Extracellular vesicle microRNA as liquid biopsy biomarkers in early stage colorectal cancer patients

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A thesis

submitted to the University of Otago

in fulfilment of the requirements for the degree of

Master of Medical Science

University of Otago

2019

Abstract

New Zealand has one of the highest rates of colorectal cancer (CRC) in the world. Current strategies to diagnose CRC, including population level stool-based screening, are flawed due to poor sensitivity in early disease and limited participation. Patients have expressed a preference for a blood-based test, or 'liquid biopsy', if it were available. A liquid biopsy that has parity with, or outperforms current stool based screening would likely lead to earlier diagnosis of CRC and improved patient outcomes. Extracellular Vesicles (EVs) are produced in abundance by CRC cells, their contents also reflect their parent cell of origin. microRNA (miRNA) are small non-coding RNA molecules, which are dysregulated in CRC cells compared to normal cells. Identifying EV miRNA in the bloodstream of early stage CRC patients that are sufficiently different from healthy patients may enable identification of CRC from a liquid biopsy. Our study examined four miRNAs, miR-19a, miR-23a, miR-183 and miR-1246, in both tumour tissue and plasma EVs that have previously been reported as dysregulated in CRC.

miRNA expression of the candidate miRNAs was in examined in CRC tumour tissue vs normal colonic mucosa, and in plasma EVs of stage I and II CRC patients vs healthy controls, by RT-qPCR. Changes in expression in tissue were assessed to establish if they would translate to circulating EVs. Furthermore, any differences between the miRNA expression in plasma EVs were examined to assess for a capacity to differentiate early stage CRC patients from healthy controls and be useful as a liquid biopsy biomarker in CRC.

From our results, miR-1246 and miR-183 were significantly overexpressed in tumour tissue compared to normal colonic mucosa. However, neither miRNA was consistently expressed in the circulating EVs. In the plasma analysis, miR-19a was significantly downregulated in EVs of CRC patients. It demonstrated significant differences even when stage I patients alone were compared against controls and it also had superior sensitivity to CEA in our cohort. This appears to be the first study to document a significant decrease in miR-19 in the EVs of early stage CRC patients. From our results, significant variance from the current literature was seen. Some possible factors for this variance may include differences in methodology and control selection.

Although, plasma EV miRNA display great promise as liquid biopsy biomarkers, there are a number of challenges in identifying a marker specific for CRC. Standardisation of methodology may help identify candidate miRNAs that offer the greatest potential. Finding a liquid biopsy biomarker for CRC that is equally effective, or outperforms, current faecal-based methods would likely increase uptake in screening. The subsequent effects would include decreased healthcare costs due to earlier diagnoses of CRC with improved patient outcomes for the growing millions of people who will suffer from this disease.

Acknowledgements

Firstly, I would like to sincerely thank my two supervisors, Associate Professor Elizabeth Dennett and Dr Kirsty Danielson. Thank you for your patience, advice and encouragement over the past year. Liz, I would specifically like to thank you for all of the amazing coaching I received prior to presenting for the Louis Barnett prize. I am aware that you took a lot of extra time out of your busy schedule to help me and I think it made a huge difference.

Kirsty, I am so grateful for all of your help this year. Taking someone without a science background into your group, and all of the extra work that goes with it, is a huge undertaking. Your door has always been open to me and no problem has ever been too small for you to help with, particularly when I have been having a nightmare with stats. I could not have asked for two better supervisors and I feel very lucky to have worked with both of you.

I would like to thank my colleagues in the lab group, Bianca Black, Annabelle Greenwood, Olivia Buchanan and Emma Symonds. All of you have been a wonderful help over the past year especially with your assistance in collecting samples for the biobank while I was away on leave. Thank you for helping learn everything that goes into working in the lab, particularly considering I had negligible experience. I have really appreciated your teaching, assistance and encouragement over the past year.

The members of the Surgical Cancer Research Group have been more than accommodating in helping us collect samples for the biobank, without which none of this work would be possible. Mr Ali Shekouh and Mr Alex Dalzell have had their clinics, endoscopy lists and operating theatres interrupted to recruit patients and collect samples. I am grateful to all staff involved in all of these settings, we appreciate things can be extremely busy, and we have never been made felt anything other than welcome. I would also to thank Mr John Keating, who stopped in and offered coaching and advice prior to the Louis Barnett prize presentation, even though he primarily works in another hospital.

This year would have been much more stressful without the help of Sara Farrant, Colorectal CNS. You have constantly helped with notifying me of new patients, finding patients in clinic and almost everything else I could need assistance with.

Most patients have been so well informed by you about the research that consenting them for research is painless. You are fantastic at what you do and we are all lucky to work with you.

The entire department of Surgery & Anaesthesia have been nothing but welcoming from my first day. I have been made feel entirely at home and it has been a pleasure to work with all of you. You are all incredibly hardworking, intelligent and most of all hilarious. I have no doubt that each and every one of you will continue to do great things in your chosen field.

I was extremely grateful to be given funding in the form of a 'Research for Life' grant from the Wellington Medical Research Foundation. This funding has enabled me to carry out this research exactly as I would have hoped, as we were able to purchase enough materials to assess all of the markers that we had identified.

I would like to thank my family and friends who have been a constant source of encouragement during this past year. Finally, to my partner Jess, the number of things I need to thank you for are too numerous to count. You are amazing and constant source of inspiration. Thank you for always supporting and encouraging me even when you are the one who often has to lose out. I am so grateful to have you behind me in everything I do, your support means the world to me.

Contents

List of tables

List of figures

Abbreviations

5-FU	5-Fluorouracil
AFC	Automated Fraction Collector
ANOVA	Analysis of Variance
AUC	Area under the curve
CEA	Carcinoembryonic antigen
CRC	Colorectal cancer
CRP	C-Reactive Protein
CT	Computed Tomography
C_T	Cycle Threshold
(c)DNA	(Complementary) Deoxyribonucleic acid
ED	Emergency Department
EDTA	Ethylenediaminetetraacetic acid
EMVI	Extramural venous invasion
EV	Extracellular Vesicle
FBC	Full Blood Count
FFPE	Fixed-formalin Paraffin-embedded
FIT	Faecal Immunochemical Test
FOBT	Faecal Occult Blood Test
FOLFOX	Folinic acid, Fluorouracil, Oxaliplatin chemotherapy
LVI	Lymphovascular invasion
MDM	Multidisciplinary Meeting
MMR	Mismatch Repair
miRNA	micro-RNA
MRI	Magnetic Resonance Imaging
MSI	Microsatellite instability
NBSP	National Bowel Screening Programme
NLR	Neutrophil to Lymphocyte ratio
NOS	Not otherwise specified
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PNI	Perineural invasion
SCRG	Surgical Cancer Research Group
RNA	Ribonucleic acid
ROC	Receiver Operating Characteristic
RT	Reverse Transcription
RT-qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
SD	Standard Deviation
SEC	Size Exclusion Chromatography
SST	Serum-separating tube
TNM	Tumour Nodes Metastasis staging
UC	Ultracentrifugation

Chapter 1: Introduction

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1.1 Colorectal cancer

Colorectal cancer (CRC) is a significant health issue, both internationally and in New Zealand. Globally, CRC is the third most common cancer with 1.8 million new cases diagnosed annually (1). Additionally, it is the second leading cause of cancer related mortality with 881,000 deaths estimated in 2018 (1). A decline in both incidence and mortality has been observed in some high-income countries (2), however the global burden of CRC is expected to increase significantly in coming decades. Given demographic projections, in just over a decade it is predicted that there will be an increase to more than 2.2 million new CRC diagnoses and 1.1 million deaths by 2030 (3).

New Zealand and Australia have some of the highest rates of CRC in the world (1). International trends are reflected in New Zealand where CRC is the second most common cancer after non-melanoma skin cancer, with approximately 3200 new cases diagnosed and 1200 deaths annually (4). Although, overall population rates of CRC have started to decline, it still places significant resource demands on our health system. Due to both population growth and aging, the numbers of new CRC diagnoses in New Zealand are likely to increase (4). Cases of CRC are forecast to increase by approximately 26% by the year 2026. This will likely lead to a proportional increase in demand for resources required for the diagnosis, treatment and follow up of CRC patients such as colonoscopy, surgery, chemotherapy, radiotherapy and outpatient appointments (4). The predicted yearly costs for CRC is expected to rise from $83.6 million in 2014 to $100 million in 2026 (4). As less intensive treatments are required for CRC if diagnosed at an earlier stage, measures that help increase the rate of diagnosis of early stage CRC may help to mitigate some of these expected cost increases.

One of the most important predictors of survival in CRC is the extent of disease at diagnosis. The most widely used classification to quantify the extent of CRC is the tumour, nodes and metastases (TNM) classification (5). This measures the depth to which the tumour has invaded the bowel wall (T), presence and number of involved lymph nodes (N) and the presence of distant metastases (M) (see supplementary

Table S1 for further detail on TNM staging in CRC). In general, adjuvant or neoadjuvant treatments, in the form of chemotherapy and radiotherapy are reserved for patients with more advanced CRC. For the majority of patients diagnosed at a very early stage, surgery alone will be sufficient. In addition to requiring less intensive treatment, lower TNM stage is significantly associated with a benefit for most clinical outcomes including, lower rates of local or distant recurrence, improved disease free and overall survival (6-8).

Early diagnosis is key to reducing CRC related mortality (9). Five-year survival for stage I colon cancer is 80%, which drops to 6% for stage IV disease (Table 1; 10). Prior to the introduction of a bowel-screening programme, New Zealand has lower rates of detection of early stage CRC when paralleled to some other comparable health systems that have established screening programmes. Stage I colon cancer only makes up 12% of diagnosed cases in New Zealand, in the UK rates of stage I diagnoses have been reported as high as 18% (4, 11). The proportion of stage IV disease for both colon (19%) and rectal (24%) cancers are higher in New Zealand compared to Australia (17-19%) and the UK (18%; 4, 11). This data further demonstrates that any strategies which aid earlier diagnosis of CRC should improve patient outcomes and reduce CRC related mortality.

	Colon Cancer		Rectal Cancer	
	New diagnoses (%)	**Five-year survival (%)**	**New diagnoses (%)**	**Five-year survival (%)**
Stage I	503 (12%)	80%	1066 (76%) (Stage I-III)	65% (Stage I-III)
Stage II	1139 (27%)	71%		
Stage III	1035 (25%)	(N1) 63%		
		(N2) 50%		
Non-metastatic NOS	228 (5%)			
Stage IV	991 (24%)	6%	271 (19%)	10%
Unknown	297 (7%)		64 (5%)	
Total	4193 (100%)		1401 (100%)	

Table 1*- Colon and rectal cancer rates of diagnosis by stage* (4) *and five-year survival by stage* (10) *in New Zealand. (N1 = Metastasis in 1-3 regional lymph nodes, N2 = metastasis in 4 or more regional lymph nodes, NOS = not otherwise specified).*

The PIPER project included 5667 patients from across New Zealand over a 3 year period. It provides an insight into the landscape for CRC in New Zealand prior to the introduction of screening. The average ages for colon and rectal cancer were 67.9 and 71.4 years respectively (12). In the total recorded population, 75% were diagnosed with colon cancer and 25% with rectal cancer (12). With regard to ethnicity, 8% were Māori, higher proportions of metastatic disease at diagnosis are observed in Māori and Pacific populations. Rates of diagnosis for stage IV colon cancer being 32% and 35%, and for rectal cancer being 29% and 22% for Māori and Pacific patients, respectively (4). In non-Māori, non-Pacific patients the rates of metastatic disease at diagnosis were 23% and 18% for colon and rectal cancer respectively. The emergency department (ED) was the initial mode of presentation for 34% of patients with colon cancer, which is again higher for Māori (44%) and Pacific patients (51%). In contrast, only 20% of CRC patients are diagnosed through an emergency presentation in the UK (13). Methods that aid in the earlier detection

of CRC that are easily accessible in a primary care setting may contribute to decreased rates of metastatic and emergency presentations of CRC. This may contribute to decreasing the overall morbidity and mortality of CRC in the population and addressing inequalities in healthcare.

1.1.1 Presentation and diagnosis of colorectal cancer

Currently, a diagnosis of CRC is made following symptomatic presentation, due to the bowel-screening programme, or as an incidental finding during investigations performed for unrelated reasons. Symptoms include weight loss, change in bowel habit, and perirectal bleeding; however, patients are often asymptomatic at early stages of the disease (14). Colonoscopy is the gold standard investigative and diagnostic tool in CRC. Patients in New Zealand currently have access to endoscopy through surveillance if they are at increased risk, if they are symptomatic of CRC or if they have a positive bowel-screening test.

During colonoscopy, any suspicious precancerous polyps are removed, and should a cancer be found, biopsies can be taken for histological evaluation. Despites it's obvious utility, colonoscopy has some limitations and disadvantages including: extensive pre-procedure preparation, patient discomfort, and high healthcare costs that hinder its universal application as a screening tool (15). Patients who undergo polypectomy may occasionally develop post-polypectomy haemorrhage, including from extremely low risk lesions that would be unlikely to ever cause harm, and which would not have been seen by other investigative modalities (16). New Zealand data on colonoscopy complications show a rates of post-colonoscopy bleeding requiring hospital presentation and perforation of 0.79% and 0.12% respectively (17).

Ideally, a colonoscopy would only be performed on patients that stand to benefit from the procedure. However, due to its nature as an investigative tool, there are a significant number of entirely normal colonoscopies performed. Data from the National Bowel Cancer Screening Programme pilot study states that 45% of positive screening tests result in an entirely normal colonoscopy (17). These patients are unnecessarily exposed to some of the risks of colonoscopy in addition to consuming a limited resource. Any diagnostic test for CRC with increased specificity compared

to current methods may help decrease the number of these unnecessary colonoscopies and assist in more efficiently deploying finite resources.

Once a diagnosis of CRC is made, it is necessary to promptly stage the disease. This includes a full medical history and physical examination including digital rectal examination and rigid sigmoidoscopy for rectal cancer. Blood tests including complete blood count, liver and renal function tests, and carcinoembryonic antigen (CEA) are performed. Radiological imaging including a Computed Tomography (CT) scan of the chest, abdomen and pelvis, in addition to Magnetic Resonance Imaging (MRI) of the pelvis for rectal cancer. The management of CRC is guided by both patient and disease factors. It is recommended that all patients with CRC are discussed in a colorectal multidisciplinary meeting (MDM) (18). At that stage a decision is made regarding planning of treatment (Figure 1).

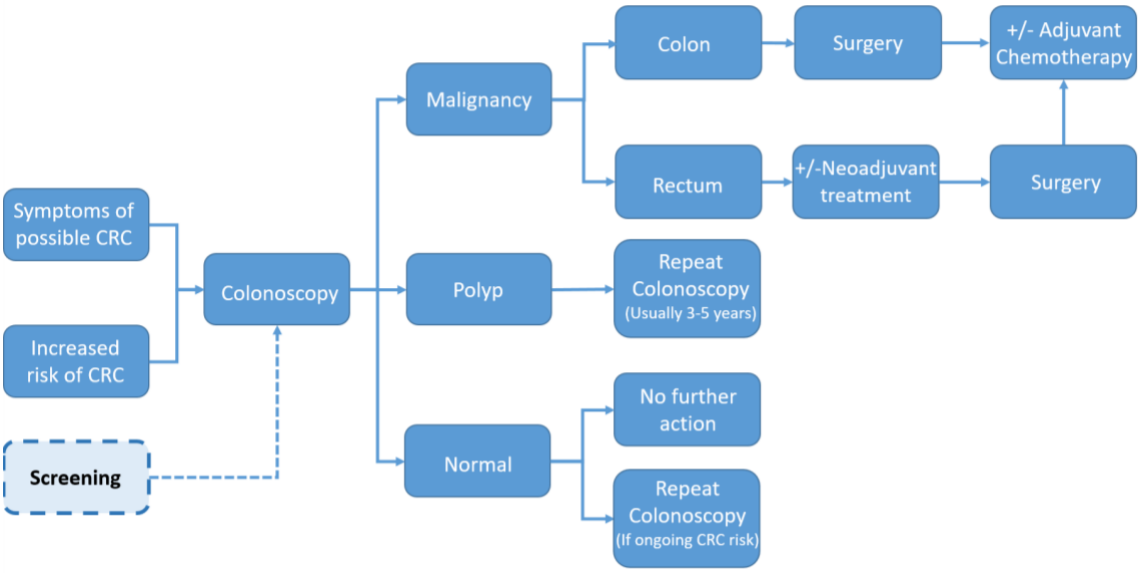

Figure 1*- Simplified current diagnosis and treatment pathway for non-metastatic (stage I-III) CRC in New Zealand.*

If the disease is non-metastatic (stage I-III), surgical resection is the standard of treatment in colon cancer. For those diagnosed with rectal cancer, there is a possibility of neoadjuvant treatment in the form of either radiotherapy alone or chemo-radiotherapy in order to downstage the disease prior to surgery. The decision to choose neoadjuvant therapy depends on many factors including both the extent of disease at diagnosis and the patients' suitability to undergo treatment. Less advanced rectal cancer is less likely to require neoadjuvant treatment.

