cancer tissue (54, 55, 62). In a study by Li et al, miR-143 relative expression in rectal tissue from surgical specimens (snap frozen) was decreased 4.82 fold in colon cancer, but did not change significantly in rectal cancer (55). This potentially highlights the heterogeneity in the biology of these cancers.

MiR-143 and miR-145 are reported to have tumour suppressive functions in CRC (38). Both have been shown to regulate cell growth and proliferation in in vitro studies. MiR-143 has been shown to inhibit translation of KRAS (63). There is some evidence that p53 regulates miR-145, and that miR-145 modulates multiple oncogenes including insulin receptor substrate-1 (IRS-1), EGFR, MUC-1, and SOX2. The tumour suppressive function of miR-145 is related to regulation of cell proliferation via its targeting of IRS-1 (64).

Arndt et al (62). analysed the expression of miRNA in both clinical CRC samples (n=45) and 8 CRC cell line models, of which they found 37 miRNAs that were differentially expressed. They focused on miR-143 and miR-145 and transduced miR-143 or miR-145 vectors into SW620 CRC cells and analysed their cell proliferation, differentiation and anchorage-independent growth. There is possibly some function of miR143 that has a tumour suppressor effect in metastatic CRC cells, and that conversely miR-145 has some oncogenic effect in metastatic CRC cells with increase in cell proliferation and metabolic activity, and an increase in anchorage-independent growth (62).

In terms of predictive tests, Drebber et al. (44) examined relative expression of miR-21, miR-143 and miR-145 in tissue of forty patients with advanced rectal cancer, and assessed tumour response to neoadjuvant therapy with TRG. The authors found that miR-143 and miR-145 were significantly upregulated in tissue after LCCCR. A significant correlation between miR-145 expression and tumour regression was also seen, in that patients with a low intratumoral post-therapeutic expression had a worse response to neoadjuvant therapy AUC of ROC curve 0.696 (p = 0.031). Regarding circulating levels,
there is one recent prospective study looking at the association of TRG to LCCR with serum miR-143, in which the authors report it to be an independent predictor of good response in locally advanced rectal cancer patients, which is very encouraging (47).

1.8 Conclusion

Rectal cancer is an important problem in New Zealand. Patients with locally advanced disease are currently managed with neoadjuvant therapy and surgery. Due to the varied patient response to neoadjuvant therapy, there is a need to determine which patients will benefit prior to administering this treatment. Currently there are no clinically used blood biomarkers to predict patient response. There is a growing appreciation for the role of miRNAs in CRC and new research into how these could be used to predict response to neoadjuvant therapy. Developing these tests would allow patients to receive optimal therapy and avoid ineffective and potentially harmful treatments.
Hypothesis and Aims

We hypothesise that circulating plasma miR-21, miR-29a, miR-143 and miR-145 could act as biomarkers for the detection of CRC and as predictors of response to neoadjuvant chemoradiation therapy.

Our specific aims are:

1. To determine whether plasma levels of miR-21, miR-143, miR-145 and miR-29a have altered expression in patients with stage II/III rectal cancer compared to controls. Target miRNA levels will be measured in plasma samples from rectal cancer patients and control patients using RT-qPCR.

2. To determine whether plasma levels of miR-21, miR-143, miR-145, and miR29a, taken prior to neoadjuvant treatment can act as predictive markers of response to neoadjuvant therapy. We will compare baseline plasma target miRNA expression from stage II/III rectal cancer patients who respond to SCR and LCCR vs non responders.

3. To investigate changes in plasma levels of these miRNA in response to neoadjuvant therapy as markers of therapeutic response. We will compare baseline plasma target miRNA expression from stage II/III rectal cancer patients who respond to SCR and LCCR with post therapy expression to determine whether circulating miRNA levels change as an indicator of response to therapy.
Chapter 2:

Methods
2.1 Patient recruitment

2.1.1 Colorectal Cancer Biobank

Patients with CRC were recruited for donation of blood and tissue for a Colorectal Cancer Biobank under ethical approval by Health and Disability Ethics Committee (‘Establishment of human tissue bank of surgical cancers for future unspecified research, ref: 15/CEN/143). MicroRNA measurement for the proposed study was approved by the Health and Disability Ethics Committee (‘Molecular biomarkers in colorectal cancer’, ref: 18/CEN/138). Consultation was also undertaken with the Ngai Tahu Research Consultation Committee for this project. All work was performed in accordance with the Declaration of Helsinki and all patients provided written informed consent at the time of recruitment.

The biobank was established in October 2016, and patient recruitment is ongoing. For this project, data collected up to September 2018 was utilised.

Demographic patient data such as gender, ethnicity, age at diagnosis, presenting symptoms, medical comorbidities and current medication was collected. Diagnostic information such as endoscopic findings, biopsy results, staging CT and MRI results, and surgical information such as operation details and pathological findings were also noted. Study data was collected and managed using REDCap electronic data capture tools hosted by Otago University (65).

Patients who were included in the Biobank were over the age of 18 and diagnosed with CRC on endoscopy at Wellington Hospital, or they were referred to Wellington Hospital from Hutt Valley and Wairarapa Hospitals.

Exclusion criteria were; patients who had care transferred to a private hospital, patients who had more than one concurrent malignancy, those who had malignant polyps removed endoscopically on presentation, patients under the age of 18, those who
underwent emergency surgery, and finally those who had significant cognitive impairment and were not able to provide informed consent.

Patient recruitment and sample collection was carried out by a team of researchers at the University of Otago. Recruitment involved identification of patients, consent, data entry, database audit, blood collection, and assistance with collecting tissue at time of surgery. Patients suitable for recruitment were identified from MDMs, general surgical outpatient clinics and elective theatre lists. The author recruited patients between December 2017 and June 2018.

