BIOMARKERS AND LYMPH NODE MORPHOLOGY IN EARLY-STAGE COLON CANCER

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Abstract

New Zealand has one of the highest rates of colorectal cancer (CRC) in the world, resulting in approximately 1200 deaths per year. Optimising the use of adjuvant chemotherapy is one key area to reduce CRC-related mortalities. Stage II colon cancer patients are clinically and biologically heterogenous. Despite 60-80% of these patients being cured from surgery alone, a subset of these patients have a recurrence risk approximating stage III disease. Current risk stratification for the administration of adjuvant chemotherapy fails to accurately detect subgroups with different prognostic and treatment sensitivities. The discovery and use of molecular biomarkers could be used to improve the power of this risk stratification.

The aim of this exploratory, pilot study was to characterise the expression of potential prognostic biomarkers, CD147, microRNA-29a and microRNA-21, in a small pilot cohort of stage II colon cancer patients. These markers are strongly associated with aggressiveness and progression in later-stage CRC. However, their prognostic potential in early stage colon cancer has not been well defined. Additionally, these markers are expressed on tumour-derived extracellular vesicles (tEVs) which can home to nearby lymph nodes and promote the spread of cancer. Considering the lymph nodes are the first site for dissemination of tumour cells in CRC, tEVs may be particularly relevant to stage II colon cancer patients who undergo recurrence. Therefore, the overexpression of these markers could be risk-factors in the tumour and draining lymph nodes of these patients.

Tumour, normal mucosa, and the draining lymph nodes were collected from 13 stage II colon cancer patients at the time of surgical resection. Immunohistochemical and quantitative reverse transcription PCR techniques were optimised for the detection of CD147, miR-21 and miR-29a in all tissue types. The histological morphology of the lymph nodes was also explored.

We found CD147 was overexpressed in 60% of tumours while miR-21 was overexpressed in 50% of tumours. When combined, CD147 and miR-21 potentially highlight specific subsets of patients. Tumour-associated expression of miR-21 was dysregulated in the lymph nodes, while tumour-

specific CD147 expression was not detectable due to immunological-associated expression. Instead, histomorphological findings suggested a large variation in the number, size and shape of lymph node B cell compartments. Specifically, changes in follicle and germinal centre size density and size were associated with pathological risk factors such as, the presence of lymphatic invasion and T stage.

CD147 and miR-21 are considerably dysregulated in stage II colon tumours and combined with our knowledge of their functionality in preclinical and clinical studies, they represent potential prognostic biomarkers for this population. We have also demonstrated how the heterogenous histomorphology of B cell compartments, within the lymph nodes, could be a reflection of the observed clinical heterogeneity in these patients. Altogether, these potential biomarkers could be used to strengthen the current risk stratification in stage II colon cancer patients. While our preliminary data warrants validation in a future, larger cohort, our findings demonstrate the clinical feasibility of detecting potential biomarkers and direct the research design of future studies.

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List of Abbreviations

µl	microlitre
µM	Micrometre
5-FU	Fluorouracil
$A_{260/280}$	Absorbance at 260 and 280 nanometres
Ab	Antibody
ABC transporters	ATP-binding cassette transporter
BMDCs	Bone marrow-derived cells
CD147/EMMPRIN	Cluster of differentiation 147/extracellular matrix metalloproteinase inducer
cDNA	Complementary DNA
CEA	Carcinoembryonic antigen
COX-2	Cyclooxygenase 2
CRC	Colorectal cancer
Ct	Cycle threshold
DFS	Disease-free survival
dMMR	Deficiency in mismatch repair
DPX	Distyrene, tricresyl phosphate and xylene
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ESMO	European Society of Medical Oncology
EMT	Epithelial-to-mesenchymal transition
etoh	Ethanol
FFPE	Formalin-fixed paraffin-embedded
GCs	Germinal centres
H&E	Haematoxylin and Eosin
HER2	Human epidermal growth factor 2
HR	Hazard ratio
IHC	Immunohistochemistry
IRS	Immunoreactivity score
ISH	*In situ* hybridisation
KLF4	Kruppel-like factors
LNA	Locked nucleic acid
miR-21/29a	MicroRNA-21-5p/29a-3p
miRNA	MicroRNA
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
MSS	Microsatellite stable
NCCN	National Comprehensive Cancer Network
NFkB	Nuclear factor kappa-light-chain-enhancer
ng	Nano gram
oncomiR	Oncogenic microRNA
OS	Overall survival
PBS	Phosphate-buffered saline
PDCD4	Programmed Cell Death 4
PFA	Paraformaldehyde
PMN	Pre-metastatic niche

PTEN	Phosphatase and tensin homolog
REDCap	Research Electronic Data Capture
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SCRG	Surgical Cancer Research Group
TDLNs	Tumour-draining lymph nodes
TDSFs	Tumour-derived secreting factors
tEVs	Tumour-derived extracellular vesicles
TMA	Tissue Microarray
TME	Total mesorectal excision
TME	Tumour microenvironment
VEGF	Vascular endothelial growth factor

CHAPTER 1: INTRODUCTION

1.1 COLORECTAL CANCER – PREVALENCE, TREATMENT AND OUTCOMES

1.1.1 PREVALENCE OF COLORECTAL CANCER

Colorectal cancer (CRC) is the second most frequently diagnosed malignancy in New Zealand (1). Over 3000 people are diagnosed with CRC every year, claiming approximately 1200 lives. More than 75% of these diagnoses occur in people 60 years and older (1). Therefore, with an ageing population these statistics are expected to worsen (1, 2). In addition to this, New Zealand and Australia have the highest per capita rate of CRC in the world (1). This highlights the burden of CRC in the clinic and the tremendous value improved patient outcomes would have for New Zealanders and our health system.

1.1.2 COLORECTAL CANCER AND PATHOLOGICAL STAGING

CRC is a malignancy of the colon or the rectum whereby colon cancer represents approximately 74% of CRC's with rectal cancers making up the minority (1). Anatomically, the colon and the rectum make up the majority of the large intestine with the colon being proximal to the rectosigmoid junction and the rectum being distal (3). More than 90% of colorectal carcinomas are adenocarcinomas originating from epithelial cells of the mucosa (4) and are characterised by glandular structures with varying abnormalities. Other CRC tumours can arise from neuroendocrine, squamous, spindle and other cell types, although they are particularly rare (4).

The TNM staging system is the single strongest prognostic indicator for CRC which assists in treatment decision making (1). Histological examination determines the depth of invasion through the bowel wall (T), the number of lymph nodes involved (N) and presence of distant metastasis (M), ultimately reflecting the extent of disease (*5*) (Table 1). For stage I the primary tumour has invaded the submucosa or the muscularis propria while for stage II it has invaded into the subserosa or visceral peritoneum (Table 1). By stage III the cancer has spread to a number of lymph nodes and by

stage IV the cancer has spread to distant organs (Table 1). According to the New Zealand PIPER Project Statistics (2018), patients diagnosed with stage I colon cancer have an 80% long-term survival rate which drops to 6% in stage IV patients, reflecting the prognostic significance of this system (6).

Table 1: TNM Staging System for Colorectal Cancer (5)	
Primary Tumour (T)	
T_x	Tumour cannot be assessed
T_{is}	Carcinoma in situ
T_1	Tumour invades submucosa
T_2	Tumour invades muscularis propria
T_3	Tumour invades subserosa
T_{4a}	Tumour directly invades visceral peritoneum
T_{4b}	Tumour invades or has attached to adjacent organs/structures
Regional Lymph Nodes (N)	
N_x	Lymph nodes cannot be assessed
N_0	No lymph node metastases
N_1	Metastases in 1-3 lymph nodes
N_2	Metastases in ≥4 lymph nodes
Distant Metastasis (M)	
M_x	Metastases cannot be assessed
M_0	No distant metastases
M_1	Distant metastases present
Stage	
I	T_1-T_2
IIA	T_3
IIB	T_{4a}
IIC	T_{4b}
IIIA	T_1-T_2, N_1/N_{1c} or T_1, N_{2a}
IIIB	T_1-T_2, N_{2b} or T_2-T_3, N_{2a} or T_3-T_{4a}, $N_{1/1c}$
IIIC	T_{4b}, N_1-N_2 or T_2-T_3, N_{2a} or T_3-T_{4a}, N_1-N_2
IV	$T_{1\text{-}4}$, $N_{1\text{-}2}$, M_1

1.1.3 TREATMENT OF NON-METASTATIC COLON CANCER

Surgical resection of the primary tumour is the mainstay of treatment for CRC cancers. However, surgical approaches and targeted secondary treatments for colon and rectal cancers are distinct (7, 8) suggesting they should be considered separately.

The type and extent of surgery performed largely depends on the location and stage of the tumour and the draining lymph nodes (9). For non-metastatic colon cancers where surgeries are performed

with curative intent, typically the surgical approach is a right/left hemicolectomy, transverse colectomy or subtotal colectomy (10). All remove part or the entirety of the colon and draining mesenteric lymph nodes. Alternatively, the surgical approach for rectal cancers involves a total mesorectal excision (TME) which removes the entire mesorectum and lymph nodes and the intact enveloping fascia (11). Consequently, the rate of complications, including anastomotic leakages, blood clots and infections, are higher in rectal cancer patients (7). Despite this, associated mortality rates are higher in colon cancer patients due to more severe complications post-surgery in this group (7).

Differences also exist in the use of systemic and targeted secondary treatments that aim to reduce the risk of recurrence and death between colon and rectal cancer patients (9). For many non-metastatic colon cancers, treatment post-surgery is limited to chemotherapy while rectal cancer surgery can be preceded by neoadjuvant radiotherapy or chemoradiation and less commonly adjuvant chemotherapy (9) (figure 1). This is largely due to the high risk of locoregional recurrence in rectal cancers as a result of closely surrounding organs and the difficulty of achieving wide surgical margins (9). Additionally, colon and rectal cancers are varied in their response to these treatments and their long-term recurrence rates (9). While these two cancers are often referred to in combination, this study has focused on colon cancers due to disparities in treatment and thus we will refer to colon cancer from here on.

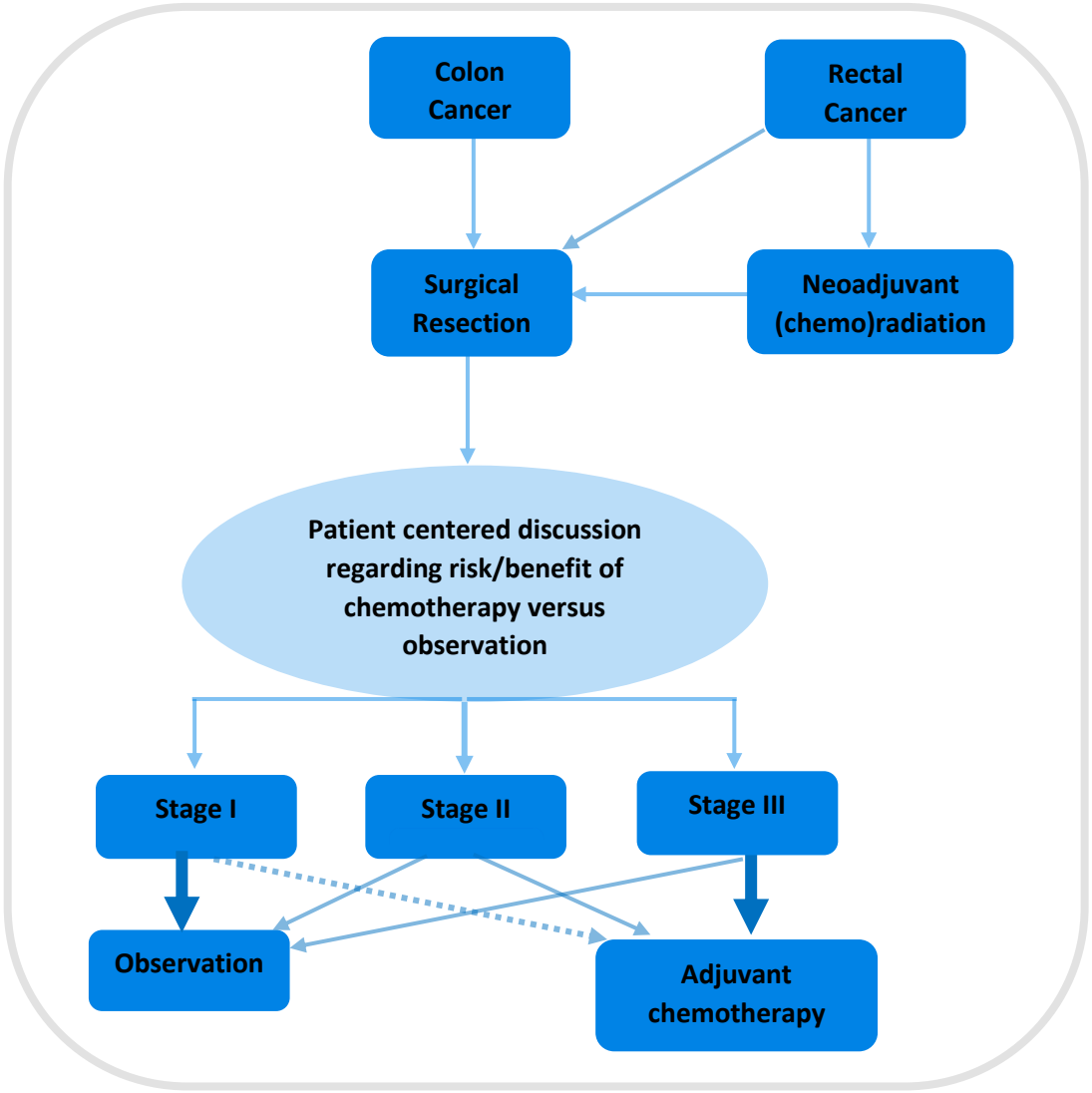


Figure 1: Basic flow diagram of treatment in non-metastatic CRC.
Colon and rectal cancer patients undergo surgical resection of the primary tumour. Rectal cancer patients may be preceded by neoadjuvant (chemo)radiation before surgery. TNM stage is then confirmed after surgical resection. The choice of whether CRC patients undergo adjuvant chemotherapy comes down to an informed patient/clinician discussion. Stage I patients rarely undergo adjuvant chemotherapy while most stage III patients undergo chemotherapy. The decision to administer chemotherapy to stage II patients is usually less straightforward.

1.1.4 RISK ASSESSMENT IN STAGE II COLON CANCER PATIENTS

Recurrence following curative-intent surgery and adjuvant therapy in colon cancer is often the ultimate cause of death (12). Complete recovery for stage I patients is largely achieved by surgical resection alone with these patients having a 5-year survival rate of approximately 90% (13). stage II and III patients experience survival rates of approximately 60-80% and 45-80% respectively (13) therefore the addition of adjuvant chemotherapy in these patients is considered (figure 1).

Despite this, the decision to use chemotherapy, particularly for stage II patients is complicated and variable (figure 1). Stage II patients are clinically heterogenous. While most stage II patients only require surgical resection for a cure from the disease, a subset of these patients have a survival rate approximating or even beyond stage III disease (13). This suggests the TNM staging system alone is limited. Therefore, high-risk clinicopathological features are also considered in stage II patients to assess their risk of recurrence and guide the administration of chemotherapy. Although no consensus exists on the definition of high-risk stage II colon cancer, international guidelines include features such as T4a and T4b stage, poorly differentiated pathology, perforation, bowel obstruction, perineural invasion, lymphovascular invasion, less than 10-12 lymph nodes examined and positive surgical margins (13). But even with incorporation of high-risk features, inconsistencies exist – we know that 'average risk' patients can recur whilst some 'high risk' patients do not (14). Taken together it appears the current risk assessment for stage II patients is limited. This is reflected by contradictory evidence from clinical trials and discrepancies between international guidelines over treatment decisions.

1.1.5 GUIDELINES FOR TREATMENT DECISIONS

In contrast to stage II patients, decision making regarding the use and lack of use of adjuvant chemotherapy in stage I and III colon cancer patients is mostly straightforward. Stage I patients typically only require surgery for complete recovery. Therefore, New Zealand's Standards of Service Provision for Bowel Cancer Patients do not recommend the administration of adjuvant chemotherapy to these patients (Table 1) (15). This is consistent with major international guidelines such as The National Comprehensive Cancer Network (NCCN) (13) and The European Society of Medical Oncology (ESMO) (12) (Table 1). Similarly, the decision to use adjuvant therapy in stage III is also relatively uncomplicated. All guidelines strongly recommend the use of adjuvant therapy in all stage III patients (Table 1).

Despite this, discrepancies exist between major guidelines over the use of adjuvant chemotherapy in stage II colon cancer patients. The NCCN guidelines recommend observation, a clinical trial or a course of adjuvant therapy for all stage II patients regardless of high-risk features (although the presence of these features should weight the decision towards pro-adjuvant therapy) (13). Fluoropyrimidine-based regimens are recommended with the addition of Oxaliplatin only for high-risk patients (Table 1). ESMO are more conservative in their recommendations that adjuvant chemotherapy should not be administered to stage II patients and only considered for patients with high-risk features, however no specific requirements of the chemotherapy regime are given (12). Similarly, the New Zealand Bowel Cancer Tumour Standards recommend the use of adjuvant chemotherapy only for high-risk stage II patients (15).

Across all guidelines there is a general agreement that the decision to use adjuvant chemotherapy in stage II patients should incorporate informed discussions between the patient and physician. This involves consideration of prognostic aspects of the disease itself as well as non-disease related characteristics such as comorbidities, fitness and age (12). It cannot be absolutely recommended one way or another whether to use chemotherapy.

Table 2: International and New Zealand Guidelines for treatment of stage I-III colon cancer patients.

Guidelines	Stage I	Stage II	Stage II high-risk	Stage III
NCCN 2018 (13)	No adj chemo	No adj chemo or Clinical trial or 5-FU/LV[a] or Capecitabine[b]	5-FU/LV or Capecitabine or FOLFOX[c] or CapeOx[c] or FLOX[c] for patients with at least one high-risk factor	FOLFOX or CapeOx or FLOX or 5-FU/LV or Capecitabine
ESMO 2013 (12)	No adj chemo	No adj chemo	Adj chemo (type not specified) for patients with at least one high-risk factor	FOLFOX or FOLFOX-4[c] or CapeOx or FLOX or 5-FU/LV or Capecitabine
NZ Standards of Service Provision for Bowel Cancer Patients 2013 (15)	No adj chemo	No adj chemo	Offered same adj chemo regimen as stage III patients	Oxaliplatin-based

[a]5-FU; fluorouracil and leucovorin calcium combined
[b]Capecitabine; 5-FU prodrug
[c]FOLFOX, FOLFOX-4, CapeOX, FLOX; Oxaliplatin-based therapies

1.1.6 EVIDENCE FOR THE USE OF ADJUVANT CHEMOTHERAPY IN STAGE II COLON CANCER

Contradictory evidence from clinical trials has led to discrepancies in guidelines for the use of chemotherapy in stage II colon cancer patients. Prior to 2007, there was no compelling evidence for

the use of adjuvant chemotherapy in these patients. Some landmark trials (including IMPACT B2, Ontario, MOSIAC and NSABP C 07), failed to find any statistically significant benefit for overall survival (OS) in stage II patients (16, 17, 18, 19). Trials comparing Fluorouracil (5-FU) with no chemotherapy found no benefit in OS however, a trend towards benefit was noticed (16,17, 19). In contrast to this, the QUASAR trial, which randomly assigned patients to 5-FU and no chemotherapy, found a small but significant 2.9% increase in OS in the treated group (20*).* Furthermore, the results of a 2016 analysis of over 150,000 stage II colon cancer patients, supported the QUASAR trial and found treatment was statistically associated with improved survival (HR, 0.76; *P*<0.001) (21*)*. These conflicting results make it difficult to derive the true benefit chemotherapy has for stage II colon cancer patients.

For high-risk stage II colon cancer patients, the role of chemotherapy is still debated. Clinical and pathological data required to subgroup patients in large clinical trials are not complete or precise enough to evaluate treatment in small patient subsets (20, 22). Therefore, the idea that chemotherapy should be administered to high-risk patients is based off high-risk features that are only moderately prognostic of outcome in stage II colon cancer. The rationale for providing chemotherapy to high risk stage II is the clear benefit seen in stage III colon cancer. A recent meta-analysis found the 5-year disease-free survival (DFS) in stage III colon cancer patient was 49% for those not treated with chemotherapy and 63.6% for those who were (19). Because high-risk stage II patients have a 5-year OS closer to stage III disease (13), it may be worth exposing only these patients to chemotherapy as opposed to all stage II patients. This is the perspectives of the ESMO and NZ guidelines (12, 15): if there is a benefit from treatment in stage II patients it is likely to be small and only applicable a subgroup.

Interestingly, the NCCN suggests chemotherapy be an option for all stage II patients, regardless of risk status, because the evidence base for the benefit of fluorouracil (FU)-based therapy in stage III patients is clear (13). However, inconsistencies exist within these guidelines. The addition of

Oxaliplatin to fluoropyrimidine therapies has a clear benefit in the OS of stage III patients. While there is no such evidence in stage II patients the NCCN recommend the addition of Oxaliplatin to fluoropyrimidine therapies only to high-risk stage II patients as opposed to all stage II patients (13).

1.1.7 LIMITATIONS OF THE CURRENT RISK-ASSESSMENT FOR STAGE II PATIENTS

The ultimate decision of whether to administer adjuvant therapy to stage II patients appears to come down to an imperfect risk assessment and non-disease related characteristics. As suggested, stage II colon cancer patients represent a clinically heterogeneous group. No large-scale study has thoroughly assessed the prognostic and predictive impact of all risk features together. However, clinical observations suggest these risk features are only moderately prognostic and lack the ability to predict responders from non-responders to chemotherapy in stage II colon cancer patients. This suggests the current definition of high-risk patients requires refinement or revaluation. In an ideal situation, prognostic indicators would more accurately stratify stage II patients to prevent the over or under use of chemotherapy.

1.2 PROGNOSTIC AND PREDICTIVE MOLECULAR BIOMARKERS IN COLON CANCER

The search for molecular biomarkers for the management of patients with stage II colon cancer has been the focus of a significant amount of intensive research (20, 23, 24). This is largely based on the poor predictive power of current pathological and clinical factors, as previously discussed. The heterogeneous response to adjuvant chemotherapy seen in this population is likely a reflection of heterogeneity at the biological level. Exploring the underlying biology may allow us to detect more powerful prognostic and predictive factors (25). For this reason, molecular biomarkers are a particularly promising development. Molecular biomarkers could also be additive to clinicopathological factors currently used to stratify stage II patients and improve the accuracy of this stratification.

Indeed, molecular markers already routinely used in the clinic for CRC, such as Carcinoembryonic antigen (CEA), KRAS and microsatellite instability status (MSI), have been evaluated for their prognostic significance in the context of stage II colon cancer patients. However, only MSI status appears to have any clinical relevance to this patient group and is now thus routinely used in the clinic for this population. Conflicting evidence exists as to the effectiveness of CEA and KRAS and their associations with recurrence are likely weak (23, 24, 26). Importantly, they have not been shown to add any more prognostic/predictive information to current clinicopathological risk features (23, 24), this is reflected in their infrequent use specifically for stage II colon cancer patients (12, 13). Candidate biomarkers such as gene-expression profiling have also emerged in the literature. However, these markers seem to also be limited by their weak associations (27) and our poor understanding of how their underlying biology is relevant to stage II colon cancer patients.

1.2.1 MSI – A VALIDATED PRO-NO ADJUVANT PROGNOSTIC BIOMARKER

MSI is an example of a successful functional biomarker (currently implemented in the clinic) that has significant prognostic ability in stage II colon cancer patients. MSI status is a molecular signature of deficiency in the mismatch repair (dMMR) proteins and as such, MMR proteins are routinely measured by immunohistochemistry (IHC) in the clinic as a surrogate for MSI (28). MSI status has mostly been used for the detection of Lynch syndrome, an inherited form of a dMMR system (13). The identification of this is particularly important to manage the risk of other cancers for the patient and their family members (13). Additionally, this status has been found to hold valuable prognostic and predictive information in sporadic cancers which constitutes more than 75% of all MSI tumours (29).