Similarly, adjuvant chemotherapy is given to selected patients following surgery that are thought to be at increased risk of recurrence due to more advanced TNM staging. Methods that assist in the earlier diagnosis of CRC may lower the numbers of patients requiring neoadjuvant and adjuvant treatment and help decrease the overall cost of treating this disease while also improving patient outcomes.

1.1.2 Bowel Cancer Screening

Population level bowel cancer screening programmes have been demonstrated to decrease mortality from CRC (19, 20). Additionally, screening programmes have also been found to identify CRC at an earlier stage, which leads to decreased costs as less intensive treatments are required (21). The New Zealand Ministry of Health is currently implementing the National Bowel Screening Programme (NBSP). This will be introduced to District Health Boards on a staged basis, and is planned to be in place nationally by 2021. Screening programmes have been shown to be either cost effective or cost saving wherever they have been implemented (22). Recent data on the Australian bowel-screening programme has predicted that CRC-related mortality and overall healthcare costs can both be decreased with any increase in the screening participation rate (23). While colonoscopy has been adopted as an effective first line screening tool in a number of countries, issues with resources and invasiveness, as discussed in the previous section, have precluded its universal adoption (24). An increasing number of countries, including New Zealand, have instead opted for stool-based screening methods (24).

Stool-based population screening programmes have been adopted in at least twelve countries (24). Stool-based testing includes Faecal Occult Blood Testing (FOBT) and Faecal Immunochemical Testing (FIT). FOBT is a guaiac based test that detects occult blood in the stool, FIT are immunoassays specific for human haemoglobin (25). FIT is preferable over FOBT for population based screening due to its higher sensitivity (79% vs. 71.2%) and comparable specificity (94% vs 93.6%) for CRC detection (26-29). The major limitation of FIT as a screening tool is its limited sensitivity for detection of adenomas and early stage CRC. FIT has a higher rate of false-negative results for carcinoma *in situ* and T1 cancer when compared to more advanced CRC, with a test sensitivity as low as 66.7% for these early lesions (30). A diagnostic or screening test with improved sensitivity compared to FIT may

increase pick-up rates of early stage CRC and thus contribute to both decreased CRC-related mortality and decreased overall healthcare expenditure.

While the adoption of population based screening is a positive step toward reducing CRC-related mortality, screening uptake is not universal and in New Zealand will only be available between the ages of 60-74 (24). A considerable number of patients with CRC are either not eligible or do not participate in screening, likely only being diagnosed after becoming symptomatic following progression of disease (31). Uptake rates of stool-based screening have been reported at only 41-57% (17, 32, 33) and research suggests that there is a patient preference for blood-based, compared to faecal-based, screening if it were available (34). From a recent NBSP pilot study, a participation rate of 56.9% was seen in a New Zealand population (35). A study from 2012 found that 78% of those surveyed would prefer to give a blood sample for CRC screening over a faecal-based test (34). These findings are compounded by recent data from Australia suggesting that if the screening participation rate were to rise from 40% to 60% there would be significant decreases in CRC-related mortality as well as significant healthcare cost savings (23). Were a blood-based screening test, or 'liquid biopsy', available it may serve to increase participation rates and thus take advantage of some of these potential benefits.

1.2 Liquid Biopsies

The concept of a liquid biopsy stems from the discovery that cancer cells secrete molecules into the circulation that contain signature markers of their cell of origin (36, 37). The advantages of blood-based screening include minimal invasiveness, repeatability, and possible improved uptake compared to stool based methods (34). If an effective liquid biopsy biomarker were established it might have a role in augmenting, or replacing, the current stool-based screening strategies.

Currently, no liquid biopsy markers are routinely used for screening or diagnosis of CRC. CEA is commonly used to monitor for recurrence; however, issues with sensitivity and specificity negate its use in diagnosis or screening (38). The US Food and Drug Administration has recently approved a blood-based marker that measures methylated Septin9 DNA by real-time polymerase chain reaction (PCR)

for CRC screening. While early case-control studies of this test reported a 70% sensitivity and 90% specificity for CRC detection, testing in an asymptomatic cohort found only 35% sensitivity with 91% specificity for the detection of stage I CRC. This would hinder its viability for population-based screening for early stage cancers (39-41). Another blood-based marker in CRC is the neutrophil-to-lymphocyte ratio (NLR), increased NLR at diagnosis has been significantly associated with poorer tumour differentiation and overall survival and it may have a role as a prognostic marker (42, 43). For any potential liquid biopsy biomarker to replace current stool-based screening methods, it would need to have at least comparable sensitivity and specificity to strategies already in use.

The development of liquid biopsy biomarkers with higher sensitivity for early detection of CRC has the potential to improve rates of CRC-related mortality in addition to possible cost-saving benefits. Novel diagnostic and screening strategies may be used either in conjunction with, or as a replacement for, current stool-based screening programmes. Various molecules have been identified as having potential as liquid biopsy markers. These include circulating tumour cells that originate from primary or metastatic sites, circulating cell-free tumour DNA, as well as microRNA (miRNA) which can be free in the circulation or encapsulated in extracellular vesicles (EVs) (44, 45).

A significant amount of ongoing research is focused on the rapidly developing field of EV biomarkers. EVs are stable in the circulation and under various storage conditions (46, 47). The contents of EVs are protected in the circulation by encapsulation within a membrane; in addition, EV contents reflect the unique profile of their cell of origin (46, 47). There has been increasing interest in examining the non-coding RNAs within these EVs, particularly miRNA, as candidates for liquid biopsy biomarkers.

1.2.1 Extracellular Vesicles

EVs are a diverse group of membrane bound particles released from all human cells (48). They have become of particular interest to cancer biomarker researchers due to the ability of tumour cells to secrete large amounts of EVs in comparison to normal cells, which also contain protected tumour-specific cargo (49-51). EVs also

have a role as functional mediators of cancer cell biology. They can act in a paracrine fashion locally within the tumour microenvironment, and in an endocrine manner at distal sites via the circulation (46, 52, 53).

EVs are divided into 3 main classes based on biogenesis: exosomes (~40-100nm) originate in multivesicular bodies from the cell's endosomal system; microvesicles (or ectosomes/microparticles; 100nm-1µm) are formed from outward budding of the plasma membrane (54-56); and, apoptotic bodies (1-5µm) arise from dying cells undergoing apoptosis (57-59). In addition to these classes, some cancer-specific subtypes of EVs have been identified. Oncosomes (100-400nm) are produced by non-transformed cells and their contents can produce oncogenic effects (60, 61), whereas large oncosomes (1-10µm) arise from malignant cells and are more atypical in morphology (62-64).

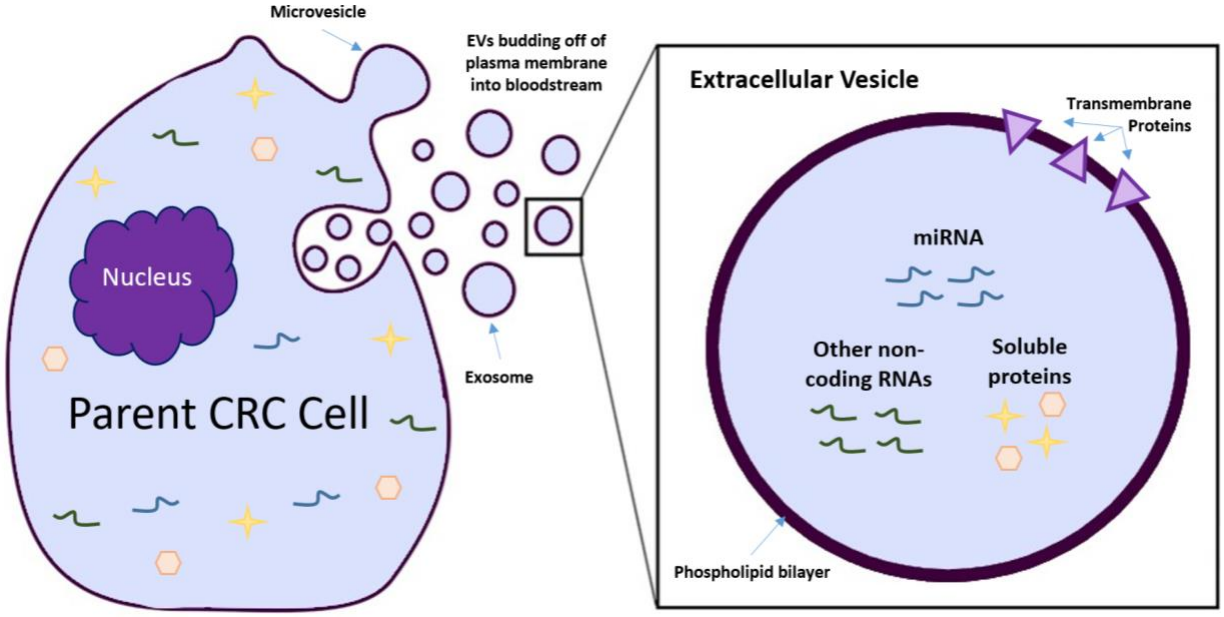

***Figure 2**- Origin of Extracellular Vesicles (EVs) demonstrating the formation of EVs from the parent cell and the encapsulation of their contents, including miRNA, within a membrane in the circulation (adapted from de Jong et al., 2019 (65)).*

EVs contain a range of contents including proteins, lipids, and RNA that directly reflect the parent cell of origin (Figure 2; 46, 47). EVs are robust within the circulation, thus their unique contents offer an attractive target as a possible liquid biopsy biomarker. It also appears that cancer EVs contain higher amounts of certain molecules such as miRNA compared to EVs from other cell types (66). Additionally,

within tumour cell EVs it has been observed that pre-miRNA can be processed to mature-miRNA, a feature that is not present in EVs from non-cancerous cells (66). This may further add to the differentiation and specificity of the unique constituents of tumour cell EVs that adds to their potential as biomarkers in CRC.

1.2.2 Extracellular Vesicle miRNA

miRNA are single stranded, non-coding RNA molecules of approximately 18-22 nucleotides that have been implicated in a host of normal biochemical processes including cell differentiation, proliferation, and apoptosis (67-70). miRNA levels are significantly dysregulated in CRC tumour tissue compared to normal colonic mucosa (71). These abnormally expressed miRNAs may play a role in tumour suppression, tumorigenesis and progression (72, 73). As the profile of miRNA that is produced by tumour cells is significantly different to healthy cells they offer an attractive target as possible liquid biopsy biomarkers.

In addition to dysregulation in tumour tissue, accumulating research points to the existence of unique miRNA signatures in body fluids that may function as diagnostic and prognostic biomarkers for cancer (74-76). Circulating miRNAs remain stable in serum or plasma under a host of unfavourable conditions including extreme temperatures and repeated freeze-thaw cycles (77, 78). The stability of miRNAs in the circulation is due to a number of factors protecting them from degradation by RNAses, this includes their encapsulation in EVs (Figure 2), in addition to their association with carrier molecules such as Argonaute-2 and lipoprotein complexes (78-80). This notable stability increases the appeal of these molecules as potential biomarkers in CRC and other diseases.

There is some evidence that EV-specific miRNA may be of greater utility with regard to developing a liquid biopsy in CRC compared to total circulating miRNA levels. This is based on the theory that EV miRNA profiles are more specific for tumour-derived signatures than total circulating miRNA due to the large volume of EVs released by tumour cells (81). While sources of circulating EVs include platelets, red blood cells, and immune cells, in addition to tumour cells (82), CRC tumour cells have been shown to release EVs in abundance *in-vitro* (83). This data suggests that EV miRNAs have significant potential as liquid biopsies due to both their robustness

in the circulation and subsequent storage in addition to the specificity of their contents in reflecting the genetic makeup of their cell of origin.

1.3 EV-miRNA in colorectal cancer

EV-miRNA are dysregulated in numerous cancers and have recently become the focus of an extensive amount of work aiming to identify novel biomarkers of disease (71, 81, 84). There have been a substantial number of circulating miRNAs suggested as potential CRC biomarkers; examples include miR-1246, miR-19a, miR-23a and miR-183 (85-87). Studies have focused mainly on EV-miRNA derived from primary CRC tissue culture, *in-vitro* CRC cell lines, serum or plasma. Focusing specifically on EV miRNA that become dysregulated in stage I and II CRC may have greater clinical relevance to developing a blood-based test for diagnostic or screening purposes. The earlier CRC can be detected the lower the overall mortality as well as overall healthcare expenditure.

1.3.1 miR-1246

miR-1246 has attracted interest as a possible biomarker in CRC. EV miR-1246 levels have been shown to be elevated in serum, plasma and CRC cell lines, but with conflicting results in tumour tissue (81, 85, 86, 88-91). miR-1246 may have a role in promoting tumour progression. Yamada et al. have demonstrated that miR-1246 and transforming growth factor (TGF-β) are transported in microvesicles from CRC cells to endothelial cells. Their subsequent effect on Smad 1/5/6 signalling modulates the tumour environment to promote angiogenesis and tumour growth (90).

miR-1246 in tissue and CRC cell culture

miR-1246 has been observed to be both elevated and suppressed in CRC tumour compared to normal colonic tissue (90, 91). Scarpati and colleagues (2014) examined CRC tissue from 57 CRC patients, of which 24 were stage I and II, compared to normal stroma. Their results found miR-1246 to be upregulated relative to normal colonic tissue, with a 2.12 fold change (p<0.0001; 91). Yamada et al. (2014) conversely, demonstrated a mild decreased expression of miR-1246 in tumour tissue compared to normal colonic tissue in 33 CRC patients of which 13

were stage I and II (90). Methodology differed in the processing of tissue samples between these studies. Scarpati and colleagues (2014) liberated tissue samples from formalin-fixed, paraffin-embedded (FFPE) slides, whereas Yamada et al. (2014) immediately froze the CRC tissue in liquid nitrogen (90, 91). This may be a possible reason for conflicting findings.

Yamada and colleagues also examined EVs isolated from CRC culture media from DLD-1, WiDr, SW480 and COLO201 cell lines (90). Their findings demonstrated a significant enrichment in expression of miR-1246 within the EVs when compared to intracellular levels ($p<0.01$; 90). A further study also observed a similar pattern, miR-1246 was found to be elevated in CRC cell culture media EVs when isolated from the LM1863 cell line when compared to the cell lysate (88). The findings of these studies suggest that miR-1246 may be packaged into EVs and subsequently released from CRC cells. This finding offers promise that miR-1246 may be elevated in EVs in the bloodstream.

miR-1246 in plasma and serum EVs

EV miR-1246 has been observed to be elevated in stage I and II CRC in both serum and plasma in a number of studies (81, 85, 86, 90). Serum EV miR-1246 was found to be elevated 2.23 fold in a cohort of 209 patients, of which 107 were stage I and II, when compared to 28 healthy controls (85). Serum EV levels have also been observed to decrease following resection of CRC tumours (86). Additionally, significantly elevated serum EV levels have also been associated with liver metastases in patients with CRC compared to healthy controls (85).

In a study by Ogata-Kawata et al. (2014) examining serum EV levels of miR-1246 to differentiate 88 CRC patients of all stages from healthy controls found a 95.5% true positive and 9% false negative rate. Using Receiver Operating Characteristic (ROC) curves, a corresponding Area Under the Curve (AUC) of 0.948 was demonstrated. A further small validation cohort in the same study demonstrated a 90% sensitivity for differentiating stage I CRC patients alone from healthy controls (86).

In addition to dysregulation in serum, altered plasma EV miR-1246 levels have also been observed. Plasma EV miR-1246 was significantly elevated when comparing 46 stage II CRC patients to 50 healthy controls ($p<0.0001$; 81). Additionally in murine

models transfected with human CRC, elevation of plasma EV miR-1246 was demonstrated in 61% of samples (90). Circulating EV miR-1246 has been demonstrated to be dysregulated in both serum and plasma. In addition it appears to have an ability to discriminate early stage CRC patients from healthy controls, its role as a possible liquid biopsy biomarker warrants further examination.

1.3.2 miR-19a

A growing body of research is establishing the functional role miR-19a has to play in the development of CRC. Functional markers that become more dysregulated along with progression of disease are another possible target for potential liquid biopsy biomarkers. miR-19a is part of the miR-17-92 cluster consisting of six miRNA genes, miR-17, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a (92).