2.1.2 Patient selection

From the Colorectal Biobank, patients with Stage II and Stage III rectal cancer who had neoadjuvant treatment were identified for this study. Patients were enrolled at the time of diagnosis of rectal cancer, prior to surgery. The decision as to whether patients would undergo either short or long course neoadjuvant therapy was made at the Wellington Hospital Colorectal Multi-Disciplinary Meeting.

Control patients were selected via elective colonoscopy lists. Selected patients were chosen to age match the CRC patients. Consent was obtained prior to endoscopy. All control patients were symptomatic of either change in bowel habit, anaemia, or overt rectal bleeding. To be included as a control, the resulting endoscopy was required to be normal with no pathological findings of colorectal cancer, Ulcerative Colitis, or Crohn’s disease. Grossly normal endoscopy findings with abnormal biopsies were considered a reason for exclusion (e.g. ileitis).

2.2 Sample collection

Patients undergoing short course neoadjuvant treatment typically received 25 Gy in 5 fractions. They then underwent early surgery, within 3-7 days of finishing radiation. Patients undergoing long course chemoradiation generally received 50.4 Gy in 28 fractions over 6 weeks with concurrent oral Capecitabine 825 mg/m² twice daily. They
then underwent surgery, typically within 6-8 weeks of finishing neoadjuvant treatment (Figure 1). Venous blood samples were donated at the time of recruitment (Timepoint A) and again after completing neoadjuvant therapy, prior to surgery (Timepoint B). For short course neoadjuvant patients, blood was drawn on the day of surgery prior to any anesthetic agent being given. For long course neoadjuvant patients, blood was drawn after completing therapy, on the day of surgery prior to receiving anaesthetic agents.

![Diagram](image)

**Figure 1 Typical time course structure for (A) short course and (B) long course neoadjuvant therapy**

Blood samples were obtained from patients at the abovementioned time points. Twenty millilitre venous blood specimens were collected using a 21G BDTM vacutainer blood collection set into sodium citrate collection tubes (3.2%, 0.109 M) then centrifuged within 30 minutes of withdrawal at 2,000 g for 10 minutes to separate blood into plasma and serum. Plasma samples were then stored in a -80°C freezer in multiple aliquots. Venous blood was also collected into EDTA tubes and SST II Gel tubes for biochemistry and hematological tests by the hospital laboratory.
2.3 Quantitative PCR

2.3.1 Sample preparation and RNA extraction

Plasma samples were thawed at room temperature then centrifuged at 1500 g for 10 minutes to remove any remaining cell or platelet debris. RNA was extracted starting with 300 μL of plasma using the miRNeasy Serum/Plasma Advanced Kit using RNeasy UCP MinElute columns (Qiagen) according to the manufacturer’s instructions.

During the lysis step, the samples were spiked with an exogenous miRNA as an extraction efficiency measure, using 50 fmol cel-miR-39-3p spike (5'UCACCGGGUGUAAUAUCACGUUG).

The final product was eluted in 30 μL of nuclease-free water and stored at -80°C.

2.3.2 Reverse transcription

An LNA-based reverse transcription system - miRCury LNA RT kit (Qiagen) was used to synthesise cDNA, using 2 μL of RNA for each sample. Reverse transcription (RT) was performed according to the manufacturer’s protocol. The RT reaction was as follows; incubation at 42°C for 60 minutes, heat inactivation at 95°C for 5 minutes, then immediately cooled to 4°C. Samples were then stored at -20°C.

2.3.3 Quantitative PCR

cDNA samples from the above RT reaction were diluted 1:2 with nuclease-free water. Semi-quantitative real-time PCR was then performed with the SYBR Green PCR master mix (ExiLENT, Exiqon) on a Corbett RotorGene 6000 with miRCURY LNA PCR primer sets specific for hsa-miR-21-5p, hsa-miR-29a-3p, hsa-miR-143-3p, hsa-miR-145-5p (Table 4). Control primer sets were cel-miR-39-3p (exogenous control) and hsa-miR-345-5p (endogenous control). The total reaction volume of each sample was 10µL, which included 1 µL of diluted cDNA. Negative reactions containing water instead of cDNA
were included in every experiment. All reactions were performed in duplicate and $C_T$ values within 0.5 were considered acceptable. The following cycling conditions were utilised: 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 s, and 60 °C for 1 min.

*Table 3 Primers used for quantitative PCR*

<table>
<thead>
<tr>
<th>Primer target</th>
<th>miRNA sequence 5'-3' - Forward</th>
</tr>
</thead>
<tbody>
<tr>
<td>cel-miR-39-3p spike</td>
<td>5'UCACCGGGUGUAAAAUCAGCUUG</td>
</tr>
<tr>
<td>miR-21-5p</td>
<td>5'UAGCUUACAGACUGAUGUGA</td>
</tr>
<tr>
<td>mirR-29a-3p</td>
<td>5'UAGCACCAUCUGAAAUCGGUUA</td>
</tr>
<tr>
<td>miR-143-3p</td>
<td>5'UGAGAUGAAGCACUGUAGCUC</td>
</tr>
<tr>
<td>miR-145-5p</td>
<td>5'GUCCAGUUUGGCCCCAGAAUCCCU</td>
</tr>
<tr>
<td>miR-345-5p</td>
<td>5'GCUGACUCCUAGUCCAGGGCUC</td>
</tr>
</tbody>
</table>

Melt curve analysis was conducted at the end of cycles to analyse the purity of PCR products. This consisted of increasing the temperature from 50°C - 99°C, rising by 1°C each step every 5 seconds. As the temperature of the reaction rises, double stranded DNA melts apart at a specific temperature depending on the sequence, resulting in a reduction in fluorescent signal from intercalated SYBR green. This is then graphed as a first derivative of relative fluorescence units over temperature vs temperature of the reaction mixture. Specific PCR products should produce a distinct peak in the graph (Figure 2).

