# Characterisation of MicroRNAs in Ulcerative Colitis and Colitis Associated Cancer For Use As Potential Biomarkers

**Thesis submitted in accordance with the requirements of the University of Leicester for the degree of Doctorate of Medicine**

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## Declaration

This thesis is being submitted in partial fulfilment for the degree of Doctorate of Medicine at the University of Leicester. This thesis is a product of my own work and investigation, carried out at the University of Leicester during the periods: October 2010 – October 2012 and October 2013 - October 2014.

All work is original except where otherwise stated and all other sources are acknowledged by explicit reference. None of the following work has been submitted for another degree at this University or elsewhere.

# Abstract

## Characterisation of MicroRNAs in Ulcerative Colitis and Colitis associated cancer for use as potential biomarkers

**Miss Maleene Patel**

### Introduction

Patients with Ulcerative Colitis (UC) are at increased risk of developing Colorectal Cancer termed Colitis Associated Cancer (CAC). They are monitored via a colonoscopy based screening programme. This is resource intensive, invasive, potentially harmful and can miss dysplastic precursor lesions. Accurate biomarkers are therefore required for surveillance purposes in order to prompt early surgical intervention. MicroRNAs (miRNAS) are short non-coding RNAs responsible for regulating genes at a post-transcriptional level that have shown the ability to act as diagnostic biomarkers.

### Aim

Conduct a feasibility study to profile microRNAs in the colonic mucosa and plasma of patients with Ulcerative Colitis and identify aberrantly expressed miRNAs related to disease progression.

### Methods

A retrospective cohort of patients with CAC was first characterised to ensure suitability for sequential biomarker discovery from archived colonic mucosal samples. High throughput miRNA microarray profiling was employed as a screening tool to identify differentially expressed miRNAs in a discovery cohort. MiRNAs of interest were subsequently validated using RT-qPCR in an independent cohort. The experiments were repeated using two cohorts of plasma samples collected prospectively from patients undergoing colonoscopic surveillance.

### Results

Within the colonic mucosa the expression of miR-21 and miR-135b was significantly increased in both dysplastic and CAC tissues when compared to normal and chronic UC tissue. MiR-18a and miR-31 appear to distinguish the CAC cases from normal. MiR-375 expression was significantly up-regulated in the circulation of patients with CAC. Colonic tissue and plasma profiles were found to differ.

### Conclusion

There is differential expression of miRNAs in both the colonic mucosa and plasma of patients with UC when compared to CAC, Combining different miRNAs increased their ability to differentiate between the disease states. MiRNAs show biomarker potential, and future potential screening blood test will likely be based on a panel of miRNAs. Further validation in larger cohorts is required.

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*“To know that we know what we know, and to know that we do not know what we do not know, that is true knowledge”*

**Nicolaus Copernicus (1473-1543)**

*“ The true method of knowledge is experience”*

**William Blake (1757-1827)**

*“Quality is never an accident; it is always the result of high intention, sincere effort, intelligent direction and skilful execution; it represents the wise choice of many alternatives.”*

**William A. Foster**

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## List of Abbreviations and Symbols

| | |
|---|---|
| ACF | Aberrant crypt foci |
| ALM | Adenoma like mass |
| AUC | Area under the curve |
| 5ASAs | Aminosalicylates |
| APC | Adenomatous Polyposis Coli |
| API5 | Apoptosis Inhibitor 5 (gene/ protein) |
| AV | Dr Ajay Verma (Specialist Doctor Gastroenterology) |
| BAX | Bcl-2-associated X protein |
| BCL-2 /Bcl-2 | B-cell lymphoma 2 (gene/protein) |
| BRAF | Serine/threonine-protein kinase B-Raf |
| BSG | British Society of Gastroenterology |
| CAC | Colitis associated cancer |
| CD | Crohns Disease |
| cDNA | Complementary deoxyribonucleic acid |
| CIMP | CpG Island methylator phenotype |
| CIN | Chromosomal instability |
| CK-1 | Casein kinase 1 |
| CMV | Cytomegalovirus |
| C-Myb | Cellular protein product of myleoblastosis oncogene |
| COX-2 | Cyclooxygenase-2 |
| CpG | Cytosine-phosphate-Guanine |
| CRC | Colorectal cancer |
| CRP | C reactive protein |
| CSK | C-src tyrosine kinases |
| C-src | Protein oncogene tyrosine-protein kinase (gene/protein) |
| CT | Cycle threshold |
| DALM | Dysplasia associated lesion-mass |
| DCC | Deleted in colon cancer |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphate |
| DPC4 | Deleted in pancreatic cancer 4 - tumour suppressor gene |
| dsRNA | Double stranded ribonucleic acid |

| | |
|---|---|
| EBV | Epstein Barr Virus |
| EDTA | Ethylenediaminetetraacetic acid |
| EGFR | Epidermal growth factor receptor |
| FAP | Familial adenomatosis polyposis |
| ffpe | Formalin fixed-paraffin-embedded |
| FOXO3 | Protein product of forkhead box O3 gene |
| G | Gravitational force |
| GSK-3β | Glycogen synthase kinase 3-beta |
| GTPase | Enzymes that hydrolyse guanosine triphosphate |
| H&E | Haematoxylin and eosin |
| HGD | High grade dysplasia |
| HIV | Human immunodeficiency virus |
| HNPCC | Hereditary non-poyposis colorectal cancer |
| IBD | Inflammatory bowel disease |
| IBDU | Inflammatory bowel disease unclassified |
| IBS | Irritable bowel syndrome |
| IC (IDC) | Indeterminate colitis |
| IL | Inter-leukin |
| IMS | Industrial methylated spirit |
| IND | Indeterminate for dysplasia |
| IRAS | Integrated research application system |
| IRS-1 | Insulin receptor substrate 1 |
| ISH | In situ hybridisation |
| KRAS | V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog |
| KW | Consultant Histopathologist Dr Kevin West |
| LOH | Loss of heterozygosity |
| LGD | Low Grade Dysplasia |
| LP | Linda Potter (Senior bio technician – Cancer Studies) |
| MALT | Mucosa associated lymphoid tissue |
| MAPK | EGFR-mitogen activated kinase |
| MCC | Mutated in colon cancer |
| MDM2/ mdm2 | Murine double minute 2 (gene/protein) |
| MEK2 | MAPK-ERK-2 |
| miR/miRNA | Micro Ribonucleic acid |

| | |
|---|---|
| MIP-2α | Macrophage inflammatory peptide-2-alpha |
| MgCl | Magnesium Chloride |
| MLH1 | MutL homolog 1 |
| MMR | Mis-Match Repair |
| mRNA | Messenger Ribonucleic acid |
| MSH2 | MutS homolog 2 |
| MSI | Microsatellite Instability |
| MSI-H | Microsatellite Instability-high |
| MSI-L | Microsatellite Instability-low |
| NFκB | Nuclear factor kappa B |
| NHS | National health system |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| NREC | National research ethics committee |
| p | Short arm of chromosome |
| p16 | Protein 16 |
| p53 (TP53) | Tumour protein 53 (gene/protein) |
| PCR | Polymerase chain reaction |
| Pri-miRNA | Primary transcript |
| Pdcd4 | programmed cell death gene 4 |
| PI3K | Phophatidylinositol 3-kinase |
| PK | Protein kinase |
| PSC | Primary sclerosing cholangitis |
| PTEN | Phosphatase and tensin homolog |
| q | Long arm of chromosome |
| qRT | Quantitative real time |
| PUMA | P53 up-regulated modulator of apoptosis |
| R&D | Research and development |
| RNA | Ribonucleic acid |
| RISC | RNA-inducing silencing complex |
| RhoB | Ras homolog gene family, member B (protein) |
| RONS | Reactive oxygen and nitrogen species |
| RPM | Revolutions per minute |
| RT | Reverse transcriptase |

| | |
|---|---|
| SCRC | Sporadic colorectal cancer |
| SDS | Sodium dodecyl sulphate |
| SMAD | Human homologs of Drosophila melanogaster Mad gene |
| SMAD4 | SMAD family member 4 |
| sno | Small nucleolar |
| SNOMED | Systemised nomenclature of medical (clinical terms) |
| SOP | Standard operating procedure |
| SNP | Single nucleotide polymorphrism |
| TA | Tubular adenoma |
| TEM | Transanal Endoscopic Muscosal (resection) |
| TGFβ | Transforming growth factor beta |
| TMN | The Tumour-Lymph Node-Metastasis Classification System |
| TNF | Tumour necrosis factor |
| TNFα | Tumour necrosis factor alpha |
| TRIS | Tris(hydroxymethyl)aminomethane |
| TVA | Tubulovillous Adenoma |
| UC | Ulcerative Colitis |
| UHL | University Hospitals of Leicester |
| UoL | University of Leicester |
| upH2O | Ultra-pure water |
| UTR | Untranslated region |
| WHO | World Health Organisation |
| WNT | Wingless signalling pathway |
| Wt | Wild-type |
| ∆ CT | Delta cycle threshold |
| ∆∆ CT | Delta delta cycle threshold |
| ng | Nanogram |
| µl | Microlitre |

# Chapter 1. Background To Project

## 1.1 Ulcerative Colitis and Colitis Associated Cancer

### 1.1.1 Introduction to Inflammatory Bowel Disease: The Clinical Problem

The term Inflammatory Bowel Disease (IBD) encompasses two major and distinct idiopathic entities known as Ulcerative Colitis (UC) and Crohn's Disease (CD). Both conditions are characterized by inflammation of the gastrointestinal tract and have a range of overlapping pathological features. In 10-15% of cases it is difficult to differentiate between the two resulting in a diagnosis of IBD type unclassified (IBDU) or indeterminate colitis (IC), as it has traditionally been known.

The precise aetiology of these diseases remains unclear but has been hypothesised to arise from a complex series of interactions between susceptibility genes, the environment and the immune system. Rare forms of microscopic IBD include the subsets collagenous colitis and lymphocytic colitis. Non-idiopathic forms of IBD result from radiation, drugs, ischemia, parasitic infection and intestinal resection with subsequent diversion. These will not be considered here.

Collectively UC and CD affect over 250,000 people in the United Kingdom (UK) (1). A recent systematic review estimated the incidence of UC varied from 0.6-20 people per 10,000 person years in Europe and North America with a worldwide prevalence of up to 500 people per 100,000 worldwide (2). Evidence suggests that these rates are increasing over time. UC is the most common type of IBD with an incidence of 10 per 100,00 and a prevalence of 243 per 100, 000 in the UK. Between 6,000 and 12,000 cases of UC are diagnosed every year. UC has no cure; without surgery, it remains a life long disease and thus poses a significant health problem and financial burden to the National Health Service (NHS). The cost to the NHS has been estimated at £720 million per annum with an average cost of £3,000 per patient per year. Furthermore due to its chronic nature, UC inflicts socioeconomic and psychological burden upon patients, impacting upon their quality of life (3-5).