Studies have consistently shown patients with MSI tumours in colon cancer have a better prognosis compared to stable tumours (MSS) (30, 31, 32). MSI status is a more relevant prognostic factor in stage II patients, as MSI tumours are more common in stage II affecting approximately 22% of stage II patients and only 12% of stage III patients (21). Additionally, using data from the PETACC-3 trial,

MSI was potentially more powerful in terms of OS and DFS for stage II patients compared to stage III patients treated with adjuvant chemotherapy (33). Stage II had a hazard ratio (HR) for OS of 0.16 [95% CI 0.04-0.64, P=0.001] while stage III patients had a HR of 0.70 [95% CI 0.44-1.09, P=0.04], which was statistically significant (*33*).

MSI status may also be a predictive marker of treatment to 5-FU chemotherapy in stage II patients. This is primarily because the recurrence rate is considered too low to justify adjuvant chemotherapy in patients with MSI tumours (34). Additionally, some studies have indicated MSI tumours have a decreased benefit to adjuvant therapy (31, 35). In a retrospective analysis, stage II MSI patients who received chemotherapy did not experience an increased benefit compared to MSI patients who did not receive chemotherapy. However, MSS patients did see a benefit with the addition of chemotherapy (*33).* Further, no DFS benefit was detected with a trend towards worse outcomes in patients with MSI treated with Fluoropyrimidine monotherapy (*33*). This indicates 5-FU based chemotherapy may be detrimental to MSI tumours in stage II patients.

The exploration of the underlying biology of MSI status further strengthens this association. MSI tumours often arise through disruption of the MLH1, PMS2, MSH2 or MSH6 genes which results in changes in pathological and molecular features not seen in MSS (36). The abundance of MSI-induced frameshift peptides are frequent targets of the immune system in MSI patients (36). Many of these responses have subsequently been shown to be mediated by tumour infiltrating lymphocytes (36). Furthermore, MSI tumour's checkpoint proteins are upregulated compared to MSS tumours, which means they are less likely undergo immune evasion, an important hallmark of cancer progression (35).

While MSI status appears to have powerful prognostic significance only 22% of stage II colon cancer patients have MSI tumours (21). This leaves a considerable number of patients with unpredictable treatment responses. Therefore, there is still a pressing need for more biomarkers like this to further stratify stage II patients and guide their treatment decisions.

Taken together, the success of this marker highlights two important points. Firstly, there is a great need for more biomarkers like MSI status in this population of patients. Secondly, MSI status highlights the factors necessary for clinically relevant biomarkers. As implied, this includes a strong association with prognosis that is independent of the TNM staging system and other clinicopathological risk factors, as well as the ability to predict outcome to treatment. Further, a clinically feasible way of detecting these markers is necessary. For example, dMMR can effectively be measured by IHC without the requirements of a molecular laboratory (29). Functional relevance of biomarkers to stage II colon cancer also provides confidence and can be used to form hypothesis for future drug targets. The effect of MSI on the surrounding tumour-microenvironment (TME) as described earlier, is clear, while for example gene-expression assays, developed through statistical approaches, do not provide any reassurance of their biological role (37).

More recently, microRNAs and functional proteins associated with tumour-derived extracellular vesicles (tEVs) have emerged as potential biomarkers for stage II colon cancer patients. Their feasibility as a biomarker in the clinic and their functional relevance to stage II colon cancer patients makes them an attractive approach, as will be discussed.

1.3 MIR-21, MIR-29A AND CD147 AS MOLECULAR BIOMARKERS IN CRC

1.3.1 MIRNAS: DEFINITION AND FUNCTION IN CARCINOGENESIS

MicroRNAs (miRNA) are small, non-coding, regulatory RNAs comprised of 21-24 nucleotides (38). They typically function by interacting with specific mRNAs through complementary base-pairing to influence the translation or stability of the target mRNA molecule (38). These RNA fragments are involved in many intracellular processes and remarkably, a single miRNA species is capable of binding and repressing multiple mRNA targets (38). As such miRNAs have been implicated in several biological and pathological processes, including carcinogenesis (38). The dysregulation of miRNAs in cancer cells has been found to influence a diverse range of intracellular pathways in cancer cells namely cell proliferation, apoptosis, differentiation and inflammation (39). For this reason, many

miRNAs have been termed “oncomiRs” or “tumour suppressive miRs” for their roles in carcinogenesis (39).

1.3.2 MIRNA-21 AND MIR-29A AS BIOMARKERS IN COLORECTAL CANCER.

Several aspects of tumour-associated miRNAs make them attractive as a new class of prognostic biomarkers in early-stage CRC. Most striking is their stability in biofluids partially due to their presence within membranous nano-micro sized particles called “tumour-derived extracellular-vesicles” (tEVs) (40). Further, their aberrant expression profiles vary among difference tumour types making them specific to CRC (40). At least 37 different miRNA species have been found to be dysregulated in tumour tissue (41). Included among these are miR-21-5p (miR-21) and miR-29a-3p (miR-29a). Both are significantly upregulated in CRC tissue and in the blood of CRC patients (42, 43, 44, 45). More importantly, studies over the last 10 years have gone on to find significant associations with these miRNAs and clinical risk factors, namely lymph node positivity and distant metastasis (41, 44, 46). Subsequently, the functional role of miR-21 and miR-29a and their targets have been elucidated, further strengthening their potential as functional prognostic biomarkers in colorectal cancer (47, 48, 49). Despite this, the potential for these miRNAs as biomarkers in stage II colon cancer has been less well explored.

miR-21 represents one of the most intensively studied oncomiRs due to its abundant overexpression in the vast majority of cancers including CRC (50). In CRC, miR-21 is arguably one of the most dysregulated miRNAs (41). One of miR-21’s most characterised roles is its ability to induce stable activation of *PTEN*, an important tumour suppressor. In turn, this stably induces the NFkb inflammatory pathway (47, 48) which is known to foster conditions to promote cancer progression, invasion and metastasis (51).

Unsurprisingly, elevated miR-21 has consistently been associated with lymph node positivity, advanced TNM stage, poorly differentiated tumours and other clinical risk factors (41, 42, 46, 52) in populations consisting of all TNM stages. Furthermore, miR-21 is an independent predictor of DFS

(41, 46, 52). However, few studies have focused primarily on the prognostic impact of miR-21 in stage II CRC. Interestingly, miR-21 expression increases from early to late stage CRC and elevated levels have even been detected in adenomas (46). This suggests miR-21 overexpression could be an early event in progression of cancer making this miRNA particularly relevant to stage II colon cancer patients.

Similarly, miR-29a is dysregulated in the majority of cancer types including CRC. Interestingly, this miRNA is downregulated in most cancer types (53) but significantly upregulated in tumour tissue and the blood of CRC patients (54) compared to healthy controls. This is consistent with its specific functional role in CRC where inhibition of E-cadherin, promotes epithelial-to-mesenchymal transition (EMT), thereby promoting cell invasion and metastasis (38). In contrast to miR-21, its prognostic potential has been less well explored in CRC tissue however, it is a well-known secretory miRNA that has both diagnostic and prognostic potential in the blood of CRC patients (44). Further, this marker has been associated with distant metastasis and poor OS (44). Taken together, miR-29 could also be an important prognostic tumour biomarker in stage II colon cancer.

1.3.3 CD147 AS A BIOMARKER IN COLORECTAL CANCER

In addition to miRNAs, protein markers are particularly important as they provide a more dynamic reflection of the impact of the cell's genetic aberrations (55). CD147 is a transmembrane protein and like miR-21 and miR-29, it is released extracellularly in tEVs and plays an important role in tumour progression and aggressiveness (56). CD147/EMMPRIN (extracellular matrix metalloproteinase inducer), is best known for its role as a matrix metalloproteinase (MMP) inducer (57). MMPs are key mediators for extracellular matrix (ECM) and basement membrane degradation and therefore promoters of invasion and metastasis in cancer (57). More recently, CD147 has been shown to be involved in angiogenesis by enhancing VEGF levels in cancer cells and the mesenchyme (58), and other features such as hypoxia, anti-apoptosis and chemoresistance through a complex network of interactions (58, 59).

Notably CD147 has been detected in the vast majority of human cancer types including CRC (60) and is one of the most highly expressed proteins in disseminated cancer cells (61). Several publications have reported increased relative CD147 expression in CRC tumour compared to normal adjacent tissue and some have subsequently found associations with lymph node positivity, distant metastasis, as well as DFS (62, 63). Despite this, few studies have explored CD147 expression in stage II CRC alone. Those that have are small and variable in their outcomes with some reporting an association with cumulative survival and others failing to validate this (63, 64). This makes forming definitive conclusions difficult.

1.4 SURROGATE MARKERS FOR EXTRACELLULAR VESICLES

The functional relevance of potential biomarkers, including miR-21, miR-29a and CD147, may go beyond their expression in tumour tissue and be utilised in the tumour-draining lymph nodes (TDLNs) of stage II colon cancer patients. This is not in the context of detecting occult lymph node metastasis, however. Perhaps preliminary to this is the detection of tEVs.

As mentioned, tEVs are bioactive, nano-micro sized particles, released from normal cells and tumour cells but in greater abundance (65). EVs are the generic term for many diverse, secreted vesicles including exosomes, microparticles and microvesicles (65). They are central to intercellular communication by carrying specific nucleic acids and proteins from their donor cell. They are abundant throughout the body, travelling through several bodily systems, including the lymphatics and blood and are capable of transferring molecular cargo to recipient neighbouring and distant cells (65). Consequently, tEVs have recently emerged as biomarkers and functional mediators of tumourigenesis in TDLNs and distant organs of cancer patients (65).

One mechanism by which tEVs contribute to tumorigenesis is by formation of the pre-metastatic niche (PMN) in the draining lymph nodes (66). miR-21, miR-29a and CD147 have mostly been characterised as blood-based tEV markers however, their potential to detect tEVs in the lymph nodes is highly relevant to high-risk stage II colon cancer patients for two reasons: Firstly, the TDLNs

are typically the first place for dissemination of tumour cells (67) and secondly, detecting tEVs in surgically resected TDLNs is technically restricted. There is currently no standardised or validated methodology for isolating tEVs from human tissue samples let alone in a clinically feasible manner. Therefore, these markers could act both as biomarkers themselves and surrogate biomarkers for tEVs.

1.4.1 TEVS AND THE PRE-METASTATIC NICHE (PMN)

The PMN is a term relatively new to the field of metastatic research; however, it is thought to at least be partially responsible for metastatic formation (68). Intensive research efforts in animal models have discovered factors necessary for PNM formation including tumour-derived secretion factors (TDSFs) that are capable of conditioning distant sites in the body by regulating immunosuppression and hypoxia. This allows the recruitment and survival of metastatic cells within these sites (68). Along with TDSFs, tEVs are now emerging as potential mediators of PNM formation. Peinado *et al* (2016) demonstrated the mechanistic role for melanoma-derived EVs to condition the PMN (66). Using a mouse model, they demonstrated the ability for tEVs, isolated from metastatic melanoma cell lines, to educate bone marrow-derived cells (BMDCs). This promoted vascular leakiness, a hallmark of pre-metastatic formation, at pre-metastatic sites (66). While tEV-mediated PMN formation has not been investigated in CRC it has been demonstrated in animal models for breast, renal, pancreatic and other cancers (69, 70, 71). This suggests PNM formation could be a phenomenon in CRC.

1.4.2 EVS AND CONDITIONING OF THE LYMPH NODES

In CRC the TDLNs are the first place for dissemination of tumour cells (67) and lymph node metastasis is the most important prognostic factor differentiating stage II and III CRC (72). Therefore, certain molecular patterns and subsequent morphological changes in TDLNs of stage II patients may provide evidence of pre-metastatic spread (73, 74). tEVs present in the lymph nodes could be one of the first steps involved in metastatic spread to distant organs. Strikingly, melanoma-derived EVs

have been shown to support PNM formation in the lymph nodes by enhancing melanoma cell recruitment and potentially regulating the extracellular matrix and vascular proliferation (75) (Figure 2). This animal study demonstrated the ability of melanoma EVs to home to sentinel lymph nodes and present molecular signals to influence the recruitment of melanoma cells as well as extracellular matrix deposition and vascular proliferation, ultimately facilitating metastatic spread (75). This fulfils the "seed and soil" hypothesis whereby the tEVs act as the "seed" and the lymph nodes the "soil" in preparation for metastasis (76) (Figure 2). Following up from this, Hu *et al* (2015) tracked melanoma EVs to specific regions of lymph nodes using MRI *in vivo* (77). Formation of PMN's specifically in lymph nodes via tEVs has also been demonstrated in gastric, breast and ovarian cancer animal models (78, 79, 80) however, there is no such evidence in colon cancers.

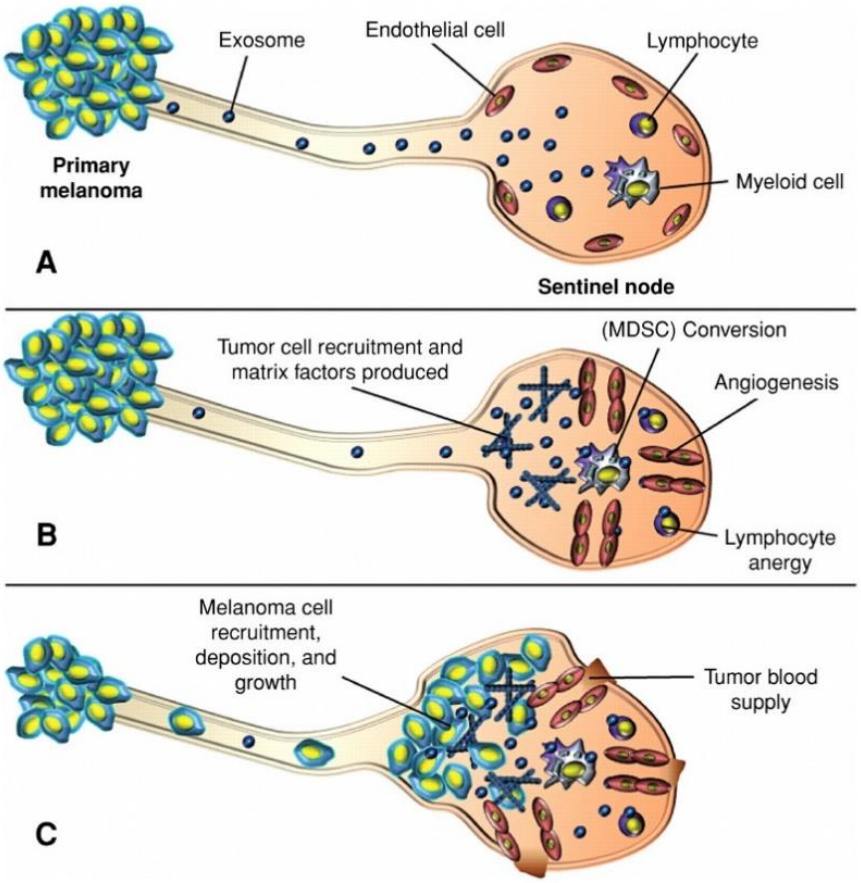


Figure 2: Melanoma-derived tEVs homing to lymph nodes
A) Exosomes or tEVs shed from the primary melanoma and home to sentinel lymph nodes. B) Exosomes release molecular signals in the lymph nodes to change the extracellular matrix and promote angiogenesis. C) Exosomes promote the recruitment of melanoma cells to lymph nodes and support their deposition and growth through the changes made. From Hood et al 2011 (75).

1.4.3 LYMPH NODE TEVS AS PROGNOSTIC AND/OR PREDICTIVE BIOMARKERS IN STAGE II COLON CANCER

The prevalence of this phenomena across several types of cancers suggests it could be active in colon cancer. Extending from this, is the idea of using tEVs or surrogate markers within lymph nodes as prognostic and/or predictive biomarkers for clinical use. Stage II and III colon cancer are stratified based on the presence of tumour cells in the lymph nodes. Given tEVs direct site-specific metastasis through the lymph nodes, (81) detection of tEVs in the lymph nodes could be indicative of higher risk stage II disease. Compared to other emerging candidate biomarkers, where some are limited by lack of understanding of their mechanistic role, tEVs represent attractive biomarkers. This information could be used to add value to TNM staging and other clinical risk factors to more accurately stratify stage II patients. Additionally, they may have predictive value in terms of response to adjuvant chemotherapy, under the hypothesis that patients with micro-metastasis or early-stages of cancer spread might respond to this type of treatment.

1.4.4 DETECTING TEVS IN LYMPH NODES USING SURROGATE BIOMARKERS

One major limiting aspect of tEVs is their detection in humans. Their identification and characterisation remain a challenge in this field and any current techniques remain impractical in clinical settings (82). However, cancer-specific markers such as miR-21, miR-29a and CD147 are currently being used as surrogates for blood-based detection of tEVs in CRC patients. This was recently demonstrated by Yoshika and colleagues (2015) using antibodies against CD147 in the blood of CRC patients. They found this simple method could be used to detect CD147 positive tEVs (56). Likewise, Tsukamoto and colleagues (2017) detected miR-21 through exosomal plasma in CRC patients finding significant correlations with tissue levels as well as associations with clinical factors (83). This was achieved through microarray analyses in exosomal plasma.

CD147 is detectable in the invaded lymph nodes of CRC patients using IHC in formalin-fixed paraffin embedded (FFPE) samples (62, 63). As mentioned previously, this method is also used on tumour

samples for the clinical detection of MSI. Furthermore, miRNA can now be extracted from FFPE samples and subsequently quantified by quantitative reverse transcription PCR (RT-qPCR) (84). Therefore, it seems plausible and clinically appropriate that tEVs present in the draining lymph nodes could be detected through IHC and RT-qPCR techniques in FFPE samples, after surgical resection. This would also avoid compromising pathological assessment of these lymph nodes for diagnostic confirmation of the disease.

miR-21, miR-29a, CD147 or any tumour-specific biomarker encapsulated in tEVs in the lymph nodes of stage II colon cancer patients has not been investigated to date but is certainly an interesting research question. In contrast to other candidate biomarkers, in particular, gene-expression profiling, these markers along with tEVs may have a clearer functional role. This is particularly pertinent to early-stage colon cancer where patients typically do not undergo neo-adjuvant therapy, which is known to disrupt the morphology of lymph nodes in rectal cancer patients (85).

The detection of miR-21, miR-29a and CD147 in the tumour and lymph nodes of stage II patients could provide additional prognostic power. Success in this area may pave the way for more accurate stratifying of patients in the clinic, which would significantly improve the health outcomes of many New Zealanders. Stage II colon cancer patients are clinically heterogenous and therefore stratification and subsequent treatment of these patients is a challenge. MSI status is currently the only successful biomarker that aids in decision making for treatment of these patients and paves the way for more biomarkers to be used in the clinic. Analogous to MSI status, miR-21, miR-29a, and CD147 may have a clear, functional role in tumour biology. Specifically, these markers are involved in the initiation and progression of CRC. Further their association with tEVs, which are involved in priming the lymph nodes for metastatic spread, make these markers particularly relevant for detecting high-risk stage II colon cancer patients. Lastly, detection of these biomarkers, both on their own and as surrogates for tEVs, through IHC and RT-qPCR in FFPE samples, allows for their ease of translation into clinical practice.

1.5 AIMS AND OBJECTIVES

There is an apparent need for more prognostic and predictive biomarkers to improve the risk assessment and adjuvant chemotherapeutic strategies in stage II colon cancer patients. The necessary factors for a clinically successful biomarker include: A prognostic and/or predictive impact that is more informative than current clinicopathological risk-factors and a clinically feasible way of measuring the marker. Further, functional relevance to the specific populations can support the use of biomarkers. miR-21, miR-29a and CD147 have recently emerged as potential biomarkers in CRC. Due to their functionality in the initiation and development of CRC and their association with tEVs we hypothesise these markers could provide important prognostic information for stage II colon cancer patients.

The overall aim of this exploratory pilot study was to characterise the expression of potential biomarkers: miR-21, miR-29a and CD147 in the tumour and draining lymph nodes in a small pilot cohort of stage II colon cancer patients. The use of antibodies specific for CD147 in FFPE samples represents a simple, sensitive and clinically relevant approach. Further, molecular technologies, such as RT-qPCR, provide a strong quantitative tool with a high level of precision and reproducibility to detect small differences in expression levels of miRNAs (86). The specific objectives of the study were as follows;

1) To establish and optimise the molecular techniques necessary to detect miR-21, miR-29a and CD147 in the tumours, normal mucosa tissues and TDLNs of stage II colon cancer patients. Optimisation of IHC for the detection of CD147 and RT-qPCR for the detection of miR-21 and miR-29a was performed in formalin-fixed tissues and/or specimens preserved in RNAlater.

2) To begin to characterise the expression levels of these markers in tumour and normal mucosa in a small pilot cohort of stage II colon cancer patients. This was to determine whether these markers were dysregulated in the tumours and to what extent. Further, to explore the prognostic significance

of these markers we compared the expression status of these markers to certain pathological risk features in the cohort.

3) To explore the expression levels of these markers in the TDLNs in the cohort. This was to determine if tumour-associated expression of these markers was present in the TDLNs and could be indicators of pre-metastatic spread.

We hypothesised that cancer-associated miR-21, miR-29a and CD147 expression levels would be measurable in the tumours and TDLNs of stage II colon cancer patients. Further, we believed expression differences between tumour and normal mucosa tissue samples would be detectable.

The results of this pilot study will allow us to understand whether CD147, miR-21 and miR-29a have any prognostic utility in stage II colon cancer patients, form hypotheses and determine whether the detection of these markers is clinically feasible. Further, the results will determine whether tumour-associated expression is present in the TDLNs of these patients and has any prognostic potential. Ultimately, this study will inform larger, future prospective cohorts which will validate the prognostic significance of these markers to determine whether they are more informative than current clinicopathological risk-factors and could add power to the current risk stratification of stage II colon cancer patients.

CHAPTER 2: MATERIALS AND METHODS

2.1 ETHICAL CONSIDERATIONS AND CASE SELECTION

2.1.1 ETHICS APPROVAL AND CONSENT TO PARTICIPATE

At the beginning of the study period, the primary researcher selected patients (to be in the current study) from the web-based application Research Electronic Data Capture (REDCap), a database for the Surgical Cancer Research Group (SCRG) Colorectal Cancer Biobank. This multi-disciplinary research group collects and stores blood and tissue samples from CRC patients at Wellington Hospital who have consented to be a part of "genetic and molecular research". Patient demographics and clinicopathological features are also collected prospectively from hospital records and stored within the REDcap database. Informed consent to collect tissue samples for banking and future, unspecified research was obtained from each participant. Ethical approval for this biobank and the current study was obtained from the Central Health Ethics Committee (15/CEN/143).

2.2.2 ETHICAL CONSIDERATIONS

Because the patients had already consented into being a part of the biobank, this altered the ethical considerations of the current study. However, it was essential that the primary researcher was aware of some aspects that were outlined during the recruitment process. For example, the voluntary nature of participation, which means patients are free to withdraw from the study at any time. Further, the researcher had access to a secure database that contained specific patient details. Therefore, it was a requirement of the researcher to adhere to de-identification and the confidentiality of these patients.

2.2.2 TE ARA TIKA

It was also important that the primary researcher was cognisant of the ethical considerations surrounding Māori participation. Respect for and incorporation of Tikanga was paramount. For example, Te Whakahoki i te Taonga (return of the gift) which refers to providing access for donors to

research reports and raw data when requested, was discussed at the time of recruitment. Further, disposal of tissues by a karakia (blessing) was offered at the time of recruitment and if chosen, adhered to. Specifically, the author of this study treated all samples with respect during the research protocols and kept in mind at all times the importance tikanga.

2.2.3 CASE SELECTION AND SAMPLE COLLECTION

A search strategy was applied based on the following inclusion/exclusion criteria: TNM stage II colon adenocarcinomas (5), excluding those within the rectosigmoid junction and patients that have received neo-adjuvant therapy. Patients with a previous history of malignancies or inflammatory bowel conditions were also excluded. Of note, the biobank excludes patients with emergency presentations due the time constraints and ethical considerations of obtaining informed consent in a short time frame.