Expression levels of miRNA were quantified using the Rotor-Gene software version 1.7.75. Cycle thresholds were set to 0.09767 across all samples (Figure 2).
Figure 2 Representative data from quantitative PCR experiments

(A-G) Representative trace images (left) and melt curve profiles (right) for (A) miR-21, (B) miR-29a, (C) miR-143, (D) miR-145, (E) miR-345, (F) cel-miR-39 (G) negative water controls. All samples were run in duplicate
2.4 Tumour specimens and pathologic response grading

Surgical specimens were analysed by trained Pathologists at Wellington Hospital. Pathological staging was performed using the AJCC TNM classification (66). This data was available via the Wellington Hospital medical records. For patients who underwent short course neoadjuvant therapy, a single pathologist analysed the slides and graded the pathological response using the Modified Ryan scheme AJCC 8th edition tumour regression grading system (67). For the patients who underwent long course neoadjuvant therapy, the tumour regression grade was part of the synoptic pathology report. Patients that had a score of 0, or 1, were considered to have a “good” response, and those with a score of 2 or 3 were considered to have a “bad” response (Table 5).

Table 4 Modified Ryan Tumour Regression Grading system

<table>
<thead>
<tr>
<th>Responder</th>
<th>TRG Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Good&quot;</td>
<td>0</td>
<td>No viable cancer cells, complete response</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Single cells or small groups of cancer cells</td>
</tr>
<tr>
<td>&quot;Bad&quot;</td>
<td>2</td>
<td>Residual cancer with evident tumour regression but more than single cells or small groups of cancer cells</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Extensive residual cancer with no evident tumour regression</td>
</tr>
</tbody>
</table>

2.5 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 7.00 software. Results were expressed as mean ± standard deviation. For comparisons of numerical data, either paired or unpaired t tests were used. Comparisons of categorical data were analysed using χ² tests. Values of p < 0.05 were considered statistically significant.
The suitability of the housekeeper control primers (endogenous marker miR-345 and exogenous cel-miR-39) was evaluated by comparing the raw C\(_T\) values of the control patients vs the cancer patients, and then the pre treatment vs post treatment values. Raw C\(_T\) values were of Gaussian distribution. Unpaired \(t\) tests were performed.

The relative expression values of target miRNAs (miR-21, miR-29a, miR-143, and miR-145) were calculated using the \(2^{-\Delta\Delta C_T}\) method (68). To calculate \(\Delta C_T\) the geometric mean values of the housekeeper miRNA was subtracted from the average \(\Delta C_T\) value of the miRNA of interest. \(\Delta\Delta C_T\) was calculated by subtracting the average \(\Delta C_T\) value of the control group from the group of interest. Unpaired \(t\) tests were performed to compare target miRNA relative expression means.

When looking at paired samples pre and post neoadjuvant therapy, raw C\(_T\) minus the average of the housekeepers (\(2^{-\Delta C_T}\)) was used. Paired \(t\) tests were performed for statistical analyses. To compare fold change in relative expression, \(\Delta C_T\) of target miRNA from samples prior to neoadjuvant therapy was subtracted from \(\Delta C_T\) post therapy and then logged. Again, paired \(t\) tests were then performed for statistical analysis.

Correlation of miRNA raw C\(_T\) values were calculated using the Pearson calculation for \(r\). The Pearson calculation was also used to analyse the correlation of miRNA relative expression with CEA, NLR and CRP tests. NLR and CRP values were logged due to a tailed distribution.
Chapter 3:

Results
3.1 Patient demographics

3.1.1 Colorectal Biobank

From October 2016 to September 2018, a total of 195 patients had been recruited to the Colorectal Cancer Biobank. Of the total Biobank patients, the average patient age was 68.0 years (range 27-88 years). The majority of patients are of New Zealand European ethnicity (86.3%), followed by Māori ethnicity (5.3%). Of these, 23 are “healthy” control patients who have no evidence of CRC detected on endoscopy. The remaining 171 patients have CRC, of which 63 have rectal cancer (32%). Forty-one of the patients with rectal cancer have Stage 2 or Stage 3 disease and underwent neoadjuvant treatment as recommended by the Colorectal MDM, making them eligible for this study. Comparing this to New Zealand (NZ) data, the proportion of patients with rectal cancer in our Biobank is higher (32%) than that described in the PIPER project (24%)(7). Again, regarding the PIPER data, the majority of patients were NZ European ethnicity, followed by Māori ethnicity (8%).

3.1.2 Rectal cancer patients

From the CRC Biobank patients, a total of 53 patients were included in this neoadjuvant study across two groups; control patients ($n = 19$) and rectal cancer patients ($n = 34$). Of the 34 patients with rectal cancer, 16 underwent SCR neoadjuvant therapy, and 18 underwent LCCCR therapy (Table 6). Two patients did not proceed to surgery after neoadjuvant therapy, one due to frailty, and one patient declined surgical intervention, therefore surgical specimens were not available for these patients.

Seven rectal cancer patients were excluded from this study due to the following: care transferred to a private hospital (2), the patient was deemed unfit for major abdominal surgery (2), post operative pathology specimen benign (1), technical exclusion (RNA not able to be extracted from plasma) (1), and one patient who had a pelvic lymph node radiated rather than tumour. One control patient was excluded from this study due to the finding of terminal ileitis on endoscopy biopsy.
Of the 16 patients who underwent SCR, one did not have a blood sample from prior neoadjuvant therapy starting (Timepoint A - Figure 1). Of the 18 patients who underwent LCR, four did not have a blood sample at Timepoint A. These patients were not used for pre/post matched sample analysis or for analysis of response to treatment.