### 1.1.2 Clinical Features and Treatment of Ulcerative Colitis

Ulcerative colitis most commonly starts in young adults, aged 20-35, with a second incidence peak in the seventh decade. The disease affects both sexes equally. The predominant symptoms of UC include bloody diarrhoea, abdominal pain and can be accompanied by systemic features such as weight loss, anaemia, pyrexia, and sepsis during an acute presentation. UC is also associated with a plethora of ocular, dermatological, rheumatological, hepatobiliary and vascular conditions. These extra-intestinal manifestations are not necessarily related to disease activity.

The extent of disease in UC can vary significantly between patients and may be described using the Montreal Classification (Table 1).

**Table 1. Montreal Classification of Distribution of Ulcerative Colitis**

| Term | Distribution | Description |
|---|---|---|
| E1 | Proctitis | Limited to the rectum alone |
| E2 | Left Sided Colitis | Limited to the rectum, sigmoid and descending colon |
| E3 | Extensive Colitis | Colitis beyond the Splenic flexure (pancolitis) |

Adapted from report of a Working Party of the 2005 Montreal World Congress of Gastroenterology (6)

Pancolitis normally terminates at the ileocecal junction; however a minority of patients will also exhibit a small degree of continuous ileal inflammation termed backwash ileitis. Severity of disease in UC also differs markedly between patients. Over ten different disease activity indices exist which attempt to objectively quantify disease activity based on clinical, endoscopic and quality of life parameters. A common scoring system is the Truelove and Witt's criteria (7) or the modified version, which categorises episodes as mild, moderate and severe. Disease remission is defined as complete resolution of symptoms and mucosal healing (8). Overall it can be said that the clinical course of disease activity in UC follows a complex relapsing and remitting pattern. Patients may experience bouts of acute symptoms with complete resolution between episodes. A small proportion will present with continuous unremitting disease. It is important to establish both extent and severity of disease to chose the optimum treatment modality and determine subsequent follow-up and surveillance intervals (9). The mainstay of treatment focuses upon inducing and

maintaining disease remission by using a variety of pharmacological agents (10-12). Classes of drugs used in the management of UC include, 5-aminosalicylates (5ASAs), corticosteroids, immunosuppressant's and biological therapies. Surgical treatment is reserved for those patients who develop an acute complication (colonic perforation or toxic megacolon) or fail to respond to medical treatment. In these cases the choice of bowel resection is a total colectomy (resection of the large bowel only). In patients who have developed complication secondary to chronic UC such as dysplasia or malignancy the operation of choice is a panproctocolectomy (resection of large bowel and rectum) (13-15).

### 1.1.3 Histopathological features of Ulcerative Colitis

Histology plays an important part in the diagnosis and management of IBD. An in-depth review can be found in the British Society of Gastroenterology biopsy reporting guidelines (16). In summary, there are a number of microscopic and macroscopic histopathological features that distinguish UC from CD. The histological changes are often graded and used as a tool to assess the severity of the disease.

#### Macroscopic Features

The sites of inflammation are different in UC and CD. Crohns Disease may affect any portion of the small or large bowel and is characterised by discontinuous transmural inflammation (skip lesions). In contrast UC related disease is limited to the colon and rectum and is frequently characterised by contiguous mucosal inflammation of the colon, starting at the rectum and progressing proximally for variable distances. A hallmark feature of CD is non-caseating granulomas; which are always absent in UC. Another classical gross feature of UC is mucosal ulceration with subsequent mucosal bridging. Following a degree of healing and according to disease severity, multiple filiform inflammatory pseudopoylps may develop. Furthermore healing may occur in an irregular manner, giving rise to a heterogeneous mucosal appearance not dissimilar to CD, this can be the case following treatment.

During an acute episode of UC, mucosal haemorrhage, erythema, friability and granularity will be endoscopically visible.

**Microscopic Features**

Microscopic features of UC are that of structural mucosal damage and can be described in terms of architectural, inflammatory or generalised changes. Architectural disturbances include irregular mucosal surface, crypt deformity (shortening and budding) accompanied by a decrease in crypt number. Inflammation of crypts (cryptitis) can also lead to the presence of crypt abscesses. A substantial amount of inflammatory infiltrate is seen throughout the lamina propria accompanied by a focal then diffuse increase in plasma cells around the crypts (basal plasmacytosis). During severe disease neutrophils will be seen in the epithelium. Mucin depletion and paneth cell metaplasia is also seen during active disease.

**Histological Changes in Chronic Disease and Following Treatment**

It is important to note that the appearances can vary according to disease duration, activity and reversal of some changes in response to treatment is well documented. In chronic or quiescent disease crypt disruption is more apparent and lymphoid follicles and aggregates can be found in the deeper aspects of the mucosa. The inflammatory infiltrate also changes composition as the disease progresses to include more neutrophilic and eosinophilic cells.

### 1.1.4 Ulcerative Colitis and Colitis Associated Cancer

UC is a well-established risk factor for Colorectal Cancer (CRC) also known as colitis associated cancer (CAC) (17-21). Inflammatory bowel disease ranks as the third highest risk for development of CRC, following familial polyposis coli (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). This risk was first acknowledged in 1925 by Crohn and Rosenberg and has been confirmed by a large number of studies since.

A landmark meta analysis study by Eaden et al in 2001 (18) estimated the cumulative risk for any patient with UC to be 2% at 10 years, 8% at 20 years and 18% at 30 years, following the onset of symptoms. Further population studies thereafter have reported different risk rates and some studies even suggest that the overall risk of CAC maybe declining (22-27). A Copenhagen cohort study looking at the risk of CRC in UC also found low rates of 0.4% at 10 years, 1.1% at 20 years and 2.1% at 30 years (28). A 30-year Hungarian study reported the cumulative risk of CRC to be 0.6% after 10 years, 5.4% at 20 years and 7.5% at 30 years, with an overall CAC annual incidence of 0.15% (29). Another 30-year prospective study from St Marks Hospital, UK reported their cumulative incidence as 2.5% at 20 years, 7.6% at 30 years and 10.8% after 40 years (21). Several theories attribute this decreasing rate to regular use of maintenance therapies and improved surveillance programs.

A recent Korean study showed higher incidence rates of 0.7% at 10 years, 7.9% at 20 years and 33.2% at 30 years with a mean age at time of diagnosis with CRC was 49.6 (30). These and other studies are summarised in Table 2.

**Table 2. Summary of a selection of Population and Hospital Studies Investigating Risk Between Ulcerative Colitis and Colitis Associated Cancer**

| Study | Duration | Disease | Population | IBD Patients | Study Design | CAC cases | Incidence or SMR | Cumulative Risk | Important Points |
|---|---|---|---|---|---|---|---|---|---|
| Risk of Ulcerative Colitis Associated Colorectal Cancer in China<br>Gong et al 2012 (31) | 1998-2009<br>11 Years | UC | Guangzho<br>China | 3922 | Observational Study | 34 | - | 10 years -1.15%<br>20 years -3.56%<br>30 years: 14.36% | Risk factors include disease duration, extensive colitis and dysplasia |
| The Risk of Inflammatory Bowel Disease-Related Colorectal Carcinoma is Limited<br>Baars et al 2011 (32) | 1990-2006<br>16 years | UC & CD | Netherlands | - | Case Control Study | 173 | IR 0.04% | - | Risk factors = PSC, Older age, pseudopolyps and duration of disease |
| Trends of Ulcerative Colitis Associated Colorectal Cancer in Korea<br>Kim et al 2009 (30) | 1970-2005<br>35 years | UC | Seoul<br>Korea | 7061 | Observational Study | 26 cases | - | 10 years -0.7%<br>20 years -7.9%<br>30 years: 33.2% | Dysplasia observed in 56.2% of resected specimens |
| Risk Factors for Ulcerative Colitis Associated Cancer<br>Lakatos et al 2006 (29) | 1974-1985<br>30 years | UC | Veszprem<br>Hungary | 723 | Observational Study | 13 | - | 10 years -0.6%<br>20 years -5.4%<br>30 years: 7.5% | Average duration of UC at presentation =19 years<br>Median age at presentation – 51 years<br>Risks include disease duration, extent, PSC and dysplasia |
| Risk of Intestinal Cancer in IBD<br>Jess T et al 2006 (33) | 1940-2001<br>60 years | UC & CD | Minnesota<br>America | 692<br>(378 UC) | Observational Study | 6 | SIR 1.1 | - | Median age at presentation – 51 years<br>Risks include pancolitis |
| Survival After Colorectal Cancer in Patients with Ulcerative Colitis<br>Jensen et al 2006 (34) | 1977-1999<br>23 years | UC | Denmark | - | Case Control Study | 279 | Overall MRR for UC patients<br>1 year 1.24<br>5 years 1.17 | - | Mean age at diagnosis 62.6<br>Patients with CAC and SCRC have similar stage distribution |

| Study | Duration | Disease | Population | IBD Patients | Study Design | CAC cases | Incidence or SMR | Cumulative Risk | Important Points |
|---|---|---|---|---|---|---|---|---|---|
| Risk of Colorectal Cancer in Ulcerative Colitis in India<br>Venkataraman et al 2005 (35) | 1978-2002<br>25 years | UC | Vellore<br>India | 532 | Observational Study | 5 | - | 10 years – 0%<br>20 years – 2.3%<br>30 years 5.8% | All patients with CAC had pancolitis and duration >10 years |
| Survival & Cause-Specific Mortality in UC<br>Winter et al 2003 (36) | 1962-1987<br>25 years | UC | Copenhagen<br>Sweden | 1160 | Observational Study | 13 cases | 50 vs 71<br>SMR 0.7 | - | - |
| Cancer Risk in Patients with IBD<br>Berstein et al 2001 (22) | 1984-1997<br>13 years | UC & CD | Manitoba<br>Winnipeg | UC 2672 cases (19,665 person yrs) | Case Control Study | Colon- 36<br>Rectum 13 | IRR Colon: 2.75<br>IRR Rectum 1.9 | - | - |
| Increased Risk of Cancer in Ulcerative Colitis<br>Karlen et al 1999 (25) | 1955-1984 | UC | Stockholm<br>Sweden | 1547 | Case Control Study | 30 cases | SMR 4.1 | - | Median age at CAC diagnosis = 45 years and median duration of disease at diagnosis= 12.5 years |
| Ulcerative Colitis and Colorectal Cancer<br>Ekbom et al 1990 (37) | 1922-1984<br>52 years | UC | Sweden | 3117 | Observational Study | 92 | SIR 5.7 | Absolute risk = 30% for patient s with pancolitis after 35 years | Risk increases with disease extent and age at diagnosis and disease extent where strong independent risk factors for CAC. |

UC - Ulcerative Colitis, CD - Crohns Disease, CAC – Colitis Associated Cancer, PSC – Primary Sclerosing Cholangitis, IRR – Incidence Rate Ratio, SMR – Standardised Mortality Ratio, Standardised Incidence Ratios, MMR – Mortality Rate Ratio,

Factors that independently increase the risk of developing CRC in UC are listed in (Table 3). The two most significant risk factors are the duration and the extent of the disease secondary to the presence of long-standing diffuse inflammation (field-effect). Interestingly several papers report a highly increased cancer risk in those patients who have Primary Sclerosing Cholangitis (PSC), however evidence to the contrary can also be found (38,39). Patients who have had a liver transplantation for PSC still retain their increased risk (40-43).