The same search strategy was applied but with the inclusion of stage III patients for a positive control. The database was further searched for a negative control patient with surgically resected lymph nodes of the colon, who previously had a high suspicion of cancer, (and therefore falls under the ethical requirements of this study) but was found to be negative for cancer.

Available colonic tumour and normal mucosa samples of selected patients were collected from the biobank of surgical samples. Samples frozen in RNAlater or fixed in 4% paraformaldehyde (PFA) were collected. The FFPE TDLN's were also collected for the corresponding patients after pathological assessment.

2.2.4 PATIENT CLINICOPATHOLOGICAL CHARACTERISTICS

Patient demographics were collected from the REDCap database. This included; age, gender and ethnicity. Selected clinicopathological characteristic were also collected. This included; T staging, tumour cell type, grade, CEA level, extramural vascular invasion, perineural invasion, lymphatic

invasion, presence of multiple polyps, dMMR status and the number of lymph nodes surgically resected.

2.2 IMMUNOHISTOCHEMICAL DETECTION OF CD147

2.2.1 PARAFFIN PROCESSING OF TISSUE

Tumour and normal mucosa samples that were previously fixed in 4% PFA for 16-24 hours were prepared for paraffin infiltration using the Sakura Tissue Tek Processor overnight in cassettes. Briefly, tissues were dehydrated through a series of graded ethanol baths to displace water and then infiltrated with paraffin wax.

Specifically, tissue was processed as follows:

- Dehydration in 70% ethanol (etoh), 1-hour (hr)
- 95% etoh, 1-hr
- 100% etoh, 1-hr
- 100% etoh, $1_{1/2}$-hrs
- 100% etoh, $1_{1/2}$-hrs
- 100% etoh, 2-hrs
- Xylene (clearing agent), 1-hr
- Xylene, 1-hr
- First paraffin wax, 1-hr
- Second paraffin wax, 1-hr

Tissues processed into paraffin were then embedded into smooth paraffin blocks using the Sakura Tissue Tek Embedding Centre. Tissues were placed in a 58◦C paraffin bath for 15 minutes to melt surface wax away. A mold was chosen that best corresponded with the size of the tissue. Tissue was placed into the mold (cut side placed face down) and molten paraffin was subsequently dispensed into the mold. The mold was transferred to a cold plate to allow paraffin to solidify and hold tissue in

the desired orientation. The labelled tissue cassette was placed on top of the mold as the backing. More molten paraffin was added to cover the face of the plastic cassette. Paraffin blocks were allowed to solidify on ice for 30 minutes. The paraffin block was then popped out of the mold and the tissue was checked for any cracks or air bubbles. If artefacts were present, the blocks were melted and re-embedded in paraffin.

2.2.2 SECTIONING TISSUES FOR REPRESENTATIVE H&E SLIDES

Tissue blocks were sectioned using the Sakura Tissue Tek microtome. In preparation, tissue blocks were placed face down on ice for 10 minutes. Blocks were then sectioned at 4µM. Sections laid on top of a 37◦C water bath to de-wrinkle sections and subsequently picked up on adhesive/positively charged slides. Slides were then allowed to air dry for at least 1 hour and then incubated at 60◦C for 1 hour until paraffin was transparent.

Sections were stained with Haemotoxylin and Eosin (H&E) as follows:

- Dewaxed in xylene, 3x 5-minute washes (min)
- Rehydration in 100% etoh, 2x 5-min
- 95% etoh, 2x 5-min
- 80% etoh, 2x 5-min
- 70% etoh, 2x 5-min
- Tap water, 2x 5-min
- Harris' Haemotoxylin,10mins
- Tap water, 20 seconds (sec)
- 1% Acid alcohol, 5-sec
- Scotts tap water, 2-min,
- Eosin, 2-min
- Tap water, 20-sec.

- Rinsed in distilled water

Slides were then blotted dry around the sections. DPX mounting media was distributed evenly over the tissue and slides were coverslipped.

2.2.3 TMA CONSTRUCTION

Tumour, normal mucosa and lymph node FFPE samples from all patients that were available were represented in 7x Unitma pre-made tissue microarray (TMA) blocks as 3mm diameter cores using the Quick-Ray™ Manual Tissue Microarrayer (figure 3). Representative areas from the donor blocks were chosen based on the density of tissue (using the H&E reference slides) (figure 3). TMA recipient blocks were then incubated at 60°C for 45 minutes (or until transparent) in an embedding mold. Paraffin was poured over the TMA block and solidified to a cassette on a cold plate. 4µm sections were cut and transferred to adhesive-coated slides using a 47°C and 55°C water bath respectively. Sections were air dried and then placed in a 60°C oven for 60 minutes.

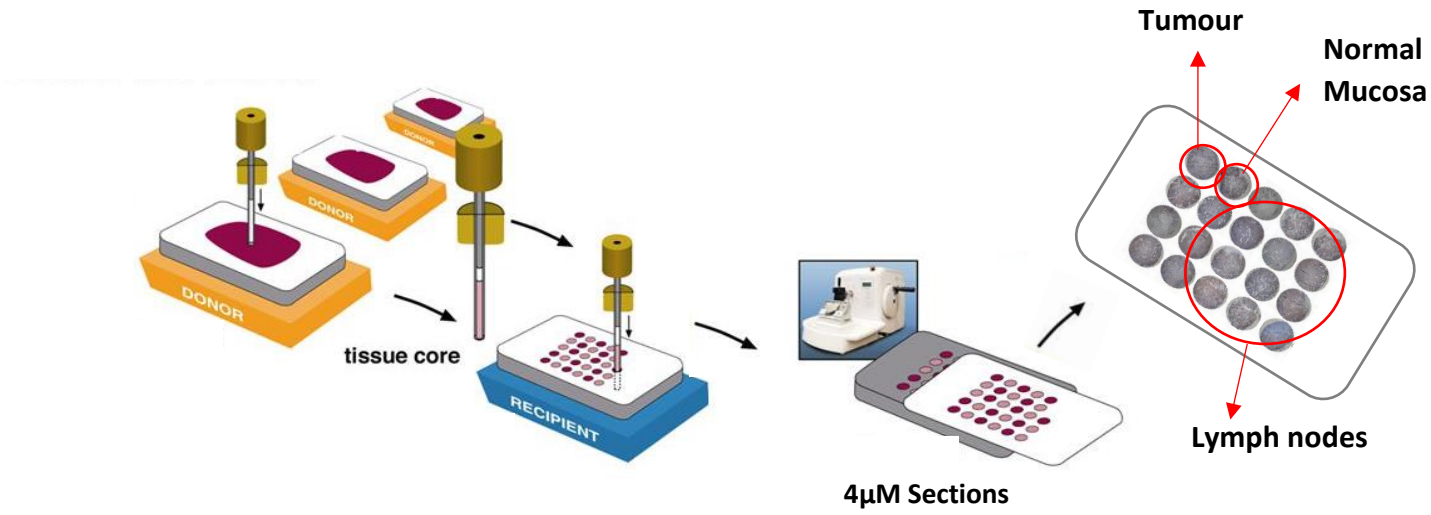


Figure 3: Tissue Microarray construction
3mm cores were extracted from the densest area of donor tissue blocks (tumour, normal mucosa and lymph node samples), using the reference H&E sections. Cores were then punctured and organised into recipient tissue microarray (TMA) blocks. Typically, each block contained a tumour, normal mucosa sample and corresponding lymph nodes for each patient. 4µM sections were cut from the TMA blocks using a microtome. Adapted from Brown 2014 (87).

2.2.4 CD147 IMMUNOHISTOCHEMISTRY

The expression of CD147 was analysed by IHC staining of the TMA sections. The following methods were based off recommendations from the Manufacturers protocol (Novus Biologicals). Sections were deparaffinised and rehydrated as follows:

- Xylene 3x 5-min
- Rehydration in 100% etoh, 2x 10-min
- 95% etoh, 2x 10-min
- 70% etoh, 2x 10-min
- 50% etoh, 2x 10-min
- Deionized water, 2x 5-minute

Antigen retrieval was trialled using both a microwave and pressure cooker method. Spare sections were placed in 1mM EDTA and 0.05% Tween solution (pH 8.0) and either microwaved for 3x 5-min bursts or placed in the pressure cooker on high for 5-min. After optimisation, all samples were placed in antigen retrieval solution in the pressure cooker on high for 5 minutes. Sections were cooled to room temperature in antigen retrieval solution.

R&D Systems Goat VisUCyte™ HRP Polymer-DAB Cell & Tissue Staining Kit for detection of goat IgG Antibodies was used in combination with R&D Systems Human EMMPRIN/CD147 Affinity Purified Polyclonal Antibody (Ab). The manufacturers protocol was followed for the blocking, IHC and chromogenic staining procedure. Conditions for the primary Ab were optimised using spare TMA sections. 3 different concentrations were trialled: 1, 0.5 and 0.2 µg/ml. In the final optimised protocol all TMA sections were stained at 0.5 µg/ml for 1 hour at room temperature on 2 separate occasions. PBS controls for each TMA were also included.

All TMA sections were counterstained with Haematoxylin as follows:

- Water 5-min

- Haematoxylin ‘dips’
- Tap water gently ‘dippping’, 30-sec
- Dehydrated in 75% etoh, 1-min
- 90% etoh, 1-min
- 100% etoh, 1-min
- Xylene 2x 1-min washes

Sections were then air dried and coverslipped with DPX mounting media as described earlier.

4x, 10x and 20x field of view images for each sample within the stained TMA sections were taken using a confocal microscope. Evaluation of staining intensity was performed while blinded to cohort and clinicopathological characteristics. Evaluation was strictly qualitative and a modified version of a previously described scoring system (62). A score of 0 indicates no difference, 1 indicates a moderate increase and 2 indicates a high increase in staining of the tumour compared to normal mucosa. Tumours with a score of 1-2 were considered positive and a score of 0 was considered negative for CD147 overexpression.

2.3 MIRNA ANALYSIS

2.3.1 TOTAL RNA EXTRACTION FROM FFPE LYMPH NODE CORES

During TMA construction, additional 3mm cores were taken from the same FFPE lymph nodes and frozen at -20°C until use. Cores were then deparaffinised in xylene and washed with 100% ethanol twice. Homogenisation of spare cores was trialled using a mortar and pestle in 100% ethanol however, this proved inefficient and instead the cores were homogenised in 100% ethanol using a hand-operated motor driven grinder. Total RNA was prepared from the cores using the Qiagen RNeasy FFPE kit following the manufacturers protocol. Total RNA was quantified using the Nanodrop spectrophotometer.

2.3.2 TOTAL RNA EXTRACTION FROM TISSUES STORED IN RNA*LATER*

Corresponding tumour and normal mucosa samples stored at -20°C in RNA*later* solution (non-toxic reagent stabilises and protects cellular RNA) were collected and 50mg of tissue was cut from the original samples. Samples were washed in PBS 3x and minced using surgical blades to assist the homogenisation process. The minced tissue was homogenised in QIAzole Lysis Reagent using a hand-operated motor driven grinder. Total RNA was prepared from the samples using the Qiagen miRNeasy kit following the manufacturer's protocol. Total RNA was quantified using the Nanodrop spectrophotometer.

2.3.3 CDNA SYNTHESIS

10µg of total RNA from the tumour and normal tissue of patient samples that were available was used to synthesise double-stranded cDNA. Additionally, total RNA from 23 randomly selected lymph nodes across all patients were used to synthesise cDNA. The TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems) was used for this purpose following the manufactures protocol.

2.3.4 QUANTITATIVE REAL-TIME PCR-BASED DETECTION OF MIR-21 AND MIR-29A

miRNA expression of hsa-miR-21-5p, hsa-miR-29a-3p, hsa-miR-345-5p and hsa-miR-16-5p was examined by real-time PCR using the TaqMan™ Fast Advanced Master Mix, miRNA assays (Applied Biosystems) and the RotorGene 6000 detection system. The manufacturers protocol for detection was followed.

miRNA was measured using threshold cycle values (C_t). The threshold was set at 0.06529 during the log phase of replication for each run (figure 4). Each sample was run in duplicate to obtain 2 C_t values. Duplicate C_t values were less than 0.5 units apart (figure 4). Averaged C_t values of miR-345 and miR-16 were used to normalise miR-21 and miR-29a average C_t values. The fold change for each sample was calculated using the 2^{-ddCt} method (88).

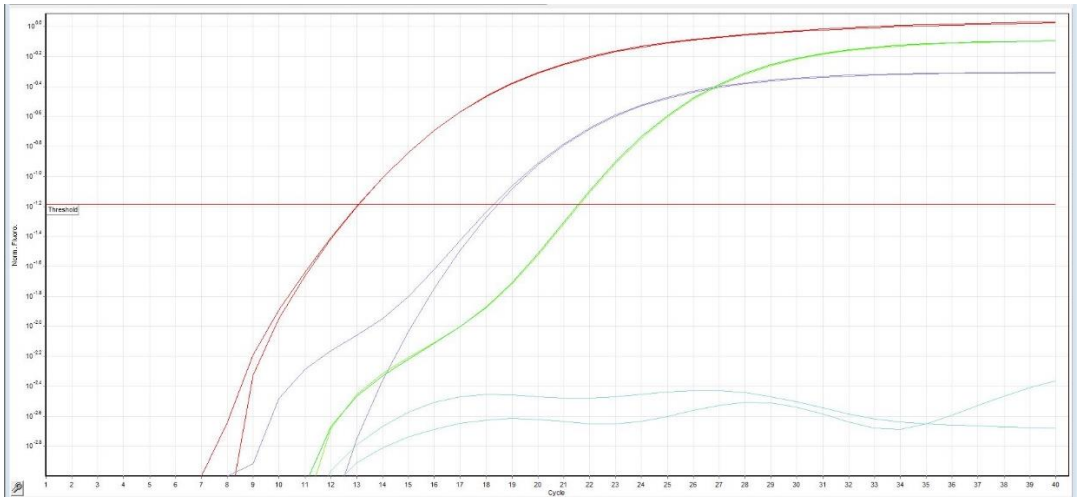


Figure 4:Representative RT-qPCR amplification curves for miR-21, miR-29a and miR-345 in a tumour sample, performed in duplicates.
Threshold bar set at 0.06529 to obtain threshold cycle values.

2.4 HISTOMORPHOLOGICAL ANALYSIS

The following evaluation procedure was based on previous methodology (73). Morphometrical analysis was performed on 4x (field of view) lymph node images stained for CD147 (figure 5). All lymph nodes compartments were annotated using ImageJ software (ImageJ 1.52a Wayne Rasban, National Institutes of Health, USA). Specifically, germinal centres (GCs) (characterised by CD147 (brown) staining), follicles (characterised by an intense blue ring around the GCs) and primary follicles (intense blue compartments with no GCs) were quantified (figure 5). Follicle and GC density, were calculated as the average number of follicles/GCs per lymph node and follicle and GC size were calculated by averaging the circumference of the 3 largest follicles/GCs in each LN. Further, primary follicle density was calculated as the average number of follicles minus the average number of GCs per lymph node.

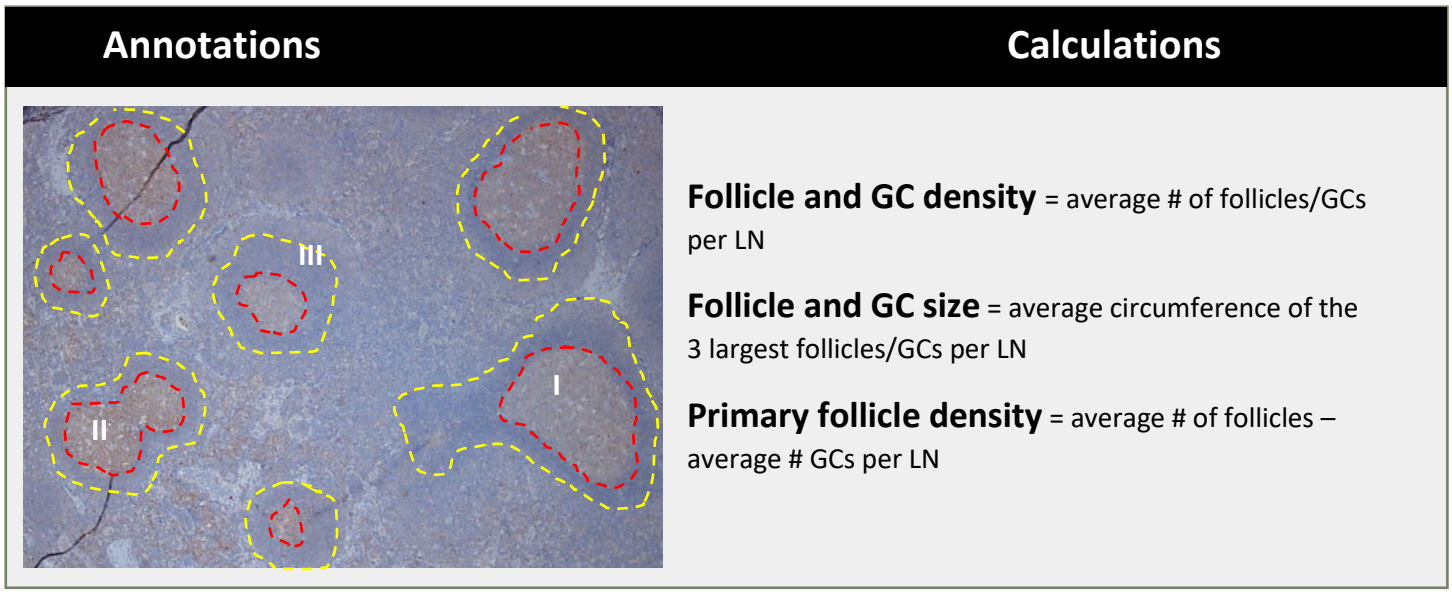


Figure 5:Evaluation procedure for lymph node histomorphological analysis

Left: All lymph nodes were annotated for B cell compartments (red and yellow dashed lines) using ImageJ. (I) Circular Germinal centre (GCs), (II) Non-circular/fused GC, (III) follicle. Right: Calculations for follicle and GC density (number of follicles/GCs per lymph node), follicle and GC size and primary follicle density (number of primary follicles per lymph node).

2.4 ASSOCIATIONS WITH PATHOLOGICAL FEATURES

miR-21, miR-29a and CD147 tumour expression levels were compared to certain pathological risk factors. While OS and DFS are considered gold-standard endpoints (89) in investigations of prognostic biomarkers, it was not possible to examine these end points as the mean follow-up period since the time of surgery was 8 months (table 3). While DFS at 3 years is considered appropriate for assessing prognosis in colon cancer patients, OS at 5 years is the most quoted metric for this purpose (89). Current pathological risk factors used in stage II colon cancer patients are not completely accurate (14) however, correlations of potential biomarkers with these factors could be used to form hypotheses regarding the prognostic utility of these biomarkers.

The risk factors considered included moderate-poor tumour differentiation grade, T4 tumours (size or extent of the primary tumour), presence of multiple polyps, mucinous tumour type, lymphatic invasion, perineural invasion and extramural vascular invasion. All have been associated with a worse prognosis in stage II colon cancer patients and are recognised as risk-factors by several expert

panels including ASCO and ESMO (12, 13). However, due to a small cohort size, only tumour grade, T stage and lymphatic invasion were compared to expression levels of miR-21 and miR-29a. Of note, CD147 was not considered in any comparisons due to a small sample size. For the lymph node morphological features follicle and GC density and size were compared to tumour grade, T stage, lymphatic invasion and dMMR status.

2.5 STATISTICAL ANALYSIS

For descriptive statistics and statistical testing, GraphPad Prism software (GraphPad Prism 7.00 software, Inc) was used. For comparisons between tumour and normal mucosa one-sided paired testing was used because it was a within-subject comparison and data was normally distributed. While unpaired testing was used for comparisons between two independent groups. Appropriate adjustments were made when data was not normally distributed or standard deviations were not equal using Mann-Whitney, Welch's and Wilcoxon t-tests. It must be noted however, that due to the small samples size in this study, statistical power was limited.

CHAPTER 3: RESULTS

3.1 COHORT CHARACTERISTICS

Based on the inclusion/exclusion criteria applied to the REDCap database of the SCRG CRC Biobank, 13 cases of TNM stage II and 6 cases of TNM stage III colon adenocarcinomas were available from the report output. A stage III patient was chosen as a positive control that closely matched the median age of all 13 stage II patients and where surgical samples were available. Further, a patient with diverticulosis (previous high suspicion of CRC) was chosen as a negative control.
Frozen and formalin-fixed tumour and normal mucosa samples were available for 10 stage II patients and for the stage III positive control. Further, FFPE TDLNs were available for all stage II patients, the stage III patient and mesenteric FFPE lymph nodes from the non-cancer patient (negative control).

A detailed listing of cohort and clinicopathological characteristics of all cancer patients in the study are listed in table 3. Of the entire patient cohort (n=14), age at the time of diagnosis was 67 years (± 14), with an even number of males to females. The cohort was predominately European (n=13) with one Māori patient. This closely resembles the incidence of CRC in Māori in New Zealand which is approximately 6% (2).

Of the selected tumour characteristics and risk factors, all tumours were adenocarcinomas with only 2 cases being mucinous adenocarcinomas. As expected, majority of the tumours were T3 stage (n=9) and had well differentiated pathology (n=9) with few patients having T4a/b tumours (n=5) and moderate to poorly differentiated tumours (n=5). The presence of extramural vascular, perineural or lymphatic invasion was only present in the stage III patient and 5 stage II patients. 3 stage II patients had evidence of multiple polyps along with the primary tumour. 3.5ng/ml was chosen as the cut-off value for the preoperative CEA (private communication). 8 patients had a CEA value more than 3.5ng/ml. Lastly, 11 tumours were tested for dMMR with 5 of these patients having evidence of dMMR.

Of note, perforation and obstruction are also considered risk factors however, the biobank excludes emergency presentations. Therefore, these patients are not represented in this study.

Table 3: Cohort and tumour clinicopathological characteristics

Characteristic		N (%)	Median (range)
Cases		14	
Age[a]		7 (50)	67 ± 14 (34-78)
Gender	Male	7 (50)	
	Female	7 (50)	
Ethnicity	European	13 (93)	
	Māori	1 (7)	
Stage	II	13 (93)	
	III	1 (7)	
T stage	T3	9 (64.3)	
	T4a	3 (21.3)	
	T4b	2 (14.3)	
Cell type	Adenocarcinoma	12 (86)	
	Mucinous adenocarcinoma	2 (14)	
Grade	Well differentiated	9 (64)	
	Moderately differentiated	1 (7)	
	Poorly differentiated	4 (29)	
CEA[b]	<3.5	6 (43)	1.95 ± 0.5707 (1.1-2.4)
	≥3.5	8 (57)	6.5 ± 11.62 (5.5-32.4)
Extramural vascular Invasion	Yes	2 (7)	
	No	12 (86)	
Perineural Invasion	Yes	2 (7)	
	No	12 (86)	
Lymphatic Invasion	Yes	5 (36)	
	No	9 (64)	
Multiple Polyps	Yes	4 (29)	
	No	10 (7)	
MMR deficiency[c]	Not tested	3 (21)	
	No evidence	6 (43)	
	Evidence	5 (36)	
Lymph nodes examined[d]	≥12	13 (93)	21 (5-39)
	<12	1 (7)	
Follow-up period since surgery (months)			8 (3-15)

[a]The median was used for the age cut-off value
[b]CEA level 3.5ng/ml cut-off (private communication)
[c]Deficiency in at least one of MSH2, MSH6, PMS2, MLH1
[d]<12 resected lymph nodes is considered a risk-factor due to under sampling (13)

3.2 OPTIMISATION OF TECHNIQUES FOR THE DETECTION OF CD147

To characterise the expression levels and distribution of CD147 in stage II colon tumour samples using a clinically feasible approach, IHC was applied to 10 pairs of colon cancer and normal mucosa formalin-fixed tissues. This required establishment and optimisation of this technique to the specified tissues.

3.2.1 ANTIGEN (OR EPITOPE) RETRIEVAL

Two heat-induced antigen retrieval methods were used to reverse fixation-dependent protein crosslinking and masking of antigenic sites. Both a microwave and pressure cooker were trialled to determine the best technique for antigen retrieval. The microwave method was deemed inappropriate due to uneven staining of the top half of the sections compared to the bottom half, suggesting uneven antigen retrieval (figure 6). The pressure cooker method produced more consistent staining across sections.