Among the miRNAs within the miR-17-92 cluster, miR-19a is an important oncogenic miRNA (93). Elevated expression of miR-19a has been associated with activation of the Wnt/β-catenin pathway. This activation appears to have an effect on tumour proliferation, invasion, progression and angiogenesis that has been demonstrated in a number of studies (94-96). However, a recent study by Chen et al. (2018) has contradicted these findings and suggested that overexpression of miR-19a inhibits CRC angiogenesis and thus progression by inhibiting KRAS (97). Whilst the exact role of miR-19a remains to be fully defined, it can be seen from the current data that altering its expression has an effect on CRC tumours.

miR-19a in CRC cell culture and tissue

miR-19a has been observed to be enriched in EVs isolated from CRC cell culture models. It appears to be upregulated in A33 positive EVs isolated from culture from the LM1863 CRC cell line (88). It has been found to be upregulated in tissue samples from CRC liver metastasis when compared to primary stage I and II CRC tumours (98). The current data on miR-19a in tissue appears to be limited and may merit further assessment.

miR-19a in serum EVs

Serum EV miR-19a is upregulated in both early and late stages of CRC (85, 99). It was able to discriminate 107 stage I and II CRC patients from 16 healthy controls

(p<0.001; 85). Elevated levels of serum EV miR-19a were associated with serosal invasion, lymphatic invasion, lymph node and liver metastasis, in addition to higher TNM stage and poorer overall survival (85). It has also been observed by Chen et al. (2013) to be significantly increased in serum of CRC patients resistant to FOLFOX chemotherapy when compared to patients with a good response (*p*=0.009; 99).

The current evidence suggests that miR-19a is dysregulated in the serum EVs of early stage patients. It may have a role in tumour development, as well as the response to chemotherapy. miR-19a appears to play an important functional role in CRC and may be a promising candidate for a liquid biopsy biomarker.

1.3.3 miR-23a

miR-23a has been observed to be dysregulated in CRC tissue and cell culture, additionally altered expression has been seen in EVs in both cell culture media and from the circulation (36, 86, 100-102). There is an increasing body of data regarding its possible role as a biomarker and it appears to be a potential candidate for a liquid biopsy. miR-23a forms a cluster with miR-27a and miR-24-2 (103). It has been suggested that 23a may promote the migration and invasion of CRC cells and also appears to have a role in promoting resistance to chemotherapy agents such as 5-FU in at least two studies (103-105).

miR-23a in tissue and CRC cell culture

miR-23a has been shown to be upregulated in CRC tissue samples from patients of all stages in a number of papers, with at least a 2 fold change in expression, when compared to matched normal colonic mucosa (100-102). These findings have been reflected in CRC cell lines (DLD-1, HCT116, SW620, WiDr and SW480) where miR-23a has been found to be overexpressed compared to the normal colonic tissue cell lines CCD18Co, FHC and CCD-841 CoN (101, 102). Furthermore, its presence has been found in EVs isolated from LIM1863 CRC cell culture media (88).

Interestingly, it appears that miR-23a may be preferentially upregulated in earlier stage CRC tumour tissue. miR-23a had increased expression in CRC tissue from stage I and II patients when compared to both adenomas and carcinoma *in situ* (*p*=0.0001). Elevated miR-23a expression was specific to early stage CRC tissue,

as tissue from stage III and IV patients had lower miR-23a levels (p=0.0001; 103). While a biomarker which is elevated in early stage disease is promising, if it were to decrease again in late stage disease this may affect its utility as a liquid biopsy for screening as patients presenting with more advanced cancers may be missed.

miR-23a in serum and plasma

miR-23a has also been found to be elevated in EVs in CRC from the circulation (36, 85, 86, 88). Plasma and serum EV levels have been shown to be upregulated in stage I and II CRC and to decrease post resection (36, 86). In addition, serum EV miR-23a appears to have potential in differentiating CRC from healthy controls with an AUC of 0.953, when comparing 88 CRC patients of all stage, of which 40 were stage I and II, against 11 healthy controls (86). miR-23a was found to be elevated 2.7 fold by Matsumara et al. (2015) when comparing serum EV-miRNA from a group of 209 CRC patients, of which 107 were stage I and II, against 28 controls (85).

A number of studies have demonstrated miR-23a may be dysregulated in early stage CRC. Furthermore, the literature has suggested it may have an ability to differentiate early stage CRC patients from healthy controls; this makes it an attractive target possible for a liquid biopsy. Although, a caveat of its use include the possibility of it decreasing in later stage disease.

1.3.4 miR-183

miR-183 has been demonstrated to promote proliferation and invasion of CRC in *in-vitro* models (106). It has also been found to be overexpressed in CRC types with mismatch repair (MMR) deficiency (107). With regard to clinical outcomes, it has a correlation with elevated TNM stage, lymph node and distant metastasis, in addition to an association with decreased disease free survival and overall survival (108, 109). It appears to be consistently dysregulated in tissue, but a role as a possible circulating EV biomarker does not appear to have been thoroughly investigated.

miR-183 in tissue and CRC cell lines

A study by Nagy et al. (2017) found miR-183 to be overexpressed in both colonic adenoma and CRC compared to normal colonic tissue (110). Other studies have demonstrated a significant elevation in CRC tissue compared to normal mucosa in

studies examining 94, 42 and 48 patients of all stages respectively (108, 109, 111). Its presence in EVs from CRC cell culture does not appear to have been established.

miR-183 in the circulation

miR-183 levels have been found to be significantly elevated in total plasma of CRC patients when compared to controls. It has been shown to decrease following resection and is associated with tumour recurrence (87). miR-183 appears to be consistently dysregulated in CRC tissue and its total levels in the circulation also appear to be elevated in the context of CRC. However, levels of expression in EVs in CRC does not appear to have been examined thoroughly. It is possible that miR-183 may have potential as a novel EV miRNA biomarker.

1.3.5 miR Panels

An obstacle in finding a single EV miRNA that can function as a liquid biopsy biomarker is that all of the EV miRNAs discussed have been implicated in multiple pathologies, both malignant and benign. Increased levels of circulating miR-1246 have been found in other cancers including oesophageal squamous cell carcinoma, metastatic cervical cancer and multiple myeloma (112-115). Dysregulation of miR-19a is observed in oesophageal, bladder and gastric cancers, in addition to asthma and rheumatoid arthritis (116-120). The miR-23a~27a~24 cluster has been associated with acute leukaemia, bladder, hepatocellular, gastric, and pancreatic cancers (121-125). Furthermore, miR-183 has been implicated in a host of diseases including Acute Myeloid Leukaemia, Parkinson's disease as well as a number of malignancies (126-128). From this data, it appears that many miRNA are involved in a host of different diseases. Another consideration is that, by its nature, CRC tumorigenesis is not an organised process. There may be significant diversity of EV miRNA profiles produced by different CRC tumours. Therefore, it is possible that finding a single EV miRNA marker that is both sensitive and specific for CRC will prove difficult. Combining a number of markers in a panel of EV miRNAs have been employed to attempt to mitigate against these factors.

A number of studies that have examined a panel of miRNA together. This is to establish if their capacity to act as a liquid biopsy is superior when combined to any single miRNA alone. Yuan et al. (2016) developed a six EV miRNA panel that

compared 100 CRC patients (25 for each stage I-IV) as well as 50 controls. Their panel was able to differentiate CRC from healthy controls with an AUC of 0.68 and 0.77 for stages I and II, respectively. This was superior to any lone EV miRNA (129). Similarly, Ogata-Kawata and colleagues (2014) studied seven EV miRNA panel, all were elevated in serum EVs from 88 CRC patients, of which 20 each were stage I and stage II, compared to 11 controls (86). Combined use of this panel did not show more diagnostic power than two miRNA alone, miR-1246 and miR-23a, which had AUC values of 0.948 and 0.953 for all stages of CRC respectively (86). While the concept of an miRNA panel is promising, data from the above studies is not definitive. Further examination of a different combination of EV miRNA markers may provide a route to an effective liquid biopsy.

1.4 Summary

The global burden of CRC is set to increase significantly over the next decade. Although New Zealand has one of the highest prevalence of CRC in the world, it appears to underperform internationally when it comes to diagnosing CRC at an early stage. The earlier a diagnosis of CRC is made, the better the patient outcomes and lower overall cost of treatment. Screening improves pick-up rates of earlier cancers with a corresponding decrease in the rates of more advanced cancers. However, current stool based screening methods are not without issue, including limited sensitivity in early stage disease, in addition to sub-optimal participation rates. A blood-based screening test in the form of a liquid biopsy is an attractive proposition that may help address these concerns.

EVs are robust in the circulation and are produced in abundance by CRC cells. The contents of EVs directly reflect their cell of origin and examining these contents, such as miRNA, may provide a good target for a liquid biopsy. Identifying EV-miRNA within the circulation that are both sensitive and specific for CRC could help develop a blood-based screening test in the form of a liquid biopsy. This may contribute to earlier diagnosis of CRC, which could significantly help with decreasing both the overall morbidity and healthcare costs of this very common disease.

1.5 Aims and Hypothesis

1.5.1 Aims

The overall aim of this study is to establish whether the microRNA miR-1246, miR-19a, miR-23a and miR-183 can be used to differentiate early stage CRC from controls in both tissue and plasma EVs. This study will be performed in a well-defined cohort of stage I and II CRC patients with matched CRC tumour tissue and normal colonic mucosa from the same patient. Plasma samples will include all patients in which tissue is examined in addition to further stage I and II patients for whom only plasma is available. Control plasma EVs will be sourced from a matched cohort of patients who have had an entirely normal colonoscopy to rule out any polyps or other colonic abnormalities that may affect expression of the candidate miRNAs. If these microRNAs are differentially expressed in tissue and circulating EVs then they may have utility as liquid biopsy biomarkers.

The specific aims are:

1. To determine whether there is differential expression of miR-19a, miR-23a, miR-183 and miR-1246 in tumour tissue from stage I and II CRC patients compared to matched normal colonic mucosa from the same patients.

2. To determine if there is differential expression of miR-19a, miR-23a, miR-183 and miR-1246 in plasma EVs from the above stage I and II patients in addition to further patients from whom only plasma is available, when compared to plasma EVs from healthy controls who have had an entirely normal colonoscopy.

1.5.2 Hypothesis

We hypothesise that the microRNAs miR-19a, miR-23a, miR-183 and miR-1246 will be differentially expressed in tumour tissue in stage I and II CRC patients when compared to matched normal colonic mucosa from the same patients. That miR-19a, miR-23a and miR-183 will demonstrate increased expression in tissue as shown in previous research, and miR-1246, which has been shown to be both over and underexpressed in tissue, will demonstrate dysregulation in either direction. Subsequently, changes in CRC tissue will lead to altered expression in plasma EVs released from CRC cells into the circulation when compared to plasma EVs from

healthy controls. Furthermore, we expect that this difference between the miRNA expression in the EVs of stage I and II CRC patients will differentiate CRC patients from healthy controls and be useful as a blood-based liquid biopsy biomarker in CRC.

Chapter 2: Methods

Chapter 2: Methods

2.1 Patient Recruitment

Patients were recruited for donation of blood and tissue for the Surgical Cancer Research Group (SCRG) CRC 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 Ngāi 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 CRC biobank was created in October 2016, patients continue to be recruited on an ongoing basis. Data collected up to June 2019 was used. Patient demographic data was collected including; age, gender, ethnicity, presenting symptoms as well as co-morbidities and medications. Information relating to their diagnosis and treatment of CRC was also collected. This included colonoscopy results, biopsy findings, imaging, surgical operation notes and pathology reports. Study data was collected and stored using REDCap data capture system hosted by Otago University in a secure database (130).

Inclusion criteria for the CRC group were patients over the age of 18 who were being treated for CRC at Wellington Hospital. Patients were diagnosed on endoscopy at Wellington Hospital, or were referred to Wellington Hospital following diagnosis in Hutt Valley or Wairarapa Hospitals.

Exclusion criteria were patients who had their surgery performed in a private hospital, those who were under the age of 18, emergency presentations and patients who had significant cognitive impairment that precluded them from providing informed consent.

Recruitment of patients and collection of samples was carried out by a team of researchers at the University of Otago, Wellington. Recruitment involved identification of patients, consent, data entry, database audit, blood collection, and

assistance with collecting tissue at time of surgery. Suitable patients were found through MDMs, outpatient clinics and elective theatre lists. The author was responsible for patient identification, recruitment, blood sample collection and processing between January and December 2019.

2.1.1 Patient Selection

From the Colorectal Biobank, patients with stage I and stage II CRC were identified for the study, usually from outpatient clinics. Patient enrollment was at the time of diagnosis of CRC, prior to any treatment. Stage I and II patients with matched tissue and normal mucosa were included. Tissue from patients who had received neoadjuvant radiotherapy was not included as this has been shown to effect miRNA expression levels in CRC tissue (131).

All stage I and II CRC patients that had plasma samples available were also identified, this included all the above patients who provided tissue samples. In a number of cases there was no tissue available but plasma alone had been collected. Staging status was based on post-operative staging as recorded at the Wellington Hospital Colorectal MDM. All plasma samples were collected at the initial clinic appointment following diagnosis and preoperative staging was also recorded. If any change in staging occurred following neoadjuvant treatment, these patients were excluded from the plasma analysis, as they may have had more advanced disease at the time of plasma collection.

Control patients were selected from those who were referred to endoscopy with a suspicion of CRC but were found to have an entirely normal colon following a full colonoscopy. They were age and sex matched to the CRC patients. Consent was obtained prior to colonoscopy. All control patients that were recruited were symptomatic in the form of abdominal pain, weight loss, change in bowel habit or perirectal bleeding. Control patients were required to have a complete colonoscopy to the caecum without any evidence of CRC, adenomatous polyps or inflammatory conditions of the colon such as Ulcerative Colitis, Crohn's Disease or infective colitis. This was to ensure there was no other colonic pathology occurring which may have an effect on the expression of the candidate miRNAs.

2.2 Sample Collection

Venous blood samples were taken from patients at the time of recruitment. This was always prior to any neoadjuvant treatment or surgery. Blood samples were collected using a 21G BD™ vacutainer blood collection set into sodium citrate collection tubes (3.2% 0.109 M) then centrifuged within 30 minutes of withdrawal at 3000 x g for ten minutes to separate the plasma. Plasma samples were separated into aliquots and stored in a -80°C freezer. EDTA and SST II Gel tubes were also used to collect venous samples that were used to perform biochemical and haematological tests in the hospital laboratory. This included Full Blood Count (FBC), CEA and C Reactive Protein (CRP). Blood sample tubes from which plasma EVs were isolated were taken following the samples that were sent to the hospital laboratory. This was in order to avoid cellular and skin debris from the initial venipuncture contaminating plasma EV preparations.

Tissue samples were collected immediately following surgical resection of the specimen in the operating theatre. Once the specimen was resected a samples of central tumour tissue as well as a section of normal colonic mucosa. These samples were then immediately stored in RNAlater Stabilization Solution™ (ThermoFisher Scientific) according to manufacturer's protocol.

2.2.1 Tissue RNA extraction

Tissue samples of tumour tissue and normal colonic mucosa were weighed to quantify their mass in milligrams (mg), the manufacturers recommendations were for 30-100µg of tissue, depending on the yield and purity of RNA being obtained. Samples were then homogenized in QIAzol Lysis Reagent™ solution using a mortar and pestle until all visible tissue particles had dissolved. RNA was then extracted using the miRNeasy Mini™ kit with the supplied miRNeasy Mini™ spin columns (all Qiagen) using a phenol-chloroform based extraction method according to the manufacturer's instructions. The final RNA product was eluted in 30 µL of nuclease free water and stored at -80°C. The concentration and quality of RNA was assessed using a NanoDrop™ 2000 Fluorospectrometer (ThermoFisher Scientific). The concentration of RNA in ng/µL was recorded for tumour tissue and normal colonic mucosa. In addition to concentration, quality of the RNA extracted was assessed by

recording the 260/280 values. This is a ratio of absorbance at 260 nm and 280 nm on a fluorospectrometer, with a value of 2.0 considered of ideal purity of RNA, and values between 1.8 and 2.2 deemed of acceptable quality (132).

2.2.2 Plasma Extracellular Vesicle isolation and RNA extraction

Plasma extracellular vesicles were isolated using size exclusion chromatography (SEC) columns. qEVoriginal 70™ columns were used in conjunction with an automated collector; the Izon Automated Fraction Collector™ (both Izon Science). Five-hundred microlitres of plasma was added to the column and fractions seven, eight, nine and ten were collected using the automated fraction collector according to the manufacturer's instructions. EVs within the collected fractions were subsequently pooled and added to a thin walled 17 mL ultracentrifuge tube (ThermoFisher Scientific). The remaining volume of the tube was filled with 12 mL 1 x Phosphate Buffered Saline (1 X PBS). These tubes were then placed in a swing bucket rotor ultracentrifuge (ThermoFisher Scientific) at 4˚C, at 100,000 x g for two hours to form an EV pellet.