**Table 3. Factors That Increase Cancer Risk of Colitis Associated Cancer**

| Risk Factors | References |
|---|---|
| • Duration of disease / young age of onset | (18,22,26) |
| • Anatomical extent of disease | (37) |
| • Severity of disease | (44) |
| • Primary Sclerosing Cholangitis | (45-51) |
| • Family history of colorectal cancer | (52,53) |
| • Stricturing Disease | (54,55) |
| • Post-Inflammatory Polyps | (56,57) |

### 1.1.5 Ulcerative Colitis, CAC and Dysplasia

Tumourgenesis in CAC is initiated by multiple episodes of acute on chronic inflammation (44,58). Levels of nitric oxide (NO) are increased, inducing extensive oxidative stress related genomic damage. Over a long period of time chronic inflammation and failure to repair subsequent deoxynucleic acid (DNA) damage may result in accumulating genomic mutations leading to dysregulated cell division and cellular abnormality such as dysplasia. Therefore, CRC in colitis arises via a specific inflammation-dysplasia-carcinoma sequence differing from the well-characterised developmental pathway of the majority of sporadic colorectal cancers. This has been well reviewed (59).

A histological diagnosis of dysplasia is defined as unequivocal malignant transformation of the colonic epithelium without invasion into the lamina propria (60). Dysplasia is also termed intraepithelial neoplasia (IEN) (World Health Organisation (WHO) classification). The presence of dysplasia forewarns the potential development of CAC. It is characterised by nuclear, cellular and architectural changes within epithelial cells. Macroscopically dysplastic colonic

mucosa occurring in UC can be divided into flat or raised lesions. Raised lesions can be further described by appearance: plaque, mass, stricture, nodule or polyp. A raised lesion, which is endoscopically visible but not amenable to endoscopic resection, is specifically termed dysplastic-associated lesion or mass (DALM) (61). The finding of a DALM warrants further action as they are associated with con-current malignancy (61,62). Furthermore a DALM must be distinguished from sporadic adenomas (adenoma like mass (ALM)), which can also occur within the colonic mucosa affected by UC. An ALM can safely be excised endoscopically as long as the specimen, resection margins and the adjacent mucosa are free of dysplasia, thereby eliminating the need for colectomy (63). A further subset of lesions is the sporadic adenomatous polyps occurring in normal non-inflamed mucosa. These can be managed in a similar manner to those arising in non-UC patients.

Once found, dysplasia can be graded according to severity into low-grade dysplasia (LGD), high-grade dysplasia (HGD) or indeterminate for dysplasia (IND) (60). It is believed that CAC occurs via progression of normal mucosa to IND, then LGD, HGD and finally invasive carcinoma (64). However this model maybe unrealistically simplistic as it has also been shown that CAC can develop from any stage of dysplasia or directly from normal mucosa (65-67). CRC in UC tends to be of an aggressive nature and synchronous lesions are often present (10-30% of cases). This is a consequence of a pathological inflammatory field change effect, in which the entire colon becomes susceptible to the development of CAC due to diffuse molecular and cellular changes.

### 1.1.6 Clinical Presentation of Colitis Associated Cancer

CAC tends to arise 15-20 years earlier than that of sporadic CRC in the general population (25,37,68). The average age of diagnosis has been estimated between 43.2 and 50.9 years. In another study a quarter of cancers were diagnosed before the age of 40 (69). Macroscopically CAC may present in a manner of ways: lesions can be well differentiated, irregular, stricturous, ulcerating or appear as an inflammatory polyp. Microscopically there are higher proportion of mucinous and signet ring cell pathology (70), which can correlate

to an aggressive tumour type. In contrast some lesions have also been found to be flat and remarkably discreet (71).

### 1.1.7 Clinical Classification of Colitis Associated Cancers.

As with sporadic CRC, both the Dukes classification system (Table 4) and the TNM classification of malignant tumours (TMN) system for malignant solid tumours (Table 5) have been used to classify the spread of CAC tumours. This information is used to calculate a stage (0-4) for guiding treatment and predicting prognosis. Definitive staging occurs after surgery. The Dukes system has been largely been replaced by the TMN system. Tumours can also be classified according to their grade; this reflects the degree of tumour cell differentiation. Well-differentiated tumours retain their original appearance, grow slowly and are termed low-grade. Poorly differentiated tumours tend to be invasive, rapidly growing and are termed high grade.

**Table 4. Modified Dukes Staging System for Colorectal Cancer**

| Stage | Description |
|---|---|
| **Dukes A** | Invasion of primary tumour into (but not through) the bowel wall (mucosa only) |
| **Dukes B** | (B1) Invasion of primary tumour into the muscle layer (muscularis propria) |
| | (B2) Invasion of primary tumour through the muscle layer (muscularis propria) |
| **Dukes C** | (C1) Invasion of primary tumour into the muscle layer (muscularis propria) with nodal involvement |
| | (C2) Invasion of primary tumour through the muscle layer (muscularis propria) with nodal involvement |
| **Dukes D** | Distant metastases of primary tumour |

The Dukes system also has associated 5-year survival rates (Dukes A – 90%, B – 70%, C – 20/30%, D - <5%).

**Table 5. TMN Clinical Classification System for Colorectal Cancer**

| TMN Classification | | Description |
|---|---|---|
| **T = Primary Tumour** | **Tx** | Primary tumour can not be assessed |
| | **T0** | No evidence of primary tumour |
| | **Tis** | Carcinoma in-situ – intraepithelial or invasion of lamina propria (not used to describe CRC) |
| | **T1** | Invasion of submucosa |
| | **T2** | Invasion of muscularis propria |
| | **T3** | Invasion of subserosa, non-peritonised pericolic or peri-rectal tissues |
| | **T4** | Invasion of other organs, or structures |
| **N = Regional Lymph Nodes** | **Nx** | Regional lymph nodes can not be assessed |
| | **N0** | No regional lymph node metastasis |
| | **N1** | Metastasis in 1 to 3 regional lymph nodes |
| | **N2** | Metastasis in 4 or more regional lymph nodes |
| **M = Metastatic Disease** | **Mx** | Metastatic disease can not be assessed |
| | **M0** | No metastatic disease |
| | **M1** | Metastatic disease |

### 1.1.7 Surveillance Programs for Ulcerative Colitis

Prevention of CAC is centred on early detection of dysplasia through regular periodic colonoscopy (9,72-74). The evidence for enrolling patients onto a surveillance programme is based upon a range of retrospective studies carried out in the last several decades (24,75,76). Periodic colonoscopy was traditionally initiated 8-10 years after disease diagnosis in patients with pancolitis and 12-15 years after the start of left-sided colitis. However recent guidelines developed by the British Society of Gastroenterology (BSG) suggest all patients with UC undergo a colonoscopy after 10 years of disease in order to stratify risk and based on a combination of disease extent, endoscopic and histological findings (77) (Figure 1).

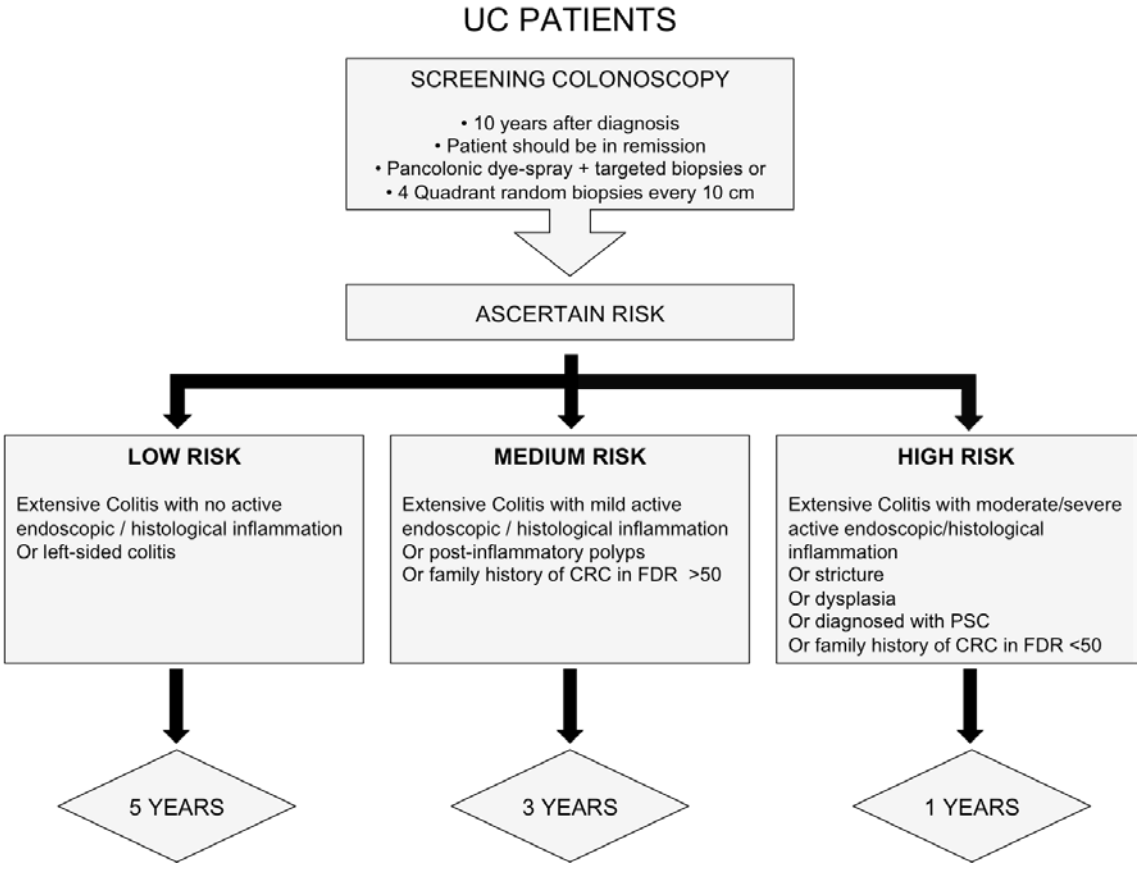



**Figure 1. Surveillance Intervals For Patients With Ulcerative Colitis**

Patients with Ulcerative Colitis are first screened 10 years from diagnosis. Subsequent intervals are dependant on risk stratification, patient's age, and personal preferences. UC – Ulcerative Colitis, CRC – Colorectal Cancer, FDR – First Degree Relative. Adapted from guidelines Cairns,S.R. et al (77).