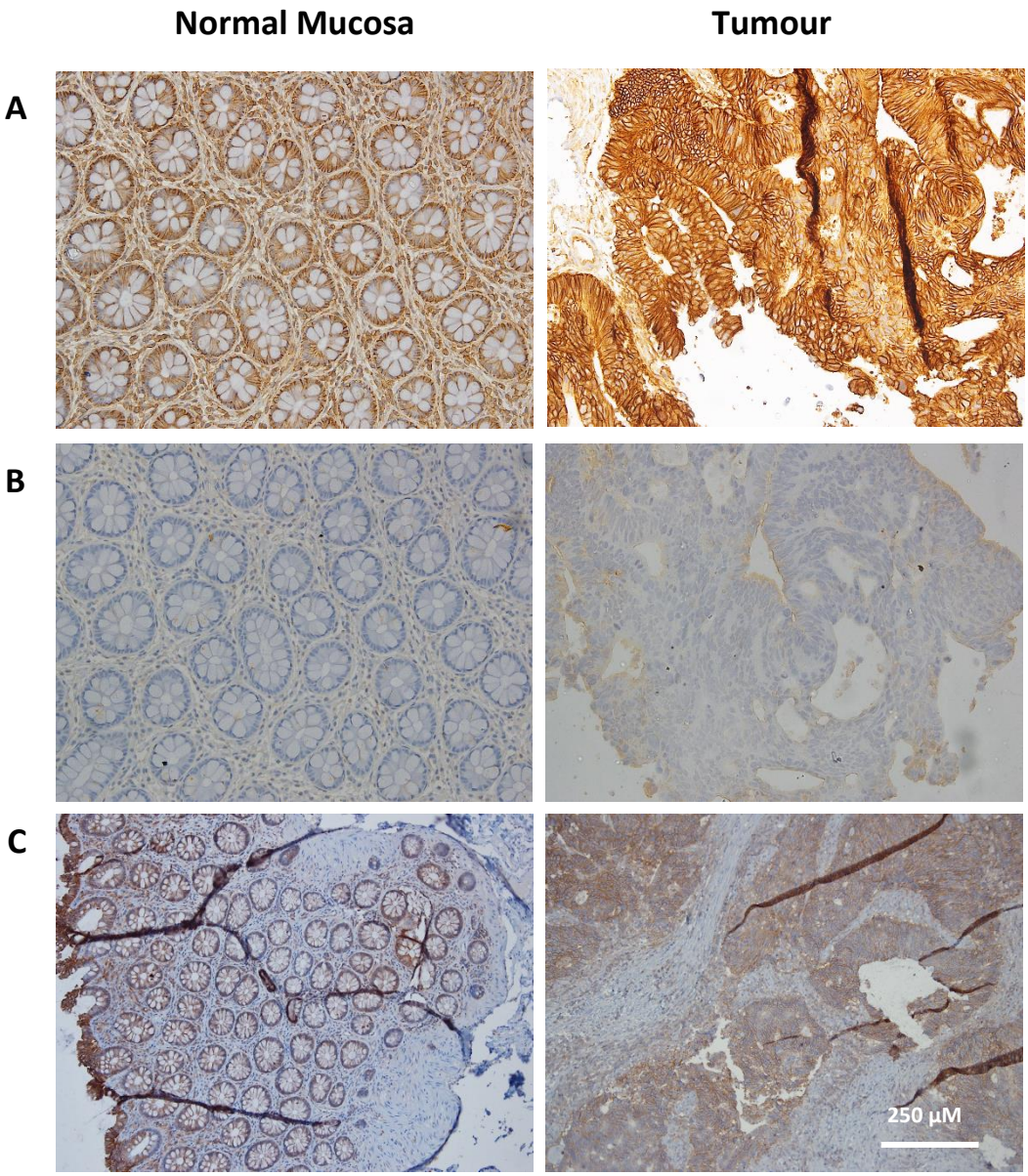


Figure 6: CD147 staining using the microwave and pressure cooker method of antigen retrieval. *A) Overstained normal mucosa and tumour tissue using microwave method. B) Under stained normal mucosa and tumour tissue using microwave method. C) Consistent staining of normal mucosa and tumour using the pressure cooker method.*

3.2.2 PRIMARY ANTIBODY CONDITIONS FOR TMA SECTIONS

To visualise specific CD147 staining while minimizing non-specific background signals the appropriate conditions for the primary antibody were determined by trialling 3 different concentrations at a single exposure time of 1 hour. The antibody was trialled against TMA sections containing stage III and II tumours, normal mucosa and lymph node samples at time 1, 0.5 and 0.2 µg/ml for 1 hour at room temperature (figure 7) along with PBS controls. These conditions were based on the manufacturer's recommendations (R&D Systems). Of all antibody concentrations,

0.5µg/ml appeared to be the most appropriate, showing less background staining, greater membrane localisation signal and sufficient intensity of staining compared to 1µg/ml and 0.2µg/ml in both tumour and normal mucosa tissues (figure 7). Further, low to moderate intensity of staining was still observed in the normal mucosa tissue at 0.5µg/ml, which is representative of physiological levels of CD147 in the colon. Despite this, intensity of staining in tumour tissues was visually distinguishable to normal mucosa tissue (figure 7C). Therefore, tumour tissues with overexpression of CD147 could be visually detected.

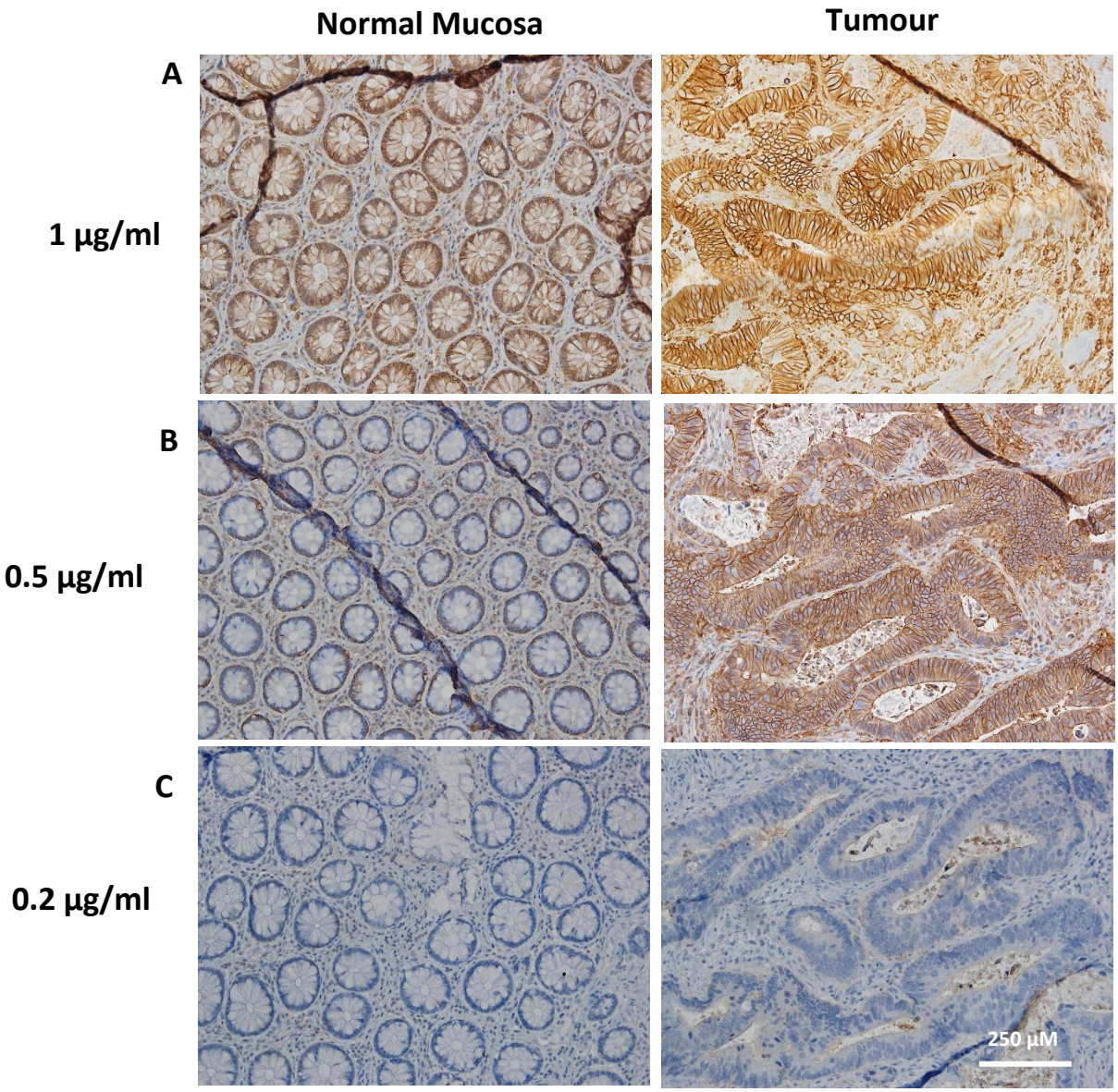


Figure 7: Primary antibody concentration optimisation for IHC detection of CD147
A) No difference between normal mucosa and tumour CD147 staining and haematoxylin cannot be seen using 1 µg/ml primary antibody. B) Greater intensity of CD147 staining in tumour compared to normal mucosa and haematoxylin provides contrast using 0.5 µg/ml. C) No CD147 staining visible in normal mucosa or tumour using 0.2 µg/m of primary antibody. Scale bar=250 µM.

3.3 OPTIMISATION AND QUALITY CONTROL OF TECHNIQUES FOR THE DETECTION OF MIR-21 AND MIR-29A

3.3.1 HOMOGENISATION TECHNIQUES FOR THE PREPARATION OF NORMAL MUCOSA, TUMOUR AND LYMPH NODE TISSUE

Initially, mechanical disruption with a mortar and pestle was trialled against tissue samples. However, this process proved inefficient with large chunks of tissue still present after 10 minutes of grinding action. Instead, a motorized mortar and pestle homogeniser was trialled which successfully homogenised all tissue types, although tumour and normal mucosa tissues required additional mechanical mincing using surgical blades prior to homogenisation. Sufficient RNA yields and sample purity were gained for all tissue types (table 4). At least 10ng/µl were required for downstream miRNA quantification (TaqMan applied biosystems). Only RNA with a ratio of absorbance at 260 and 280nm ($A_{260/280}$) between 1.6 and 2.2 were used (Qiagen RNeasy kit).

Table 4: Representative RNA yields (ng/µl) and ratio of absorbance at 260 and 280nm ($A_{260/280}$) from tumour, normal mucosa and lymph node samples.

Tumour		Normal Mucosa		Lymph node	
RNA (ng/µl)	$A_{260/280}$	RNA (ng/µl)	$A_{260/280}$	RNA (ng/µl)	$A_{260/280}$
1093.5	2.10	164.3	2.06	2518.5	1.99
790.5	2.09	575.5	2.08	967.4	2.02
670.6	2.08	195.9	2.05	598	1.96
692.3	2.10	82.3	2.00	1485	1.96
1075.9	2.09	98.1	2.06	1246.5	1.94
1307.7	2.09	1239.9	2.07	369.5	1.96
728.9	2.09	1501.5	2.09	1467.8	2.03
1260.3	2.08	808.3	2.08	107.4	1.96
446.1	2.06	226.1	2.06	1286.7	1.99
696.8	2.07	153	2.09	801.8	2.02

3.3.2 EVALUATION OF ENDOGENOUS CONTROL (REFERENCE) MIRNAS FOR NORMAL MUCOSA, TUMOUR AND LYMPH NODE TISSUE

miR-345 and miR-16 were trialled as endogenous controls for normalisation of miR-21 and miR-29a RT-qPCR in tumour and normal mucosa tissue and/or the lymph nodes (figure 8). For tumour and normal mucosa tissues, Ct values of miR-345 and miR-16 were all within 2 Ct values and no statistical differences in terms of mean Ct values and standard deviations were detected between normal mucosa and tumour samples (figure A, B). Therefore, the geometric mean of the two miRNAs was used to normalise tumour and normal mucosa samples (figure 8C). Again, no statistical differences were detected between normal mucosa and tumour samples.

Mean Ct values and standard deviations between the non-cancer (negative control) and stage II lymph nodes were not statistically different for both miR-345 and miR-16 (figure D, E). However, a wide range of Ct values for miR-345 (24.75-32.85) and miR-16 (16.55-30.59) for all lymph nodes was observed.

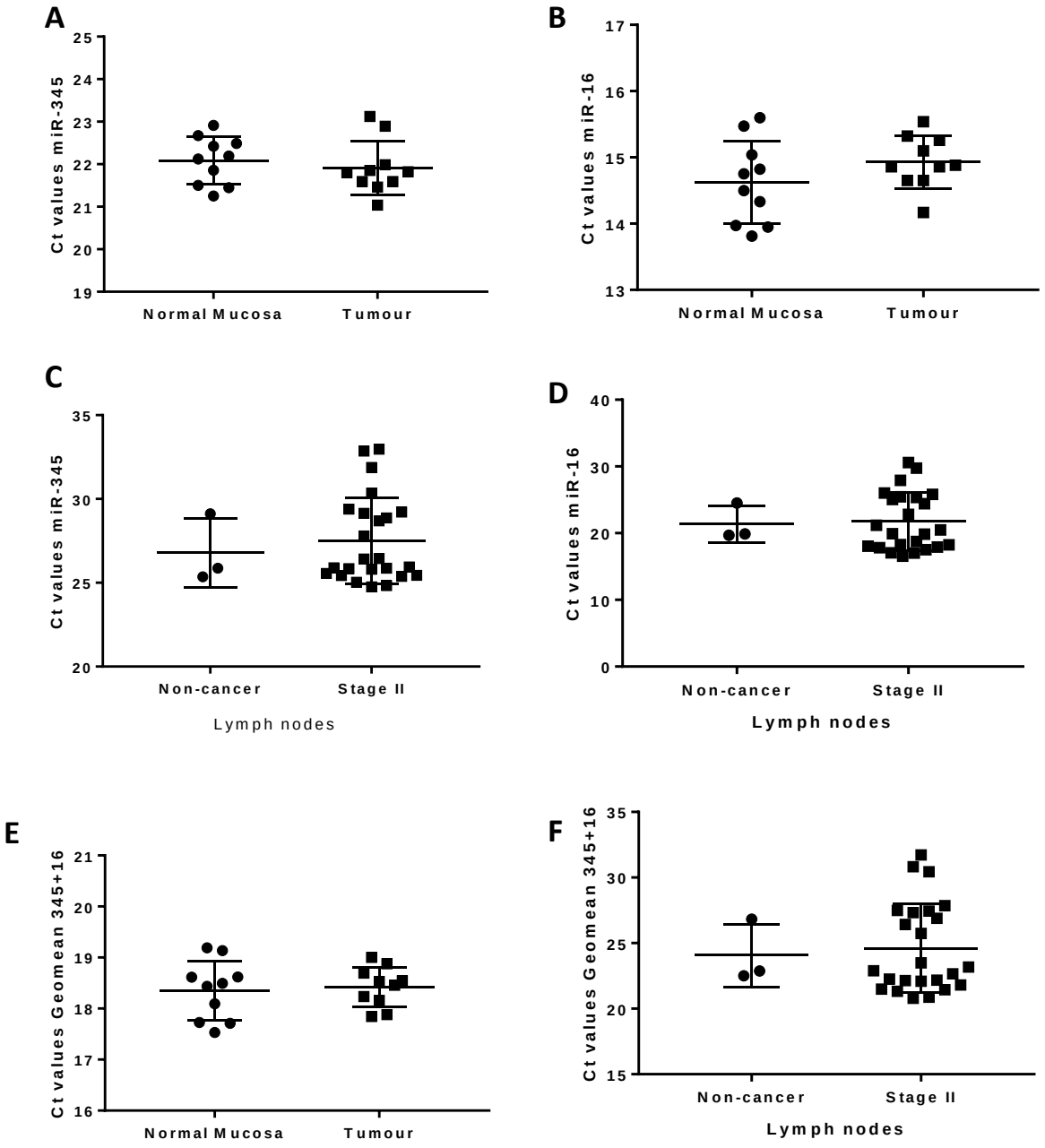


Figure 8: RT-qPCR Ct values of miR-345 and miR-16 endogenous controls for tumour, normal mucosa and lymph node tissues.

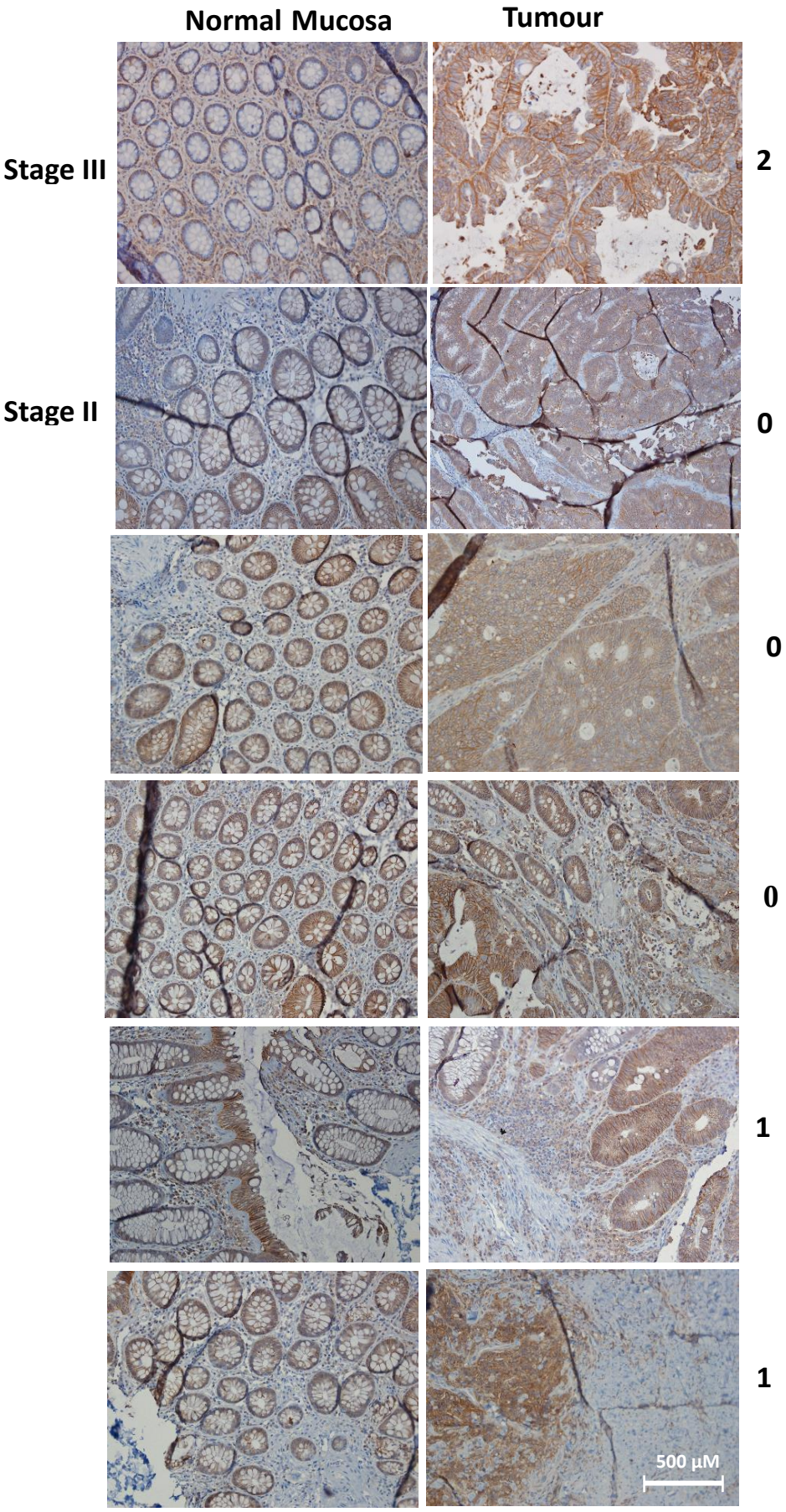
A, B and E) No differences were observed between normal mucosa and tumour tissue for Ct values of miR-345, miR-16 or the geometric mean. All samples were within 2 Ct values. C, D and F) No differences were observed between the non-cancer and stage II lymph nodes for Ct values of miR-345 and miR-16. However, a large range of Ct values were observed in both groups for miR-345 (24.75-32.85) and miR-16 (16.55-30.59)

3.4 CHARACTERISATION OF CD147, MIR-21 AND MIR-29A IN STAGE II TUMOURS

3.4.1 IMMUNOHISTOCHEMICAL CHARACTERISATION OF CD147 IN STAGE II COLON CANCER TUMOURS

After optimisation of IHC to stage II and III TMA sections, the protocol was applied to 10 patient tumour and normal mucosa samples available as 3mm cores within recipient TMA blocks. The protocol was also performed on PBS controls (no primary antibody) and repeated on a separate occasion.

CD147 immunostaining was both membranous in tumour cells and the intestinal glands of normal mucosa tissues (figure 9). CD147 was abundant in both tumour and normal mucosa tissues however, intensity of staining was generally lower in normal mucosa tissues. The overexpression of CD147 protein in each tumour was qualitatively evaluated based on the intensity of staining relative to the matched normal mucosa (figure 9). Of the 10 tumour samples, 6 were positive and 4 were negative.


Normal Mucosa
Tumour
Stage III
2
Stage II
0
0
0
1
1
500 µM

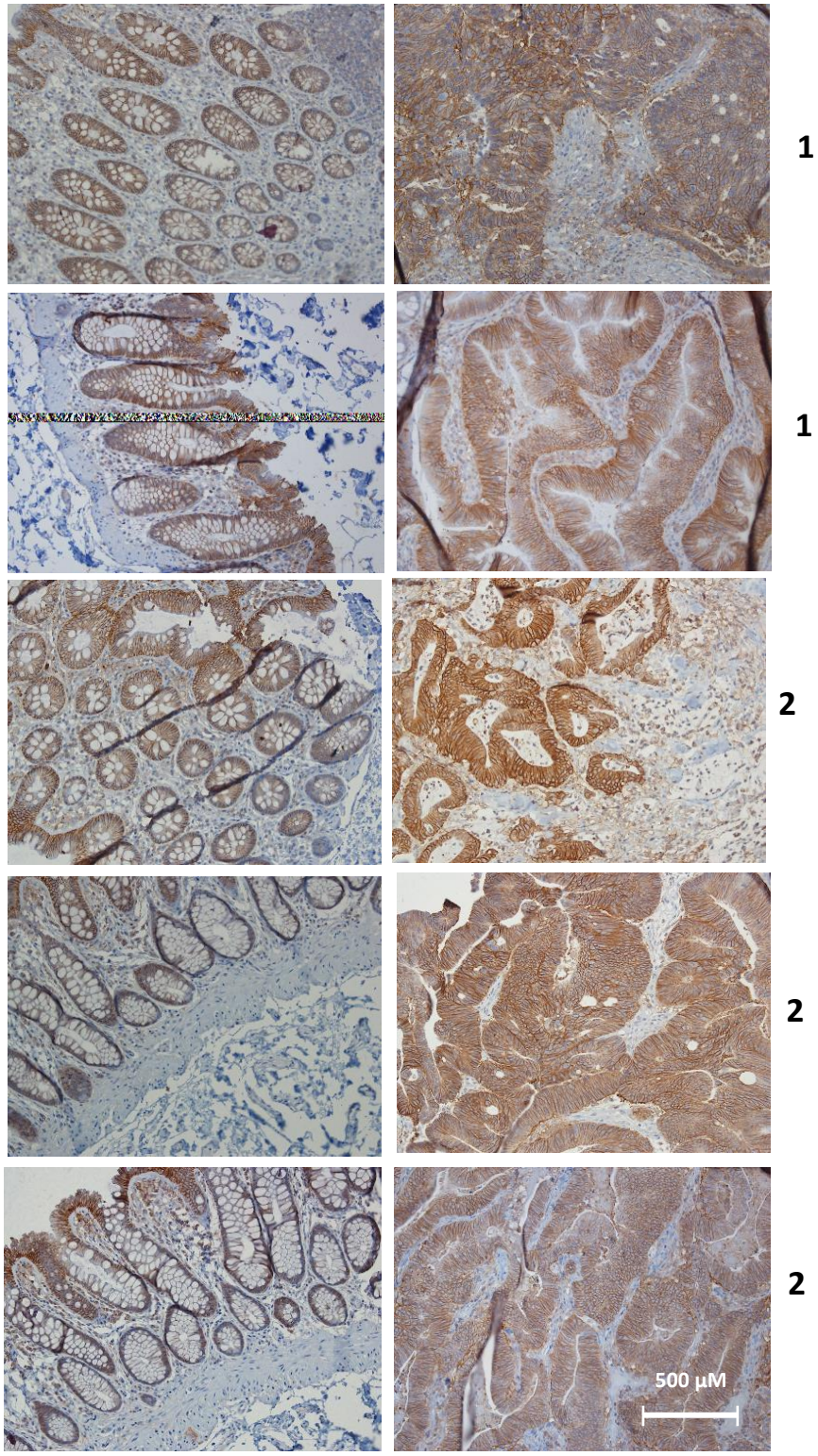


Figure 9: Qualitative IHC evaluation of staining intensity for CD147 in paired stage III (positive control) and stage II tumour and normal mucosa tissues.