The mean age at diagnosis for the rectal cancer patients was 63.55 (±13.9) years. The mean age of the SCR patients was significantly higher than for the LCCR patients, 70.6 (±9.9) years vs 57.3 (±13.9) years respectively, \( p = 0.003 \) (Table 6). When comparing to the general NZ population, the mean age of patients with rectal cancer in the PIPER study was 71.4 (±12.2) years and the majority of patients were male (62.5%). Only a third of the patients in the PIPER study were completely staged, so it is difficult to compare the stage of presentation to our patients, however they were able to state 76% of their patients had stage I-III disease(7). The majority of patients in our study had stage III disease (\( n = 25 \)).
**Table 5 Rectal cancer patient demographics**

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Short course</th>
<th>( p )</th>
<th>Long course</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total ((n))</strong></td>
<td>19</td>
<td>16</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mean age at diagnosis (Std. dev)</strong></td>
<td>68.2 (11.5)</td>
<td>70.6 (9.9)</td>
<td>0.56</td>
<td>57.3 (13.9)</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Gender (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>12 (63.2)</td>
<td>7 (43.8)</td>
<td>0.25</td>
<td>9 (50)</td>
<td>0.42</td>
</tr>
<tr>
<td>Male</td>
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<tr>
<td>3</td>
<td></td>
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*Age is expressed as mean +/- standard deviation. Pre-operative radiological stage is based on staging pelvic MRI radiology reporting.*

The time from completion of neoadjuvant therapy to the day of surgery was calculated for each patient. In the SCR group, one patient had a markedly extended delay to surgery after completing neoadjuvant treatment of 47 days. This was a planned delay and discussed in the Colorectal MDM as the patient was not felt to be able to withstand LCCR due to frailty. With this patient excluded, the average time to surgery after SCR was 3.43 (±1.05) days. In the LCCR group, the average time to surgery after completing LCCR was 51.87 (±7.40) days.
3.2 Quality control of qPCR

The relative expression of miRNA in plasma were analysed using RT-qPCR. First, endogenous miR-345 and exogenous cel-miR-39 were compared across the groups. There was no statistical difference in the mean raw $C_T$ for either miR-345 or cel-miR-39 when comparing those in control patients with those with cancer (Figure 3). Comparisons were also performed for pre and post treatment, and for the geometric means of miR-345 and cel-miR-39 together (Figure 3). Again, there was no significant differences, and thus these two miRNA could be used as housekeepers for further analysis.
Figure 3. Analysis of endogenous and exogenous housekeepers.

A) Raw $C_T$ values for cel-miR-39 for the control vs. cancer groups ($p = 0.7305$), B) Raw $C_T$ values for miR-345 for control vs cancer groups ($p = 0.6056$), C) Raw $C_T$ values for cel-miR-39 pre and post treatment ($p = 0.1189$) D) Raw $C_T$ values for miR-345 for the pre and post treatment groups ($p = 0.2770$), E) Geometric means comparing controls vs. cancer patient groups ($p = 0.9282$), F) Geometric means comparing pre and post neoadjuvant therapy ($p = 0.1292$). Tx = treatment. All means compared using unpaired t test.
3.3 MicroRNA expression in rectal cancer patients vs. controls

Prior research has demonstrated that miR-21 and miR-29a are upregulated in rectal cancer tissue, whilst miR-145 is downregulated in rectal cancer tissue and miR-143 is downregulated in colon cancer tissue (55). Studies have shown similar changes in expression in plasma which seem to be specific to cancer stage (58, 69, 70). Therefore, we compared the relative expression of miR-21, miR-29a, miR-143 and miR-145 in plasma for the control patients vs. stage II/III rectal cancer patients (prior to neoadjuvant therapy) to determine the difference in expression. There was no statistically significant change in relative expression between the control group vs. cancer patients (Figure 4). miR-21 was overexpressed compared to the average of the controls in 35% of patients and there was one patient with very high expression. miR-143 and miR-145 were overexpressed in 21% of patients, whilst miR-29a was overexpressed in 17% of patients. When looking at miR-143 and miR-145 relative expression in the controls, there was one patient with very high expression of both of these miRNAs.

3.4 The effect of neoadjuvant therapy on circulating target miRNA expression

Pre and post neoadjuvant treatment miRNA relative expression levels (paired samples) were analysed to determine whether there was a treatment effect which would be clinically relevant. There was a statistically significant increase in miR-143 after short course radiation. There were no other statistically significant changes in relative expression (Figure 5). The average fold changes in expression were also analysed. For miR-21 the average fold change after SCR was 1.36 (±1.45), and after LCCR was 1.42 (±1.06). MiR-29a had an average fold change of 1.16 (± 0.967) after SCR and 1.152 (±0.79). For miR-143 the average fold change after SCR was 2.00 (±1.53) and after LCCR was 3.04 (±5.26). The average fold change was the greatest for miR-145 which after SCR was 5.2 (±8.409) and after LCCR 3.64 (±9.01). Unpaired, group averages pre and post treatment were also analysed and there was no statistically significant difference in relative expression.
Figure 4 Relative expression of target miRNA, comparing control patients to those with cancer.

A) miR-21 ($p = 0.5036$), B) miR-29a ($p = 0.7260$), C) miR-143 ($p = 0.2433$), D) miR-145 ($p = 0.5965$).
Figure 5 Target miRNA relative expression for paired data comparing pre and post neoadjuvant therapy.

A) miR-21 relative expression after SCR (p = 0.3289) and LCCR (p = 0.8794). B) Relative expression of miR-29a after SCR (p = 0.2470) and LCCR (p = 0.8626) for miR-29a, C) miR-143 relative expression was significantly different after SCR (p = 0.0282) but not after LCCR (p = 0.4719). D) miR-145 relative expression after SCR (p = 0.1860) and LCCR (p = 0.5838).
3.4 miRNA correlations

Raw miRNA C\textsubscript{T} values for each patient (at Timepoint A) were analysed using Pearson’s correlation (Figure 6). The strongest correlation was between miR-21 and miR-29a (r = 0.8107), then between miR-29a and miR-143 (r = 0.7219), followed by miR-21 vs miR-143 (r = 0.7044), miR-21 vs miR-145 (r = 0.6805), miR-29a vs miR-145 (r = 0.5371), and lastly the weakest correlation was between miR-143 and miR-145 (r = 0.4865).