Furthermore some clinicians may opt to initiate screening earlier in high-risk patients such as those with UC and PSC and in those with severe or extensive disease and repeated on an annual basis (9). Some guidelines also suggest that screening intervals should also decrease as duration of disease and subsequent risk increases. Patients with pancolitis should be screened every three years in the second decade of disease, every two years in the third decade of disease and every year from the forth decade onwards.

### 1.1.8 Endoscopy Techniques and Limitations

Authorities recommend that non-targeted four quadrant mucosal biopsies be taken every 10 cm during colonoscopy from the entire colon (78). Additional targeted biopsies should also be obtained from any suspicious lesions. However this technique only approximately samples a minuscule 1% of the entire bowel mucosa and it has been estimated that a minimum of 33 biopsies are required for a 90% chance of detecting dysplasia (79,80). An increased sensitivity of 95% would require a minimum of 64 biopsies. The usefulness of this technique has been the subject of much on going debate. Some studies advocate the surveillance programme, showing a reduced incidence of death from CRC in patients with UC (76,81-83). Some studies feel surveillance colonoscopy coupled with habitual non-targeted biopsies offers no benefit (84,85). Furthermore there may also be a high degree of variation in actual clinical practice (86,87) and despite being enrolled on a surveillance programme, a portion of patients will still go on to develop high-grade dysplasia or carcinoma in-between colonoscopies (interval cancers) (88).

Colonoscopy in UC may prove challenging, as flat dysplastic lesions may not be macroscopically distinguishable from the surrounding inflamed mucosa. It is therefore recommended that surveillance colonoscopies take place during a period of disease remission. Difficulties in detection result in a significant degree of inter and intra-observer variability and subsequent high sampling errors and potential diagnosis may still be missed (89-91). Good communication between the endoscopist and pathologist may make the process far more effective (92).

The introduction of chromo-endoscopic aided surveillance has been shown to increase the sensitivity and specificity of targeted biopsies (93-102). The technique utilises a combination of methylene blue dye to stain normal mucosa and indigo carmine dye to stain abnormal mucosa. The application of dye spray enhances aberrations in mucosal anatomy and thus highlights suspicious areas allowing the endoscopist to take targeted biopsies (100). Recent guidelines now recommend the use of chromoendoscopy to aid the detection of abnormal lesions in conjunction with a 'tailored' surveillance period based upon the patient's risk rather than disease duration. However its role has not been fully established and its practice is not yet wide spread as the technique requires sufficient training. Furthermore, despite the use of chromoendoscopy potential suspicious lesions still may be missed. Other emerging imaging modalities include narrow band imaging and confocal laser endomicroscopy.

Microscopic classification of dysplasia into LGD, HGD and IND also poses a diagnostic challenge to the histopathologist and it is advised that such a diagnosis should only be made and confirmed only by experienced histopathologists (9,103). The complexity of diagnosis has also made it difficult to report the true incidence of dysplasia in patients with UC.

Detection of HGD or multifocal LGD warrants surgical intervention. Studies have shown that HGD has a high progression rate to cancer (up to 25-45% in some studies) and therefore those patients who are found to harbour HGD in their biopsy specimens are advised to undergo a prompt panproctocolectomy with or without ileal pouch anastomosis (9,21,104). Early evidence to support this includes a study in which five patients out of nine underwent colectomy for HGD and were found to concurrent carcinoma (105). Further information comes from a review of ten surveillance studies encompassing 1225 patients. The evidence showed that from 40 patients undergoing colectomy for a DALM 43% had a co-existing cancer in the resected specimen (10 of 24 for HGD and of 16 for LGD), (62). However much controversy exists regarding the management of a unifocal flat LGD lesion. There is no unified consensus regarding whether it is best to proceed directly to surgery or enrol patients back onto an aggressive surveillance programme. As dysplasia is often multifocal, the diagnosis of LGD

can be suggestive of further areas of dysplasia or even concurrent neoplasia. Studies have shown a 22% prevalence of CRC in colectomy specimens undertaken for LGD. A further 11% of specimens contained HGD. A high proportion of patients who were found to have dysplasia and did not receive surgical intervention went on to develop HGD or CAC (64%). In contrast several schools of thought advise against panproctocolectomy, deeming the procedure as unnecessarily radical (104,106). In these studies a lower rate of progression from LGD to HGD or CAC was found therefore favouring adherence to a stringent follow up program instead. A recent study of 35 patients being followed for LGD or IND also showed a low rate of progression to HGD or CAC, with a total incidence rate of 2.7 cases of HGD or CAC per 100 person-years at risk (107).

Thus there are many problems associated with surveillance colonoscopy as a screening tool including user error, sampling error, diagnostic inaccuracy and risks pertaining to the test itself (colonic perforation). Surveillance colonoscopy programmes are resource intensive and represent a huge financial strain upon the NHS. Surveillance colonoscopy is also completely dependent upon patient understanding and compliance. Furthermore a recent Cochrane database evidence based review of all relevant literature (1966-2005) debates the benefits of surveillance on overall survival in patients with UC (19) and screening programs still remain a contentious issue. Nonetheless, a screening programme for CAC is currently utilised in the UK.

An ideal screening test would be sensitive, specific, minimally invasive, cost-effective, within the capacity of the hospital, fast and acceptable to the patient. In the context of UC, it would be useful if such a test could distinguish between background inflammation, adenomas, dysplasia and CRC, thereby allowing the clinician to monitor disease and ascertain risk of malignant transformation.

### 1.1.9 Molecular Basis of Colitis Associated Cancer

As described before, chronic inflammation is a strong risk factor for the development of cancer. This occurrence is classically illustrated in several conditions including colorectal cancer in inflammatory bowel disease, pancreatic cancer in chronic pancreatitis, hepatic cancer in chronic hepatitis and oesophageal cancer in Barrett's oesophagus (this has been well reviewed (108)).

Ulcerative colitis is characterised by a relapsing remitting disease pattern resulting in repeated episodes of acute on chronic inflammation of the large bowel epithelium. The continuous state of chronic inflammation leads to a constant state of cellular regeneration and repair in tandem with tissue destruction. The complex molecular mechanisms underlying these processes are interrelated and over activation of these processes are pro-tumourgenic. The molecules involved include nuclear factor kappa B (NFκB), reactive oxygen and nitrogen species (RONs), cytokines and prostaglandins. A barrage of immune cells including macrophages, neutrophils and eosinohpils are activated in the inflammatory process and release pro-inflammatory cytokines such as interleukin (IL)-1α IL-β and tumour necrosis factor (TNF) alpha (TNFα). As a result cyclooxygenase-2 (COX-2) and the inducible enzyme nitric oxide synthase (NOS) are increased in acutely inflamed tissue generating high levels of free radicals (nitric oxide (NO)) and prostaglandins, which consequently inflict physiological stress and oxidative damage upon DNA (109-112).

Under normal circumstances cells with abnormal DNA, such as DNA containing double strand breaks, point mutations and inappropriate cross-linking, are dealt with accordingly by a variety of mechanisms. One such molecule involved in the regulatory cascade mechanism is tumour protein 53 (p53), which acts as a tumour suppressor. The naturally occurring, non-mutated form of p53 is referred to as the wild type p53 (wt-p53). The regulation of p53 is highly complex. In the context of inflammation, increasing levels of NO, increase the levels of p53, which ultimately act to reduce the levels of NOS and NO through a feedback loop. However in the presence of mutated p53 (or absence of functional p53),

this feedback mechanism is disrupted leading to pro-tumourgenic conditions that promote genomic instability and predispose to cell proliferation, clonal expansion, and eventual malignant transformation.

CAC tumours exhibit a similar molecular picture as that of CRC with of 85% of tumours arising due to chromosomal instability (113,114) and 15% displaying microsatellite instability. However, the timings of the underlying molecular events differ considerably and has been reviewed in detailed (115) (Figure 2).

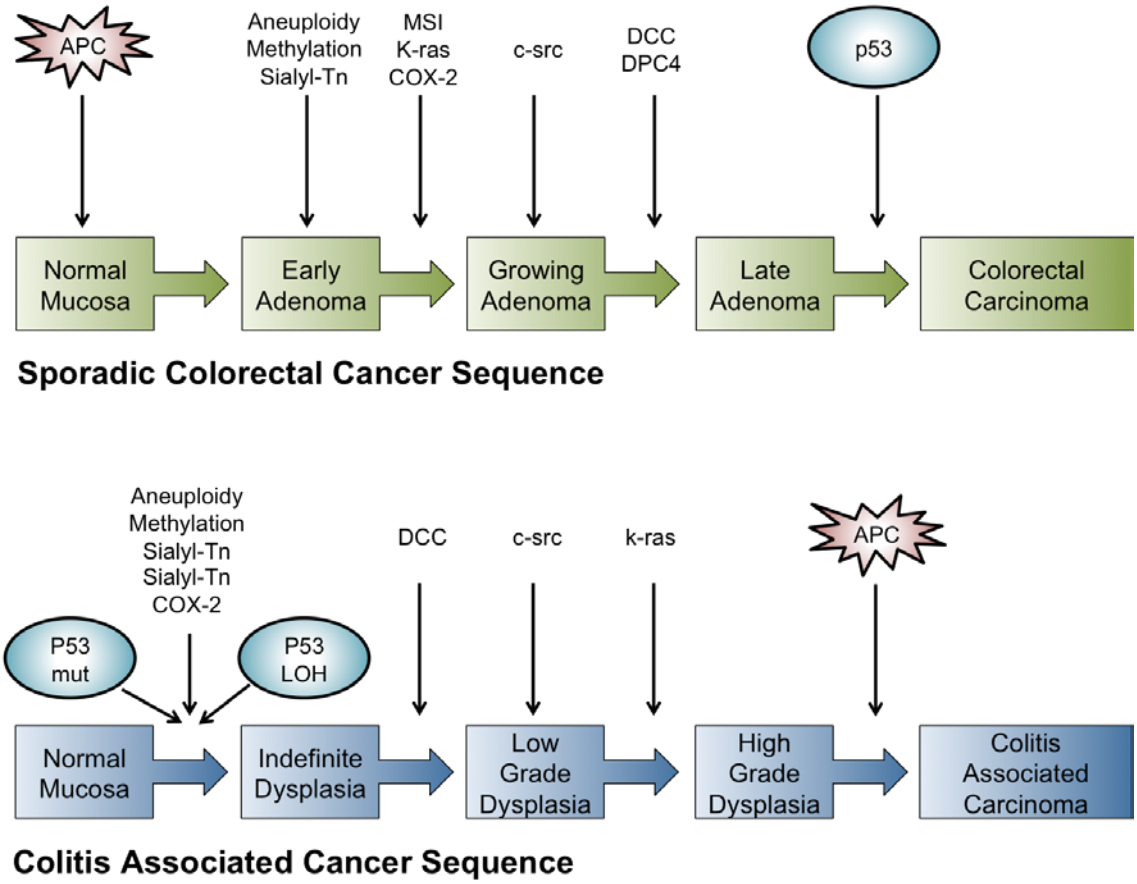



**Figure 2. Comparison of Molecular Basis of The Development of Sporadic Colorectal Cancer and Colitis Associated Cancer.**