Intensity score of 0= no difference in staining intensity between paired tumour and normal mucosa, 1= Moderate increase in staining intensity of tumour compared to matched normal mucosa, 2= High increase in staining intensity of tumour compared to matched normal mucosa. Scale bar=500 µM.

3.4.2 TUMOUR CD147 EXPRESSION AND PATHOLOGICAL RISK FACTORS

The number of patients in the present study was too small to compare CD147 status and pathological factors such as T stage, pathological differentiation and lymphatic invasion. However, of note is the absence of patients with both a low CD147 expression and T4a/b stage (table 5).

Table 5: T stage, tumour grade and lymphatic invasion verse CD147 expression in stage II colon tumours

Pathological feature	CD147 overexpression	
	Positive n (%)	Negative n (%)
T3	3 (42.86)	4 (57.14)
T4a/b	3 (100)	0 (0)
Low grade	4 (66.67)	2 (33.33)
High grade	2 (50)	2 (50)
Lymph invasion	2 (50)	2 (50)
None	4 (66.67)	2 (33.33)

3.4.3 CHARACTERISATION OF MIR-21 AND MIR-29A IN STAGE II COLON CANCER TUMOURS USING QUANTITATIVE RT-PCR

To characterise and quantify the expression levels of miR-21 and miR-29a in stage II colon tumours, quantitative reverse-transcription PCR (RT-qPCR) was applied to the same tumour and normal mucosa pairs.

miR-21 was upregulated in 50% of tumours compared to normal mucosa samples (figure 10A). Higher miR-21 relative expression levels were observed in the tumour samples (1.631 ± 1.029) compared to normal mucosa samples (1.045 ± 0.3358). This association did not reach statistical significance, but a trend was observed (p=0.071).

Greater variability was also observed for the tumours compared to normal mucosa samples (figure 10A). There was a statistically significant difference (p=0.0027) between the standard deviations of the tumours and the normal mucosa samples.

miR-29a was upregulated in the tumour of 1 patient and downregulated in the tumours of 8 patients compared to normal mucosa samples (figure 10B). Overall lower miR-29a relative expression levels were observed in the tumour samples (0.8894 ± 0.6428) compared to the normal mucosa samples (1.054 ± 0.3378). However, this association did not reach statistical significance, but a trend towards statistical significance was observed (*p*=0.0967). No difference in variability was observed between the tumours compared to normal mucosa samples.

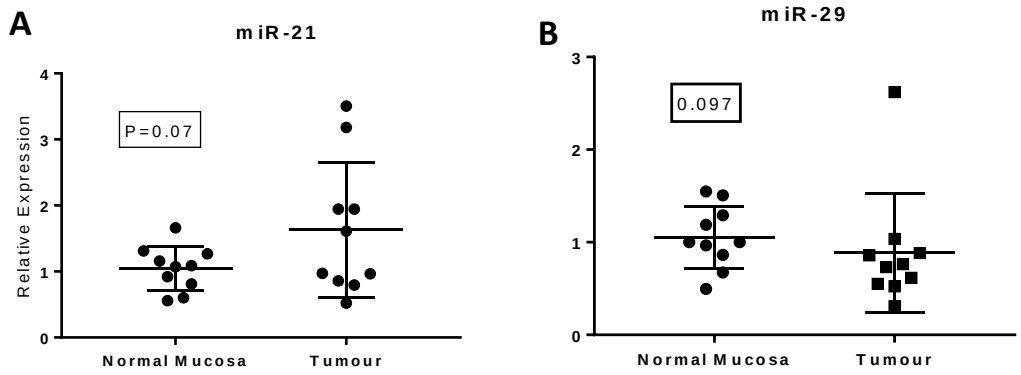


Figure 10: Relative expression of miR-21 and miR-29a in matched tumour and normal mucosa sample of stage II colon tumours.
A) miR-21 relative expression normal mucosa verse tumour (p=0.071, paired t-test). B) miR-29 relative expression normal mucosa verse tumour (p=0.0967, Wilcoxon t-test).

3.4.4 TUMOUR MIR-21, MIR-29A EXPRESSION LEVELS AND PATHOLOGICAL RISK FACTORS

Due to a small cohort size, only tumour grade, T stage and lymphatic invasion pathological factors were compared to expression levels of miR-21 and miR-29a (figure 11).

Lower miR-21 relative expression levels were observed in patients with a high T stage (T4a/b) (0.7634 ± 0.1313) compared to patients with a low T stage (T3) (1.615 ± 0.3214) (figure 11A). This association trended towards statistical significance (*p*=0.0681). Further, patients with low grade tumours had significantly lower miR-29a relative expression levels (median=0.5833) compared to high grade tumours (median=0.959) (*p*=0.0048) (figure 11F). No other trends or associations were observed between miR-21, miR-29a and pathological factors (figure 11B, C, D, E).

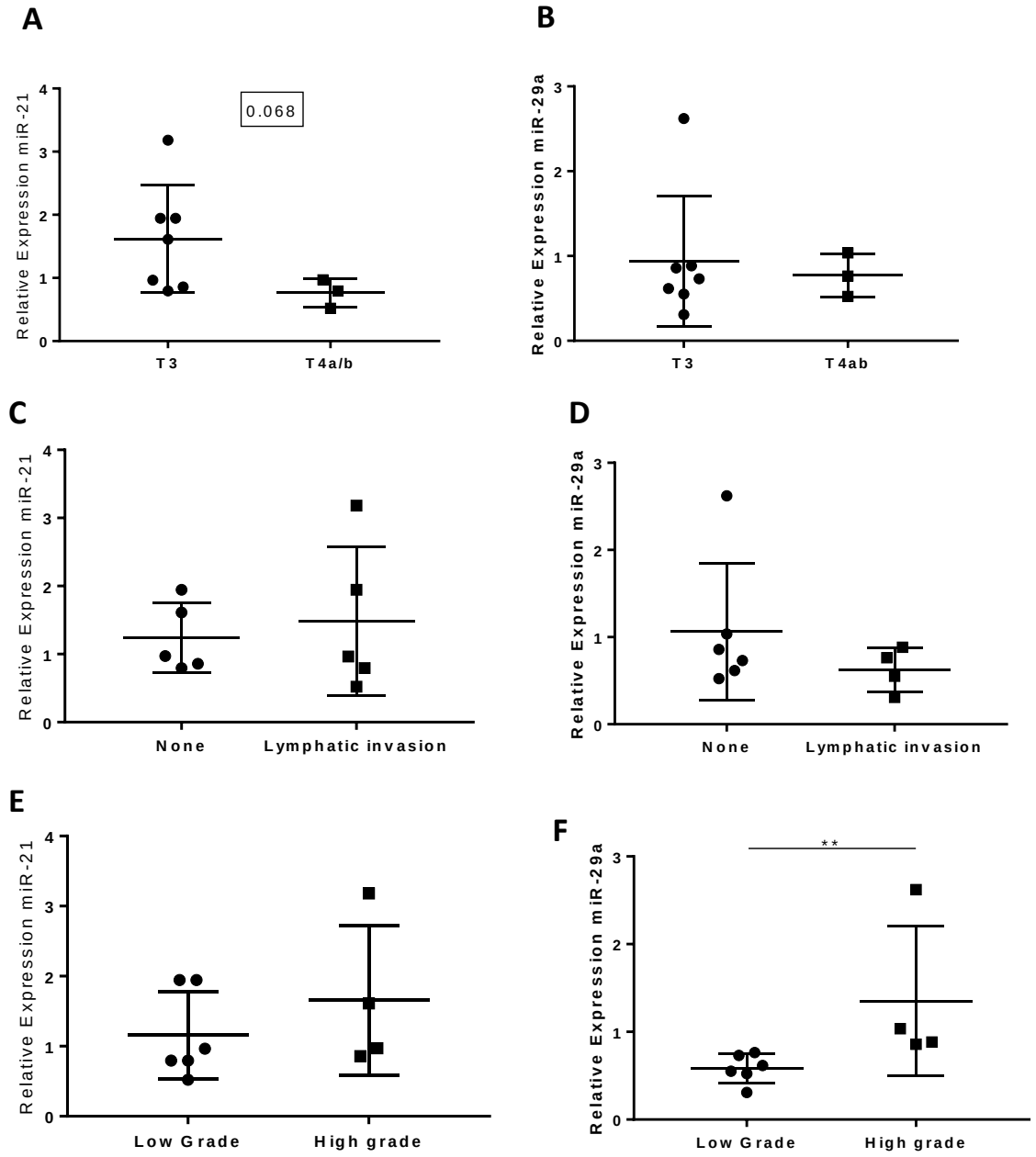


Figure 11: Tumour miR-21, miR-29a relative expression and pathological factors.
*A) miR-21 relative expression in T3 verse T4a/b tumours (p=0.0681, unpaired t-test). F) miR-29a relative expression in low grade verse high grade tumours (p=0.0048, Mann-Whitney t-test). B, C, D, E) No other trends or associations were found between miR-21, miR-29a and T stage, lymphatic invasion and tumour grade. ** = p value < 0.01.*

3.4.5 Association between miR-21, miR-29a and CD147 tumour LEVELS

The expression levels of CD147, miR-21 and miR-29a were compared to determine if there was a relationship between the markers (figure 12).

Tumours with positive CD147 protein levels had lower miR-21 relative expression levels (1.01 ± 0.1988) compared to tumours with negative CD147 protein levels (1.884 ± 0.4954) (figure 12A). This

association was statistically significant (*p*=0.0481). Interestingly, all patients with positive CD147 tumours had low tumour expression of miR-21 except for one patient who had a high expression of both markers. No trends or associations were detected between miR-29a and CD147 or miR-21 tumour expression levels (figure 12B, C).

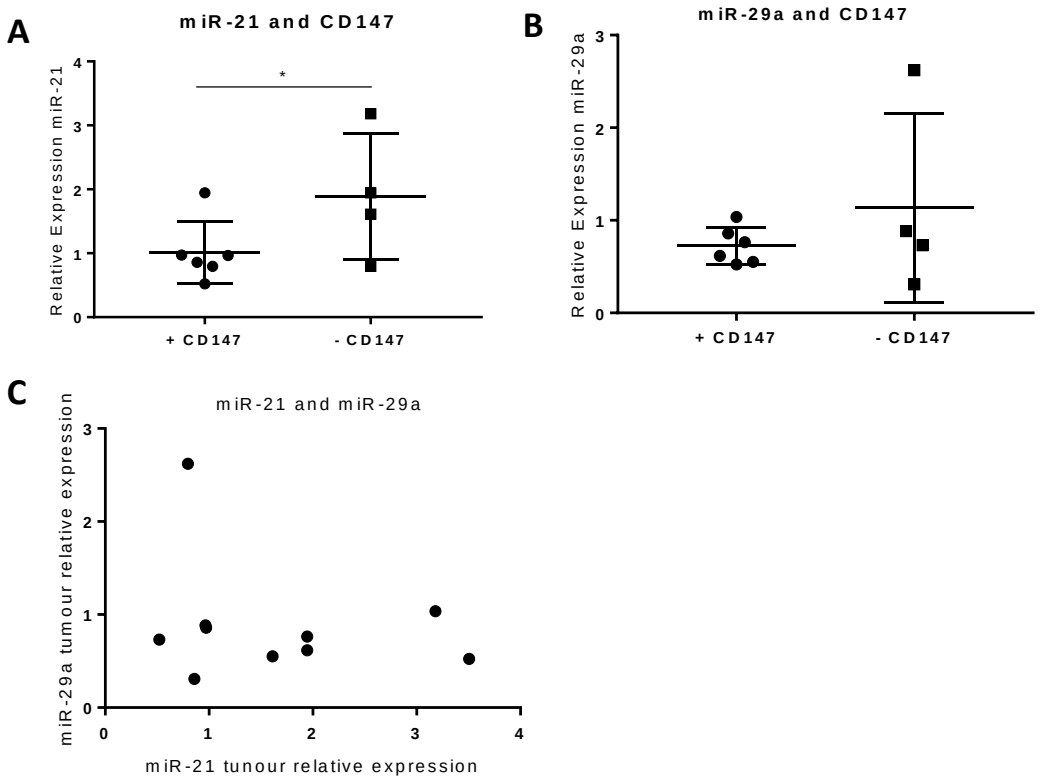


Figure 12: Relative expression levels of miR-21 and miR-29a and histological protein expression of CD147.

*A) Tumour relative expression of miR-21 in CD147 positive verse negative tumours (p=0.0481, unpaired t-test). B) No associations were detected between miR-29a and CD147. C) No correlation was detected between miR-21 and miR-29a. * = p value < 0.05.*

3.5 IMMUNOHISTOCHEMICAL EVALUATION OF CD147 STAINING IN THE STAGE II LYMPH NODES

3.5.1 IMMUNOHISTOCHEMICAL EVALUATION OF CD147 STAINING IN STAGE II COLON CANCER LYMPH NODES

As mentioned previously, the optimised IHC protocol was applied to stage II and III patient samples, including the TDLNs, available as 3mm cores within recipient TMA blocks. Lymph nodes from a non-cancer patient (diverticulosis) were also stained for CD147.

A substantial amount of staining was observed in all lymph node samples, including the non-cancer lymph nodes (figure 13D). This staining was predominantly present in the B cell GCs of the lymph nodes. Initially this staining pattern was not expected because according to the human protein atlas (90), CD147 is not expressed in unstimulated or stimulated lymph nodes (characterised by the formation of follicles and GCs) of the gastrointestinal tract (figure 13A, B). However, upon further investigation it is more likely that CD147 is expressed in activated T cell and B cells according to the Human CD Marker Chart (91) and a previous report in the normal adjacent lymph nodes of patients with B cell lymphomas (92).

For this reason, the tumour-associated expression of CD147 could not be explored. Instead, the project shifted focus towards exploring the morphological patterns in the stage II, III and non-cancer lymph nodes. Despite, this change of direction, the ultimate aim of discovering biomarkers was still persistent.

3.6 HISTOLOGICAL MORPHOLOGY OF STAGE II COLON CANCER LYMPH NODES

A total of 146 lymph node cores from stage II colon cancer patients were examinable (where more than 50% of the 3mm cores had tissue present) with an average of 10 lymph nodes per patient. In most cases (n=8) patients had more than 10 examinable lymph nodes, in 4 cases there were 5-9 examinable lymph nodes and in 3 cases there were 3 examinable lymph nodes. There was no evidence of metastasis in the stage II TDLNs. For the stage III patient (positive control), 20 TDLNs

examinable. However, metastatic deposits were present in 80% (n=16/20) of them. For the non-cancer patient (negative control), all 3 surgically resected lymph nodes were examinable.

3.6.1 QUALITATIVE ANALYSIS OF LYMPH NODE B CELL COMPARTMENTS

All stage II TDLNs (n=126) except 1, formed B cell follicles and GCs and were therefore considered stimulated lymph nodes (figure 13D). Only one TDLN of the stage III patient formed these B cell compartments (figure 13H), the rest were infiltrated with metastatic deposits or unresponsive. All 3 of the non-cancer lymph nodes were stimulated.

Comparison of non-cancer stimulated lymph nodes compared to stage II cancer-specific stimulated lymph nodes revealed differences in the number, relative size and roundness of follicles and GCs (figure 13D, E, F). Of the 3 non-cancer stimulated lymph nodes, there was an average of 3 follicles and GCs per lymph node (figure 13C) while an average of approximately 11 follicles and 8 GCs were observed for the stage II TDLNs (table 6). While no striking differences were observed between the non-cancer and stage II lymph nodes in terms of the average follicle and GC size (table 6), some stage II lymph nodes formed large, irregular shaped follicles (figure 13E, F). This pattern was not seen in the non-cancer lymph nodes that were all consistent in shape and size (figure 13C).

We did not compare these findings to the stage III TDLNs because only one formed follicles and GCs. However, no primary follicles can be seen in the non-cancer lymph nodes, while primary follicles without GCs are evident in both stage III and II lymph nodes (figure 13H).

Table 6: Average follicle and GC density and size in non-cancer lymph nodes and stage II TDLNs

	Non-cancer lymph nodes	Stage II TDLNs
GC density	3	7.899946
F density	3	11.402
F size (mm)	0.141222	0.1431
GC size (mm)	0.059556	0.053428

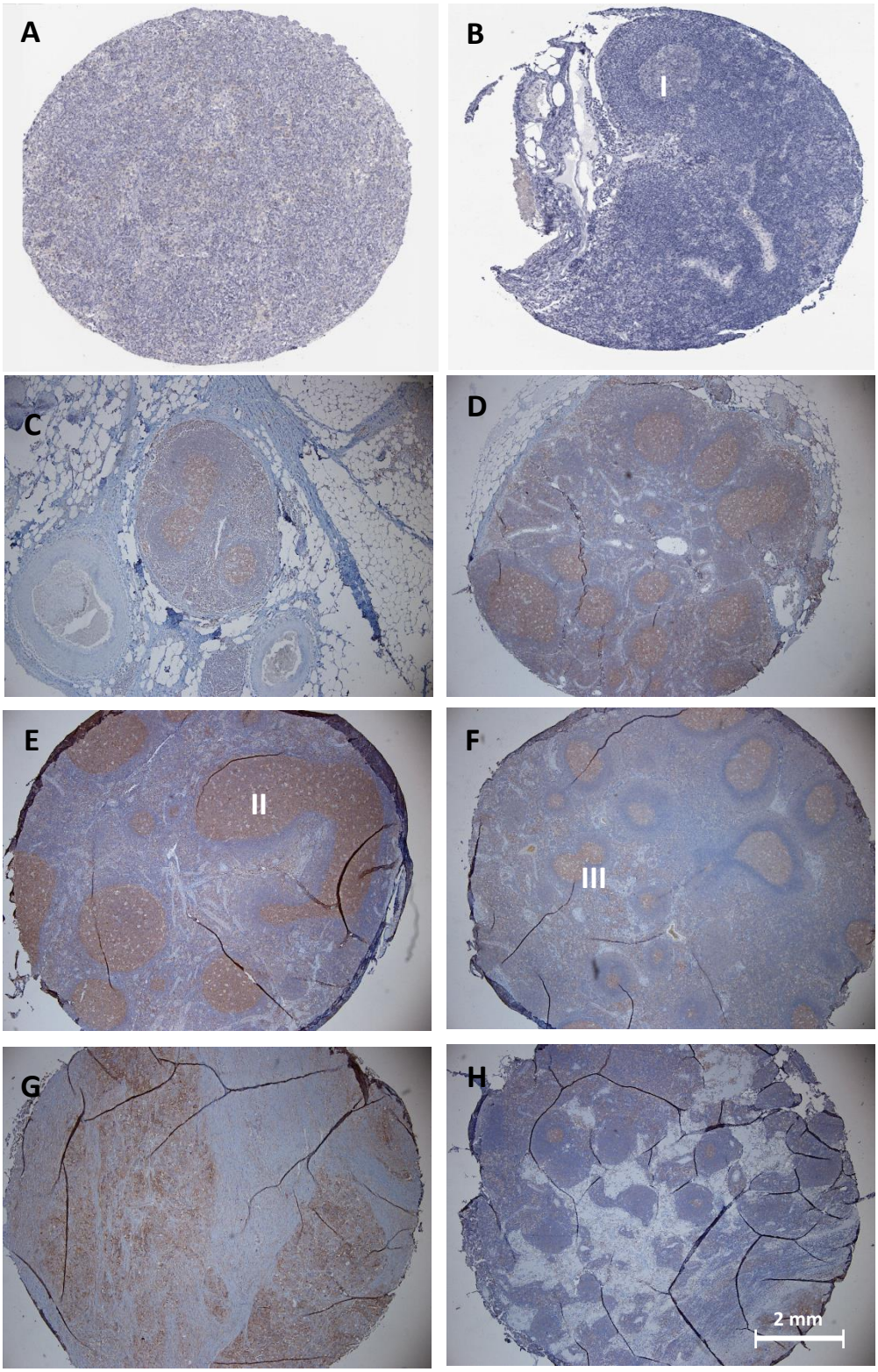


Figure 13: B cell follicles and GCs in lymph nodes from different patient origins stained for CD147. *A) Unstimulated lymph node characterised by the lack of follicles and GCs. No CD147 staining present. Data from the Human Protein Atlas (90) however, patient of unknown origin B) Stimulated lymph node from the same patient characterised by the formation of a single follicle and GC. However, there is no CD147 staining in the GC (I) while CD147 staining was present in all GC in this study. C) Non-cancer lymph nodes. D) Typical stage II lymph node. E) Stage II lymph node with a large, irregular GC (II). F) Stage II lymph node with a fused/non-circular GC (II). G) Typical stage III invaded lymph node. H) The only stimulated, non-invaded stage III lymph node.*

3.6.2 ASSOCIATIONS BETWEEN PATHOLOGICAL RISK FACTORS AND HISTOMORPHOLOGICAL DIFFERENCES IN LYMPH NODES

To address the hypothesis that histomorphological changes in the draining mesenteric lymph nodes of stage II colon cancer patients is associated with pathological risk factors the number and size of follicles and GC were correlated with T stage, dMMR status, lymphatic invasion and tumour grade (figure 14, 15, 16, 17).

Patients with T3 tumours tended to have a lower GC density (7.168 ± 1.805) compared to patients with T4a/b tumours (9.546 ± 3.375) (figure 14B). The same trend was seen for GC size where patients with T3 tumours had a lower GC size (0.03422 ± 0.004963) compared to patients with T4a/b tumours (0.09664 ± 0.02714) (p=0.0516) (figure 14C). Similarly, patients with T3 tumours tended to have a smaller follicle size (0.1153 ± 0.009043) compared to patients with T4a/b tumours (0.2057 ± 0.05046) (p=0.0851) (figure 14E).