The EV pellet was re-suspended in 300 µL 1X PBS. RNA isolation was subsequently performed using the Total Exosome RNA and Protein Isolation Kit (Invitrogen) according to the manufacturer's instructions. The final RNA product was eluted in 50 µL of nuclease free water and stored at -80°C.

2.2.3 Reverse Transcription

For both tissue RNA and plasma-EV RNA, reverse transcription (RT) was performed using the TaqMan® Advanced miRNA cDNA Synthesis Kit (ThermoFisher Scientific). For each tissue sample, 5 ng of RNA was used. For plasma EV samples 2 µL of RNA was used. For both, the protocol was followed as per the manufacturer's instructions. Briefly, an initial polyadenylation step was performed at 37°C for 45 minutes, followed by heat inactivation at 65°C for 10 minutes. This was followed by an adaptor ligation reaction at 16°C for 60 minutes. RT was then performed with incubation at 42°C for 15 minutes, with subsequent heat inactivation at 85°C for 5 minutes. The cDNA product was stored at -20°C until use. Pre-amplification was performed according to the manufacturer's instructions using an initial denaturing step at 95°C for 3 seconds followed by an anneal/extend phase at 60°C for 30

seconds, this cycle was repeated 14 times. The amplification product was also stored at -20°C.

2.2.4 Quantitative PCR

Pre-amplified cDNA samples from the above reaction were diluted 1:10 with nuclease-free water. Quantitative Reverse Transcription PCR (RT-qPCR) was then performed with the TaqMan® Fast Advanced Master Mix (ThermoFisher Scientific) on a Corbett RotorGene 6000 with TaqMan® advanced miRNA Assays (ThermoFisher Scientific). Specific assays for hsa-miR-19a-5p, hsa-miR-23a-3p, hsa-miR-183-3p, hsa-miR-1246-5p, hsa-miR-16-3p and hsa-miR-345-5p were used. Both hsa-miR-16-3p and hsa-miR-345-5p were employed as endogenous reference genes based on previously published research that has examined both CRC tissue and plasma EVs (85, 133-135). The total reaction volume of each sample was 10 µL, which included 2.5 µL of diluted cDNA. Negative reactions containing RNAse free water instead of cDNA were included in every experiment.

All reactions were performed in duplicate and cycle threshold (C_T) values within 0.5 were considered acceptable. The following cycling conditions were utilized: 95°C for ten minutes, followed by 40 cycles of 95°C for ten seconds, and 60 °C for one minute. Expression levels of miRNA were quantified using the Rotor-Gene software version 1.7.75. A threshold of 0.07657 was set across all tissue and EV experiments. A C_T cut-off value of 35 was used. For miRNA samples where this cutoff threshold was exceeded, a C_T value of 35 was assigned as the miRNA expression levels were too low to be adequately quantified when using our methods.

Endogenous controls in the form of reference genes were employed for all tissue and plasma EV samples. For tissue, miR-16 and miR-345 were used. The mean expression of both of these genes was calculated as the geometric mean. In plasma EVs, miR-16 alone was used as miR-345 did not demonstrate consistent expression across patient groups (supplementary Figure S1). miR-16 has previously been used as an endogenous reference gene in published studies examining both CRC tissue and plasma EVs in CRC (85, 133, 134). miR-345 has also been used as an endogenous control in the published literature and recommended as a stable endogenous

reference gene in CRC in a number of studies examining both CRC tissue and circulating EVs (133, 135).

2.3 Data Analysis

2.3.1 Calculating relative miRNA expression (ΔC_T)

Expression levels of candidate miRNA are expressed as ΔC_T, or the change in C_T value relative to endogenous reference genes. In tissue, the relative expression values of target miRNAs (miR-19a, miR-23a, miR-183, and miR-1246) were calculated using the $2^{-\Delta\Delta Ct}$ method (136). To calculate ΔC_T for each sample, the geometric mean values of the endogenous reference genes (miR-16-3p and miR-345-5p) was subtracted from the C_T value of the miRNA of interest. The ΔC_T value for normal mucosa was subsequently subtracted from the paired ΔC_T value for tumour tissue from the same matched patient ($\Delta\Delta C_T$), and log transformed to calculate the relative expression ($2^{-\Delta\Delta CT}$). For all candidate miRNA samples where the cutoff threshold of 35 was exceeded, a value of 35 was assigned, as the miRNA expression levels were too low to be detected by our current RT-qPCR set up.

With respect to plasma-EV miRNA, ΔC_T was calculated by subtracting the C_T value of the endogenous reference gene (miR-16-3p) from the C_T value of the miRNA being assessed for each CRC or control sample. The relative change in expression, $\Delta\Delta C_T$, was calculated by subtracting the mean ΔC_T value of the control group from the mean ΔC_T values from the CRC group for each of the candidate miRNA. Similarly to tissue, the mean fold change in expression was then log transformed as $2^{-\Delta\Delta Ct}$.

The relative changes in mean miRNA expression between tumour tissue and plasma EVs were examined. This was calculated by subtracting the ΔC_T for tumour tissue from that of EVs for the same sample and calculating the change in expression using the same $2^{-\Delta\Delta Ct}$ method.

2.3.2 Correlation of patient variables

At the time of patient recruitment, a number of patient variables were recorded. These include patient demographics such as age, gender and ethnicity, in addition to blood-based variables including CEA, CRP and Neutrophil to Lymphocyte ratio (NLR). Finally, following surgery, tissue-based pathological variables were recorded

including perineural invasion (PNI) extramural venous invasion (EMVI), lymphovascular invasion (LVI) and mismatch repair proficiency/deficiency (pMMR/dMMR) from patient notes following pathological examination of the surgical specimen. Correlation between these variables and miRNA using Pearson's and Spearman's rank correlations, for parametric and non-parametric data respectively, were evaluated. This was to assess if the expression of each variable was independent, or possibly related to, miRNA expression.

In the tissue analysis, a subset of patients existed with elevated expression of two of the candidate miRNA when compared against the rest of the tumour tissue group. In order to examine this further, these groups were subdivided into 'high expression', taken as a greater than two-fold increase, and 'normal expression' subgroups. These groups were then examined to establish if there were any significant difference in clinical or pathological variables.

2.3.3 ROC Curve Analysis

In order to assess the capacity of a variable, such as miRNA expression, to discriminate between CRC and controls, ROC curve analyses were employed. The values of a variable for CRC cases against controls were plotted into a ROC curve, subsequently the AUC was calculated. There were a number of instances where two variables were added together to create a combined ROC curve analysis, this was performed using methods described by Pepe et al. (2000; 137). This was to assess if two variables, when combined, were additive in their ability to differentiate control cases from CRC. Firstly, assessment for any correlation, as described above, was performed to ensure that expression of either variable was independent of one another. Subsequently, all the values for CRC cases and controls were listed together. From this list, any significant outlying data points were excluded using the ROUT method (138).

The remaining figures were then reviewed to ensure normal distribution, the largest value was identified and assigned an arbitrary value of 1. Subsequently all the remaining values were divided by this largest value to give the fraction relative to the highest chosen value. Any outliers that were greater than the chosen greatest figure were also given a value of 1, consequently for each variable every patient had a value between 0 and 1. This corresponded to their relative expression of the

variable relative to the largest value. Fractions for multiple variables were added together for every patient. Thus, when two variables were combined for each patient, a maximum value of two could be achieved. These combined values for CRC against controls were then used in a ROC curve analysis to assess their ability to discriminate between groups.

2.3.4 Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 8.0.2 software. Results were expressed as mean ± standard deviation. For comparisons of numerical data, either paired or unpaired *t* tests, in addition to one-way ANOVA with Tukey post hoc analysis were used. For non-numerical data, chi-squared tests were employed. Values of $p<0.05$ were considered statistically significant.

With regard to the tissue analysis paired *t* tests were performed to compare the ΔC_T of target miRNAs between CRC tumour tissue and normal colonic mucosa from the same patient. For the plasma EV analysis, unpaired *t* tests were performed to compare ΔC_T values of the target miRNA between the CRC patients and the control group. For all figures in which ΔC_T is examined (Figures 5, 7 and 8) the y-axis has been reversed. This was implemented as a ΔC_T values are in reverse log order, thus reversing the axis converts data points to reflect the actual directional change in expression.

For correlations between expression of miRNA and other variables, one variable was plotted on each of the x and y-axes. When correlating with ΔC_T the $\log_2$ of the variable was used. Subsequently, a Spearman's or Pearson's correlation was computed (*r*), then a two-tailed t-test with a 95% confidence interval was performed to assess for significance. Values of $p<0.05$ were considered statistically significant.

Chapter 3: Results

Chapter 3: Results

3.1 Patient Demographics

From October 2016 to August 2019, 321 patients were recruited to the SCRG, CRC biobank from Wellington Hospital. Of the total biobank patients, the average patient age was 67.55 years (range 27-88 years). The majority of patients are of New Zealand European ethnicity (83.8%), followed by Māori ethnicity (6.9%).

Sixty-three patients in the biobank are healthy controls without CRC. Of these, 30 patients had an entirely normal colonoscopy without any evidence of CRC, polyps or other bowel abnormalities. These patients were included as the healthy control patients in this study. Of the remaining 259 patients, 80 have stage I or II CRC (30.8%). Fourteen patients were excluded from analysis as they had down staging of their disease following neoadjuvant treatment; this left 66 stage I or II patients with plasma. Forty-two of these patients with plasma also had matched CRC tumour tissue and normal mucosa that was included for tissue analysis.

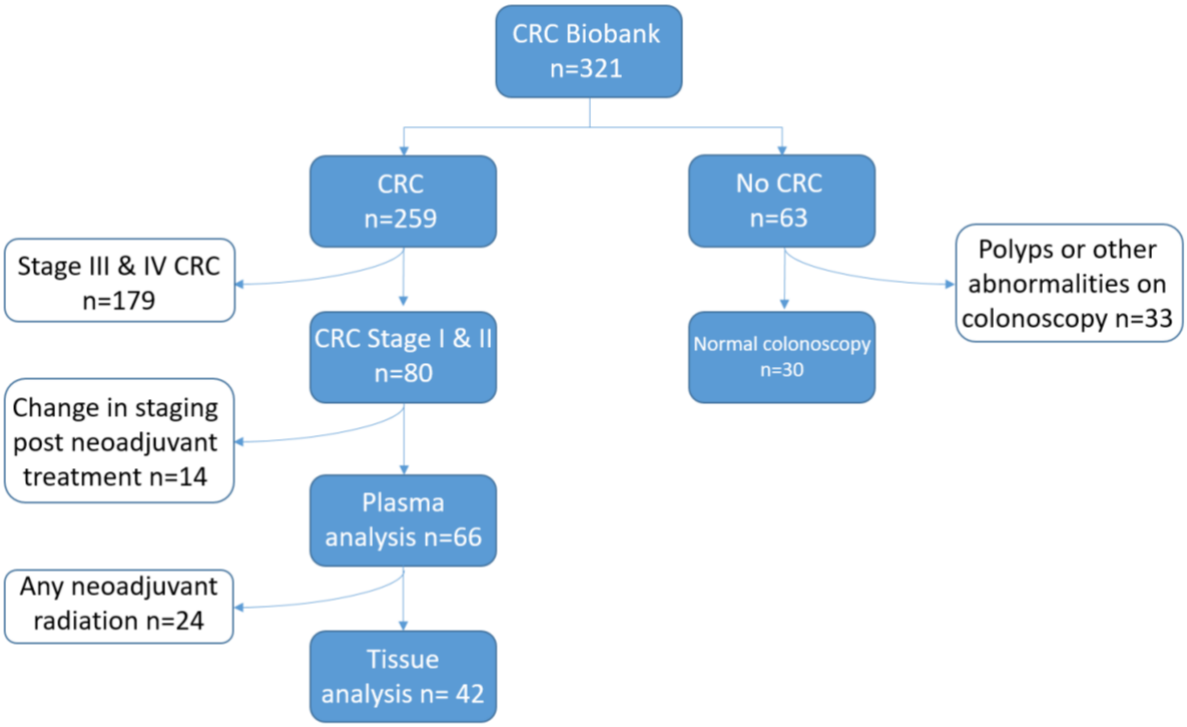

Figure 3 *- Selection of patients from CRC biobank.*

3.1.1 Patient cohort with available tissue samples

There was a total of 42 patients with matched tumour tissue and normal colonic mucosa. The mean age of this group was 68.5 (±12.56) years, 19 of the 42 (45.2%) were female (Table 2). The majority (83.3%) were of NZ European ethnicity. Nine patients (21.4%) were stage I, with the remaining 33 (78.6%) being stage II. The majority (88.1%) of CRC originated in the colon, of which 25 were 'right-sided' with regard to the relation to the splenic flexure.

Total	**42**
Mean Age (±SD)	68.5 (12.56)
Female (%)	19 (45.2)
Male (%)	23 (54.8)
Ethnicity (%)	
NZ European	35 (83.3)
Māori	2 (4.7)
Other	5 (12.0)
TNM Stage (%)	
Stage I	9 (21.4)
- T1	- 3 (7.1)
- T2	- 6 (14.2)
Stage II	33 (78.6)
- T3	- 27 (64.3)
- T4a	- 5 (11.9)
- T4b	- 1 (2.4)
Colon	37 (88.1)
- Right sided	- 25 (59.5)
- Left sided	- 12 (28.6)
Rectum	5 (11.9)

***Table 2**- Patient demographics and tumour characteristics for tissue samples.*

3.1.2 Patient cohort with available plasma samples

Patients included in the plasma group compromised all 42 patients with tissue, in addition to 24 patients from which only plasma was available (Table 3).

	CRC	**Controls**	***p* value**
Total	66	30	
Mean Age (±SD)	67.5 (11.5)	63.79 (11.1)	0.1486
Female (%)	33 (50)	18 (60)	0.3628
Male (%)	33 (50)	12 (40)	
Ethnicity (%)			
NZ European	57 (86.3)	22 (73.3)	0.2976
Māori	2 (3.0)	2 (6.7)	
Other	7 (10.6)	6 (20.0)	
TNM Stage (%)			
Stage I	20 (30.3)		
T1	7 (10.6)		
T2	13 (19.7)		
Stage II	46 (69.7)		
T3	38 (57.6)		
T4a	7 (10.6)		
T4b	1 (1.4)		
Site (%)			
Colon	52 (78.8)		
Right sided	34 (51.5)		
Left sided	18 (27.3)		
Rectum	14 (21.2)		
Neoadjuvant treatment	7 (10.6)		
No Neoadjuvant treatment	7 (10.6)		

Table 3 *- CRC patient and control demographics and tumour characteristics for plasma samples. Unpaired t-tests performed for age, chi-squared tests performed for other variables.*

For patients included in the plasma analysis, the mean age at diagnosis for CRC patients was 67.5 (±11.5) years. The mean age of the control patients was 63.8 (±11.1) years (Table 3). There was no significant difference in age between the control and CRC groups on an unpaired *t*-test (p=0.1486). Nor was there any significant differences in gender or ethnicity between the groups when a chi-squared test was performed.

The majority of patients were of NZ European ethnicity, which composed 73.3% and 86.3% for control and CRC groups respectively. The majority of CRC patients were stage II (69.7%). Most of the CRC tumours included in the study originated in the colon (66.25%). There is a higher proportion of rectal cancers in the plasma analysis (21.2%), when compared to tissue (11.9%). This is due to the exclusion of patients who received neoadjuvant therapy from tissue analysis. Of the rectal cancer patients, half received neoadjuvant treatment.

3.2 Quality control of extraction of RNA from tissue samples

To determine whether the miRNA of interest were dysregulated in tumour tissue compared to normal mucosa, total RNA was extracted from matched tissue pairs and miRNA expression was quantified by RT-qPCR.

Of the 42 matched pairs of tumour tissue and normal mucosa, RNA extraction was previously performed for 18 patients, values for the weight of these samples was not available. The author processed the remaining 24 patient samples.

Mean values of 36.09 (±16.89) mg and 33.64 (±19.87) mg for weight were comparable between tumour tissue and normal mucosa respectively. For RNA, mean concentration values of 702.5 (±663) ng/µL and 1070 (±1059) ng/µL for normal mucosa and tumour tissue respectively were also broadly similar. Quality of the RNA extracted was assessed by recording the 260/280 values, the average of which were 2.061 (±0.043) and 2.051 (±0.031) for tumour tissue and normal mucosa respectively. Individual values for weight of tissue processed and concentration of extracted RNA are included in supplementary Tables S2 and S3.