APC – Adenomatous Polyposis Coli Gene, COX-2 – Cyclooxygenase 2, C-src – Proto oncogene tyrosine-protein kinase sarcoma, DCC – Deleted in colon cancer, DCP4 – Deleted in pancreatic cancer locus 4 tumour suppressor gene, K-ras-Vi-Ki ras 2-kirsten rat sarcoma viral oncogene humalog, MSI – Microsatellite instability

In contrast to sporadic CRC, CAC develops via a pathway in which loss of tumour suppressor protein Adenomatous Polyposis Coli (APC) is an uncommon or late feature. Furthermore, p53 alterations or mutations occur at an early stage. Several studies have shown a raised level of p53 mutations in inflamed intestinal tissue from patients with UC. Such mutations are often detected in non-dysplastic normal looking mucosa. Many immunohistochemistry studies have identified an association between p53 abnormalities, dysplasia and CAC. Furthermore widespread aneuploidy is well recognised even in non-dysplastic chronic UC affected epithelium (80,116).

Several in vitro studies have revealed a causal role of oxidative stress processes contributing to the impairment of DNA mismatch repair (MMR) enzymes and subsequent hypermutability through microsatellite instability (MSI). Furthermore hypermethylation of genetic cytosine-phosphate Guanine (CpG) islands is found in UC mucosa and appears to occur prior to the development of dysplasia. However, in contrast a recent study (117) concluded that CAC does not preferentially arise via CIMP phenotype and MLH1 promoter methylation pathways. The study also found that p53 mutation (exon 4 missense mutation) was an important factor rather than V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations. This adds further evidence to the importance of p53 in CAC. A study by Cartwright (118) found the proto-oncogene C-src tyrosine kinase (CSK) to be elevated in low-grade dysplasia, suggest that it might play an early role in the development of CAC.

### 1.1.10 Summary of Ulcerative Colitis and Colitis Associated Cancer

- UC is a life long inflammatory condition of the bowel that is prevalent in the UK. The management of UC inflicts a financial burden upon the NHS and significantly impacts upon the quality of life of patients.

- UC is a well-established risk factor for developing CAC. Factors such as increasing duration, severity and anatomical extent of disease have been shown to increase the risk.

- CAC develops along an inflammation-dysplasia-adenocarcinoma pathway, differing from the development of sporadic CRC. Although the majority of CAC display CIN, the important initiating molecular events relate to P53 mutation in contrast to APC mutation that occurs in CRC.

- Patients with UC are entered into a colonoscopic surveillance program to monitor for the development of dysplastic lesions, so early surgical intervention in the form of a panproctocolectomy may be performed.

- Colonoscopic Surveillance has many limitations: it is resource intensive, associated with complications and is user dependent resulting in missed lesions. This necessitates the need for novel, non-invasive, cost-effective biomarkers that can accurately diagnose dysplasia and CAC at an early stage. Potential biomarkers and their role in UC and CAC will be discussed in the next section.

## 1.2 MicroRNAs

### 1.2.1 Background of MicroRNAs

MicroRNAs are short (19-25 ribonucleotides), single-stranded, non-coding RNA sequences (119-122). These highly evolutionary conserved molecules are involved in the control of gene expression at the post-transcriptional level thus playing a role in the regulation of cell differentiation, cell cycle progression and apoptosis. Many recent studies have confirmed their aberrant expression and role in colon, breast, lung and leukaemia carcinogenesis (123). Recent studies have also shown that many diseases express specific range of miRNAs and it has also been shown that miRNAs play a regulatory role in inflammatory processes (124). Thus miRNA profiling may potentially be used for disease diagnosis, treatment, prognosis and monitoring response to treatment.

MiRNAs were originally discovered in the early 1990's in the Caenorhabditis elegans species (125). The international Sanger miRNA database has now registered over 900 miRNAs from the human genome. Due to advancements in scientific techniques, this number is increasing exponentially as many more miRNAs are awaiting experimental validation. Bioinformatics and cloning studies have estimated that miRNAs could regulate up to 30% of all human genomic expression and each single miRNA could control hundreds of gene targets (126).

### 1.2.2 MicroRNA Biogenesis

MicroRNAs biogenesis occurs within the cell via a series of complex multistep processes (127) and will only be described briefly (Figure 3).

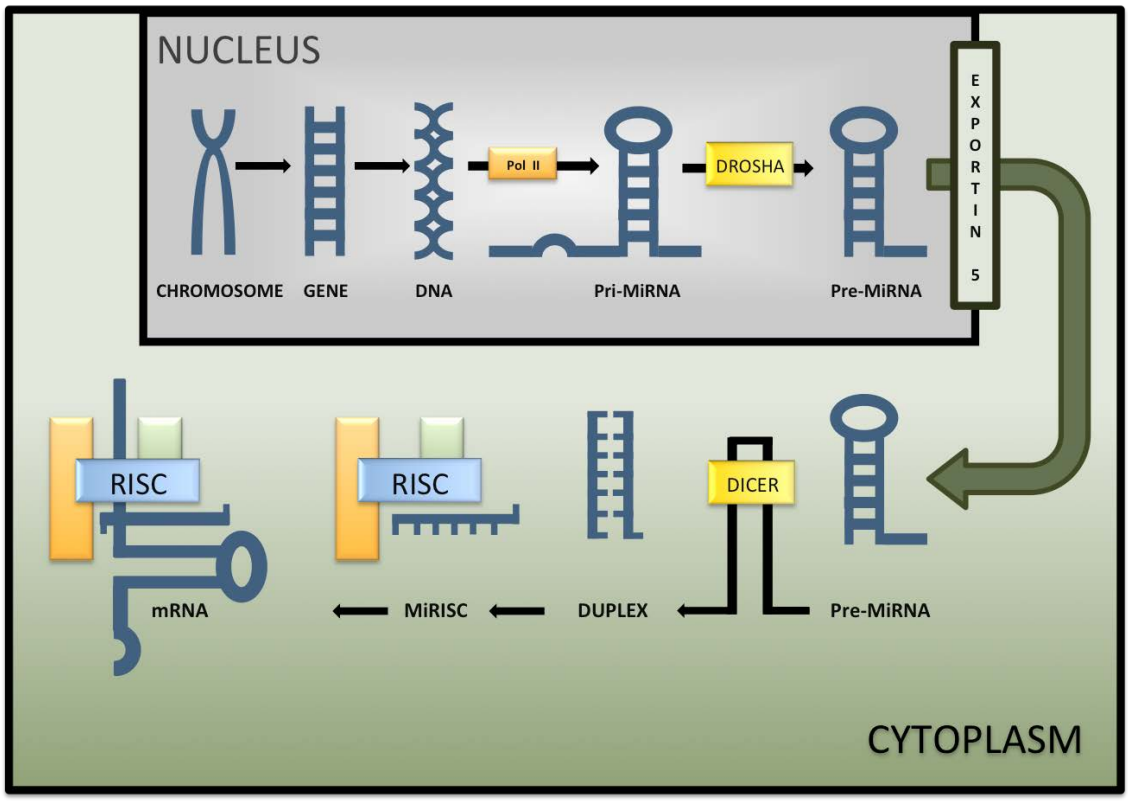



**Figure 3. Simplified Summary of MicroRNA Biogenesis**

RNA polymerase II action in the nucleus creates a primary miRNA transcript, which undergoes processing by the microprocesser RNaseIII enzyme drosha and its co-factor Pasha (128,129). A short stem 60-70 nucleotide loop structure known as pre-miRNA is formed and leaves the nucleus via the guanosine triphosphate (GTP) dependent transporter exportin 5. Once in the cytoplasm, the Pre-miRNA interacts with the RNase enzyme Dicer producing a miRNA/miRNA double stranded duplex (130,131). Further processing results in a mature miRNA strand, which is combined with Argonaute proteins to form the RNA-inducing silencing complex (RISC). The other miRNA strand is degraded. The RISC complex and its incorporated mature miRNA subsequently cleaves, inhibits or degrades target mRNAs thereby negatively regulating gene expression and inhibiting protein translation (132,133).

### 1.2.3 Circulating MicroRNAs

Mature miRNAs are released into the intercellular and extracellular environment via secreted membrane vesicles known as exosomes. This topic has been well reviewed (134) as well as their role in cell-cell communication (135-137). Such exosomes transport circulating miRNA in human plasma (138), thereby allowing miRNA to exist and function in a stable form in the peripheral blood, protected from endogenous RNase activity. The miRNA signature or 'expression profile' could be representational of a disease process occurring in distant tissues (124). They have been found to be extremely stable resisting degradation in the face of RNase activity, high temperatures and pH change (124,139).

### 1.2.4 Detection of MicroRNAs

MiRNAs are present in a range of human tissues and bodily fluids. Well-validated techniques have been developed that allow the reliable extraction and detection and analysis of miRNA from blood (plasma/serum), saliva and urine (139-142). Further advances have also lead to the development of a system that allows miRNA extraction from whole blood stored at room temperatures up to 48 hours. This expands the potential for miRNAs to act as a blood-based assay for the detection of disease. MiRNA can also be extracted from fresh frozen tissue specimens and from formalin fixed paraffin embedded samples (FFPE) (143,144).