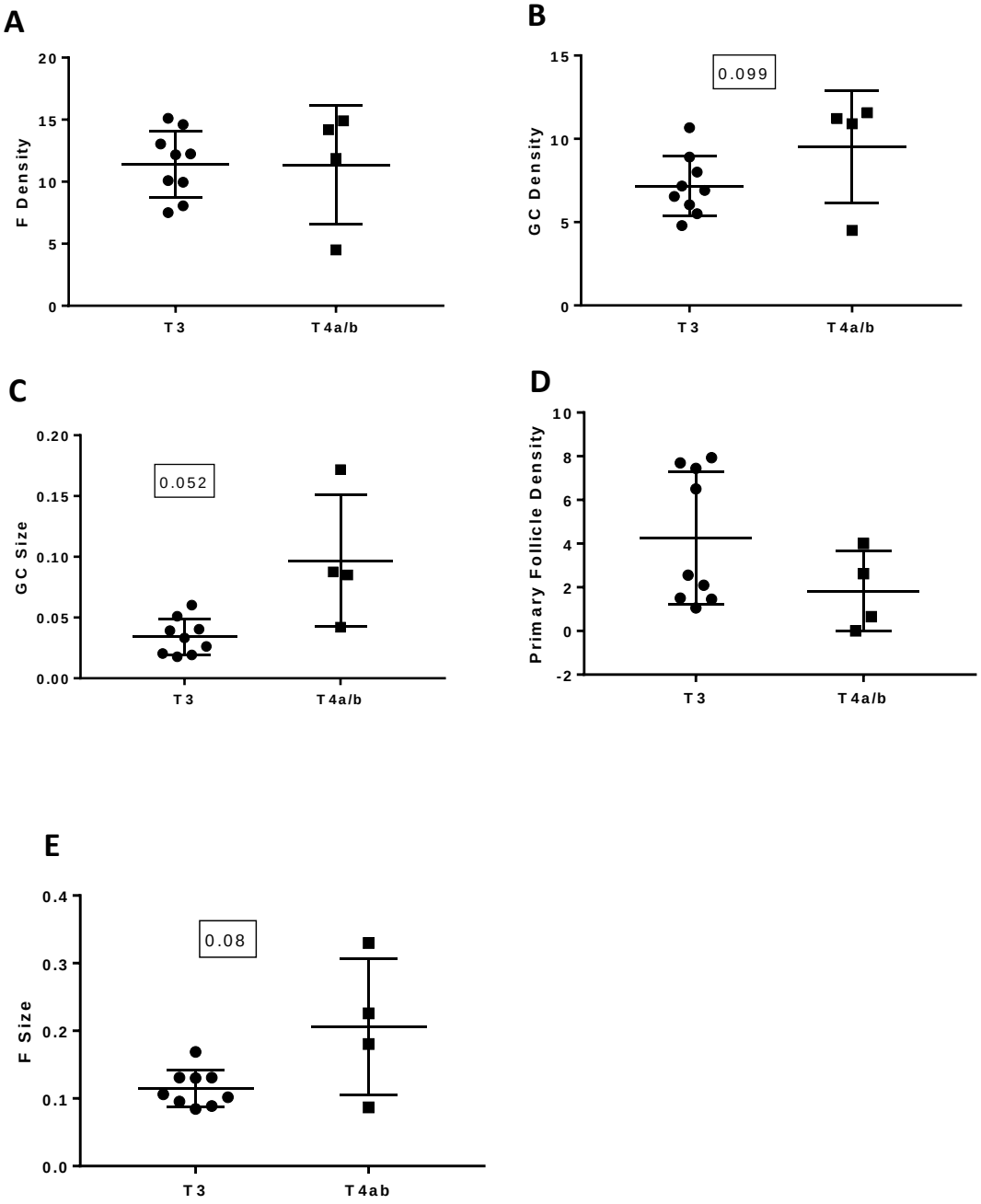


Figure 14: Lymph node morphology and T stage

A) GC density in patients with T3 tumours versus T4a/b tumours (p=0.0993 Mann-Whitney t-test. C) GC size in patients with T3 tumours versus T4a/b tumours (p=0.0516, Welch's t-test. E) Follicle size in patients with T3 tumours versus T4a/b tumours (p=0.0851, Welch's t-test). B, D) No other trends or associations found.

Patients with evidence of dMMR had a higher number of follicles per lymph node (13.05 ± 0.946) compared to patients without dMMR (9.417 ± 1.42) (figure 15b). This association was significant (*p*=0.0364). Similarly, patients with evidence of dMMR had a higher number of primary follicles per lymph node (5.385 ± 1.278) compared to patients without MSI (1.704 ± 0.3971) (figure 15C). This association was also statistically significant (*p*=0.0212).

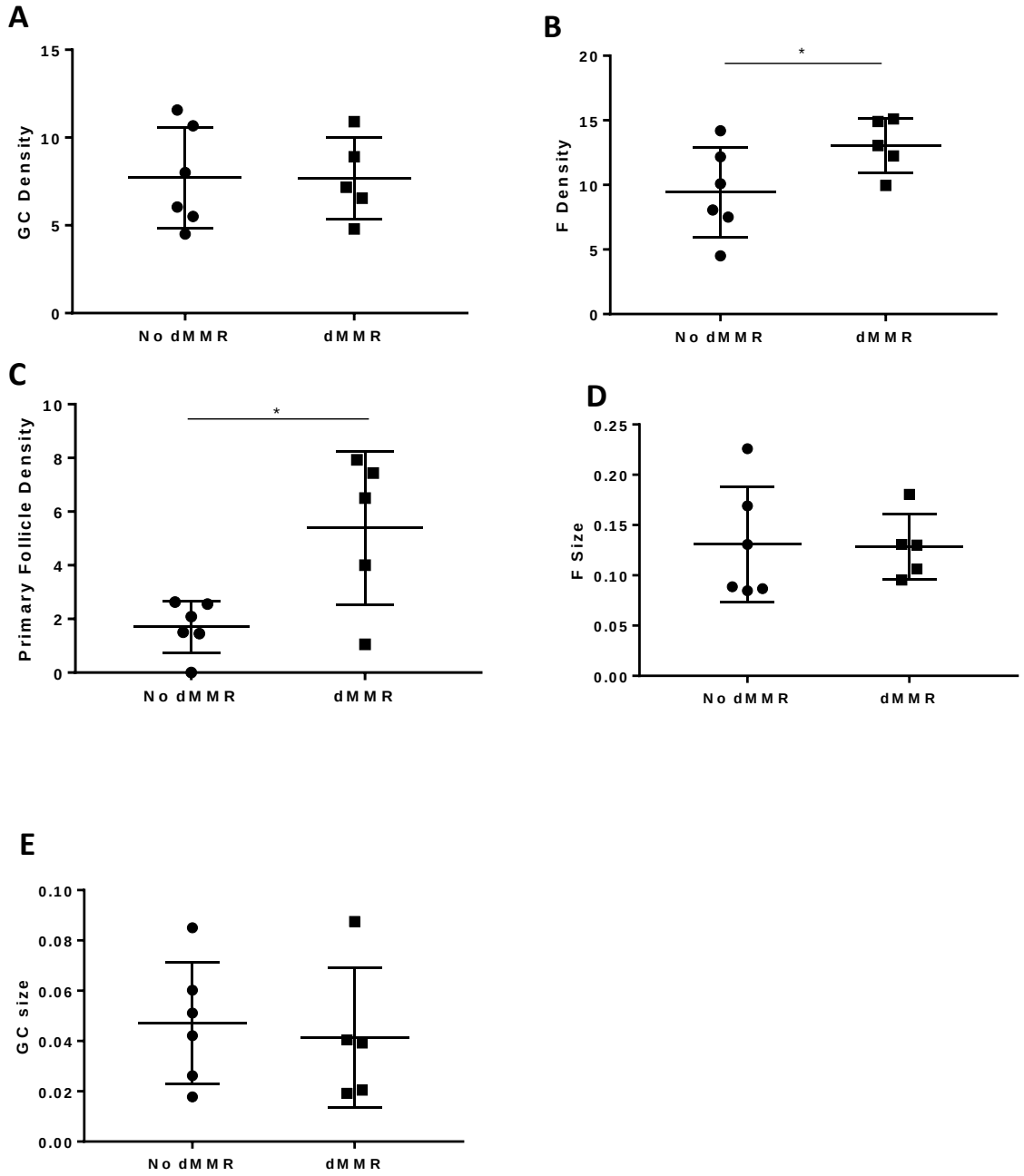


Figure 15:Lymph node morphology and dMMR status.
*B) Follicle density in patients with dMMR verse those without (p=0.0364, unpaired t-test). C) Primary follicle density in patients with dMMR verse those without (p=0.0424, Welch's t-test). A, D, E) No other trends or associations found. * = p value < 0.05.*

Patients with evidence of lymphatic invasion tended to have a smaller follicle size (0.109 ± 0.0009069) compared to patients without lymphatic invasion (0.1644 ± 0.02928) (figure 16E). This association did not reach statistical significance, but a trend was observed (*p*=0.0212). The same trend was observed for GC size, where patients with lymphatic invasion had a lower GC size (0.03486

± 0.004187) compared to patients without lymphatic invasion (0.06503 ± 0.01805) (*p*=0.0717) (figure 16D). Patients with lymphatic invasion also had a lower GC density (6.327 ± 0.7963) compared to patients without lymphatic invasion (8.883 ± 0.8775), this association was statistically significant (*p*=0.0360) (figure 16B).

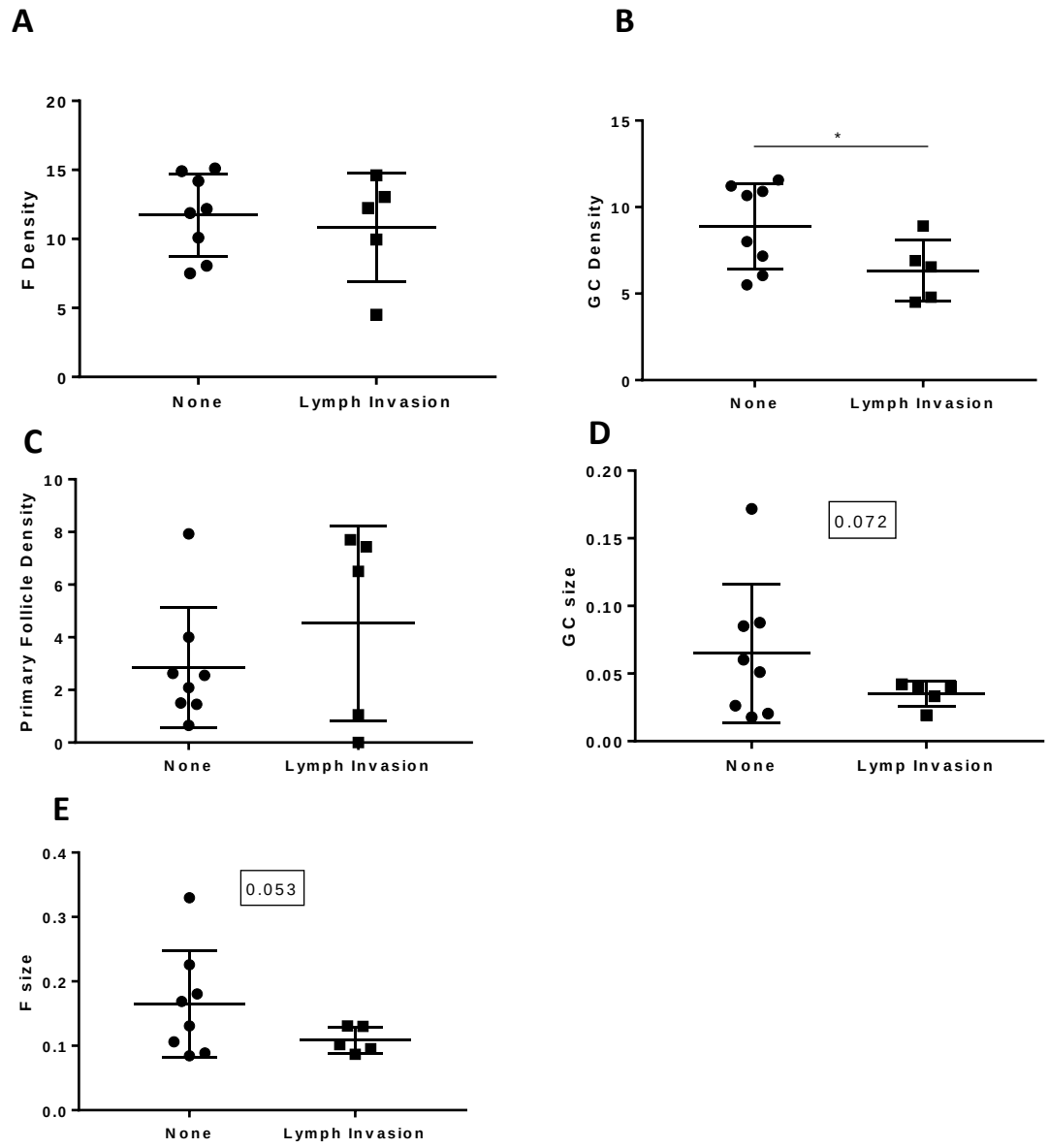


Figure 16:Lymph node morphology and lymphatic invasion.
*B) GC density in patients with lymphatic invasion versus those without (p=0.0360, unpaired t-test). D) GC size in patients with lymphatic invasion versus those without (p=0.0717, Welch's t-test). E) Follicle size in patients with lymphatic invasion versus those without (p=0.0212, Welch's t-test). A, C) No other trends of associations were found. * = p value < 0.05.*

Patients with high grade tumours had a lower primary follicle density (1.44 ± 0.4103) compared to patients with low grade tumours (4.416 ± 1.012) (figure 17C). This was statistically significant (*p*=0.0429). No other associations or trends were detected between tumour grade and GC density, follicle density, GC size and follicle size (figure 17A, B, D, E).

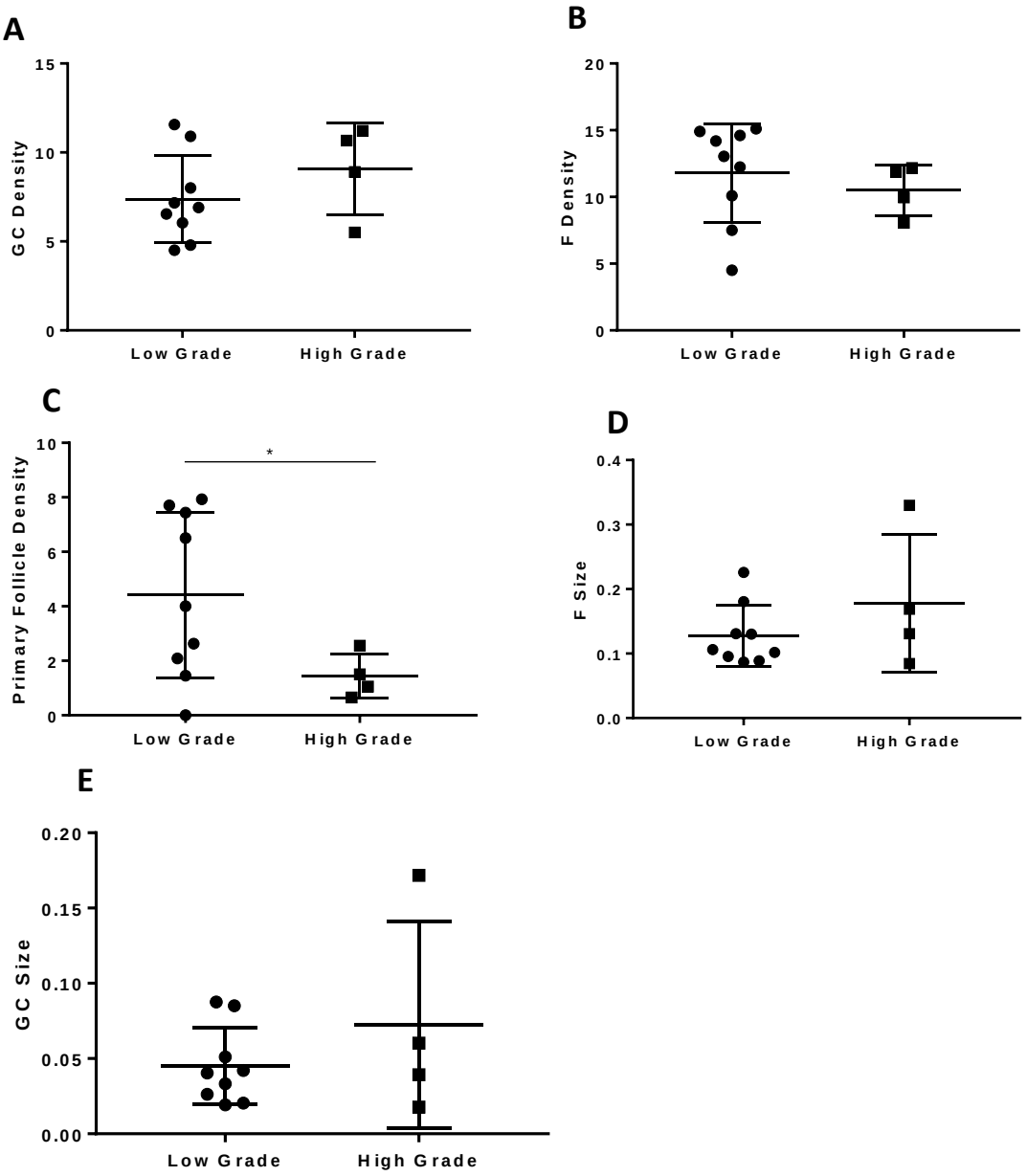


Figure 17:Lymph node morphology and pathological grade.
*High grade= moderately-poorly differentiated, low grade= well differentiated. C) Primary follicle density in patients with high grade versus low grade tumours (p=0.0429, unpaired t-test). A, B,D, E) No other trends of associations were found. * = p value < 0.05.*

3.7 MIR-21 EXPRESSION LEVELS IN THE STAGE II TUMOUR-DRAINING LYMPH NODES

To detect and characterise the expression levels of miR-21 in the lymph nodes of stage II colon cancer patients, RT-qPCR was performed in FFPE TDLNs of stage II patients and the lymph nodes of the non-cancer patient. The geometric mean of miR-345 and miR-16 was used to normalise the expression levels of miR-21. miR-29a was not measured due to time constraints. Further, RT-qPCR was not applied to all lymph nodes of the entire stage II cohort. Instead, 23 randomly chosen lymph nodes from all patients were involved in this analysis (figure 18).

A greater variation in miR-21 levels in the TDLNs of stage II patients was observed compared to the lymph nodes of the non-cancer patient (negative control) (figure 18). In particular, a 3-8-fold increase in expression levels was detected in 8 stage II TDLNs compared to negative controls (figure 18).

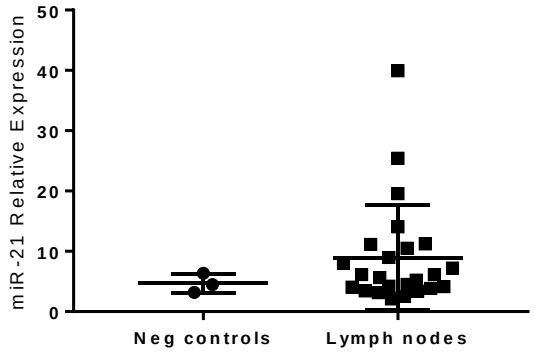


Figure 18: miR-21 relative expression levels in the TDLNs of stage II colon cancer patients and the lymph nodes of a non-cancer patient.

CHAPTER 4: DISCUSSION

4.1 KEY FINDINGS

The overall aim of the current study was the characterise the expression of CD147, miR-21 and miR-29a in the tumours and/or TDLNs in a small pilot cohort of stage II colon cancer patients. The key findings include; 1) CD147 and miR-21 are overexpressed in 60% and 50% of these patients respectively; 2) CD147 cannot be used as a tumour-associated biomarker in the TDLNs; 3) miR-21 expression levels were dysregulated in TDLNs compared to non-cancer lymph nodes; 4) In a histological manner, a large variation in the number, size and shape of B cell compartments in the TDLNs was found between stage II colon cancer patients; Here we discuss, in depth, the findings of the current study and how these findings are significant to the current field of research. We also discuss the limitations and strengths of the methodology used as well as steps to take in the future.

4.2 SAMPLE SELECTION FROM THE SCRG BIOBANK

4.2.1 PATIENT SELECTION AND COHORT SIZE

Colorectal cancer patient recruitment has been ongoing at Wellington Hospital from November 2016 with a current recruitment rate of 1-2 patients per week. At the beginning of the study period (March 2018) a maximum of 13 stage II colon cancer patients, that fitted the inclusion criteria, had been recruited into the biobank.

One requirement that significantly reduced the number of available patients was the exclusion of rectal patients due to the likelihood of them receiving neoadjuvant therapy which would introduce confounding factors. For example, neoadjuvant (chemo)radiation therapy is known to introduce morphological changes both in the primary tumour and the draining lymph nodes. Fibrous or fibroinflammatory tissue is of note, as this can obscure carcinoma cells within a tumour and morphological compartments within lymph nodes (85). Further, the design of this cohort was to simply characterise and explore the potential prognostic significance of biomarkers in patients who have not received any additional therapy other than surgical resection of the primary tumour.

While the cohort size in this pilot study was small it was sufficient enough to establish and optimise techniques in the lab for the detection of CD147, miR-21 and miR-29a for future, larger cohorts. Further, the expression of these markers could be characterised and hypotheses formed as to their prognostic significance in stage II colon cancer patients.

4.2.2 ETHICAL CONSIDERATIONS

One component of the ethical considerations was the need to ensure adequate representation of Māori within the study. Statistically Māori represent approximately 6% of all CRC cases in New Zealand (2). Fortunately, one of the thirteen patients was Māori, which meant we could more accurately represent the true population of CRC in New Zealand. A challenging aspect of this consideration in an emerging biobank though, is we can only deal with the set of patients we are given. This means we may over represent or under represent Māori. This is something that should be considered in the research design for future, prospective studies.

4.2.3 POSITIVE AND NEGATIVE CONTROLS

The tumour cell positive lymph nodes from a stage III patient and the non-cancer lymph nodes from a diverticulosis patient were particularly useful for qualitative comparison to the stage II lymph nodes. Comparisons were hypothesis-generating around the prognostic potential of histomorphological changes in the stage II lymph nodes.

Ideally, more negative controls would have been selected however, only rarely would non-cancer patients, with surgically resected lymph nodes be recruitment into the biobank. This is due to the framework of our study and ethical approval which requires that patients either have confirmed diagnosis of cancer or a high suspicion of cancer at the time of surgery. Recruitment of the non-cancer patient was only possible as there was initially a high suspicion of cancer in the clinic. However, it must be noted that the role of the positive and negative control was never to perform formal statistical analysis. Instead the purpose of these controls was more for qualitative comparisons and to explore differences.

4.2.2 SAMPLE COLLECTION AND SAMPLE TYPE

While formalin-fixed lymph nodes were retrievable for all stage II patients, fresh paired tumour and normal mucosa samples were only banked for 10 of these patients. Samples from the remaining patients in the cohort could not be taken without compromising pathological assessment due to the specimens being too small, which is sometimes the case for early-stage colon cancers (5). Therefore, samples were not available for biobanking, a limitation of the study.

Regarding the lymph nodes collected, the use of formalin-fixed tissue, as opposed to fresh, was more appropriate for two reasons. Firstly, it is difficult to bank surgically resected lymph nodes as this would comprise pathological staging of cancers therefore, the tissues were retrieved after their fixation and subsequent assessment. Secondly, for immunostaining and morphology, FFPE samples produce better quality of histomorphology compared to fresh tissues (93). FFPE samples also allow for efficient long-term storage for prospective studies (93).

4.3 ESTABLISHMENT AND OPTIMISATION OF METHODOLOGIES

Part of our first objective in the current study was to establish and optimise the molecular techniques for the detection of CD147, miR-21 and miR-29a in the tumours and normal mucosa tissues of stage II colon cancer patients. We successfully optimised primary antibody conditions to detect a strong CD147 signal that could distinguish between tumour and normal mucosa tissues without introducing non-specific staining. Using RT-qPCR, we were also able to detect miR-21 and miR-29a in tumour and normal mucosa samples frozen in RNAlater. Further, we were able to validate miR-345 and miR-16 as endogenous controls for these samples.

4.3.2 LIMITATIONS OF RNA ANALYSIS IN FFPE LYMPH NODES

Detecting tumour-associated CD147 and miR-21 in the TDLNs of these stage II patients was also an important part of our first objective. While CD147 was detectable in the TDLNs using the same IHC protocol for the tumour tissues, we discovered this signal was not tumour-specific or at least any

tumour-specific signal was not distinguishable. Non-cancer specific CD147 staining in TDLNs is discussed in more detail in chapter 4.8.