3.3 Quality Control of RT-qPCR

Endogenous reference genes were used for the tissue and plasma EV analyses. Relative expression values of the candidate miRNA is expressed as ΔC_T, which is the change in C_T value relative to the endogenous reference genes. Both endogenous reference genes have been used as controls in previous research when examining CRC tissue and plasma EVs (85, 133-135). To be effective endogenous controls there should be no significant difference in their expression between comparator groups. For tissue, both miR-16 and miR-345 were used. Mean values for each endogenous reference gene, in addition to their mean combined values, as the geometric mean, demonstrated no significant differences on a paired *t*-test (Table 4 and Figure 4 A-C).

	Normal Mucosa (±SD)	**Tumour Tissue (±SD)**
miR-16	17.09 (1.98)	17.46 (2.00)
miR-345	24.55 (2.01	24.48 (1.87)
Geometric Mean	20.85 (1.96)	20.97 (1.83)

Table 4 *- mean C_T values for endogenous reference genes in tissue samples*

In plasma EVs, miR-16 alone was used as miR-345 did not demonstrate consistent reliable expression. The mean C_T values for plasma EV miR-16 were 24.71 (±4.13) and 23.73 (±3.02) for controls and CRC patients respectively. miR-345 was only expressed in 14 of 19 (73.6%) control samples and 28 of 42 (66.7%) CRC samples. Mean C_T values were 28.17 (±1.99) and 29.19 (±1.92) for CRC and control groups respectively, these relatively high C_T values (cut-off C_T = 35) are in keeping with low levels of expression of miR-345 in plasma EVs. This would affect its suitability as an endogenous reference gene. Of the samples that did express miR-345, there was no significant difference between the groups on an unpaired *t*-test, (p=0.1194, Supplementary Figure S1).

There were no statistically significant differences in expression of the endogenous reference genes when comparing C_T values of CRC against controls in either tissue samples or plasma EVs (Figure 4). Therefore, they were suitable as endogenous reference genes to calculate the relative change in expression (ΔC_T) of the candidate miRNAs: miR-19a, miR-23a, miR-1246 and miR-183.

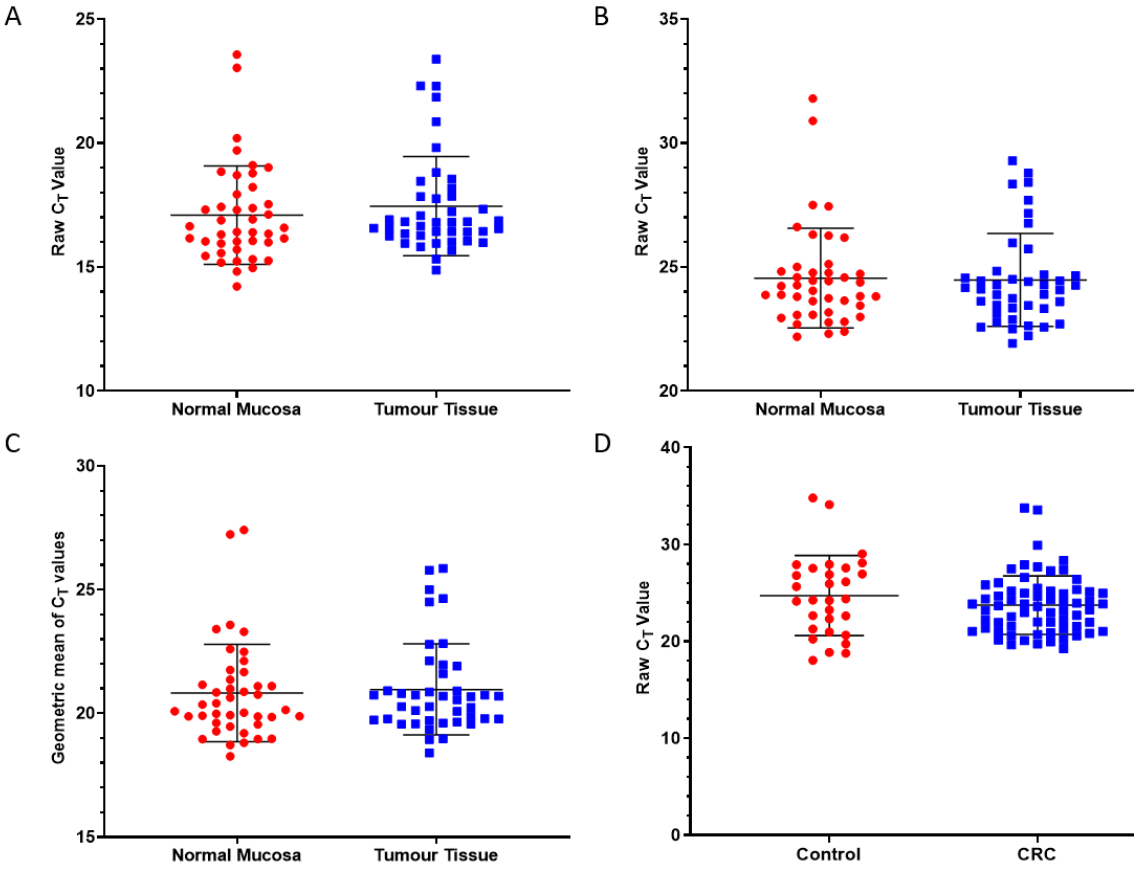

Figure 4 *- Analysis of endogenous reference genes.* ***(A)*** *Raw C_T values for miR-16 comparing normal mucosa and tumour tissue (p=0.1546, paired t-test).* ***(B)*** *Raw C_T values for miR-345 comparing normal mucosa and tumour tissue (p=0.7903, paired t-test).* ***(C)*** *Geometric mean (miR-16 & miR-345) comparing normal mucosa and tumour tissue (p=0.5857, paired t-test).* ***(D)*** *Raw C_T values for miR-16 comparing plasma EVs from controls and CRC patients (p=0.1916, unpaired t-test).*

3.4 MicroRNA expression in normal mucosa vs tumour tissue

From the RNA extracted from both tumour tissue and normal colonic mucosa, relative expression levels (ΔC_T) of the candidate miRNAs miR-19a, miR-183, miR-23a and miR-1246 relative to the geometric mean of the two reference genes (miR-16 and miR-345) was calculated (Figure 5).

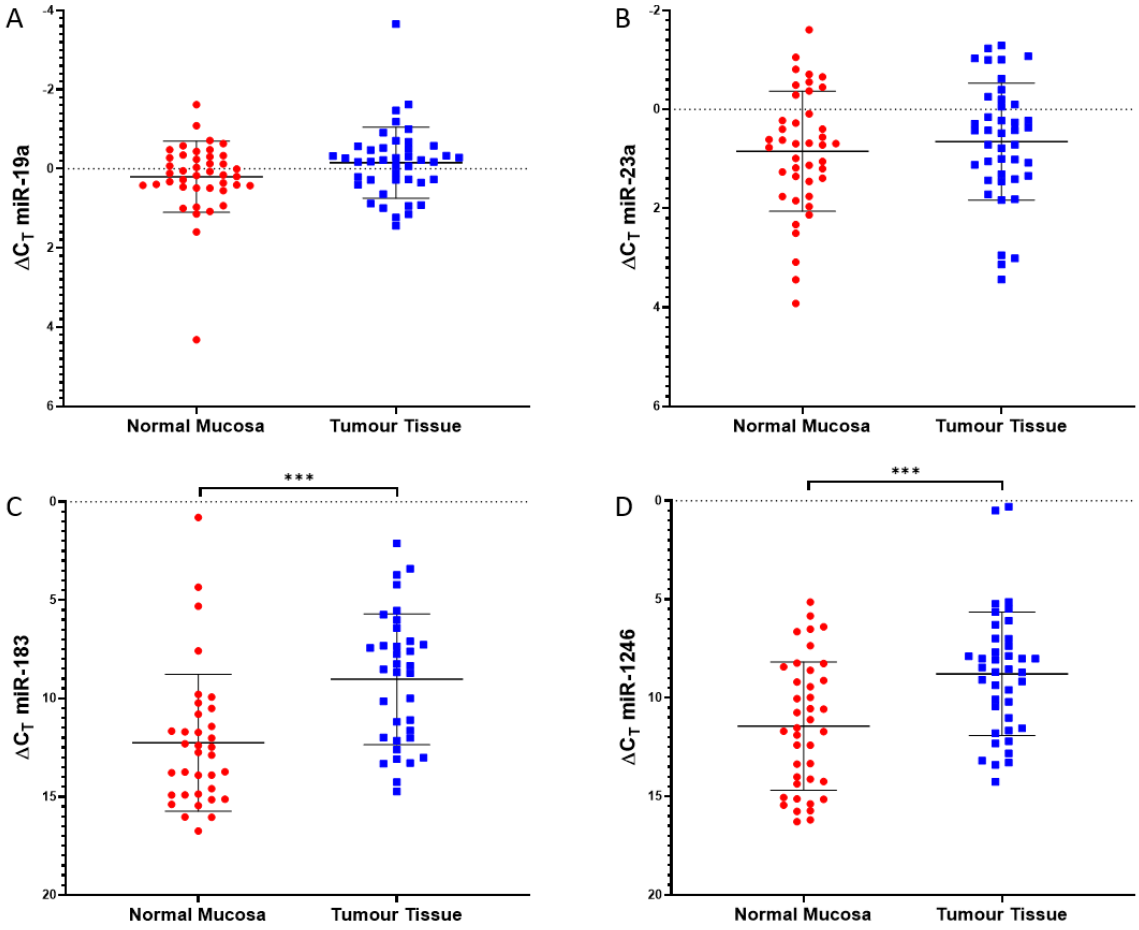

Figure 5 *– miRNA expression in normal mucosa vs tumour tissue* ***(A)*** *miR-19a* ***(B)*** *miR-23a* ***(C)*** *miR-183* ***(D)*** *miR-1246. *** = p<0.001, paired t-test; note: reversed y-axes.*

Statistically significant increases in tumour tissue miRNA expression were observed in two of the four miRNA. miR-183 was significantly increased in tumour when compared to matched normal mucosa (Figure 5 C), however it was only reliably expressed in 35 of the 42 samples, it had relatively low overall expression in tissue (mean C_T 29.52 and 32.51 for tumour and normal mucosa respectively). miR-1246 (Figure 5 D) also demonstrated a significant increase in expression in tumour tissue

and was expressed in 40 of the samples, this also exhibited low overall tissue expression (mean C_T 29.82 and 32.16 for tumour and normal mucosa respectively). For both miR-183 and miR-1246, samples that demonstrated C_T difference >0.5 on duplicate samples following three attempts at RT-qPCR were excluded from analysis. Using the $2^{-\Delta\Delta CT}$ method, the mean fold in expression was 210.6 (±575.1) and 147.9 (±494.6) fold increases in expression for miR-183 and miR-1246 respectively.

There was a trend towards an increase in expression in tumour tissue for miR-19a (Figure 5 A), however this was not statistically significant (p=0.0579). Following exclusion of a single prominent outlier from each of the tumour and normal mucosa groups, this p value increased to 0.2027.

There were no significant relationships between tissue expression of the candidate miRNA and various clinical variables including age, gender or stage of CRC (Supplementary Table S4). Additionally, there was no association between left (including rectal) or right sided tumours, nor was there a relationship with commonly used tissue-based pathological variables in CRC such as PNI, LVI, MVI and MMR deficiency (Supplementary Table S4).

For both miRNA that demonstrated overexpression in tumour tissue, the cohort was divided into 'high expression' and 'normal expression' subgroups (Supplementary Table S5 and S6). The high expression group was determined by a two-fold or greater change in expression in tumour tissue compared to normal mucosa. Females were significantly more likely to have high expression of both miR-183 and miR-1246 on a chi-squared test (p=0.0354 and p=0.007 respectively; Supplementary Table S5). Additionally, there was a significant association between high miR-1246 expression and NLR on an unpaired t-test (p=0.044; Supplementary Table S6). There were no other significant differences between high and normal expression subgroups for either miRNA in the other clinical, pathological or blood-based variables.

3.4.1 ROC Curves and tumour tissue miRNA expression

There was no evidence of correlation between the two differentially expressed miRNA in tumour tissue, which suggests these miRNA are expressed independently of each other (Figure 6 A). ROC curve analyses were subsequently compiled.

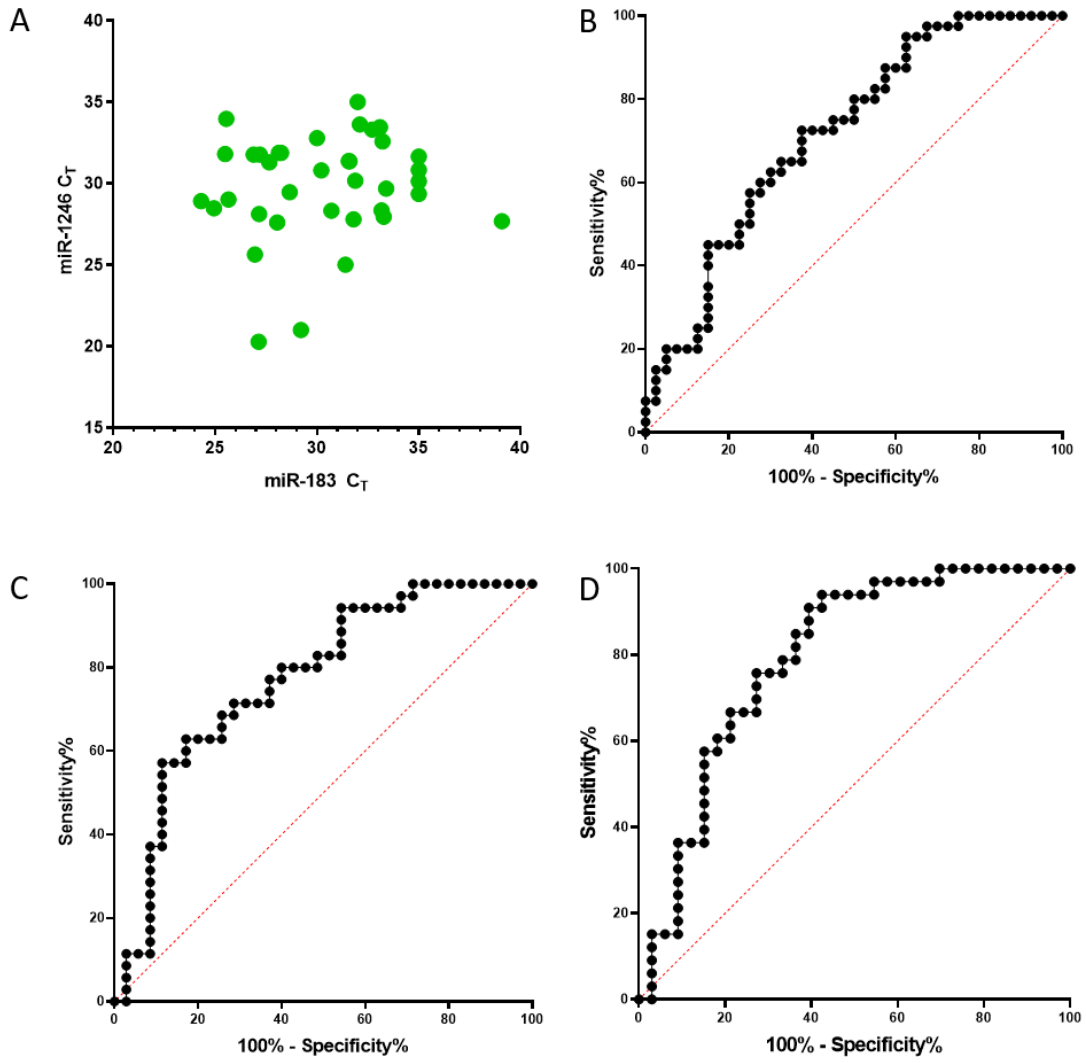

Figure 6 *– Discrimination of tumour tissue and normal mucosa by miR-1246 and miR-183 expression* ***(A)*** *Correlation of miR-183 and miR-1246 C_T values (Spearman's r=0.1168, p=0.491). ROC curve analyses for* ***(B)*** *miR-1246,* ***(C)*** *miR-183, and* ***(D)*** *miR-183+miR-1246 combined score.*

ROC curves for discriminating between tumour and normal mucosa demonstrated an AUC of 0.7175 (72.5% sensitivity, 60.0% specificity) for miR-1246, and 0.7706 (62.86% sensitivity, 85.71% specificity) for miR-183 (Figure 6 B & C). This suggests that expression levels of both miRNA have an ability to discriminate between

tumour and normal mucosa. Consequently, it was assessed if this ability was improved when values for both miRNA were added together. When combined, an AUC of 0.7943 (72.7% sensitivity, 72.3% specificity) was found (Figure 6 D), this was superior to either single miRNA. Combining miR-183 and miR-1246 was found to be additive in their capacity to discriminate between tumour tissue and normal colonic mucosa.