Several well-established methods exist for the screening and analysis of miRNA, including microarray analysis, next generation sequencing and polymerase chain reaction (PCR) (145,146). Microarray analysis enables the detection of hundreds of miRNAs from a single sample (147). However as miRNA pre-amplification, is not employed by these techniques, test sensitivity may be compromised. More recently stem-loop quantitative reverse-polymerase chain reaction (qRT-PCR) is a valid method for quantitative and qualitative analysis of miRNA allowing for the sensitive, high throughput and financially economical detection of minute amounts of miRNA (148-153). Next generation

sequencing is a relatively new modality being used for the measurement of miRNAs (154).

### 1.2.5 MicroRNA and Colorectal Cancer

It is well confirmed that the altered expression of miRNA plays a role in the pathogenesis of many diseases and tumour related processes: angiogenesis, invasion and metastasis. MiRNAs may perform this function by acting as oncogenes (oncomiRs) and tumour suppressors (tsmiRs). Many studies have used CRC tissue and blood from patients with a variety of colorectal cancers to profile miRNA expression, using real time-PCR, micro-array and mirage technologies (155-160). There are an extremely large number of papers and reviews on this topic (161-163) and only the salient points that may be pertinent to this thesis will be provided here.

An important study by Michael et al was the first of its kind to establish the role of miRNAs in colorectal cancer (164). The miRNAs miR-143 and miR-145 were found to be dysregulated and acting as potential tumour suppressors. The miR-145 may act as a tumour suppressor through the down-regulation of TGFR-II and the insulin receptor substrate 1 (IRS-1) (165,166). Since these initial reports, an explosion of further studies have investigated and confirmed the role of miRNAs in the development of colorectal cancer.

A recent study (158) demonstrated colorectal cancer tissues show stable expression of the miRNAs let-7a, miR-16, miR-26a, miR-345, miR-425 and miR-454. Another study by (157) revealed over-expression of miRNAs miR-31, miR-183, miR17-5p, miR-18a, miR-20a and miR-92 in conjunction with under expression of miRNAs miR-143 and miR-145. This study also found over expression of miR-18a to be associated with poor prognosis.

MiRNAs have been found that are implicated at each stage of the Vogelstein model of CRC pathogenesis. For example it has been shown that miR-135a and miR-135b act upon the 3'-untranslated (UTR) region of the APC gene leading to activation of the Wnt pathway (167). High expression of miR-21 in adenomas and CRC could be a potential factor in the progression of CRC

(168,169). Its oncomiR action is exerted through programmed cell death protein 4 (pcdp4) (170-172) and phosphatase and tenson homolog (PTEN), thereby causing cell migration and invasion (173).

Recently it has been shown that that miRs can be silenced by hypermethylation in the much the same way that a gene can be. This especially true for miRs that are embedded in CpG islands (miR-34b, 34c, 9-1, 129-2 and 137 and has been shown in CRC cell lines (174,175). It has also been shown that downregulation of miR-101 is involved with over-expression of Cox-2 in human colon cancer cells (176).

MiRNAs have also shown potential to be used not only for the diagnosis and staging of CRC but also as a non-invasive tool to predict prognosis (177-180) and monitor response to radiotherapy or chemotherapy. Their application in the realm of potential new therapies for cancer treatment is being currently being explored and will not be discussed here.

### 1.2.6 MicroRNAs related to p53

As mention earlier, p53 is responsible for preventing the development of cancer through regulating protective cellular mechanisms such as apoptosis and senescence. It has recently come to light that complex non-linear relationships exist between miRNAs, p53 and p53 related proteins. A bout of studies in 2007 showed that miRNAs are integral upstream and downstream components of the p53 system (181-187). In particular, the role of the highly conserved miR-34 family has been well investigated and reviewed in this context (188-190). The miRNAs miR-34a, b and c are encoded by two genes and have been shown to be the transcriptional targets of p53 and hence are crucial players in mediating the effects of p53. They are directly induced in response to genomic stress. The loss of miR-34 has been found to impair p53 related apoptosis via loss of miR-34 targets. Partial deletions of miR-34 have been found in breast and lung cancer. Ectopic expression of miR-34 induces cell cycle arrest in primary and tumour derived cell lines. A study using screenings assays and a variety of cell lines looked at wtTP53 related malignant processes. It was found that the miR-

25 and 30-d interact with the 3'UTR of the human TP gene to negatively regulate p53 expression. This in turn down regulated the target genes p21, Bax, Puma and Gadd45A resulting in a negative effect upon apoptosis and senescence (191). Another study (192) showed that p53 promotes the post-transcriptional maturation of miR-16-1, miR-143 and miR-145 (miR-143 and 145 are known to be decreased in CRC).

### 1.2.7 Summary of MicroRNAs

- MiRNAs are small non-coding nucleic acids involved in gene regulation.

- MiRNAs are present in tissues such as colonic mucosa and released into the circulation and transported within exosomes. They can be reliably extracted and detected in a variety of biological sources including fresh frozen tissue, archived tissue (FFPE tissue), plasma and bodily fluids.

- Developments in high throughput technology such as microarray and PCR mean that the screening and validation of multiple miRNAs can be carried out within the laboratory.

- Expression studies propose that specific miRNA profiles are associated with disease types and pathological mechanisms. A range of miRNAs associated with colorectal cancer and p53 function has been well characterized, suggesting their potential as diagnostic and prognostic biomarkers.

- The next section will review the miRNAs studies associated with UC.

## 1.3 MicroRNAs in Ulcerative Colitis and Colitis Associated Neoplasia

MiRNA studies concerning UC have many variables (Figure 4). This makes it very difficult to control all the different confounding factors and compare results from different studies.

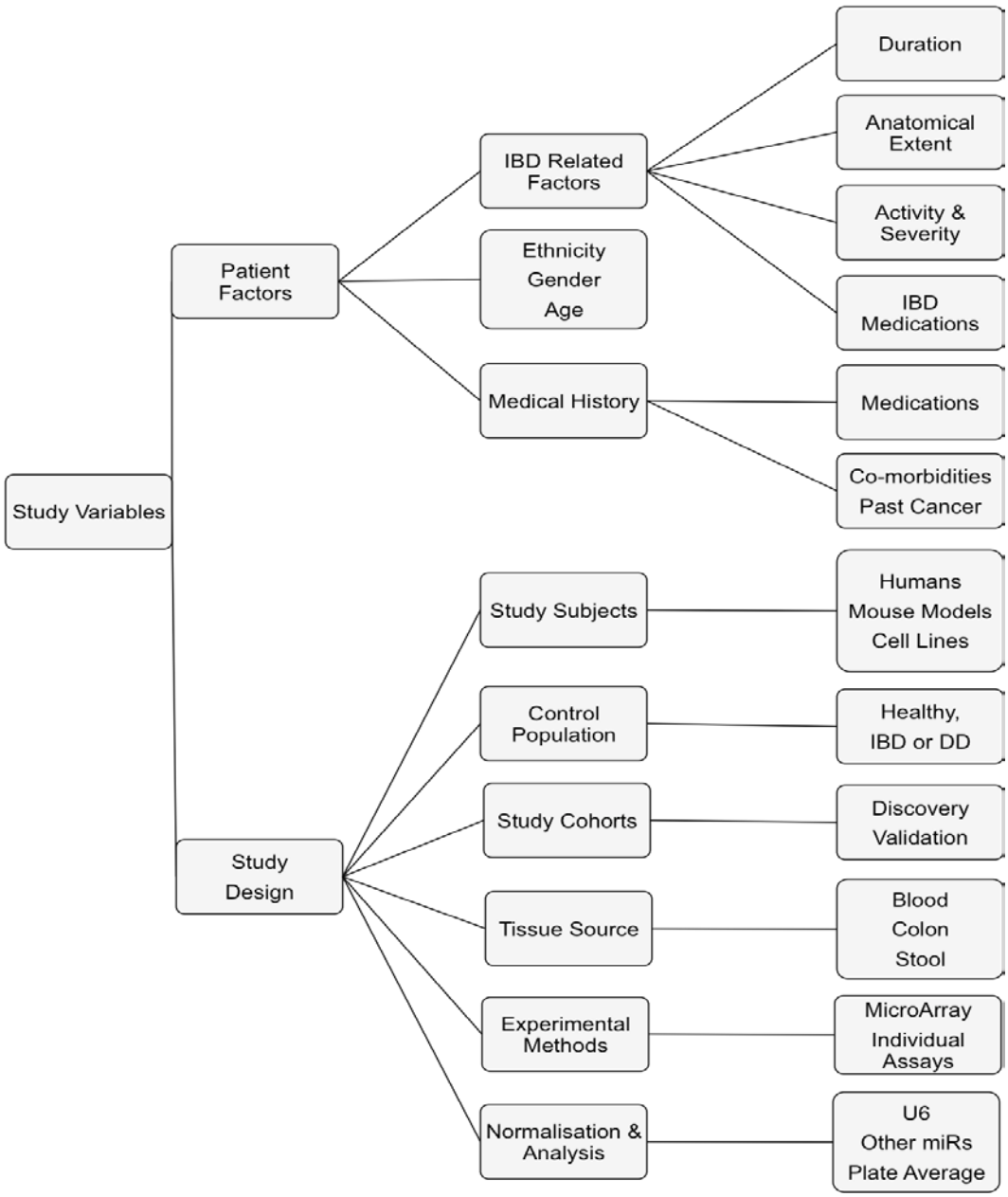



**Figure 4. Variables to be Considered When Conducting and Comparing MicroRNA Studies**

Defining and recruiting control groups poses a challenge, as the ideal control group would consist of young patients with healthy bowel and no other co-morbidities. In reality these patients would not routinely undergo a colonoscopy or require a bowel operation, thereby making it difficult to assess these patients and obtain ‘healthy’ bowel tissue. Due to the invasive nature and risks of a colonoscopy there are also ethical implications associated with obtaining additional, non-essential healthy and disease affected colonic tissue from patients for research purposes. Subsequently many of the UC based miRNA studies use patients from other disease groups that do require intervention (CD, Diverticular disease) as control cohorts. Alternatively, some studies use the inactive state of the disease or segments of colonic unaffected by disease as a ‘control’. Each approach has disadvantages and advantages and it must be noted that heterogeneity in samples can affect gene expression.

The analysis and interpretation of gene expression profiling studies should also be treated with caution. Results are often expressed in fold differences, with a two-fold change correlating to up-regulation of a gene. This could be a vast oversimplification (193) and such results should be validated several times. Furthermore, only a few studies have utilised power and sample size calculations in these experiments.