Due to the integral role of the immune system in the progression and regulation of cancer growth, we evaluated the relative expression of the miRNA with NLR and CRP. It has been previously reported that patients with a higher NLR have a lower rate of pCR (OR 1.72, 95% CI 1.26-2.33) (37). In our study, the relative expression of miR-21, miR-29a, miR-143, and miR-145 of cancer patients did not correlate with patient NLR at Timepoint A (Figure 7).

The relative expression of miR-21, miR-29a, miR-143 and miR-145 in cancer patients did not correlate with CRP at Timepoint A. This was limited by the sensitivity of the CRP assay in that all values less than three were entered as three. Even when only taking into account CRP values greater than three, (n = 12), there was still no correlation with target miRNA relative expression.
Figure 6 Correlations between miRNA raw CT values

A) miR-21 vs miR-29a, B) miR-29a vs miR-143, C) miR-21 vs miR-143 D) miR-21 vs miR-145, E) miR-29a vs miR-145, and F) miR-143 and miR-145.
Figure 7 Correlation between Neutrophil:Lymphocyte Ratio (NLR) and target miRNA expression

A) miR-21 expression and NLR ($r = 0.04897$), B) miR-29a and NLR ($r = 0.1074$), C) miR-143 expression and NLR ($r = 0.01653$), D) miR-145 expression and NLR ($r = -0.2627$).
Preoperative CEA levels in serum have previously been reported to correlate with miR-155 expression in CRC tissue (Hongliang, Shaojun et al. 2014). Whether or not there is a relationship between our target miRNA expression in plasma and CEA is previously unreported. In our study, the relative expression of miR-21, miR-29A, miR-143 and miR-145 in cancer patients did not correlate with CEA levels at Timepoint A (Figure 8).

Figure 8 Correlation between CEA and target miRNA

A) miR-21 and CEA ($r = 0.04191$), B) miR-29a and CEA ($r = -0.1335$), C) miR-143 and CEA ($r = -0.08281$) or D) miR-145 and CEA ($r = -0.02717$).
3.5 Tumour Regression Grading and miRNA relative expression

An aim of this study was to evaluate whether miRNA expression could predict TRG. Each target miRNA expression was analysed separately in patients who underwent SCR or LCCR.

3.5.1 Patients receiving SCR

Of the 16 short course neoadjuvant therapy patients, 15 proceeded to surgery and had pathology specimens analysed with TRG reported. Of these patients, 12 showed no response to radiation treatment (TRG 3), and three showed some response (TRG 2). No patients had a greater response to treatment than TRG 2. One patient did not have a plasma sample from Timepoint A and was not used in this analysis. There was no statistical difference in the relative expression of the miRNA between these two subgroups of patients (Figure 9).
Figure 9 Comparison between patients who underwent short course neoadjuvant therapy who had any response to treatment vs. no response to treatment.

A) miR-21 relative expression ($p = 0.7799$), B) miR-29a relative expression ($p = 0.1261$), C) miR-143 relative expression ($p = 0.7285$), or D) miR-145 relative expression ($p = 0.5234$).
3.5.2 Long course neoadjuvant patients

Of the 18 LCR patients, four did not have preoperative blood samples taken and one did not proceed to surgery. This left a total of 13 patients samples to be analysed when comparing miRNA expression at Timepoint A and TRG. There were no patients with pCR. Of the 13 patients who then had Timepoint A blood and TRG, 3 had a “good response” of TRG 1, and 10 had a “bad response” of TRG 2 or TRG 3. When comparing the “good” vs. “bad” response subgroups, there was no statistical difference in miRNA relative expression (Figure 10). Patient samples with a “good” and “bad” response were not further analysed comparing pre and post SCR and LCCR as planned due to the very low numbers. For example, there were only two patients in the “good” response group with paired samples making this data insignificant.

Given that previous research has demonstrated increased rates of tumour regression with prolonging the time delay from radiotherapy to surgery, it was important to review whether this impacted on TRG in our study. Regarding time from finishing neoadjuvant therapy to date of surgery, the patients were again divided into the subgroups ‘Any response’ and ‘no response’ for SCR and ‘good response’ and ‘bad response’ for LCR. The average time to surgery in these two groups was compared and there was no difference between them (for LCR; 50.7 ± 3.3 days for ‘good’ response, and 50.1 ± 8.4 for ‘bad’ response, p = 0.9082).
Figure 10 Comparison of miRNA relative expression prior to LCCR neoadjuvant therapy in “good” and “bad” responders

A) miR-21 relative expression ($p = 0.7311$), B) miR-29a relative expression ($p = 0.4917$), C) miR-143 relative expression ($p = 0.6133$), or D) miR-145 relative expression ($p = 0.8261$).
Chapter 4:

Discussion
This study has investigated circulating miRNA levels in patients who have locally advanced rectal cancer and have undergone neoadjuvant therapy. The aims of this study were to determine if patients with stage II/III rectal cancer had altered expression of the target miRNAs compared to controls, and to ascertain if these miRNA levels in plasma prior to treatment can predict response to SCR and LCCR therapy, or if changes in levels in response to neoadjuvant therapy occur as markers of therapeutic response.

The standard management for locally advanced rectal cancer is neoadjuvant therapy followed by TME surgical resection, but the response to therapy is varied. Whilst there is potential benefit to gaining complete response, on the other hand, therapy is not without risk. Patients may suffer a number of adverse side effects from treatment, and of course a delay to surgery. Despite gains in knowledge regarding the detection and management of rectal cancer, we are still not able to predict which patients will benefit from neoadjuvant therapy. Given the potential adverse effects, patients need to be selected for optimal treatment with some certainty that they would receive a benefit. The development of blood based biomarkers predictive of response to neoadjuvant therapy would allow for this early in the patient’s treatment pathway. Whilst some have been proposed to date, none have been used in a clinical setting so far.