Concerning the detection of miR-21 in the TDLN FFPE tissues using RT-qPCR, we were not able to validate endogenous miRNA controls for the normalisation of miR-21. A large variation in Ct values was observed in the lymph nodes for both mi-345 and miR-16 endogenous controls whereas, Ct values were within 2 units for the fresh tumour and normal mucosa tissue. Despite this, no statistical differences were observed between the non-cancer and stage II lymph nodes in terms of mean Ct values and standard deviations. This suggests the observed variation may be due to differences in RNA integrity in FFPE tissues versus fresh tissue. All lymph nodes were retrieved as FFPE samples from the hospital laboratory therefore, variables such as time between resection and effective fixation as well as fixation time may have affected the RNA integrity in these samples (94). Despite this, we cannot attribute sample processing to the observed variation in Ct values due to a low number of non-cancer lymph nodes for comparison.

4.3 TUMOUR CD147 EXPRESSION AS A PROGNOSTIC AND PREDICTIVE BIOMARKER IN STAGE II COLON CANCER

4.3.1 CURRENT AND PREVIOUS FINDINGS

CD147/EMMPRIN is the most overexpressed, membrane-bound protein on disseminated cancer cells (61) and is overexpressed in CRC tumours (59, 62, 63, 64) representing a potential biomarker. The second objective of this study was to characterise the expression levels of CD147 in the tumours of stage II colon cancer patients to begin to understand its prognostic potential in this population.

The current finding is that CD147 is overexpressed in 60% of stage II tumours compared to normal mucosa tissue with the location predominantly membranous. This is consistent with many publications, whereby CD147 in tumours, in populations consisting of all TNM stages, is overexpressed compared to normal adjacent mucosa (59, 62, 63, 64). While majority of these studies do not report CD147 expression in stage II patients separately, the two studies that have,

report CD147 is overexpressed in approximately 50-76% of stage II patients (63, 64). This is in line with the current finding.

Although the number of patients in the present was too small to compare CD147 status and pathological factors, previous studies have detected significant associations in support of the prognostic significance of CD147 in CRC. Associations between extramural vascular or lymphatic invasion, high grade tumours, lymph node positivity and distant metastasis in populations of all TNM stages have been detected (62, 63). Further, CD147 has been shown to be an independent prognostic factor for DFS (62, 63). While other studies have not been able to replicate this (59, 64) a meta-analysis revealed a significant association between CD147 expression and OS (58), although this was not independent of other prognostic factors. Taken together, this at least suggests CD147 is a potential risk factor in CRC patients that needs more investigation.

4.3.2 PREVIOUS FINDINGS OF CD147 IN STAGE II CRC

Existing evidence from previous studies that specifically assess the prognostic significance of CD147 in stage II CRC patients is more contradictory. While Stenzinger and colleagues (2011) found a significant difference between stage II CRC patients with high and low CD147 expression in terms of cumulative survival, Boye and colleagues (2012) failed to find any prognostic impact in stage II patients (63, 64). No apparent differences in sample size, follow-up time or methodologies were observed between these two studies however, rates of chemotherapy were not reported. Therefore, the prognostic significance of this marker in stage II colon cancer patients is unclear. However, the consistent findings that CD147 overexpression is associated with lymph node positivity, distant metastasis, metastasis-free survival and OS in populations consisting of all TNM stages suggest CD147 could still be useful in earlier CRC patients (62, 63).

4.3.3 FUNCTIONAL RELEVANCE OF CD147 TO STAGE II COLON CANCER

Evidence from preclinical studies suggest CD147 may be implicated in the recurrence of cancer supporting the potential role of CD147 as a prognostic biomarker in stage II colon cancer patients.

CD147 is directly involved in metastasis through its regulatory activity of matrix metalloproteinases (MMPs) (57). CD147 expression on the cancer cell surface stimulates the secretion of MMPs from cancer cells themselves and the surrounding stroma (57). Subsequent MMP-mediated degradation of the ECM alters its biochemical composition, promoting anti-apoptosis and removes the physical barrier promoting tumour growth, angiogenesis and importantly, invasion and metastasis. Considering CD147 is likely an important initiator of the metastatic cascade in a clinical context, areas of the tumour with particularly high CD147 expression could allow the development of micrometastasis (57). In stage II colon cancer patients with high CD147 expression, this would warrant the administration of adjuvant chemotherapy.

While the role of adjuvant chemotherapy is to eradicate micrometastatic disease after the removal of the primary tumour in stage II patients, (34) preclinical evidence also suggests CD147 may be involved in chemotherapy resistance. Xu *et al* (2014) and Peng *et al* (2015) revealed high CD147 expression decreased the sensitivity of CRC cells to 5-FU and Oxaliplatin respectively, which are two major chemotherapeutic regimens in stage II colon cancer (95, 96). While the exact mechanism for CD147 mediated drug resistance is unclear, the influence of CD147 on receptor tyrosine kinase's, ABC transporters and Monocarboxylate transporters is likely to blame (97). As of yet, no clinical associations have been made in CRC patients although, associations between CD147 and chemoresistance in bladder, ovarian and lung cancer have been detected (98, 99, 100). Taken together, CD147 could be a potential contributor to recurrence after surgery in stage II colon cancer patients, despite receiving adjuvant chemotherapy.

4.3.4 STRENGTHS AND LIMITATIONS OF THE CURRENT STUDY

One aspect of the current study that potentially limits the comparability of this study to previous studies was the inability to develop an immunoreactivity score (IRS). The first objective of this study was to establish experiment techniques for the detection of CD147 in tumour tissue. However, one issue we came across was the poor representation of dense tumour tissue as 3mm cores within

TMAs. Within these cores, only small areas of dense tumour tissue was present. While the intensity of staining was consistent across the sample area the extension or frequency of positively stained cells could not be accurately determined. Therefore, a semi-quantitative IRS, which combines both the intensity and extension of staining, could not be developed.

The extension of staining is an important parameter particularly concerning the biological heterogeneity of tumours (101). Further, considering the metastatic potential of CD147-expressing cancer cells (58), high overexpression in one area of the tumour may be more of a risk factor than moderate overexpression across the entire sample. For this reason, an IRS is the standard methodological approach for semi-quantification of CD147 (59, 62, 63, 64).

Despite this limitation, one potential advantage of our approach was the use of the intensity ratio between matched tumour and normal mucosa as opposed to a grouped comparison of all tumours compared to normal mucosa tissues. The standard approach for determining the overexpression CD147 in tumours is by calculating the IRS for each tumour and normal mucosa sample. A cut-off IRS value is then determined to group samples into positive and negative staining groups (59, 62, 63, 64). However, this does not consider normal mucosa with moderate amounts of staining relative to adjacent tumour tissue. Therefore, we believe the ratio of intensity between non-malignant and malignant tissues serves as a more informative clinical marker to determine CD147 positive tumours. This also allows for a within-subject comparison which is more rigorous than a group analysis. This statement is agreement with a previous study that also observed considerable staining in the normal tissues of CRC patients (102).

4.3.5 CONCLUSION

To summarise, this study found that CD147 is overexpressed in 60% of stage II colon tumours which is consistent with previous studies. A caveat of this work is the positivity rate is purely from qualitative assessment of staining intensity. In terms of the potential for CD147 as a biomarker in stage II colon cancer patients, associations between CD147 and pathological factors in previous

studies support this role. Further, the functional role of CD147 in metastasis, recurrence and chemoresistance in preclinical studies suggests this marker may be relevant to stage II colon cancer patients. Overexpression of CD147 may highlight patients at a higher risk of developing recurrence and those less likely to respond to adjuvant chemotherapy. While the current and previous findings are promising, further research is required to determine the clinical relevance of this biomarker in these patients.

4.4 TUMOUR MIR-21 EXPRESSION AS A PROGNOSTIC AND PREDICTIVE BIOMARKER IN STAGE II COLON CANCER

4.4.1 CURRENT AND PREVIOUS FINDINGS

miR-21 is the most consistently cited oncomiRNA (50). miR-21 is upregulated in a number of diverse human cancer types including CRC (103) and is a potential biomarker for stage II colon cancer. To achieve the second objective, which was to characterise the expression of miR-21 in the tumours of stage II colon cancer patients, we performed RT-qPCR. We found a trend towards overexpression in the tumours compared to the normal mucosa. A positivity rate (number of patients with a relative expression >1) of 50% was observed. Positive relative expression ranged between 1.61 to 3.5. Strikingly, 2 of these patients had more than a 2-fold increase in relative expression. Moreover, the considerable variation in the relative expression levels of the tumours compared to the normal mucosa tissues suggests that it's part of the molecular heterogeneity we see in the tumours of these patients and could be reflective of a molecular subtype (104).

Interestingly, three distinct populations can be seen, those with high overexpression of miR-21 (>3-fold), moderate overexpression (>1.5-fold) and those with expression equivalent to adjacent normal mucosa tissue. In a future, larger cohort if this trend held true and a multimodal distribution was observed it would be interesting to evaluate the prognostic significance of these three patient groups.

To explore the potential prognostic significance of this overexpression, we compared miR-21 levels with pathological risk factors. Unexpectedly, a trend towards statistical significance was observed between T stage and miR-21 relative expression. Patients with a high T stage (T4a/b) tended to have a lower tumour relative expression of miR-21 compared to patients with T3 tumours. However, it must be noted that only 3 patients had T4a/b tumours. Further, no other trends or associations were detected between miR-21 levels and lymphatic invasion or tumour grade. Overall, the true prognostic significance of miR-21 in stage II colon cancer patients cannot be derived from this data largely due to a small sample size.

In comparison to previous findings a significant overexpression of miR-21 in CRC tumours has consistently been described in several small clinical studies across different populations as well as some larger scale ones (42, 46, 52, 105,). These studies have additionally found associations with lymph node positivity, development of distant metastasis or advanced TNM stage and poorly differentiated tumours, strengthening the potential for miR-21 as a biomarker in this cancer type. Subsequently, several studies have found associations with OS and DFS (41, 46, 52) in populations consisting of all TNM stages. In general, a stepwise increase has been observed from adenomas to advanced TNM stage suggesting patients with high miR-21 levels at an early stage are more likely to progress to later stages of the disease (46). In other words, elevated miR-21 is a potential risk factor for stage II colon cancer patients.

4.4.2 PREVIOUS FINDINGS OF MIR-21 IN STAGE II CRC

Only a few studies have specifically analysed miR-21 expression levels in stage II CRC patients. However, all of these studies have revealed a significant overexpression of miR-21 in tumours compared to normal mucosa tissues, consistent with the current findings (46, 106, 107, 108). Of these, only two studies have reported the positivity rate of miR-21 in stage II CRC tumours. Shibuya *et al* (2010) reported 15.8% as the positivity rate in Dukes stage A, B (TNM stage I and II). While this seems relatively low, the stepwise increase in miR-21 levels suggests a slightly higher rate would be

expected for Dukes stage B alone, which is equivalent to TNM stage II. Kang *et al* (2015) reported 28.3% of stage IIA/B patients had high miR-21 levels which is more consistent with our findings. Of note however, is the exclusion of T4b tumours. Others have reported an association between elevated miR-21 levels and advanced T stage (42, 46, 52), suggesting the positivity rate is likely more than 28.3%. Further, a lack of transparency regarding cut-off values to determine positivity rates reduces the comparability of these studies to the current one.

Associations between miR-21 levels and pathological factors have further been detected in stage II CRC patients. A study in 764 stage II colon cancer patients found high miR-21 levels were associated with decreasing recurrence-free cancer-specific survival independent of clinicopathological risk-factors (108). In agreement with this was a study by Nielson *et al* (2011) and Kang *et al* (2015) that found high miR-21 levels in stage II colon cancer patients correlated with DFS. Currently, Nielson *et al* are the only authors to find a significant association with OS while others have failed to validate this likely due to insufficient power. Taken together, previous findings support the notion that elevated miR-21 is a potential risk factor for the population of interest.

4.4.3 FUNCTIONAL RELEVANCE OF MIR-21 TO STAGE II COLON CANCER

Preclinical studies implicate miR-21 in aspects of initiation, progression and chemoresistance in cancer. These findings suggest miR-21 is likely implicated in recurrence of disease which is of particular relevance to stage II colon cancer patients and supports clinical findings that elevated miR-21 is a risk factor for these patients.

miR-21 is best characterised as a proinflammatory marker in cancer (38). As such it has been implicated in an NF-kB inflammatory positive feedback loop that initiates an epigenetic switch from non-transformed cells to stable cancer cells (47). A core target of miR-21 in this loop is the tumour suppressor *PTEN* and an inverse correlation has also been detected in CRC clinical samples between these two molecules (48). The consistency of this positive feedback loop (and other inflammatory mechanisms) may also lead to a progressive increase in COX-2, which indirectly increases miR-21 to

progressively decrease its direct target, PDCD4, promoting the tumour to more malignant states (109). Again, an inverse relationship between miR-21, COX-2 and PDCD4 has been detected in human CRC tissues (109). Strikingly, aspirin use is associated with reduced recurrence and distant metastasis in sporadic CRC (110). The main action of aspirin is to target COX-2 (109). Taken together, this implicates miR-21 as being functionally relevant in early colon cancer and promoting the progression and recurrence of cancer in these patients.

Considering the number of gene targets miR-21 has (both known and unknown), the ability for miR-21 to promote chemoresistance is unsurprising (50). As such, evidence from cell culture and xenografts studies suggest miR-21 may also be involved in 5-FU resistance in CRC (49). Valeri and colleagues (2010) demonstrated the ability for miR-21 to downregulate MSH2 and MSH6, which make up the core MMR recognition protein complex. CRC cells and xenografts overexpressing miR-21 reduced 5-FU-induced G2/M damage arrest and subsequent apoptosis. A comparable response was also seen in dMMR cell lines (49). Considering MSI is a predictive biomarker to adjuvant chemotherapy (33), miR-21 could also be used to predict those patients with MSS tumours that won't respond to chemotherapeutic regimens such as 5-FU.

4.4.4 CONCLUSION

Overall, our findings support those of previous clinical studies that miR-21 is upregulated in stage II colon cancer patients. A strong trend towards overexpression was detected in this population and while the sample size was small, due to the high level of precision RT-qPCR provides us with we believe a significant association would be detected in a larger cohort. Our data also highlight distinct subsets of populations that could be used to stratify patients in future, larger cohorts. Evidence from preclinical and clinical studies also support the notion that miR-21 is a potential risk factor for stage II patients however, more research is required for conclusive evidence.

4.5 TUMOUR MIR-29A EXPRESSION AS A PROGNOSTIC BIOMARKER IN STAGE II COLON CANCER

4.5.1 CURRENT AND PREVIOUS FINDINGS

miR-29a is known to be downregulated in most cancer types and has been associated with tumour-suppressing roles (114). Conversely, it is thought to be overexpressed in CRC, (44, 45, 53) making miR-29a a particularly attractive biomarker because of its potential specificity to CRC. Like miR-21, miR-29a was characterised in tumours of the same patient cohort using RT-qPCR to achieve our second objective.

In the current study we found no significant association between tumour and normal mucosa relative expression levels. Although our sample size was small, only 1 patient exhibited a significant overexpression of this marker (>1.5-fold increase). Instead a small trend towards downregulation was observed in the remaining 9 patients. Further, the remaining 9 patient's tumour levels clustered together, analogous to the normal mucosa levels. This suggests that in early-stage colon cancer, unlike miR-21, miR-29a is not profoundly dysregulated in most patients and may not be a strong risk factor for this population. Our results show there is evidence of heterogeneity of expression of this miRNA which in a larger cohort of patients could potentially represent a subset of overexpressing patients.

Conversely, a previous report of miR-29a levels in patients of all TNM stages revealed a significant upregulation compared to normal mucosa (44), in agreement with another study that found miR29a to be significantly higher in the tumour tissue of metastatic CRC patients compared to non-metastatic CRC patients (111). Despite this, no other studies have found associations with T stage, TNM stage or other clinicopathological risk factors therefore, further research is warranted to confirm the association between elevated miR-29a levels and poor patient outcomes in CRC.

4.5.2 PREVIOUS FINDINGS OF MIR-29A IN STAGE II CRC

The only previous study to report miR-29a expression levels in stage II patients was by Weissmann-brenner *et al* (2012) where patients were stratified into two groups; those with a poor prognosis and those with a good prognosis based on the present or absence of recurrence (112). Patients with a good prognosis had a small but significant increase in miR-29a levels in the tumour compared to patients with a poor prognosis. However, miR-29a levels were not normalised to adjacent normal mucosa, a limitation of the study. Therefore, it is not known whether "high" levels of miR-29a in this study are actually overexpressed compared to normal mucosa. Perhaps downregulation of miR-29 is associated with a higher risk of recurrence as opposed to an upregulation being associated with a lower risk of recurrence.

If a downregulation is associated with a poor prognosis, the trend towards downregulation in our study was particularly small and we do not believe this would have a significant impact on patient outcomes in a larger, future cohort of stage II colon cancer patients. Based on previous findings, we believe the upregulation of this marker to be more compelling. Further, a study in stage III CRC patients found miR-29a to be significantly upregulated in tumour compared to normal mucosa (45). This is interesting considering a small subset of stage II colon cancer patients are known to have survival rates approximating stage III (13).

It cannot be concluded from this study whether the patient with elevated miR-29a is simply an outlier or is a true representation of this population due to the sample size. However, clustering of the remaining patients with a trend towards downregulation of miR-29a accentuates the patient with elevated miR-29a. Analogous to miR-21, in a future, larger cohort if a bimodal distribution was observed, it would be interesting to evaluate the prognostic difference between these two patient groups.

4.5.3 FUNCTIONALITY OF MIR-29A

What may explain discrepancies in the literature is for the ability for miR-29a to both promote and suppress tumourigenesis. The dual mechanisms of miR-29a is a theme found in many miRNAs which is unsurprising given the large number of genes influenced by a single miRNA species (113, 39). Specifically, in CRC cell lines and mouse models, miR-29a has been shown to target *KLF4*, promoting cell invasion by inhibition of E-cadherin expression (38). An inverse correlation was observed between miR-29a and *KLF4* in clinical CRC samples and both a high level of miR-29a and low level of *KLF4* was associated with distant metastasis and poor prognosis (44). Conversely, in breast cancers, an overexpression of miR-29a was significantly associated with slower growth of breast cancer cells (114). Evidence from breast cancer cell lines suggest this association may be through down-regulation of B-Myb, a regulator of cell proliferation and survival (114). Similarly, in gastric cancer, miR-29a inhibited cell proliferation and wound healing by downregulation of several cyclin-dependent kinases (115).

The role of miR-29a in CRC and whether it has a dual role in CRC has not been sufficiently described. Therefore, it cannot be concluded whether the up- and/or down-regulation of miR-29a in stage II colon cancer patients is directly associated with tumourigenesis. It has also been suggested for microRNAs with a dual role in cancers, that it is important to determine whether their net effect is oncogenic or tumour suppressive and even then, their use as biomarkers or drug targets should be cautioned (39).

4.5.4 CONCLUSION

In our small cohort of patients, it appears that miR-29a is not considerably dysregulated in this population. However, based on a previous report (44), the elevated levels of miR-29a in a single patient may be representative of a small subset of stage II colon cancer patients. Combined with a small amount of preclinical evidence, this overexpression may be functionally relevant in this patient group although, the potential duplicity of this marker needs to be determined. Taken together,

further research in a larger cohort is required to confirm a potential bimodal distribution in stage II colon cancer patients.

4.6 STRENGTHS AND LIMITATIONS OF RT-QPCR FOR THE DETECTION OF MIRNAS

A notable difference in methodologies of the current study compared to many previous studies is the use of *in situ* hybridisation (ISH) for the evaluation of miR-21 levels (46, 106, 107, 108). This limits the comparability of the present study to previous ones and highlights the advantages and disadvantages of using RT-qPCR. While RT-qPCR is a strong quantitative tool with a high level of precision and reproducibility (86), one major setback is the masking of non-cancerous tissue, such as normal mucosa, in the samples (108). Therefore, *in situ* hybridisation (ISH) using locked nucleic acid (LNA) modified by DNA probes has been employed by some researchers to semi-quantitatively measure the miR-21 signal in FFPE samples (107, 108). Of note, ISH has not been employed for the detection of miR-29a in CRC tissue likely due to the lack of publications in this area however, it has been employed in other cancer types (116). However, we believe it was appropriate to use RT-qPCR in the current study because samples were taken from central tumour and examination of H&E reference slides revealed no significant amount of non-tumour tissue.

One potential limitation however, is the inability to detect the localisation of the miRNAs. ISH allows the cellular and subcellular localisation (108) of miRNAs which could be more informative as opposed to an 'average' measure of all cell types within a tissue sample quantified by RT-qPCR.

In a clinical context ISH and RT-qPCR can both be performed on FFPE tissues to measure microRNAs and modified to be high-throughput, suggesting the cost and time-efficiency of these techniques would be similar in a clinical laboratory. Further, while ISH may be less reproducible, as it is susceptible to a certain level of observer bias, the ability to localise the mi-21 and miR-29a signal may be a more powerful prognostic measure compared to RT-qPCR. Ultimately, whether RT-qPCR or ISH is more clinically feasible and has more prognostic potential cannot be determined until both are compared across the same samples.

4.7 OVERALL SIGNIFICANCE OF TUMOUR MARKERS

4.7.1 SIGNIFICANCE OF CD147, MIR-21, MIR-29A AS PROGNOSTIC BIOMARKER IN STAGE II COLON CANCER PATIENTS

CD147 and miR-21 are relevant prognostic biomarkers for stage II colon cancer patients for several reasons. Firstly, data generated from the current study show both of these markers are considerably dysregulated in this population, with at least 50% of patients exhibiting an elevated expression of either marker. This implies CD147 and miR-21 may have functional relevance in this population. Conversely, miR-29a was only significantly upregulated in 1 stage II patient and overall, no substantial dysregulation was observed. Secondly, the stepwise increase in expression levels of these markers from early to late stage CRC, reported in the literature, suggests an elevated expression in stage II patients is a potential risk factor (46). Lastly, combined with accumulating evidence for independent associations between CD147, miR-21 and lymph node positivity, distant metastasis and DFS described above, the data presented here support the notion that the elevation of these markers are directly involved in disease advancement in stage II colon cancer patients.

Data from preclinical studies exploring the potential functionality of these markers further support their direct involvement in disease progression in stage II colon cancer patients. As suggested, functional biomarkers, such as MSI status, which has a known causal role in the behaviour of the tumour (36), allow clinicians to strategically target specific patient groups. As such, the direct role of CD147 and miR-21 in promotion of inflammation, invasion and metastasis implicates them in disease recurrence making them more valuable (38, 57). On the other hand, miR-29a appears to be both oncogenic and tumour suppressive (113) and be both upregulated and downregulated in CRC. This ambiguity suggests using miR-29a as a prognostic biomarker in stage II patients should proceed with caution.

The relevance of CD147 and miR-21 extends to their ability to detect specific subsets of stage II patients. For example, the combination of CD147 and miR-21 revealed a single patient with elevated expression of both markers. Evaluating the prognosis of patients with these characteristics, compared to patients with overexpression of only one marker, would be of interest. Further, the functional roles of these markers in chemoresistance (49, 95, 96) suggest they could be predictive of outcomes to treatment for some patients. It could be hypothesised that miR-21 may detect patients with MSS tumours that will not respond to chemotherapy. Ultimately, these markers could detect stage II patients at a higher-risk of recurrence who will also receive no benefit from adjuvant chemotherapy, reducing the mortality and morbidity associated with the overuse of chemotherapy.

Several promising biomarkers have emerged in CRC however, many have not made it to the clinic because they do not comply with daily practice. Conversely, the evaluation of dMMR has proven clinically feasible because the detection process is time and cost efficient. Importantly, CD147 represents a clinically feasible biomarker because it can be similarly evaluated by IHC in FFPE samples. RT-qPCR is not yet routine in our hospital laboratories but can be performed in FFPE tissue and technological developments mean it might not be far away.

4.8 CD147 AND MIR-21 IN THE LYMPH NODES OF STAGE II COLON CANCER

The third objective of this study was to explore the expression levels of CD147 and miR-21 in the TDLNs of stage II colon cancer patients. This was under the hypothesis that tumour-associated expression of these markers might be present in the lymph nodes and could be indicators of pre-metastatic spread. This is because miR-21 and CD147 have been characterised as blood-based tEV markers (56, 83) and tEVs are thought to be involved in the formation of the PMN in TDLNs (66). To the author's knowledge, no other study has attempted to detect tEVs in human lymph node samples. While detecting tEVs in the lymph nodes of patients is technically difficult, this study highlights the difficulty of using potential surrogate markers for tEV detection in the lymph nodes.