3.4.2 CRC stage and tumour tissue miRNA expression

To determine whether there was any increase in tumour miRNA expression with advancing stage we seperately examined miRNA expression levels in stage I and II subgroups.

	Mean Fold Change (±SD)		
	Stage I (n=9)	**Stage II (n=33)**	**Total (n=42)**
miR-19a	1.104 (0.6285)	1.716 (1.698)	1.585 (1.547)
miR-1246	19.45 (37.56)	175.14 (541.7)	147.9 (494.6)
miR-23a	1.485 (0.98)	1.526 (1.612)	1.517 (1.488)
miR-183	616.83 (1245)	126.56 (286.7)	210.6 (575.1)

Table 5 *- Mean Fold Change in tumour tissue expression of candidate miRNA by stage.*

Substantial differences are seen from stage I to stage II for both miR-1246 and miR-183 (Table 4). For miR-183 overexpression was greatest at stage I, there was a trend towards a significant decrease in overexpression from stage I to II, but this was not statistically significant (p=0.056, unpaired *t*-test). miR-1246 did also not show any significant difference in mean fold expression between stages.

3.5 Plasma EV miRNA expression in CRC patients vs controls

The second focus of the study was to examine expression of the candidate miRNA in plasma EVs. Although significant increases in expression in tumour tissue were seen for miR-183 and miR-1246, no detectable expression of these miRNA was observed below the cut-off C_T value of 35 in plasma EV samples (n=18 for miR-183 and n=8 for miR-1246). Both miR-23a and miR-19a, which demonstrated a trend towards an increase in expression in tumour tissue, were consistently expressed in plasma EVs.

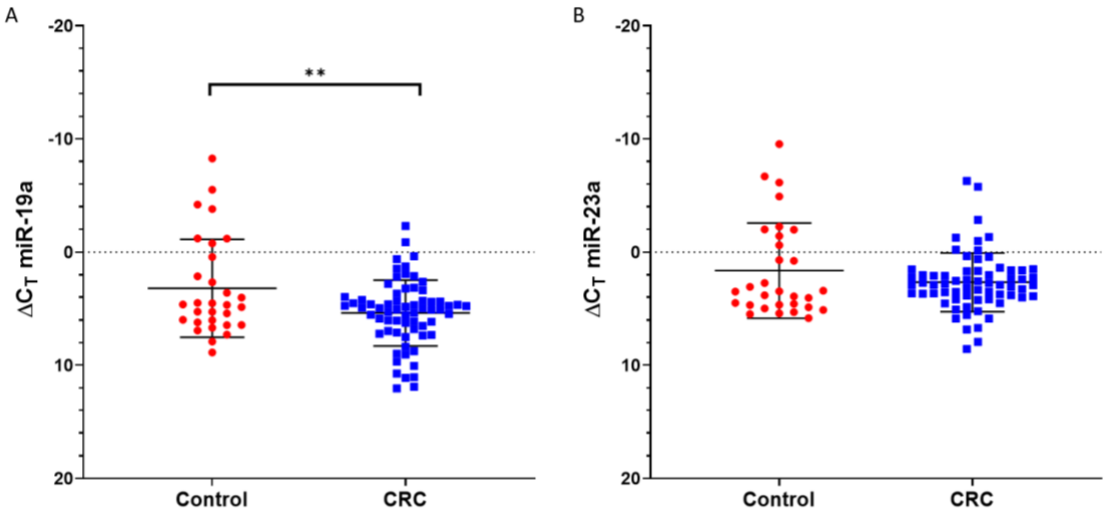

Figure 7 *- miRNA expression in plasma EVs of controls and CRC patients* ***(A)*** *miR-19a* ***(B)*** *miR-23a. ** = <0.01, unpaired t-test, note: reversed y-axis.*

miR-19a was significantly downregulated in the EVs of the CRC group compared to controls (p=0.0043; Figure 7 A). No difference was seen for miR-23a (Figure 7 B). The mean fold change in expression for miR-19a was 0.218, or a 4.6 fold decrease.

3.5.1 Plasma EV miRNA expression in CRC patients by stage vs controls

Examining differences in plasma EV miRNA dysregulation across stages may be useful to determine how early in CRC progression changes can be detected in the bloodstream of patients.

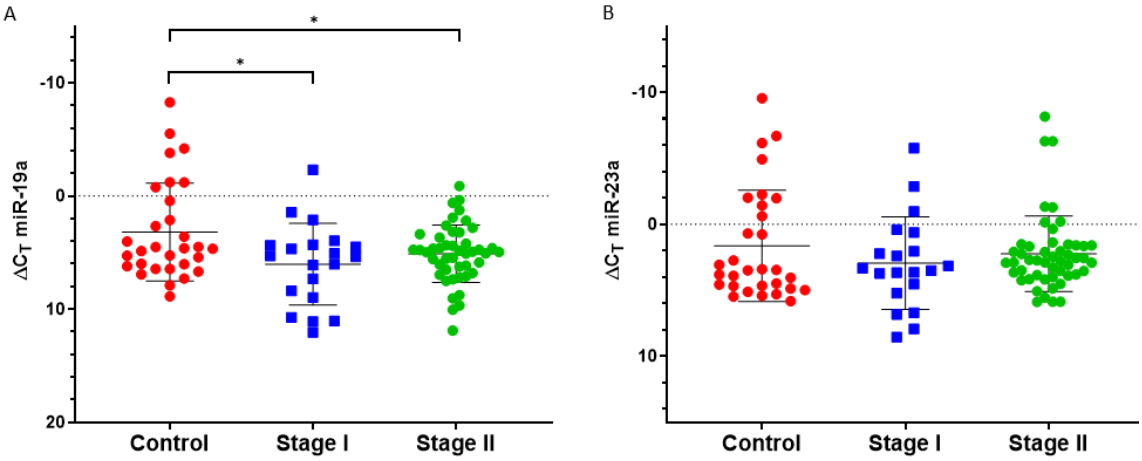

Figure 8 *- miRNA expression in plasma EVs by stage vs controls* ***(A)*** *miR-19a* ***(B)*** *miR-23a. * = p<0.05, one-way ANOVA with Tukey post hoc analysis. Note: reversed y-axis.*

Significant differences in expression of miR-19a in plasma EVs were observed between each stage and controls (Figure 8 A). Subsequently, the mean fold change in plasma EV expression was also examined. For miR-19a, a 0.139 fold change (7.2 fold decrease) in expression for stage I and 0.256 fold change (3.9 fold decrease) for stage II was observed. This suggests that downregulation of plasma EV miR-19a expression is greater at stage I than stage II; however, the differences in EV expression between stages did not reach statistical significance on pot-hoc analysis.

3.5.2 Association between plasma-EV miRNA and other blood-based markers

To determine the ability of plasma EV miR-19a to act as a liquid biopsy and differentiate between early stage CRC patients and healthy controls, a ROC curve analysis was performed. This corresponded with an AUC of 0.6104 (sensitivity 62%, specificity 53%).

As seen in the tissue analysis, two variables with independent expression may be added together to examine if it improves the ability to discriminate when combined. miR-19a demonstrated differential expression in plasma EVs. It was subsequently added to CEA, another blood based marker, which has a limited ability to identify CRC.

No correlation was observed between miR-19a and CEA (Spearman's r=-0.002, p=0.9852). Firstly, a ROC curve analysis was used for CEA alone to differentiate CRC and controls, which corresponded with an AUC of 0.6576 (sensitivity 45%, specificity 76%). Secondly, CEA was combined with miR-19a in another ROC curve analysis; however, when this was calculated the AUC declined to 0.5217 (sensitivity 57%, specificity 50%), which was inferior to either marker individually. Therefore, it does not appear that CEA and miR-19a are additive in the context of a blood based diagnostic biomarker in CRC.

Expression of EV miR-19a was correlated with other blood-based markers in CRC to assess if any relationship was observed. When using Spearman's test, a negative correlation between miR-19a and NLR was seen (r=-0.308, p=0.011). This was mirrored by a negative correlation between miR-23a, which had not demonstrated any difference in plasma EV expression, and NLR (r=-0.2561, p=0.0411). No further correlations were observed between either circulating EV miRNA and other blood-based variables (Supplementary Table S7).

3.5.3 Association between plasma-EV and tissue expression

Correlations between tumour tissue and plasma EV miRNA expression were examined to assess if differences in tumour tissue expression effected the levels in circulating EVs.

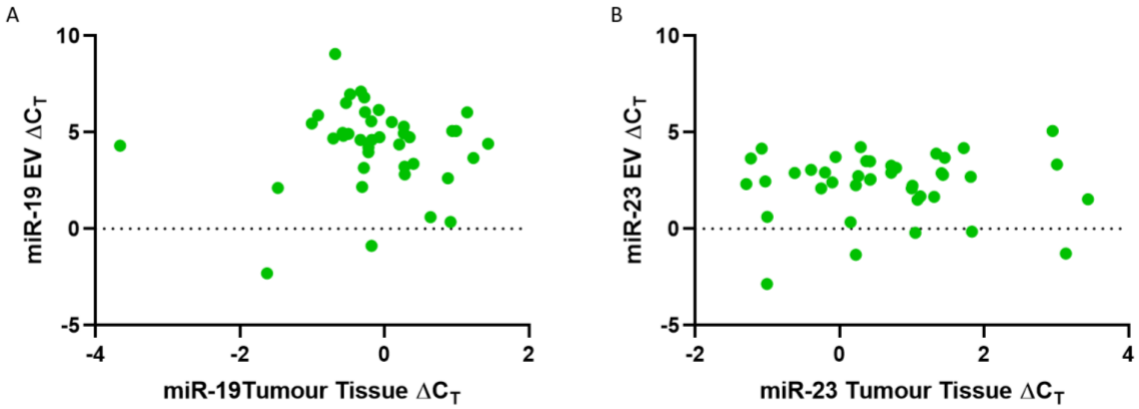

Figure 9 *- Correlation between ΔCT values for plasma EVs and tumour tissue* ***(A)*** *miR-19a (Spearman's r=-0.1808, p=0.258).* ***(B)*** *miR-23a (Spearman's r=0.0217, p=0.8927). n=41 x-y pairs.*

No correlation was observed between tumour tissue and plasma EVs (Figure 9 A). When subsequently comparing miRNA expression levels between tissue and EVs, there was a depletion in expression of miR-19a in EVs compared to tissue, with a mean fold change of 0.1916 (5.21 fold decrease).

Chapter 4: Discussion

Chapter 4: Discussion

This study has examined miRNA dysregulation in tissue and plasma EVs from early stage CRC patients, in order to assess if these miRNA are suitable liquid biopsy biomarkers. Current stool-based screening methods for CRC are imperfect, due to both limited sensitivity for detecting early stage disease and sub-optimal patient participation (30, 32-34). If a blood-based test that was as effective, or superior to, current methods it would almost certainly increase diagnosis rates of early CRC leading to decreased healthcare costs and improved patient outcomes.

CRC tumours release EVs into the bloodstream, miRNA within these EVs have shown significant potential as liquid biopsy biomarkers to help identify these patients. This study identified four candidate miRNA from the literature, all of which have demonstrated promise as biomarkers in CRC. However, these miRNA have never been examined together in a well-defined, early stage, cohort with matched tissue and plasma samples.

Our first aim was to establish evidence of dysregulation of the four candidate miRNAs in tumour tissue when compared to matched normal colonic mucosa. Two of the four candidate miRNAs, miR-183 and miR-1246, were significantly elevated in CRC tumour tissue.

Our second aim was to determine whether any dysregulation in tissue translated to altered expression in plasma EVs of stage I and II CRC patients compared to healthy controls. miR-183 and miR-1246 were overexpressed in tissue but were not reliably expressed in plasma EVs when using our methods. However, miR-19a was shown to be significantly downregulated in plasma EVs of CRC patients. A significant difference was even seen when comparing only stage I patients to controls, additionally it has superior sensitivity to CEA when identifying CRC; both of these factors suggest it may have potential as a liquid biopsy biomarker.

There a number of issues that need to be addressed in the search for a liquid biopsy biomarker in CRC. There are benefits and disadvantages to various methods of EV isolation and results in miRNA analysis can vary greatly depending on the chosen method. Furthermore, whether candidate EV miRNA are initially identified from

dysregulation in tumour tissue, or instead concentrating solely on markers in the circulation, or even on a broader discovery-based approach may be most useful. This is the first study to examine these four proposed markers together in matched tumour tissue and plasma EV samples, and significant dysregulation between CRC and controls was observed. There are substantial challenges in the search for a liquid biopsy, however the benefits to doing so are clear and obvious. An effective liquid biopsy in CRC has the potential to greatly benefit the growing numbers of people who will be affected by CRC.

4.1 Patient Demographics

There were no statistically significant differences in terms of age or gender between the CRC patient cohort and controls. When comparing the demographics of our cohort to CRC in the wider New Zealand population, the findings were broadly similar. In our study, the average age at diagnosis for colon cancer was 67.7 years and rectal cancer was 66.7 years. In the PIPER project, which is representative of a New Zealand population, the average ages for colon and rectal cancer were 67.9 and 71.4 years respectively (12). In the PIPER project overall, 75% were diagnosed with colon cancer and 25% with rectal cancer. Our figures of 78.8% and 21.2% for the plasma EV analysis appear to be more in line with what has previously been reported in New Zealand (10, 12, 139).

There were some notable differences between the tissue and plasma EV groups however. Only 11.9% of patients in the tissue analysis had rectal cancer, therefore our findings in tissue may not be directly applicable to rectal cancer. This is attributable to exclusion from the tissue analysis if a patient previously received neoadjuvant radiation. Neoadjuvant radiation has been demonstrated to alter the miRNA profile in CRC tissue (131); including both irradiated and non-irradiated CRC tumour in the same tissue analysis would introduce unknown confounding effects.

Neoadjuvant treatment similarly had a bearing on patient selection in the plasma analysis. All plasma samples were collected at the initial clinic appointment following diagnosis, prior to any treatment. Staging in our study is based on pathological examination of the resected surgical specimen, although preoperative staging is also recorded. If any change in staging occurred following neoadjuvant treatment, these patients were excluded from the plasma analysis, as they may have had more advanced disease at the time of plasma collection. Fourteen patients with rectal cancer were excluded in this manner. Because neoadjuvant treatment is only given in rectal cancer, this may further explain the difference between the higher rates of rectal cancer in New Zealand, and in the biobank overall, compared to our plasma analysis cohort.

4.2 Endogenous reference gene miRNA

For tissue, both miR-16 and miR-345 were used as endogenous reference genes, where there was no significant difference in expression between tumour tissue and normal mucosa. However, following analysis of our plasma EV data, miR-345 was not found to be suitable as an endogenous control. miR-345 has previously been recommended as a reference gene in CRC for both tissue and plasma EVs (133, 135). Danese et al. (2017) specifically endorses its use in the context of plasma EVs, this was following its successful use as a reference gene in CRC tissue by Chang et al. (133, 135). In the paper by Danese and colleagues, there is insufficient reporting of methodology, particularly the method of EV isolation, this may be a possible point of difference (135).

There are some technical considerations with RT-qPCR when identifying suitable endogenous reference genes for plasma EVs. Equal volumes of plasma were used for every sample in an attempt to ensure consistency in the quantity of RNA processed. One option may have been to use an exogenous control that is 'spiked in' (36). This may have demonstrated more reliable expression than our endogenous reference genes. However, expression of exogenous controls is dependent on the quantity of RNA extracted and does not change with natural variations in miRNA expression in the same manner as an endogenous control (140). Additionally, other studies have used RNA, such as RNU6B and RNU5A, instead of miRNA as an endogenous control, however these may not reflect natural differences in miRNA expression as accurately as a miRNA reference gene (81, 85, 91). Furthermore, employing multiple endogenous reference genes may be superior to a single endogenous control (141). Finding suitable endogenous reference genes in this context is an acknowledged issue in the literature that remains a focus of ongoing investigation (135, 142).

Any endogenous reference gene should ideally demonstrate reasonably high expression in order to ensure consistency across samples. Due to the nature of plasma EVs, the total RNA content that can be extracted is very low compared to tissue. This was exhibited in our data where the C_T values of miRNA from plasma EVs were much higher (i.e. lower relative expression) across all miRNA when compared to tissue. For example, miR-16a was expressed in all samples in the study

with no difference between CRC and control groups in either the tissue or the plasma EV analysis. However, miR-16 demonstrated a 108-fold decrease in expression between tissue and plasma EVs (mean C_T values 17.27 vs 24.04 across all tissue and plasma EV samples respectively).