MiRNA are an interesting, novel class of biomarkers that we have chosen to investigate further due to their stability in biofluids and increasing evidence that there is dysregulation in miRNA expression in cancer patients, and importantly here, in rectal cancer. We have chosen four target miRNA; miR-21, miR-29a, miR-143 and miR-145 because they are all dysregulated in CRC tissue and plasma, and miR-21, miR-143 and miR-145 have been previously associated with response to neoadjuvant therapy (71). miR-21 and miR-29a are reported to be over expressed in CRC and function as oncomiRs (38, 55), whilst miR-143 and miR-145 are under expressed and function as tumour suppressors (55). Importantly, serum miR-143 has been shown to have some ability to predict TRG after LCR (47).
In this pilot study we have found a significant increase in the relative expression miR-143 in plasma after SCR. There was no difference in expression of miR-21, miR-29a, miR-143 or miR-145 in patients with stage II and III rectal cancer compared to healthy controls. There was no difference in expression of target miRNA in patients who had a response to neoadjuvant therapy compared to those with little or no response.

This study has contributed to the small existing pool of knowledge on the use of circulating miRNA as predictive biomarkers for response to neoadjuvant therapy in rectal cancer patients. With the data we have obtained so far, it is still too early to determine absolutely if these miRNAs have a predictive use due to the small patient group size.

Rectal cancer patients at Wellington Hospital

The Wellington CRC biobank patients have a higher proportion of rectal cancers compared to that reported in the PIPER project report (32% vs 24%) (7). This is likely because the PIPER project included all CRC patients between 2006 and 2009, and recent evidence would indicate that rectal cancer incidence is increasing in patients under the age of 50, with a 13% increase in women and an 18% increase in men per decade in this age category (72). In addition to this, the Wellington biobank consists of mostly elective cases and acute rectal resection is very uncommonly performed.

When comparing the SCR and LCCR patient groups, there was a notable age difference between the short course patients (70.6 ±9.9 years) and the long course patients (57.3 ±13.9 years). It is likely this is because patients that are older are generally frailer or have more medical co-morbidities and may not be able to tolerate LCCR, making them more suitable candidates for SCR.

Given that increasing the delay after neoadjuvant therapy to surgery can improve tumour regression (73), it was important to determine the time delays for each patient. One patient did have a planned, marked delay to surgery after SCR of 47 days. Interestingly, this patient did not have a “good” response to SCR, with a TRG of 2. There
was no statistically significant difference when looking at the averages of the time delays for the “good” and “bad” responder groups.

**miRNA in rectal cancer vs. controls**

In this study, we found that there was no difference in relative expression of plasma miR-21, miR-29a, miR-143 or miR-145 when comparing control patients and rectal cancer patients. The current literature reports that the expression of miR-21 and miR-29a are upregulated in rectal cancer tissue, and that miR-143 and miR-145 are downregulated in rectal cancer tissue (55). Plasma miR-21 and miR-143 has been shown to be decreased in CRC patients compared to controls (69), and plasma miR-145 increased (71). Huang et al. report that when specifically looking at plasma, miR-29a is upregulated in CRC patients and there is a stepwise increase in expression with increasing TNM stage compared to controls (70). These authors looked at 120 plasma samples from patients with CRC which were taken prior to surgery (27 stage I, 25 stage II, 38 stage III, and 10 stage IV). Despite reporting this, there is a statistical difference when comparing control patients with patients at each stage of disease rather than when comparing each stage consecutively. This group also found that miR-29a plasma levels dropped significantly 7-10 days after surgery in a subgroup of 20 patients with CRC (p=0.04). It is interesting to note that Ng et al. (2008) found an increase in expression of both miR-17-3p and miR-92 in plasma in CRC patients but did not find an increase in expression of miR-21 or miR-29a in plasma. The main limitation with this finding was that in the first step in the discovery phase of their study they only sampled from five CRC patients compared to five healthy controls, which is a very small sample size (54).

It is possible that our study did not identify a difference in expression of our target miRNA is because we excluded patients with stage IV disease. We have specifically looked at stage II/III disease and levels of these miRNA are often reportedly higher in stage IV disease, which could potentially explain not seeing a difference. In addition to this, our sample size is small (n = 36 compared to n = 120 in the Huang study). At this stage with our results, plasma miR-21, miR-29a, miR-143 and miR-145 expression levels
prior to neoadjuvant therapy do not seem to be useful as discriminating rectal cancer diagnostic tests.

The control patients were 19 “healthy” patients and controls were confirmed after having a normal colonoscopy with normal biopsies. Despite being called “healthy”, some have underlying chronic medical conditions (for example hypertension) which could affect target miRNA relative expression to some unknown extent. The number of controls used was relatively small compared to previous studies on miRNA expression which ranged from 10 to 144 subjects (58).

miR-143 changes after neoadjuvant therapy

This study is the first to examine changes in plasma expression pre and post neoadjuvant therapy of miR-29a, miR-143, miR-145 and miR-21. We report a statistically significant increase in relative expression of miRNA-143 in plasma after SCR (Figure 4). Out of the 15 paired samples, 11 had an increase in miR-143 expression. There was no significant change in relative expression of miR-21, miR--29a, or miR-145 in either LCCR or SCR patients. Publications so far have focused on miRNA expression changes after surgery, but not at the time point prior to surgery after neoadjuvant therapy except for a recent study looking at miR-143 in serum (47). It is important to note that one possible confounder in the SCR patients in this study is that fasting status was different. Due to the nature of the venous blood collection, the Timepoint B sample is collected immediately prior to surgery when patients are fasted and could contribute to the difference seen. Ideally this would be repeated in a larger group with consistent fasting status.