As discovered, CD147 is expressed in the GCs of intestinal lymph nodes of non-cancer patient and the TDLNs of stage II colon cancer patients. However, this is likely a general process of the immune response that is not cancer associated. Data from The Human Protein Atlas originally lead us to believe CD147 is not expressed in unstimulated or stimulated (formation of GCs) (90) normal lymph nodes (unknown patient origin) and therefore could be a potential tEV marker. Conversely, the only other publication to demonstrate CD147 staining in normal lymph nodes found significant staining of GC B cells with weak to negative staining in follicle mantle zone and T cell zones (92).

While it is not possible to tell whether the lymph nodes examined by Schmidt *et al* are non-cancer, healthy controls, our results suggest CD147 GC staining is not specific to cancer patients and is a general process of the immune response. This is in agreement with the BD Biosciences Human CD Marker Chart (91), where CD147 is expressed on human B cells. Put simply, CD147 cannot be used as a biomarker to detect tumour-associated expression or as a surrogate biomarker for tEVs in the lymph nodes of stage II colon cancer patients. Perhaps, it is more valuable to measure tEVs in the circulation of patients as this approach is more feasible (56).

Interestingly, miR-21 may be more of a contender for the purpose of detecting tumour-associated expression of this marker. A key finding was the notably high expression of miR-21 in a small number of lymph nodes compared to control lymph nodes. These lymph nodes, which all came from different patients, demonstrated a 3-8-fold increase compared to control lymph nodes. Further, a greater variation in miR-21 levels was seen between lymph nodes of the same patient compared to the lymph nodes of the non-cancer patient. Importantly, previous studies investigating miR-21 in patients with B cell lymphomas have found miR-21 to be expressed at low levels in adjacent normal lymph nodes compared to B cell lymphoma tissue (117). Unfortunately, no other data can be found concerning the miR-21 expression levels of stimulated but healthy lymph nodes. Further, these findings are severely limited by the availability of control lymph nodes from a single patient.

Whether the findings that miR-21 is upregulated in some TDLNs is directly associated with the primary tumour, associated with a cancer-specific immune response or we have simply detected a non-specific immune response cannot be determined from this study. However, the association of miR-21 with blood-based tEVs (83) strengthens the hypothesis that upregulated levels in the lymph nodes of stage II colon cancer patients is cancer-specific.

4.9 HISTOLOGICAL MORPHOLOGY OF STAGE II COLON CANCER LYMPH NODES

4.9.1 INTRODUCTION

The anti-tumour response in TDLNs may be particularly relevant to stage II patients. In cancer, particularly CRC, the TDLNs either serve as effective barriers or facilitators of dissemination of the primary tumour (72). Lymph node metastasis is the most important prognostic factor differentiating stage II and III CRC patients (72). However, some stage II patients have survival rates approximating stage III disease suggesting this prognostic factor is limited (13). Considering uninvolved TDLNs are the primary site for tumour antigen presentation and T cell/B cell activation (118), it is likely the type and extent of the immune response is important in tumour evolution. The TDLNs can generate both an anti-tumour and immunosuppressive response (118) which could be responsible for heterogenous outcomes in these patients and be an important prognostic factor.

The most striking observation in the TDLNs of the stage II colon cancer patients, was the variation in number, size and shape of B cell primary follicles and GCs (secondary follicles). These spatially organised compartments, along with T cell zones and antigen-presenting cells, respond to the local tumour-associated immune signature within the draining lymph fluid (119). Depending on the signature, B cell and T cell compartments can expand or diminish and form the basis of the adaptive tumour-specific immune response (120). Specifically, the immunomodulating effects of B cell follicles and GCs change the composition of B memory and plasma cell populations that home back to the site of the tumour (120). While the effect of infiltrating immune cell populations has largely

focused on T cells, evidence is beginning to emerge for the importance of B cell mediated antitumour and protumourigenic effects (120). With a clinically feasible approach in mind, these B cell compartments were the most recognisable features and could be accurately quantified. Therefore, the focus of this project shifted to quantifying the histomorphological changes in distinct B cell compartments.

4.9.2 QUALITATIVE OBSERVATIONS IN STAGE II, III AND NON-CANCER TDLNS

Qualitative comparison of B cell compartments within the TDLNs of stage II patients, the stage II patient and non-cancer patients revealed several interesting points. Firstly, nearly all stage II lymph nodes were under immune stimulation, characterised by the presence and abundance of B cell follicles and GCs. In contrast, only 1 out of 20 of the lymph nodes of the stage III patient were activated with the majority infiltrated with cancer. Secondly, the number of B cell follicles per lymph node in the stimulated lymph nodes of the stage II patients and the stage III patient were substantially higher than the non-cancer patient. However, relative to the size of the lymph node, the stage II and III follicles tended to be smaller. Further, the stage II and III follicles appeared to be more non-circular in shape, which has previously been described as GCs tending to “fuse” (121), compared to the non-cancer follicles. Lastly, striking variations in terms of the number and size of B cell follicles and GCs, were also seen within the stage II patients.

Altogether, these qualitative observations suggest the B cell mediated immune potentiation within the TDLNs is specific and unique to stage II patients. This is in comparison to the stimulated lymph nodes of the non-cancer patient. The lack of lymph nodes that formed follicles and GCs in the stage III patient was likely to be a result of the infiltrating tumour. Further, the striking variation of the morphology of B cell compartments within the stage II patients support the idea for this being a potentially relevant prognostic factor in these patients.

4.9.3 COMPARISONS BETWEEN THE NUMBER AND SIZE OF B CELL COMPARTMENTS AND PATHOLOGICAL RISK FACTORS IN THE TDLNS OF STAGE II PATIENTS

The number and size of B cell compartments in the TDLNs in stage II patients was associated with the presence of lymphatic invasion, dMMR and the extent of tumour T stage. Firstly, patients with a lower T stage, i.e T3 compared to T4a or T4b, tended to have smaller B cell follicles and GCs. Further, patients with lymphatic invasion tended to have smaller GCs and follicles. A significant association was also found between the number of GCs per lymph node (GC density) and lymphatic invasion although no pattern was observed for the number of B cell follicles per lymph nodes (follicle density). Lastly, patients with dMMR, had a significantly lower total follicle and primary follicle density, while no trends were seen for GC density or size.

Despite this, no trends were observed between tumour grade and the number and size of B cell compartments. However, due to low patient numbers in the present study, patients with moderate tumour differentiation were considered high grade to allow comparisons between morphological features and tumour grade. Therefore, in a larger future cohort, stratification into all three groups: well, moderate and poorly differentiated tumours, may allow for more accurate associations to be seen.

4.9.4 PREVIOUS FINDINGS FOR MORPHOLOGICAL CHANGES AND PROGNOSIS IN LYMPH NODES

Morphological changes, including GC morphology, in the TDLN's of CRC patients have been observed as early as 1975 (123, 124). However, the majority of these earlier studies failed to find any significant associations with GC and follicle morphology and prognosis in CRC populations consisting of all TNM stages. Interestingly, the one study that investigated these aspects by stage found a significant association between Duke's stage B (stage II) patients with lymph nodes dominated by GCs and better survival (124). This association was not found for Duke's stage A or D (stage I and IV) suggesting specificity of this phenomena to stage II and III patients. However, the prognostic

significance of the immune-modulating effects on B cell compartments in the TDLNs has likely changed. Great advances in CRC surgical techniques and adjuvant therapy have been made resulting in a substantial increase in the survival rate in the last 20 years (124).

More recently, the current focus of CRC research in immunomodulating effects mainly addresses the role of the host immune response in the TME, such as infiltrating dendritic cells and T lymphocytes (120). The mechanisms behind morphological changes in the TDLNs- the primary site for T cell and B cell activation- is not well understood and has been poorly studied. Therefore, to our knowledge, there are no further publications investigating morphological changes in CRC. Recent insights into the lymph nodes of breast cancer and oral cancer patients however, may help us to understand how morphological patterns of B cell compartments are associated with prognosis.

The most recent findings come from Seidl *et al* (2018) (73) who investigated various TDLNs morphological changes of 206 stage I-III breast cancer patients. To our knowledge, this is the only publication to assess the association between B cell compartment morphology and pathological risk factors. Consistent with the current findings, they found GC density was significantly associated with advanced T stage. However, we could not replicate the association they detected between follicle density and T stage. Further, the size of the follicles and GCs was not reported by Seidl *et al*. While we could not detect any trends or associations with tumour grade, potentially due to low sample size, Seidl *et al* detected a significant association between a high tumour grade and an elevated follicle and GC density.

Beyond T stage and grade Seidl *et al* also explored associations between their morphological data and the molecular subtypes of breast cancer. These subtypes are based on hormone receptor and human epidermal growth factor 2 (HER2) status which are significantly associated with survival (141). The study demonstrated a significant association with the presence of oestrogen and/or progesterone receptors and lower GC and follicle density. Interestingly, both of these hormone receptors are associated with a relatively good prognosis (141). On the other hand, triple-negative

breast cancers (decrease in hormone receptors) were associated with a low density. This subtype is known to have the poorest prognosis.

Combined with the associations detected with T stage, N stage and grade, the data from Seidl *et al* support the notion that more aggressive, higher-risk breast cancer types are associated with a higher follicle and GC density. While this is consistent with the direction of association between T stage and follicle density in the current study, the rest of our findings with dMMR status and lymphatic invasion, do not support those of Seidl *et al*.

What could explain the discrepancies between these studies is the potential for lymph node morphological changes to be cancer type specific. In a study assessing the percentage of reactive follicles (equivalent to GC density) in the negative draining lymph nodes of non-metastatic oral cancer patients (stage I-III), a higher percentage of follicles was associated with a significantly better prognosis (74). Their logic behind this was the assumption that tumour-neoantigens predominately generate an anti-tumour response aimed to reduce the spread of the cancer. They also found, of all the histological features examined (LN area, capsule thickness, number of lobes, subcapsular/marginal sinuses and medullary sinuses, fibrotic and sinusoidal trabeculae), percentage of reactive follicles was the most predictive of time to death.

4.9.5 POTENTIAL FUNCTIONAL DRIVING FORCE OF HISTOMORPHOLOGICAL CHANGES IN LYMPH NODES

The exact mechanisms behind the spatial allocation and size of follicle and GC compartments and how this affects tumour immunology is not well understood. Only recently have B cells become appreciated as having an important role in tumour immunology (120). Before this, B cells were fairly out of focus and the prevailing notion was that T cells and innate immune cells primarily mediated antitumour immunity (125). The prognostic role of dense infiltration of CD8+ T cells in CRC is strong, particularly in stage I and II patients (126, 127) However, considering the lymph nodes are the

primary site for T cell activation (118), the involvement of a T cell-dependent B cell response in tumour immunology is likely.

Of particular relevance are the associations between low infiltration of cytotoxic CD8+ T cells and a medium to low grade tumour, presence of lymphatic invasion and MSS tumours (126, 128, 129). While the exact mechanisms behind these associations have not fully been elucidated, it is speculated in MSI patients, the ability for their tumours to present a large amount of mutation-generated neoantigens on the tumour cell surface stimulates T cell activity, ultimately leading to a TME rich in CD8+ T cells and T_h1 helper cells (Th1) (129). This suggests a more intense immune response in these patients is associated with a better prognosis.

In a B cell immunology context, the intense involvement of T lymphocytes suggest the B cell mediated immune response likely plays an important role in tumour evolution. Further, the association of altered T cell populations in the TME with different tumour pathological features is likely a result of altered T cell populations in the TDLNs. It is also known that specific CD4+ T cell populations are essential for the formation and maintenance of GCs as well as their output of memory cells and plasma cells (130). Taken together, it could be postulated that differences in tumour biology, with its association of altered T cell populations in the TDLN's, would also affect the spatial allocation and size of follicle and GC compartments in a T-cell B cell dependent manner in the TDLNs. As such, the output of these morphological changes in B cell compartments could be a shift in B cell populations in the TDLNs and therefore TME.

4.9.6 INFILTRATING B LYMPHOCYTES

In terms of infiltrating B lymphocytes in the TME, evidence from animal models suggest B cells have both protumourigenic and antitumour effects (120). B cells have been shown to promote carcinogenesis through providing an inflammatory TME and attenuate chemotherapeutic affects and an antitumour immune response (131, 132, 133, 134). Conversely, B cells can inhibit tumour growth and metastasis (135). The many processes of B cells are likely attributable to their multiple functions

including the ability to secrete antibodies, present antigen, induce cytotoxic killing and promote and regulate T cell responses (120).

Some evidence is beginning to emerge in CRC patients for the role of B cells in tumour biology (136, 137). For example, a dense infiltration of CD20+ B cells has been negatively associated with metastasis and positively associated with a lower T stage and an improved OS (136). An increase in a CD138+ subset has been associated with a lower grade and MSS tumours (136). Taken together, this suggests the humoral immune response is also important in tumour biology and progression. Data matching B cell populations in the TME and TDLNs is lacking. However, evidence is beginning to emerge in mouse models of CRC in terms of tumour progression and changes in B cell populations in the TME and TDLNs (138).

To briefly summarise, the role of T cell and B cell immunity in tumour biology and progression is inarguably important particularly in early stages of CRC. Thus, the primary site for T cell/B cell activation; the TDLN's, is responsible for driving many of these protumourigenic and antitumour processes. Importantly, it has been postulated that whether B cells promote or inhibit tumour growth likely depends on temporal and spatial setting and the composition of B-cell subsets in the TDLNs.

4.9.7 STRENGTHS AND LIMITATIONS

It was not part of the original aim of this study to examine histomorphological patterns in the TDLNs and we believe the use of TMAs for this purpose is a potential limitation for the data analysis. It is not possible to determine whether 3mm diameter samples of each lymph node is truly representative of the donor tissue in terms of the number and size of follicles and GCs.

We also have concerns with the approach of measuring the size of follicles and GCs. We simply measured the diameter of these compartments on digitalised slides. Whether or not this represents the volume of these compartments is unknown. We believe it would important to involve a trained pathologist in any future research in this area.

4.9.8 CONCLUSION

The data generated from the current study show the morphological patterns in B cell compartments of the TDLNs in stage II colon cancer patients have potential prognostic significance. While this area of our research is still well within the discovery phase, we have demonstrated how this potential biomarker can be detected and measured in a simple and clinically feasible approach. Further, qualitative assessments between non-cancer and stage II lymph nodes highlight the relevance of compartment morphology to stage II patients. Previous findings in breast and oral cancer and our general knowledge of cancer-immune interactions also support the potential prognostic role of compartment morphology in these patients. Again, due to our low sample size, specific associations with pathological risk factors warrant validation in a larger cohort. However, these associations raise interesting questions around the mechanisms behind the spatial allocation and size of B cell compartments and their outputs.

4.10 SIGNIFICANCE OF FINDINGS

The present study is the first in over 40 years to investigate morphological changes in B cell compartments of the TDLNs in colon cancer patients. Further, it is the first study to consider associations between these morphological changes and important pathological features including lymphatic invasion, dMMR, T stage and tumour grade. While these morphological changes as potential prognostic biomarkers are still well within the discovery phase, they are significant to this field of research for several reasons; Firstly, the TDLNs and the subsequent immune response is important and specifically relevant to early-stage colon cancer patients. Secondly, these morphological changes potentially carry information from both a B and T cell immunological perspective that could be more informative than standardised immunoscores in CRC. Lastly, this potential biomarker represents a simple, clinically feasible approach.

The most recent advancement in the prognostication of non-metastatic CRC has been the development and standardisation of an immunoscore that combines the measure of CD3+ and CD8+ T cell populations in the centre and invasive margin of the primary tumour (139). The authors demonstrated the striking prognostic significance of this score with a multivariable-adjusted HR for time to recurrence of 0.40 (95% CI 0.30-0.54) for high versus low immunoscores. Importantly, this was independent of T staging, lymph node positivity, lymphatic invasion, tumour grade and MSI status. However, this immunoscore does not necessarily encompass the complexity of immune-cancer interactions which can involve cells of the innate immune response as well as the underappreciated role of B cells, as previously discussed.

We believe the morphological patterns of B cell compartments within the TDLNs could add valuable information to the immunoscore. The TDLNs are the primary site for tumour antigen presentation and subsequent T cell/B cell activation (118). Therefore, the TDLNs are the primary source of infiltrating lymphocytes at the tumour site. Further, the morphology of B cell compartments potentially carries information that combines the complex interactions of B cell and T cell populations which could be more informative than measuring the densities of two single lymphocyte populations. Despite this, our argument lacks clinical evidence that B cell immunology has a significant role in the development of CRC. As previously mentioned, evidence is only beginning to emerge from preclinical studies, therefore a large amount of exploration and validation of our potential biomarker is warranted.

One important factor for implementation of biomarkers into clinical practice is proof of cost-effectiveness and feasibility within a clinical laboratory workflow. While the immunoscore has been standardised across different laboratories, demonstrating its cost-effectiveness and feasibility within clinical laboratories is another challenge. However, here we have demonstrated how this potential biomarker can be measured in a simple and feasible manner. Theoretically, GCs and follicles can be visualised using a simple H&E stain, without the need for antibodies as demonstrated by Seidl and

colleagues (73). Importantly, TDLNs are already observed in clinical laboratories for cancer spread to determine the TNM staging of patients. Therefore, the additional data analysis could easily be implicated in everyday practice.

4.11 OVERALL STRENGTHS AND LIMITATIONS OF THE STUDY

The overall aim of this pilot study was to characterise the expression of specific biomarkers within the tumours and TDLNs of a small population of stage II colon cancer patients. Using miR-21 as an example, a critical aspect of this characterisation was understanding the tumour overexpression of miR-21 compared to adjacent normal mucosa. However, a limitation of the study design was the small sample size and therefore lack of statistical power. Therefore, the positive and negative findings within this study are not conclusive and can only be used to form hypotheses to inform future, larger studies. However, to reiterate, it was not the primary goal of this study to perform formal statistical analysis. Instead our aim was to establish techniques for biomarker detection and begin to characterise the expression of these markers in tumour and lymph node tissue.

One important advantage of the present study was the access to fresh/frozen and formalin-fixed samples for majority of the patients recruited into the study. As mentioned, 3 out of 13 tumour and normal mucosa samples were not available due to their tumours being too small at the time of surgery. Despite this, the biobank has provided easy access to patient samples and a large amount of demographic and patient information stored within the RedCap database. Information, such as clinicopathological features, has been of high value within this study. Importantly, the value of the biobank in terms of patient numbers and the follow-up period of these patients is continuously increasing. Therefore, future, larger cohorts will be feasible within a short timeframe.

In terms of the methodology used in the study a notable issue we encountered was the use of the TMAs for the detection of CD147 in tumour tissue and evaluation of histomorphology in the TDLNs. While TMAs are the standard for validation of prognostic biomarkers (140), for the discovery phase

of biomarkers, it is unknown whether a 3mm cylinder is representative of the donor tissue. Specifically, we were not able to accurately determine the extension/frequency of CD147 staining in tumour tissues due to dominance of cores with non-tumour tissue. Further, it is unknown whether the true prognostic significance of the number and size of lymph node B cell compartment can be obtained from 3mm cores.

Similarly, while RT-qPCR has a high level of precision and reproducibility, whether it is the best suited methodology for the clinical detection and quantification of miRNAs is unknown. As mentioned, RT-qPCR values represent the 'average' expression level of miRNAs across tumour cells and cell and tissue types including normal colon tissue (108). Methods such as ISH may be more appropriate as it considers the cell and tissue types as well as the subcellular location of the miRNAs.

4.12 FUTURE DIRECTIONS

This pilot study characterised potential prognostic biomarkers in a small population of stage II colon cancer patients. However, internal validation of these markers is warranted in a larger population of stage II colon cancer patients. The data from this pilot study can be used to not only drive hypotheses, but inform the design of a future, larger cohort to evaluate the prognostic significance of miR-21, CD147 and lymph node histomorphological characteristics. For example, a sample size of 65 stage II colon cancer patients would be needed to test the following hypothesis: miR-21 relative expression levels are overexpressed in the tumours compared to paired normal mucosa tissue. This was calculated using a significance level of 0.05 and a power level of 0.9 and effect size calculated from the present study. To begin to assess the prognostication of these markers however, a bigger cohort would be required. The recurrence rate in stage II colon cancer patients is approximately only 20-25% (20). Therefore, aspects such as these need to be considered in the design of a future cohort (142).

The current study developed experimental techniques, of which some were primarily selected for their potential clinical utility, for the detection of specific biomarkers. However, as previously

mentioned, a limiting aspect of the detection of CD147 through IHC was the inability to accurately determine the extension of staining. Therefore, a critical next step would be to alleviate this problem by sampling the entire tissue as opposed to using TMAs. Some have suggested that full sections of the donor block should be used for accurate evaluation of staining frequency (140). A well-defined semiquantitative score, that combines both the intensity and frequency of staining, would not only enhance the clinical reproducibility of this technique but also partly address the issue of tumour heterogeneity (143).

A future, larger cohort would also be critical to determine IHC and histomorphological scoring systems with the most prognostic significance to standardise the quantification of biomarkers. Previous publications use a variety of ordinal scoring methods that combine the intensity and extension of CD147 staining in IHC (44, 63, 64). Therefore, an important future step would be to explore these different scoring approaches in the same cohort of samples to determine the approach with the most prognostic significance. In terms of lymph node histomorphological evaluation, the few publications in this area (73, 74), have provided a starting point for this pilot study. However, the novelty of this area warrants a significant amount of exploration of quantification approaches as well as the validity of using TMAs as previously mentioned.

For biomarkers such as lymph node histomorphology, still well within the discovery phase, understanding the mechanisms behind the spatial allocation and densities of B cell populations may help to drive hypotheses to inform evaluation and scoring approaches. This might involve staining TDLNs for B cell populations known to infiltrate the tumour such as, CD20+ and CD38+ B lymphocytes (136) and T lymphocyte populations such as CD8+ 126). A larger number of positive (stage III) and negative controls (non-cancer related) would add value to these experiments.

To address the limitations surrounding the use of RT-qPCR for the detection of miR-21, using ISH to detect miR-21 may be a future step. Some previous studies have employed ISH for the cellular and subcellular localisation of miR-21 but discrepancies exist likely due to insufficient validation of

probes (108). For example, miR-21 has been primarily detected in both stromal cells (108) and tumour cells (41). Future studies should develop and standardise ISH for the detection of miR-21 in stage II colon cancer patients. The development of this technique should determine the validity of RT-qPCR for prognostication or determine whether ISH is a more appropriate clinical test for this purpose.

Finally, independent clinical validation using standardised methodologies and data analysis in a different cohort of samples is paramount (142). This would determine the reproducibility of these assays. Reproducibility across different laboratories is also critical to demonstrate the robustness and clinical feasibility of assays. Differences in sample processing as well as interpretation of data analysis would be expected. This is particularly important for evaluation of IHC as an example, where the analysis is manual and subjective to visual assessment and therefore susceptible to a certain level of observer bias and variability.

4.13 CONCLUSION

The overall aim of this pilot study was to explore and characterise the expression levels of CD147, miR-21 and miR-29a in the tumours and TDLNs of stage II colon cancer patients. There is an apparent need for prognostic biomarkers in this clinically heterogeneous population to guide their treatment decisions. Our data show CD147 and miR-21 are considerably dysregulated in the tumours of these patients, while we did not see any substantial variation for miR-29a. In the TDLNs of these patients, miR-21 levels appeared to be dysregulated compared to lymph nodes of a non-cancer patient. Conversely, tumour-specific CD147 expression was not distinguishable from immunological expression demonstrating the difficulties of using surrogate tEV markers in these tissues. Instead, the search for clinically feasible biomarkers in the TDLNs of these patients led us to explore histomorphological changes in B cell compartments. We found a large variation in the number and size of these compartments within stage II patients suggesting their observed clinical heterogeneity could be, impart due to immunological differences. Altogether, our results demonstrate clinically

feasible approaches for the detection and discovery of biomarkers and will help guide future, larger studies to explore and validate their prognostic potential.

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