4.3 Tissue miRNA expression

Of the four candidate miRNA, all were expressed in both tumour tissue and normal colonic mucosa. miR-183 and miR-1246 demonstrated significantly increased expression in tumour tissue, both miR-19a and miR-23a did not show any significant dysregulation.

miR-1246 was found to have a 147.9 fold increase in expression in tumour tissue compared to normal mucosa. There are conflicting reports in the literature regarding its expression in tissue, where results have shown its expression to be both elevated and mildly decreased (90, 91). Findings of an overexpression of miR-1246 in tissue is consistent with work produced by Scarpati et al. (2014), where a smaller 2.12 fold increase in expression from 54 CRC tumours, of which 24 were stage I and II (90). However, it conflicts with another study by Yamada and colleagues (2014) where a mild, 1.3 fold decrease expression was found in 33 tumours, of which 13 were early stage (90). Sample processing may have affected the measured miRNA expression in the study by Yamada et al., as samples were frozen in liquid nitrogen. In contrast, in our study samples were immediately stored in RNAlater Stabilization Solution™, which may have improved RNA quality compared to freezing (143).

Our results also demonstrated a significant overexpression of miR-183 in tumour tissue compared to normal mucosa. This is in line with the published data that consistently reports an increase in expression in tumour tissue (108, 109, 111). Both miR-1246 and miR-183 demonstrated an ability to discriminate between tumour tissue and normal mucosa on a ROC curve analysis. This may have limited applications in the search for a blood-based biomarker, but it is useful to evaluate the capacity of a marker in discriminating between CRC and control tissue and examine against other known variables. Where ROC curve analysis offers greatest

benefit in the context of our research is when it is used to compare CRC patients to healthy controls on a blood-based test, to assess potential as a liquid biopsy.

There was no correlation between miR-183 and miR-1246, suggesting that expression of each miRNA was independent of one another. It is important to ensure that no correlation exists prior to combining variables in a ROC curve (144, 145). If expression of each variable is related then combining them may lead to a spurious inflation of predictive capabilities (144, 145). A combined ROC curve found the discriminatory powers of both miRNA together was better than either miRNA alone. Both miRNA were subsequently evaluated for relationships with, demographic, blood-based (CEA, CRP, NLR) and pathological (PNI, EMVI, LVI, MMR) variables, but no direct correlations were found. However, when dichotomised into high (≥2 fold change) and normal miR-1246 (<2 fold change) expression groups, an association between high miR-1246 expression and increased NLR, a prognostic marker in CRC was observed. miR-1246 has been shown to be significantly elevated in metastatic compared to primary CRC tissue, and to promote tumour growth and metastasis in *in-vitro* models (146, 147). It has not been specifically identified as a prognostic marker to date in the literature; however its association with metastatic CRC suggests a possible relationship with advanced disease.

Interestingly, for both miR-1246 and miR-183 there was a significant association with female gender and the high miRNA expression group on chi-squared tests. The significance of this is uncertain, however it should be taken into consideration for any further research regarding these miRNA. Gender differences and ethnic variations in miRNA expression have been documented (148, 149). Differences in miRNA expression between demographic variables are an important consideration, both for interpreting published data, and in developing any miRNA based biomarkers. It is possible that different liquid biopsy biomarkers may perform significantly better in a specific group and ongoing research in this field should take this into account. It is possible that certain miRNA biomarkers will be more specific depending on gender or ethnicity. One solution may be developing specific miRNA biomarkers for these demographic variables. Another strategy may to combine a number of miRNA biomarkers together in a panel that may mitigate against some of these inconsistencies in expression.

Our results demonstrated a trend towards an overexpression of miR-19a in tumour tissue when compared to normal mucosa, this trend disappeared following exclusion of a single outlier from each of the tumour and normal colonic tissue groups. Data comparing miR-19a from early stage CRC tissue to matched normal mucosa does not appear to have been published in the current literature. It has been shown to be upregulated in tissue samples from CRC liver metastasis when compared to stage I and II primary CRC tumours (85). Our data did not evaluate metastases, but no statistically significant changes in expression were observed between either stage I or II and controls. If it is associated with metastatic CRC, tissue miR-19a may have potential as a prognostic biomarker. Patients who overexpress miR-19a in tissue may have worse outcomes such as higher recurrence rates. Of note, elevated levels of serum EV miR-19a have previously been associated various adverse factors such as LVI, metastatic disease, resistance to chemotherapy and worse overall survival (85, 99). An interesting area of future research would be to evaluate both tissue miR-19a and miR-1246 as prognostic biomarkers. A limitation of our cohort is that no 5-year follow up data is currently available to examine this.

In our study, there was no significant differences in miR-23a expression between normal mucosa and tumour. This conflicts with the published literature where miR-23a has been demonstrated to be elevated at least two-fold in tumour compared to normal colonic tissue in a number of studies (100-102). These studies did not specifically identify stage I or II patients in the data and elevated levels of miR-23a that are reported may be due to more advanced stage III and IV CRC. This would not have been detected in our study.

Tumour miRNA expression of the four candidate liquid biopsy biomarkers was examined in order to establish if changes in CRC tissue translated to changes in plasma EVs. It is possible that there is selective packaging of specific miRNA from tissue into EVs but this was not observed in our data. EV miRNA dysregulation in CRC may also not originate in tumour tissue, but from some other source, such as immune cells, red blood cells or platelets. Due to this, we must question the paradigm of initial miRNA analysis in tumour tissue to identify dysregulation

translating to circulating EVs, if the goal is to develop a blood-based liquid biopsy biomarker.

4.4 Plasma EV miRNA expression

Two of the four candidate miRNA were expressed consistently in plasma EVs of early stage CRC patients and healthy controls, of these miR-19a demonstrated a significant decrease in expression. miR-183 and miR-1246, both of which exhibited significant increases in tumour tissue, were not reliably expressed in plasma EVs when using our methods.

Our results found a 4.6 fold decrease in plasma EV miR-19a between healthy controls and CRC patients. In the literature, miR-19a was found to be dysregulated in circulating EVs (85, 99). Interestingly, this study found the direction of dysregulation was opposite to our findings. Matsumara et al. (2015) found an increase in miR-19a expression in serum EVs from 107 stage I and II CRC patients, compared to 28 healthy controls, a population which is broadly similar to our cohort (85). In light of this noteworthy difference between our findings and the currently available literature, further evaluation of miR-19a in circulating EVs is warranted, especially as plasma EV miR-19a may have potential as a liquid biopsy biomarker in early stage CRC.

miR-19a demonstrated significant differences for both stage I and II when compared to healthy controls on a one-way ANOVA test. The earlier in disease progression changes are detected in the bloodstream of patients, the greater potential a biomarker may have as a liquid biopsy. A detectable difference in stage I disease is ideal if developing a liquid biopsy for screening. It aids in identifying patients at the earliest stage of disease, which requires less intensive treatment and has improved long-term outcomes. When examining mean changes in expression, no significant differences were found. However, the mean fold change in expression was greatest at stage I (0.139, 7.2 fold decrease) in comparison to stage II (0.265, 3.9 fold decrease). Examining plasma EV expression of miR-19a in more advanced stages of CRC may be beneficial to assess if there are any significant differences in expression with further CRC progression.

When the expression levels between tumour and plasma EVs were compared, miR-19a was found to be depleted in plasma EVs compared to tumour tissue. This corresponded with a 0.192 fold change in expression (5.2 fold decrease). However, no significant change in miR-19a tumour expression was found on tissue analysis. A trend towards an increase in tumour tissue was observed, that was not statistically significant ($p=0.0579$). It is possible that this is reflecting miR-19a sequestration in tumour tissue and our study population ($n=42$) was underpowered to detect this difference. The directionality of a decrease in EVs, with a possible trend towards an increase in tumour tissue is interesting and may benefit from further examination.

Another possibility is that miR-19a containing EVs are sequestered at a specific site in the body in CRC. EVs have been shown to localise to pre-metastatic niche sites in the liver (150). If EVs were to concentrate in the liver, or possibly the lymphatic system, they may demonstrate decreased levels of expression when sampled from the circulation. It is also conceivable that miR-19a in circulating EVs originates in another source from CRC tissue entirely such as immune cells, and it is the body's response to CRC that effects EV miRNA expression. The exact cause for downregulation of EV miR-19a is unclear and requires further investigation.

CEA is the only currently used blood-based marker in CRC. It is used to monitor for recurrence, but is not used in the diagnostic or screening setting due to limited sensitivity and specificity (38). It is possible that if CEA was combined with another biomarker, that had a similar capacity to identify CRC, their ability to detect CRC would be additive. Combining two markers may then reach the appropriate levels of sensitivity and specificity to be useful as a liquid biopsy. The ability of plasma EV miR-19a to act as a liquid biopsy and differentiate between early stage CRC and healthy controls was evaluated. Plasma EV miR-19a had better sensitivity but lower specificity than CEA (sensitivity 62% and 45%, specificity 53% and 76% for miR-19a and CEA respectively). A high sensitivity is very beneficial when attempting to develop a screening test, and the fact that miR-19a has been shown to outperform CEA is promising. However, when compared against FIT (sensitivity 79%, specificity 94%), it appears that substantial progress is required. When the AUC of either marker was compared, CEA was superior. However, when combined together, they

were inferior to either individual marker when it came to differentiating stage early stage CRC patients from healthy controls.

Plasma EV miR-19a was compared against a number of currently available blood-based markers, there was a significant negative correlation between miR-19a and NLR, a prognostic marker. As plasma EV miR-19a is decreased in CRC, a negative correlation implies that both change in opposite directions in the presence of CRC. Previous research has found an elevation of serum EV miR-19a to be associated with a number of poor prognostic indicators including LVI, metastatic disease, and resistance to chemotherapy (85, 99). This increase in expression conflicts with the decrease observed in our data. It is possible that miR-19a may have an association with advanced disease, although the directionality of change in our data conflicts with published literature. A weakness in our study cohort is that no long-term follow up is currently available. Assessing both plasma EV miR-19a and tissue miR-1246, where an association with NLR was similarly seen, as prognostic indicators in our cohort in the future may provide further insight into any potential prognostic applications of these miRNA.

miR-1246 was not consistently expressed in plasma EVs when using our methods, despite a number of published studies documenting overexpression in circulating EVs in CRC (81, 85, 86, 90). As miR-1246 expression levels were significantly elevated in tissue, it is possible that it is being sequestered by CRC cells and thus not present in EVs at detectable levels. The current literature suggests a promising blood-based EV biomarker that is overexpressed in early stage CRC. Previous studies have reported a significant capacity to differentiate patients of all stages from healthy controls with an AUC of 0.948, and a sensitivity of 90% was found when discriminating stage I CRC from controls in a smaller validation cohort (86). Although our results did not demonstrate reliable expression in EVs, it warrants ongoing attention as a possible liquid biopsy biomarker.

Similarly to miR-1246, miR-183 exhibited a significant increase in expression in tumour tissue that did not translate to the plasma-EV analysis. Two studies that demonstrated miR-183 elevation in tissue also found that it was elevated in whole plasma. EVs were not isolated in these studies and it is possible that it was freely circulating, rather than EV, miR-183 that was detected (87, 110). miR-183 is part of

a miRNA family which includes miR-182 and miR-96, it has been suggested that miR-182 is preferentially packaged over miR-183 into EVs. It has been documented that miR-182 was the only detectable member of the miR-183 family in EVs from multiple breast and prostate cancer cell lines and it is possible that a similar process occurs in CRC (151).

miR-23a was expressed from all 66 plasma EV samples, but no difference in expression was found between early stage CRC and healthy controls. Plasma and serum EV levels have previously been shown to be upregulated in stage I and II CRC when compared to healthy controls and to decrease post resection (36, 85, 86). Ogata-Kawata and colleagues (2014) found miR-23a was able differentiate stage I CRC from healthy controls with an AUC of 0.953, this varies substantially from our findings (86). A common theme across all of the miRNA, in both tissue and plasma EVs is an inconsistency between our findings and the published literature. A number of possible explanations have been suggested and a significant area of variance across studies may lie in methodological differences.

4.5 Methodological differences

There are numerous differences in pre-analytical variables that are not consistent with our methods, including EV isolation and control selection. Analysing these areas of variance may offer insight into the discrepancies between our results and the published literature.

Our chosen method of EV isolation was SEC columns. Advantages of this method include its ease of use, low levels of co-isolated impurities and that it can be automated to adapt to the higher throughputs required in a commercial laboratory (152). None of the referenced studies used SEC columns or an automated fraction collector (AFC), which is a significant difference from our research. An AFC offers significant efficiency benefits and can process approximately six samples an hour. Multiples of this can be processed by a single person if more than one machine is available. In our study, two machines were used, this allowed EVs to be extracted from approximately twelve samples an hour. For any liquid biopsy to become a clinically viable test, adopting methods that can readily be applied to a commercial laboratory is an important challenge to address.

One of the most common methods of EV isolation employed in the literature is ultracentrifugation (UC). It has been used as the sole method of isolation, as density gradient UC, or in conjunction with filtration (81, 85, 86). UC is limited by the number of samples, typically six, that can be processed at a time. In addition, it can take 18 hours or more to process a set of samples (81). Low-density lipoproteins outnumber EVs in the circulation. SEC has shown significantly less co-isolation of soluble proteins when compared to UC (152). SEC-based EV isolation is an effective method of removing these high-abundance blood-based proteins, however this increased purity is sacrificed for decreased EV yield (153).

Different EV isolation methods have demonstrated significant variance in the quantity and quality of EV-specific miRNA, in addition to the amount of co-isolated impurities such as proteins, when analysing the same set of samples across different isolation methods (154, 155). It is possible that in studies where UC was employed, a significant amount of the miRNA expression was coming from non-EV sources such as freely circulating miRNA. This may offer an explanation as to why some of our results are significantly different to what has been reported. For example, high levels of expression of circulating EV miR-1246 are described in the literature but it was not reliably expressed when using our methods (86).

With regard to serum or plasma, there is conflicting evidence as to which source produces a higher miRNA yield from EVs (156, 157). Wang et al. (2012) found that serum produced a greater quantity of EV miRNA, whereas McDonald and colleagues (2011) reported that the opposite to be the case (156, 157). A majority of referenced studies used serum; this may offer another significant point of difference that may explain inconsistencies between our results and the reported data. Furthermore, contamination of a sample due to haemolysis, platelets or other impurities may also contribute to varying miRNA expression findings (156, 158, 159). To control for this in our study, all blood collection and plasma processing was standardised. All samples were visually inspected for haemolysis both at the time of processing and prior to EV isolation; in addition, pre-clearing centrifugation of thawed plasma samples was performed to reduce impurities.

Control selection may also be a contributing factor. It is possible that many ‘healthy controls’ may have underlying adenoma, CRC or inflammatory bowel disease that

can only be revealed by colonoscopy. In the NBSP pilot study, 51.5% of those who underwent colonoscopy had adenomatous polyps (35). The majority of studies in the literature did not state whether control patients had a normal colonoscopy to rule out these abnormalities prior to recruitment; this is a significant difference to our methods of control recruitment. It is conceivable that a significant proportion of controls in the current literature had underlying colonic adenoma, which may have a significant, but unknown effect on miRNA expression.

4.6 Strengths and limitations

The samples size of our cohort is a limiting factor. Although our research was adequately powered to detect differences in the primary aims, there were a number of additional areas that demonstrated trends in the data that our study was not powered to detect. There was greater than twice the amount of CRC patients compared to controls, were more controls available we may have been able to observe a significant decrease in the expression levels of miR-19a, rather than only in ΔC_T values. A trend towards an increase of miR-19a in tissue was also observed, however this did not reach the threshold for significance. In light of the significant decrease of EV miR-19a in plasma, confirming overexpression in tissue by increasing the number of tissue samples analysed, would suggest that CRC tumour tissue is sequestering miR-19a and not releasing it into EVs.

The use of a single reference gene in the analysis of plasma EVs is another weakness, miR-345 demonstrated inconsistent expression and only miR-16 was used. Ideally, at least two reference genes should be used (141). Additionally, using the same two endogenous reference genes across all tissue and plasma EV samples would have ensured greater consistency in the study, as this is a point of difference between the tissue and EV analysis.

A limitation of the study is the relatively low proportion of rectal cancers compared to colon cancers in the tissue analysis. Due to the nature of rectal cancer treatment, up to 70% of patients will receive neoadjuvant radiotherapy alone or with chemotherapy (160). These patients were excluded from the study as neoadjuvant treatment may cause changes in tissue miRNA expression (131). Recent evidence suggests that the proportion of rectal cancers, as a total of CRC, is increasing (161).