Toiyama et al. looked at miR-21 expression in CRC samples in 186 patients and found that tumour and serum miR-21 expression was higher in larger tumours ($p=0.004$) (56). The authors looked at another set of 60 patients who underwent curative surgery and compared miR-21 levels pre and post surgery and found those with curative resections had a marked decrease in miR-21 expression ($p = <0.001$)(56). We also suspect circulating miRNA is from tumour bulk and that therefore, the changes seen would be
expected to be different in studies reporting changes after surgery compared to our study. We would expect that surgically excising a tumour would give different results to targeting cells with radiotherapy, as surgery would lead to a rapid decrease in miRNA expression and perhaps the increase in circulating miR-143 is due to damage to the tumour or an inflammatory response from the radiotherapy. Of note, an increase in miR-143 and other miRNA in peripheral blood have been reported after total body radiotherapy before in patients with acute myeloid leukaemia (74). These findings would also lead us to suspect that if there was a further delay after SCR we may see changes in the other target miRNA.

**miRNA correlations with other clinical markers**

The function of the immune response as a key factor in the process of cancer is an area of intense research. Given the emerging evidence that miRNA have a role to play as key modulators of the tumour immune response (75), it would seem appropriate to evaluate for correlation between miRNA expression and inflammatory markers. For example, miR-21 has a role in cell differentiation of monocytes, macrophages, T cells and dendritic cells and is thought to indirectly target Interleukin-6 (IL-6) and promote cancer cell invasion and tumour progression (76). In this study, we did not find any correlation between miRNA relative expression and NLR or CRP (Figure 7 and 8). So far, there are no published works on the correlation of miRNA and CRP in CRC, however there is some data in studies of inflammatory conditions such as Inflammatory Bowel Disease (IBD) and Rheumatoid Arthritis (RA) (77) (78). In RA patients, a change in CRP after starting combination therapy for RA seems to be weakly associated with change in miR-126-3p ($r = 0.349$) and miR-23-3p ($r = 0.360$) (77). It is possible that the reason we did not see changes in circulating miRNA’s correlating with changing CRP and NLR could be due to the fact that there is more localised inflammation within the tumour microenvironment rather than those detectable in the peripheral circulation.

In clinical practice CEA is utilised as a non-invasive tool to attempt to detect CRC recurrence as part of ongoing cancer surveillance. Even when used in this setting, it has insufficient sensitivity and specificity to be used alone, and is often coupled with
imaging studies. Some authors report that it could be advantageous to couple CEA monitoring with miRNA tests when looking for CRC recurrence (79). There is a lack of evidence that CEA can be used for prognostic purposes and in this study we also did not find any correlation between CEA and our target miRNA expression (Figure 9).

In this study there was a strong correlation of raw Ct values of miR-21 and miR-29a ($r = 0.8107$). There was a moderate correlation between miR-29A and miR-143 ($r = 0.7219$) and miR-21 and miR-143 ($r = 0.7044$). There was a weak correlation between miR-143 and miR-145 ($r = 0.4865$). This is important for future work after this pilot study because if these miRNA expression levels are very closely correlated then they might not provide additive information. Therefore, we may not need to measure all of the miRNA that we are currently targeting. Correlations between miRNA expression in plasma have not been reported previously. Prior to making these decisions however it would be imperative to test this in larger patient groups with ROC curves looking at specificity and sensitivities of the individual target miRNAs compared to multiple miRNA measures.

**Target miRNA’s ability to predict TRG**

In this study we did not find a statistical difference in target miRNA expression in plasma in patients that responded to SCR or to LCCR. Again, it is likely that the small number of patients have impacted on this finding. In a study by D’Angelo et al. (46), the authors report 11 miRNA had differential expression in CRC patients when separated into “responders” vs. “non-responders” after LCCR therapy. In this retrospective study, serum and tissue biopsies of tumour were obtained from rectal cancer patients prior to neoadjuvant therapy being started. First looking at expression in tissue, the authors identified 11 differentially expressed miRNA using microarray analysis on 38 tissue samples (3 patients had stage II disease, the rest had stage III). The authors focused on miR-125b, miR-299-5p and miR-154, and in tissues they confirmed altered expression which was statistically significant for all of these. The authors then reviewed the expression in 34 patient’s serum, and again confirmed altered expression with miR-125b significantly increased in non-responders ($p = 0.0087$) which would allow for predictive discrimination between the two groups (AUC 0.782, 95% CI 0.6123-0.9518).
In a recently published study by Hiyoshi et al. (47), the authors report the predictive capability of serum miR-143 in TRG response in rectal cancer patients. This was a prospective study in which the authors selected 18 target miRNA to look for differential expression. They collected 94 patients’ serum in total, however it is unclear as to when in the treatment this was collected. From those 94 samples, the authors selected 13 patients with pCR and near pCR as “best” responders to LCCR, and 12 patients with no response to LCCR as “worst” responders because it was thought that this would highlight differences in expression between the groups the most. In the selected subgroups the authors found that miR-143 and miR-125b were differentially expressed, but that when testing the complete group of 94 patients, only miR-143 had a statistically significant difference between responders and non-responders ($p=0.004$).

Interestingly, of an additional 18 miRNA that these authors specifically analysed, they did not find significant differences in expression of miR-145, miR-21, and miR-29a. They did not find a significant decrease in miR-125b as the previously mentioned study (46).