Despite the above issues, such profiling studies do provide a good starting point for biomarker research.

Studies investigating miRNAs in IBD fall into studies that determine expression profiles associated with disease states and functional studies that examine the role of miRNAs in pathogenesis of IBD.

### 1.3.1 MicroRNAs Expression Studies: Active and Chronic Inflammatory Bowel Disease

Only a small fraction of miRNA studies have investigated miRNA expression profiles in IBD. To date 12 studies have investigated expression profiles in adult patients with UC. These show that over 60 miRNAs have been found to be over-expressed with seventeen miRNAS being identified in more than one study: let-7g, miR-16, 21, 24, 28-5p, 29a, 29b, 31, 126, 126a, 127-3p, 151-5p, 155, 199a-5p, 203, 223, 324-3p (Table 6).

## Table 6. Summary of Studies Investigating MicroRNA profiles in Active and Inactive Inflammatory Bowel Disease

The following table describes studies that have carried out MiRNA expression profiling on colonic mucosa, serum and stool specimens obtained from patients with IBD. Results pertaining to UC patients have been specifically extracted from the studies. A combination of initial array and validation results are presented, depending upon the significance.

| | Study | Specimen | Population | Techniques | Normalisation | Over expressed MicroRNAs | Under Expressed MicroRNAs |
|---|---|---|---|---|---|---|---|
| **1.** | MicroRNAs are differentially expressed in Ulcerative Colitis and Alter Expression of Macrophage Inflammatory Peptide-2α<br>**Wu et al 2008** (194) | Colonic Mucosa (Pinch biopsies) | Control (n=15)<br>Active UC (n=15)<br>Inactive UC (n=15)<br>Infectious Colitis (4)<br>IBS (n=5)<br>Microscopic Colitis (n=3)<br>Chronic Active CD (n=5) | • **Screening**<br>Microarray<br>NCode Multi-species miRNA Microarray V2 (Invitrogen)<br>• **Validation**<br>SYBR green qRT-PCR (Invitrogen) | Let-7a, Let-7b | **Active UC Vs Control**<br>miR-16, miR-21, miR-23a, miR-24, miR-29a, miR-126, miR-195, Lef-7f | **Active UC Vs Control**<br>miR-192, miR-375, miR-422b |
| **2.** | Diagnostic MicroRNA Markers for Screening Sporadic Human Colon Cancer and Active Ulcerative Colitis in Stool and Tissue<br>**Ahmed et al 2009** (195) | Stool and Tissue (Small area biopsies) | Control (n=5)<br>CRC-stage 0-3 (n=15)<br>Active UC (n=5)<br>CD (n=5) | • qRT-PCR using 16 Individual assays (Taqman, Applied Biosytems) | 18S rRNA | **Active UC Vs. Control**<br>miR-16, miR-21, miR-126, miR-203 | **Active UC Vs. Control**<br>miR-192, miR-320 |
| **3.** | Increased Expression of microRNAs in the inflamed colonic mucosa of patients with active ulcerative colitis<br>**Takagi et al 2009** (196) | Colonic Mucosa (Pinch biopsies) | **Discovery Cohort**<br>Active UC (n=2)<br>Healthy Controls (n=2)<br>**Validation Cohort**<br>Active UC (n=12)<br>Healthy Controls (n=12) | • **Screening**<br>Microarray<br>NCode Multi-species miRNA Microarray V2 (Invitrogen)<br>• **Validation**<br>qRT-PCR (Taqman, Applied Biosytems) | U6B | **Active UC Vs. Control**<br>miR-let7a, miR-let-7c, miR-7d, miR-let7g, miR-21, miR-155, miR-923 | **Active UC Vs. Control**<br>None found |

| | Study | Specimen | Population | Techniques | Normalisation | Over expressed MicroRNAs | Under Expressed MicroRNAs |
|---|---|---|---|---|---|---|---|
| **4.** | Identification of MicroRNAs Associated with Ileal and Colonic Crohns Disease<br>**Wu et al 2010** (197) | Colonic Mucosa (Pinch biopsies) | Controls – Sigmoid (n=7)<br>Controls – Colonic (n=6)<br>CD Active– Ileum (n=6)<br>CD Active – Colonic (n=5) | • **Screening**<br>Microarray NCode Multi-species miRNA Microarray V2 (Invitrogen)<br>• **Validation**<br>qRT-PCR (SYBR green) | U6B | **CD Colon Vs. Control**<br>miR-23b, miR-106b, miR- 191<br>**CD Ileum Vs. Control**<br>miR-16, miR-21, miR-223, miR-594 | **CD Colon Vs. Control**<br>miR-19b, miR-629 |
| **5.** | Identification of restricted subsets of mature microRNAs abnormally expressed in inactive colonic mucosa of patients with inflammatory bowel disease<br>**Fasseu et al 2010** (198) | Colonic Mucosa (Pinch biopsies) | Inactive UC (n=8)<br>Inactive CD (n=8) Controls (n=10) | • **Screening**<br>The Human Early Access Release Kit (Taqman, Applied Biosystems) | U6 | **Active UC Vs. Controls**<br>miR-7, miR-29a, miR-29b, miR-31, miR-126*, miR-127-3p, miR-135b, miR-223, miR-324-3p<br>**Inactive UC Vs. Controls**<br>miR-29a, miR-29b, miR-126*, miR-127-3p, miR-196a, miR-324-3p | **Active UC Vs. Controls**<br>miR-25, miR-188-5p, miR-320a, miR-346<br>**Inactive UC Vs. Controls**<br>miR-25, miR-188-5p, miR-320a, miR-346 |
| **6.** | Peripheral Blood MicroRNAs Distinguish Active Ulcerative Colitis and Crohns Disease<br>**Wu et al 2010** (199) | Peripheral Blood | Active CD (n=12)<br>Inactive CD (n=12)<br>Active UC (n=12)<br>Inactive UC (n=12)<br>Controls (n=12) | • **Screening**<br>Microarray merCURY LNA Array chip (Exiqon)<br>• **Validation**<br>SYBR green qRT-PCR | U6B | **Active UC Vs. Control**<br>miR-28-5p, miR-151-5p, miR-103-2*, miR-199a-5p, miR-340*, miR-362-3p, miR-532-3p, miRplus-E1271, | **Active UC / Inactive UC Vs. Controls**<br>miR-505* |

| | Study | Specimen | Population | Techniques | Normalisation | Over expressed MicroRNAs | Under Expressed MicroRNAs |
|---|---|---|---|---|---|---|---|
| **7** | Circulating MicroRNA in inflammatory bowel disease **Paraskevi et al 2012** (200) | Peripheral Blood | Controls (n=162)<br>Active UC (n=88)<br>Active CD (n=128) | • qRT-PCR using 20 Individual assays<br>(Exiqon, Denmark) | U6 | **Active UC Vs. Control**<br>miR-16, miR-21, miR-28-5p, miR-151-5p, miR-155, miR-199a-5p | **Active UC Vs. Control**<br>None found |
| **8** | Genome-Wide Circulating miRNA Biomarkers for Ulcerative Colitis **Duttagupta et al 2012** (201) | Peripheral Blood | Controls (n=20)<br>UC (n=20) | • **Screening**<br>Microarray<br>Affymetrix Genechip array<br>• **Validation**<br>SYBR green qRT-PCR | RNU6B<br>miR-320b<br>miR-543<br>miR-6654-3p | **UC Vs. Controls**<br>MicroVesicles<br>miR-628-5p, miR-603, miR-221*, miR-455-3p<br><br>Platelets /MicroVesicles<br>miR-423-3p<br>Platelets<br>miR-378*, miR-146b-3p, miR-150*, miR-941, miR-27a*, miR-720, miR-550*, let-7i*, miR-501-5p, miR-92a-1*, miR-500, miR-874, miR-769-5p, miR-20b*, miR-188, miR-769-3p, miR-138, miR-1296, miR-1274b, miR-143*, miR-378, miR-31, miR-532-5p, miR-345, miR-181b, miR-330-3p, miR-362-5p, miR-422a, miR-1271, miR-22, miR-140-3p | |

| | Study | Specimen | Population( | Techniques | Normalisation | Over expressed MicroRNAs | Under Expressed MicroRNAs |
|---|---|---|---|---|---|---|---|
| **9** | MicroRNA expression patterns in indeterminate inflammatory bowel disease<br>**Lin et al 2013** (202) | Distal Colonic Mucosa<br>(Fresh frozen colectomy samples) | ID (n=16)<br>CD (n=14)<br>Chronic UC n=12)<br>Controls (n=11)<br>(Diverticular Disease) | • qRT-PCR using 5 Individual assays<br>(Applied Biosytems) | U6B | **Chronic UC Vs. CD**<br>miR-19b, miR-106a, miR-629 | |
| **10** | Identification of serum and tissue micro_RNA expression profiles in different stages of inflammatory bowel disease<br>**Iborra et al 2013** (203) | Peripheral blood Paired with Colonic Mucosa<br>(Pinch biopsies) | Active CD (n=9)<br>Inactive CD (n=9)<br>Active UC (n=9)<br>Inactive UC (n=9)<br><br>Controls (n=33)<br>(Bloods samples only) | • **Screening**<br>Microarray<br>(Taqman Human MicroRNA Array cards A + B)<br><br>No validation. | Plate averaging method | **UC Vs. Controls (Serum)**<br>miR-760, miR-423-5p, miR-650128, miR-650196b, miR-103, miR-221, miR-532-5p, miR-15b, miR-27a, miR-let-7g, miR-93, miR-let-7d, miR-598, miR-142-5p, miR-let-7e, miR-223, miR-374b, miR-19a, miR-345, miR-199a-3p, miR-24, miR-30e, miR-29a, miR-28-3p<br><br>**Active UC Vs. Inactive UC (Tissue)**<br>miR-650, miR-548a-3p | **UC Vs. Controls (Serum)**<br>miR-150<br><br>**Active UC Vs. Inactive UC (Tissue)**<br>miR-630, miR-489, miR-196b |