Only 11.9% of patients in the tissue analysis had rectal cancer, this does not represent the current, nor the likely future landscape of CRC. Therefore, caution should be exercised in interpreting the tissue findings as they are predominantly related to colon cancer.

A further question that requires attention is whether our approach in choosing candidates from the literature is the most effective strategy to identify a blood-based biomarker. The use of EV miRNA as biomarkers is a rapidly developing field. The advantage of using an EV isolation method such as SEC is a lack of contamination; this is especially useful when candidate markers have been identified. However, at this early stage of EV miRNA research there is a possibility that it is overly selective. Employing a discovery-based method in the form of small RNA sequencing, which is able to measure all small RNA species in an unbiased fashion may be more useful for identifying novel biomarkers in CRC that may be missed with SEC.

Quek et al. (2017) states that co-isolated impurities in EV isolation have little bearing on downstream miRNA quantification and that efficient, but more crude EV isolation strategies may be more useful for biomarker discovery (162). Specific methods that purify EVs above all other cell-free material, such as SEC may result in smaller libraries and worse performance when clustering is performed (154). Buschmann et al. (2018) found that EV isolation by methods such as precipitation, yields samples with decreased purity and significant co-isolated protein contamination, but states that these methods may have more potential for biomarker discovery (154). Our candidate-based approach has the potential to overlook markers that may be well suited to being a liquid biopsy in CRC.

Although there may be benefits to these methods, what makes EV related miRNA attractive as biomarkers is their potential specificity for diseases such as CRC. As previously discussed, CRC cells produce an abundance of EVs compared to normal cells, and the contents of EVs directly reflect their parent cell of origin. The literature has suggested that miRNA profiles between CRC and controls, in tissue and EVs, to be significantly different. The potential for EV miRNA to be sensitive and specific for CRC is substantial. There are many sources of miRNA within the bloodstream, including red blood cells, platelets and free miRNA. Less EV-selective methods may

analyse miRNA from a host of sources, which may defeat the purpose of isolating EVs. Future work may examine differences between whole plasma and EVs with regard to developing liquid biopsies.

Additionally, our research has found that matched tumour tissue and plasma EV samples can differ significantly in miRNA expression. It is not clear whether circulating EV miRNA dysregulation in CRC is originating from tumour tissue, or from another source entirely such as platelets, red blood cells or immune cells. If the ultimate goal is to develop a liquid biopsy for CRC, solely concentrating on markers that are dysregulated in the blood stream of CRC patients, rather than starting with tissue, may prove more beneficial. miRNA analysis in tissue may be more suited to identification of a prognostic biomarker in CRC, miRNA expression levels in tissue are much higher, in addition EVs do not require isolation. Identifying tissue miRNA that give insight into prognosis may have a substantial role to play in decision making regarding adjuvant treatment and intensity of post-operative follow up.

A strength of the study lies in the standardisation and automation of EVs from plasma using SEC columns and the AFC. Prior to the introduction of the AFC, this process involved manually collecting drops from the SEC column, this process may be inaccurate and user dependent. Another advantage is that this method has ease of scalability to meet the demands of a hospital laboratory, an important consideration for any liquid biopsy to become commercially viable.

Our recruitment identified age and sex matched controls that were symptomatic in the form of abdominal pain, perirectal bleeding or other symptoms suggestive of CRC but did not have disease on colonoscopy. This ensures that our controls are a cohort patients who would be most likely avail of a liquid biopsy test for CRC were it to become available. As these patients were recruited from endoscopy lists, it was necessary to consent prior to the procedure. Sedation is usually given during a colonoscopy and patients would be unable to give consent afterwards. Of the 53 patients recruited in this fashion, less than half were eligible controls and most of the remainder had polyps. Disadvantages of this method include the significant time investment required when many of those recruited were excluded from the control group.

This approach also had benefits however; collecting samples from a subset of patients with known polyps to the CRC biobank has potential for future research. Adenomatous polyps are the initial stage in the adenoma-carcinoma sequence (163). Up to 51% of patients who undergo a colonoscopy looking for CRC will have polyps, as evidenced in the NBSP pilot study (35). Circulating EV miRNA dysregulation has been reported to commence once a polyp is formed (164, 165). It will be possible to compare healthy control, polyp and CRC subgroups to further assess the effect polyps have on plasma EV miRNA expression. A liquid biopsy that can identify polyps prior to developing into CRC has obvious benefits. Furthermore, control patients used in this study can be used across a range of CRC research that is conducted in the SCRG.

4.7 Future directions

There are a number of challenges in developing EV miRNA as liquid biopsies in CRC. Much research has focused on total circulating, rather than EV-specific, miRNA levels. An advantage of this strategy is that there is no requirement for EV isolation, this has efficiency benefits with regard to sample processing, but potentially has lower specificity for CRC. It is also apparent that all the miRNA discussed are dysregulated in multiple disease processes, in addition to possible changes in expression across demographic variables such as gender or ethnicity. Combining a number of miRNA together in a panel may be a useful way to offset this lack of specificity.

It is not currently obvious how to best identify candidate EV miRNA biomarkers. It is possible a discovery based approach may detect novel markers which have the required sensitivity and specificity for a liquid biopsy. If this approach is taken, then consideration of the EV isolation methods is necessary as SEC may be overly selective. Additionally, as changes in tissue miRNA expression do not appear to have a predictable effect on EV miRNA expression, the utility of tumour tissue analysis in the search for a liquid biopsy for diagnostic or screening purposes should be questioned.

The literature may be inconsistent in part due to differences in pre-analytical variables and there appears to be insufficient reporting of methodology in some

cases. There is a need to standardize methods and develop technologies that aid in the isolation and detection of EVs to make it practical to perform in a hospital laboratory. Our methods are possibly a step closer to becoming clinically feasible, as the capacity to process samples can readily be increased. Global adoption of the MISEV 2018 guidelines across EV research may be a useful starting point to address some of these methodological issues (166).

It is necessary to evaluate how EV miRNA liquid biopsy biomarkers can be integrated into a modern healthcare system. Ideally, a blood-based biomarker would outperform current faecal-based assays on all parameters for screening. Identifying EV miRNA biomarkers that are both sensitive and specific to CRC may prove challenging. Using biomarkers in conjunction with current screening methods may provide an alternative avenue for their adoption to current clinical practice. Implementing blood-based screening in the first instance, followed by a stool-based test if suggestive of CRC is one option. Another possible route is performing the liquid biopsy following a negative faecal-based assay in the context of ongoing symptoms suggestive of CRC.

4.8 Conclusions

New Zealand has some of the highest rates of CRC in the world, but it is also a growing global issue. Current strategies to diagnose CRC, such as population level stool-based screening, are flawed. If a blood based screening test for CRC was available, it would have substantial benefits including an improvement in patient outcomes. EV associated miRNA appear to have great potential as liquid biopsy biomarkers in CRC that may help address this. Our study examined four miRNAs in both tissue and plasma EVs based on promising data in the literature.

Plasma EV miR-19a was significantly downregulated in EVs of CRC patients. It demonstrated significant differences even when stage I patients alone were compared against controls, it also had superior sensitivity to CEA in our cohort. These factors are promising in the context of developing a liquid biopsy marker to screen for CRC. However, its specificity was poor and it requires further examination to establish any efficacy.

Questions remain over the initial analysis of miRNA dysregulation in tissue in order to observe translation to EVs. Our results did not demonstrate any relationship between EVs and tissue. The benefits of tissue analysis may lie in developing prognostic biomarkers that can help in post-surgical treatment planning. Additionally, considering the current evolving landscape of EV miRNA biomarker research, whether a selective candidate-based approach or a more broad discovery-based approach are the most effective methods of identifying potential markers remains to be seen.

Plasma EV miRNA display great promise as liquid biopsy biomarkers, but currently there are a number of challenges in identifying a marker specific for CRC. Finding a liquid biopsy biomarker for CRC that is as effective, or outperforms, current stool-based screening methods may lead to significant increases in screening participation rates. The subsequent effects would likely include increased rates of diagnosis of early CRC, with decreased healthcare costs and improved clinical outcomes for the millions of people who will suffer from this very common disease.

Supplementary Material

Supplementary Tables

Stage	T	N	M
0	T_{is}	N0	M0
I	T1/T2	N0	M0
II	T3/T4a/T4b	N0	M0
III	T1/T2/T3/T4	N1a/b/c/N2a/b/c	M0
IV	Any T	Any N	M1a/M1b

Table S1 *– Colorectal Cancer TNM Staging (AJCC 7th edition)* T_{is} *= carcinoma in situ, T1 = tumour invades submucosa, T2 = tumour invades muscularis propria, T3 = tumour invades into pericolorectal tissues T4 = tumour penetrates to the surface of the (a) visceral peritoneum or (b) directly invades, or is adherent to other organs or structures, N0 = No regional lymph node metastasis, N1 = Metastasis in 1-3 lymph nodes (a= 1, b= 2-3, c = no lymph nodes but deposits in subserosa, mesentery or non-peritonalised perirectal/mesorectal tissues,, N2 = 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).*

Normal Mucosa (mg)	Tumour Tissue (mg)
18.8	48.9
65.3	68
45.9	28
33.8	12.6
38.7	58
36.8	16.6
19.2	36.8
33.2	36.8
26.2	36.1
40.5	55.2
17.8	21.7
41.4	10.6
24.8	6.5
29.2	9.5
66.6	32.6
32	19.2
47.8	24.8
36.1	46.7
78.2	64.3
53.3	66.4
15.2	10.7
19.5	14.1
31.3	54.3
14.6	28.9
Mean weight (±SD) **36.09 (16.89)**	**33.64 (19.87)**

Table S2 – *Values for weight of matched tissue used in analysis (n=24).*

Normal Mucosa (ng/uL)	Tumour Tissue (ng/uL)
51.7	1884.7
666.2	302.4
1465.2	620.9
3281	5074.7
1323.9	938.5
2121.8	895.7
75.1	4825.5
665.9	473.1
82.3	288.8
337.4	702.6
226.1	446.1
1239.9	1307.7
808.3	1260.3
1486.7	2072.6
252.8	623.8
1501.5	728.9
459.7	190.4
308.3	145.1
575.1	790.5
164.3	1093.5
153	696.8
451.2	238.5
313.5	128.1
278	1249.4
319.1	489
1177.2	1115
155.1	580.2
1388	1521.8
1092.8	794.4
422.5	1492.5
125.7	481
1158.5	1735.7
353.3	1887.5
1694.3	2627.1
388.2	220.3
140.9	185.3
450.2	598.7
257.7	989.2
666.8	168.7
430.5	455.9
608.7	1355
3854	1266
Mean Conc. (SD)	**702.5 (663.5)**	**1070 (1059)**

Table S3 – *concentration of RNA from matched normal colonic mucosa and tumour tissue (n=42).*

	Mean fold change in expression (±SD)			
	miR-19a	miR-23a	miR-183	miR-1246
EMVI Present (n=3)	0.67 (0.19)	0.86 (0.45)	11.47 (18.57)	18.28 (7.19)
EMVI Absent (n=39)	1.65 (1.58)	1.57 (1.53)	1165 (3356)	197.2 (561)
p value	0.296	0.437	0.567	0.589
LVI Present (n=4)	1.51 (1.14)	0.35 (0.11)	1.08 (0.59)	80.15 (130)
LVI Absent (n=38)	1.59 (1.59)	1.64 (1.51)	1105 (3272)	195.1 (569.4)
p value	0.925	0.100	0.646	0.693
dMMR (n=9)	1.32 (0.84)	1.05 (0.63)	27.24 (17.86)	394 (890)
pMMR (n=33)	1.65 (1.69)	1.64 (1.63)	1229 (3448)	114.8 (366)
p value	0.578	0.296	0.502	0.180
PNI Present (n=3)	0.68 (0.19)	0.86 (0.44)	11.47 (18.57)	18.28 (7.19)
PNI Absent (n=39)	1.65 (1.58)	1.57 (1.53)	11.47 (18.57)	197 (561)
p value	0.296	0.434	0.567	0.589
Female (n=19)	1.68 (1.88)	1.95 (1.92)	445.5 (951)	355 (766)
Male (n=23)	1.50 (1.24)	1.15 (0.89)	1740 (4694)	36.17 (84.36)
p value	0.719	0.082	0.360	0.726
Left sided CRC (n=17)	1.6 (1.67)	1.67 (1.75)	438 (885)	279 (699)
Right sided CRC (n=25)	1.56 (1.38)	1.28 (1.00)	2123 (5346)	55.9 (129)
p value	0.941	0.411	0.254	0.217
	Correlations (Spearman's rank coefficient)			
Age (Spearman's *r*)	0.019	-0.055	-0.143	-0.159
p value	0.905	0.727	0.534	0.347

Table S4 *- mean fold changes in tumour tissue expression and clinical and pathological variables, all unpaired t-test, other than age where Spearman's test is used.*

	miR-183 normal (n=11)	miR-183 overexpressed (n= 24)	miR-1246 normal (n=14)	miR-1246 overexpressed (n=26)
EMVI Present	1	0	1	0
EMVI Absent	10	24	13	26
p value		0.257		0.350
LVI Present	2	2	2	1
LVI Absent	9	22	12	25
p value		0.575		0.2763
dMMR	3	5	4	4
pMMR	8	19	10	22
p value		0.685		0.4162
PNI Present	1	1	2	1
PNI Absent	10	23	12	25
p value		0.536		0.2763
Female	2	14	2	16
Male	9	10	12	10
p value		**0.035**		**0.007**
Left sided CRC	6	8	7	10
Right sided CRC	5	16	7	16
p value		0.283		0.521
Stage I	2	4	4	3
Stage II	9	20	10	23
P value		0.999		0.214

Table S5 *– High and low tumour tissue expression subgroups for miR-183 and miR-1246 with relationships to clinical and pathological variables. Chi-squared test for all. Significant p-values underlined.*

	miR-183 normal (n= 11)	miR-183 overexpressed (n= 24)	miR-1246 normal (n=14)	miR-1246 overexpressed (n=26)
Age (mean ±SD)	71.55 (11.76)	66.58 (13.94)	70.14 (12.52)	66.65 (12.61)
p value		0.3136		0.4079
CEA (mean ±SD)	3.96 (6.31)	5.63 (6.57)	4.04 (5.75)	5.13 (6.37)
p value		0.4867		0.5977
CRP (mean ±SD)	7.7 (5.14)	10.96 (20.89)	15.92 (26.72)	9.15 (9.49)
p value		0.6322		0.2513
NLR (mean ±SD)	3.01 (1.35)	2.85 (1.14)	2.45 (0.86)	3.21 (1.22)
p value		0.700		**0.0444**

Table S6 *- High and low tumour tissue expression subgroups for miR-183 and miR-1246 with relationships to blood-based variables. Unpaired t-test for all. Significant p values underlined.*

	miR-19a	miR-23a
CEA	0.034	0.019
p value	0.793	0.875
CRP	-0.157	-045
p value	0.242	0.721
NLR	-0.276	-0.308
p value	**0.032**	**0.011**

Table S7 *– Correlations of plasma EV miRNA expression and blood-based variables. Spearman's rank coefficient (r). Significant p values underlined.*

Supplementary Figures

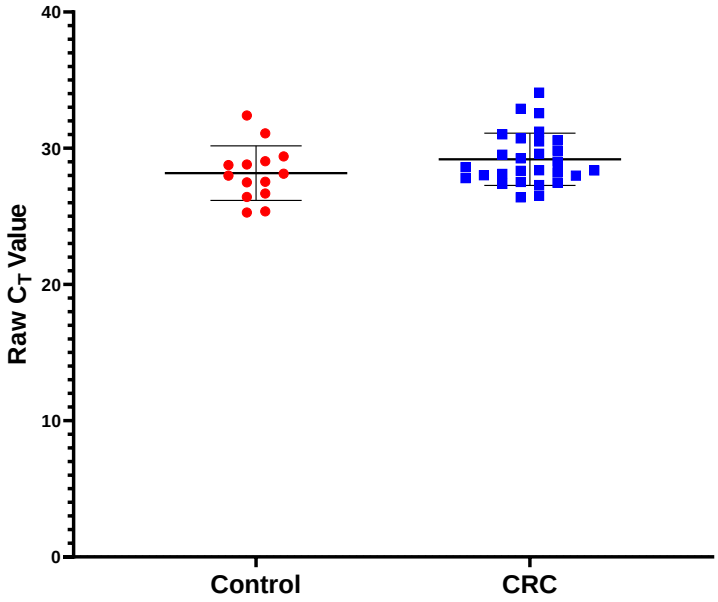

Figure S1 *- miR-345 raw C_T value (control n=14, CRC n=24, p=0.1194, unpaired t-test)*

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