Of note this study did not have an endogenous control, which is important for minimalising experimental error. Endogenous controls are important to normalise for variation in starting material volumes, reaction efficiency and sample purity (80). The authors did use cel-miR-39 as an exogenous control. As mentioned previously, this group looked at samples after neoadjuvant therapy prior to surgery in 76 of their patients and found that miR-143 expression was significantly decreased after LCCR prior to surgery. When separating out the patients into responders vs. non responders, they found that serum miR-143 was only significantly altered in the non-responder group. This does raise the question of whether testing miRNA relative expression after LCCR could help to determine whether or not a patient should proceed to surgery, and this relates to our findings in that patients with an increased miR-143 after SCR could indicate a therapeutic response to therapy.

It is important to note that miR-345 has been reported to be differentially expressed in LCCR responders vs non-responders by Yu et al. (81). This group reviewed 20 tissue samples post operatively (and post LCCR) and reviewed miRNA in their serum and found a decrease in expression in the LCCR responders ($p=0.002$). Despite this, in our
validation of our housekeeper miRNA, we found no difference in miR-345 expression pre and post treatment.

4.1 Strengths and limitations

There were a number of strengths to this study including; its prospective design, the use of both endogenous and exogenous controls, a group of well screened controls, and using both SCR and LCCR patients. The limitations were mostly regarding small patient numbers leading to a poorly powered study.

There were clear benefits to the prospective nature in which the data was gathered. First, plasma obtained before and after treatment allowed for the assessment of paired samples for miRNA levels pre and post neoadjuvant therapy. We had the benefit of standardised blood collection with high quality specimens which were processed quickly. It also allowed for the collection of demographic data and other clinically useful tests such as CRP, CEA, and NLR which could then be correlated with miRNA levels. Data on these correlations is lacking and therefore we will be able to add to the pool of research in this area (42). In addition to this, patients in the biobank will have further blood samples obtained and can be followed for outcomes such as either local recurrence, rates of metastatic disease, or disease free survival.

Regarding standardisation of RT-qPCR, this study used both endogenous and exogenous controls as housekeepers. cel-miR-39 is a reliable exogenous control to ensure accurate extraction efficiency. miR-345 has been reported to be stable in plasma and to have the same expression in CRC patients vs controls in tissue, plasma, and exosomes (53), except as noted in the study mentioned previously by Yu et al. (81). There was no difference between miR-345 or cel-miR-39 Ct values in control patients vs. cancer patients or in cancer patients pre vs. post neoadjuvant therapy and therefore we consider both of these to be adequate controls for the present study. We used both miR-345 and cel-m-39 because there are still very few reports that detail robust identification and validations strategy for suitable reference genes for normalisation.
(82), despite reliable controls being important for the reliability and accuracy of circulating miRNA biomarker research (83).

Short course radiation neoadjuvant therapy is not often considered in predictive miRNA studies. This is likely because it is unusual to see a significant amount of tumour regression when the time to surgery after radiation is short. It is therefore an interesting finding that miR-143 has increased after SCR therapy. The Stockholm III trial has demonstrated that a delay to surgery after SCR can lead to increased tumour regression with statistically significant higher rates of pCR and Dworak grade 4 regression (73). In this study, we looked at time to surgery after neoadjuvant therapy ended to see if any delays had confounded our results. It would also be interesting to compare post SCR levels with post surgical levels and see if they decrease dramatically.

A clear limitation to this study is the small data set. This is due to the frequency of patients presenting with rectal cancer requiring neoadjuvant therapy at Wellington Hospital. Waiting for long course treatment then the stand down period prior to surgery has allowed for this sample size. Having more patients and therefore more plasma samples will be beneficial in that it will reflect the natural heterogeneity of the disease (42). Having said this, previous publications do have smaller or similar data sizes (46) and this is a pilot study.

Importantly, another limitation was that we did not have any patients with pCR. Unfortunately, this is not something that could have been altered and is likely a reflection of this being a small study. This could mean that our samples are likely to reflect a subtler phenotype difference than the other studies we are comparing our results too.

In this study we have used a hypothesis driven approach rather than discovery driven approach. Therefore, we have analysed expression of only four target miRNA’s. It would be ideal to have performed microarray or deep sequencing to “discover” which miRNA had altered expression first but we have used this study design due to available time and resources.
Regarding patient outcomes, what is most clinically applicable to the patients undergoing SCR is local recurrence. In this study we have used TRG as a surrogate for recurrence due to the reasonably short length of time that the biobank has been operating. It is interesting that circulating miR-29c has been reported to be increased in patients who have early recurrence within one year of surgery ($p = 0.012$) (84). As patient follow up continues, recurrence data will be available to be analysed.

4.2 Future directions

The target miRNA we have chosen have not yet shown statistically significant differences in our patient population in plasma regarding predicting TRG. I would continue to investigate these target miRNA in a larger patient population.

With the samples we are collecting for the CRC biobank, we will be able to look at recurrence rates and survival outcomes with this patient group and see if there is any correlation with our target miRNA expression levels. It is possible that there is some clinical significance to the change in miRNA-143 after therapy. As previously stated it would be interesting to further analyse this group to see what the outcomes are for these patients clinically.

Given promising findings with miRNA in serum, further work should be focused on blood based biomarkers where possible.

4.3 Overall significance

New Zealand has a high incidence of CRC by international standards, with over 1200 deaths annually (7). Specifically, rectal cancer contributes to a significant proportion (25%) of these cases (7). Despite advances in the detection and surgical management of rectal cancer, we still lack the ability to predict which patients will respond to neoadjuvant therapy. This thesis has examined the levels of miR-21, miR-29a, miR-145 and miR-143 in plasma in patients with rectal cancer undergoing neoadjuvant therapy.
This is the first study to use patients’ plasma to evaluate the expression of these miRNA and examine if this is related to TRG. This is important because blood based biomarkers are a much more accessible and potentially clinically useful tool for prediction than tumour biopsy. Findings from this study will contribute to the pool of research on the use of miRNA’s in CRC for diagnostic and predictive tests. Future samples being collected from these patients may also contribute to knowledge on recurrence.
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