| | Study | Specimen | Population/Cohort | Techniques | Normalisation | Over expressed MicroRNAs | Under Expressed MicroRNAs |
|---|---|---|---|---|---|---|---|
| **11** | miR-20b, miR-98, miR-125b-1* and let-7e as new potential diagnostic biomarkers in ulcerative colitis<br>**Coskun et al 2013** (204) | Distal Colonic Mucosa<br>(Pinch biopsies) | **Discovery Cohort**<br>Active UC (n=2)<br>Quiescent UC (n=2)<br>Active CD (n=2)<br>Quiescent CD (n=2)<br>Controls (n=2)<br><br>**Validation Cohort**<br>Active UC (n=20)<br>Quiescent UC (=19)<br>Controls (n=20) | • **Screening**<br>Microarray<br>miRNA MicroArray Geniom Biochip (Febit, GmbH)<br><br>• **Validation (7 assays)**<br>qRT-PCR<br>(Taqman, Applied Biosystems) | RNU6B | **Active Vs. Inactive UC**<br>miR-20b, miR-99a, miR-203, miR-26b, miR-98<br><br>(Only miR-20b, 125b found to be significantly up-regulated in the active UC validation cohort and let-7e*, miR 98 in inactive UC) | |
| **12** | Novel Specific microRNAs biomarkers in idiopathic inflammatory bowel disease unrelated to disease activity<br>**Lin et al 2014** (205) | Distal Colonic Mucosa<br><br>(fresh frozen tissue and FFPE specimens from colectomies) | **Discovery Cohort**<br>Active UC (n=10)<br>Active CD (=9)<br>Controls (n=18)<br>(Inactive Diverticular disease)<br><br>**Validation Cohort**<br>UC (n=36)<br>Quiescent UC (=26)<br>Infectious Colitis (n=12)<br>Ischemic Colitis (n=19)<br>Controls (n=29)<br>(Inactive Diverticular disease) | • **Screening**<br>Illumina next-generation sequencing of small cDNA libraries<br><br>• **Validation (9-assays)**<br>qRT-PCR<br>(Taqman, Applied Biosystems) | RNU6B | **UC Vs. Controls**<br>miR-31, miR-206, MiR-424, miR-146a<br><br>(Only miR-31 found to be significantly up-regulated in the validation cohort) | |

### 1.3.2 MicroRNAs Expression Studies: IBD related Dysplasia and CAC

At the time of writing, only 4 studies have investigated miRNAs directly associated with CAC and the inflammation-dysplasia-cancer pathway (Table 7).

**Study 1: Olaru et al** (206)

At the time of writing, this has been the most comprehensive study upon miRNAs associated with neoplastic transformation in IBD. This multi-centre study utilised 175 fresh frozen specimens including 11 IBD dysplasia and 37-IBD cancer cases. Of these there were 30 CAC cases and 9 cases of UC-related dysplasia or DALMS. Microarray analysis was performed upon eight chronic UC cases and eight rectal dysplastic cases. Twenty-two miRNAs were found to be up-regulated of which Mir-31 was found to track neoplastic progression. MiR-31 expression was 5.5 fold higher in Normal-IBD tissues versus normal tissues ($P=0.08$ Students t-test) and 8.2 fold higher in inflamed-IBD tissues versus normal ($P<0.001$). No difference in Mir-31 expression was found between IBD-dysplasia and IBD-carcinoma, however when dysplastic cases were grouped with carcinoma cases (IBDN) there was a 61.38 fold increase when compared to normal colon and a 11-fold increase when compared normal IBD-tissues ($P< 0.001$). The authors concluded that Mir-31 is increased in a stepwise manner as disease progresses. Furthermore Mir-31 was increased (19.8 fold) in SCRC specimens compared to normal tissues but was significantly higher in IBDN (3-fold difference SCRC versus IBDN). Receiver operating curves evaluating miR-31 ability to distinguish IBDN from normal tissue produced an AUROC of 0.997. The study also identified the role of miR-31 in negatively regulating factor inhibiting inducible factor 1 (FIH-1). A comparison of miR-31 expression across six colonic segments showed no difference arising as a result of anatomical variation.

**Study 2: Necela et al** (207)

This study utilised Dextran sodium Sulphate and APC $^{Min/+}$ mouse models to compare tumours of inflammatory and genetic origins. Individual miRNA assays were used to validate the results from the original and were tested in additional independent cohort (4 CAC tumours, 8 controls). Six miRNAs were found to be

differentially expressed, of which four (215,708, 135-b and 31) were common to both tumour types. A limit of the study was the relatively small sample size, which was attributed to reagent costs and the time taken to generate the CAC mouse models.

**Study 3: Kannan et al** (208)

Six, immunotherapy naïve patients, undergoing bowel surgery for IBD associated cancers were used in this study. All resected patient specimens harboured all three-tissue types (non-neoplastic, dysplasia and cancer), and were thereby able to act as their own controls. Laser capture microdissection was used to obtain tissue from the FFPE blocks. The false discovery rate was set to 10%. The combined analysis of UC and CD patients showed differential expression of miR-193b, miR-373, let-7e, miR-15b, and miR-372 from non-neoplastic tissue to dysplasia and dysplasia to cancer ($P<0.001$).

**Study 4: Tan et al** (209)

This used the dextran sodium sulphate and 1,2-diformlhydrazine (DMH) mouse model to investigate the miRNAs associated with UC and the development of CAC. The levels of seven miRNAs were up-regulated. These results should be interpreted with caution as the entire rodent colon was milled and used as a tissue source, with no attempt to isolate epithelial cells. Subsequently miRNAs expressed in other tissues may confound the results.

In summary 30 miRNAs have been found to be up regulated in IBD-neoplasia, of which miR-31, 135b, 192, 194, and 215 have been reported to be up-regulated by more than one study. A striking feature of the four studies described above is that only two studies have used human tissue, illustrating the difficulty in obtaining large number of CAC and UC related dysplastic specimens. Furthermore, none of the studies have utilised plasma samples collected from patients with IBD neoplasia. Importantly, miRNAs expression was found to be stable when comparing different anatomical areas of bowel. It is also difficult to extrapolate the findings from mouse studies and in vitro studies to humans, as simulated conditions may not mimic the exact pathological mechanisms taking place in human disease.

**Table 7. Summary of Studies Investigating MicroRNA Profiles in Inflammatory Bowel Disease dysplasia and Colitis Associated Cancer**

| | Study | Specimen | Population / Specimens | Techniques | Normalisation | Over expressed MicroRNAs | Under Expressed MicroRNAs |
|---|---|---|---|---|---|---|---|
| 1. | Dynamic Changes in the Expression of MicroRNA-31 During Inflammatory Bowel Disease associated Neoplastic Transformation<br>**Olaru et al 2011** (206) | Colonic Mucosa | Control (n=55)<br>Chronic UC (n=35)<br>Inactive UC (n=22)<br>Dysplasia (n=11)<br>CAC (n=37)<br>SCRC (n=15) | • **Screening**<br>Microarray<br>(Agilent Technologies)<br>• **Validation (6 assays)**<br>qRT-PCR<br>(Taqman, Applied Biosystems) | U6 | **Inflamed UC Vs Dysplasia**<br>miR-552, miR-31, miR-31*, miR-203, miR-215, miR-135b, miR-200b*, miR-200a, miR-200c, miR-194, miR-200b, miR-192, miR-192*, miR-141, miR-96, miR-194*, miR-200a*, miR-429, miR-375, miR-424*, miR-183, miR-224 | **Inflamed UC Vs. Dysplasia**<br>miR-892b, miR-122, miR-223, miR-501-5p, miR- miR-146b-5p, miR-142-3p, miR-139-5p, miR-155, miR-1288, miR-490-3p |
| 2. | Differential Expression of MicroRNAs in Tumours from Chronically Inflamed or Genetic ($APC^{Min/+}$) Models of Colon Cancer<br>**Necela et al 2011** (207) | Rodent Colonic Distal Epithelium | Normal Epithelium (n=4)<br>Acute Colitis (n=4)<br>Chronic Colitis (n=4)<br>CAC tumours (n=4)<br>APC epithelium (n=4)<br>$APC^{Min/+}$ tumours (n=7) | • **Screening**<br>Microarray<br>(Taqman Rodent MicroRNA array V2.0)<br>• **Validation (8 assays)**<br>qRT-PCR<br>(Taqman, Applied Biosystems) | Sno-135<br>miR-25 | **CAC Vs. Inflamed Tissue**<br>miR-133a, miR-467d, miR-218, miR-31, miR-135b, miR-708 | **CAC vs. Inflamed Tissue**<br>miR-215 |

| | Study | Specimen | Population / Specimens | Techniques | Normalisation | Over expressed MicroRNAs | Under Expressed MicroRNAs |
|---|---|---|---|---|---|---|---|
| 3. | Differential MicroRNA Expression Tracks Neoplastic Progression in Inflammatory Bowel Disease-Associated Cancer<br>**Kanann et al 2012** (208) | Colonic Mucosa | CD associated cancer (n=4)<br>UC associated cancer (n=2) | • **Screening**<br>Microarray<br>(Human Cancer Arrays – SuperArray Biosciences)<br>• **Validation**<br>qRT-PCR<br>(Taqman, Applied Biosystems) | U6<br>Plate average | **Dysplasia Vs. IBD Cancer (CD)**<br>miR-181a, miR-146b-5p, miR-let-7e, miR-17 | **Dysplasia Vs. IBD Cancer (CD)**<br>miR-let-7e, miR-17, miR-143 |
| 4. | Screening of Differentially Expressed microRNAs in Ulcerative Colitis Related Colorectal Cancer<br>**Tan et al 2013** (209) | Rodents<br>Colons | Control mice (n=20)<br>UC mice (n=30)<br>CAC (n=30) | • **Screening**<br>Microarray GeneChipR hybridisation<br>(Affymetrix)<br>• **Validation**<br>qRT-PCR<br>(Shanghai Biotech Ltd) | 5s rRNA | **CAC Vs. Control**<br>miR-194, miR-215, miR-93, miR-192, miR-92a, miR-29b, miR-20a | **CAC Vs. Control**<br>miR-1231, miR—195, miR-143, miR-145 |

Results pertaining to colitis associated cancer and dysplasia have been selected from the studies. Results from both initial array and validation studies are presented, in order of significance or fold difference

### 1.3.3 MicroRNA Functional Studies Related to IBD Pathology

The aetiology of IBD remains unknown; it is the likely result of complex interactions between environmental, immunological, bacterial and genetic factors, mediated by a broad range of biological mechanisms. This has prompted a variety of studies assessing the role of miRNAs in these mechanisms (Table 8). For miRNAs to act as biomarkers, it is not entirely necessary to elucidate the mechanism by which they are produced or determine their role. Nonetheless, functional studies are important as miRNAs involved in the pathology of IBD could be released into the circulation, making detection of a signature panel of miRNAs a viable option.

**Table 8. Summary of Studies Investigating the Function of MicroRNAs in the Pathology of Ulcerative Colitis**

| | Study | Specimen | Population / Specimens | Techniques | Findings |
|---|---|---|---|---|---|
| 1 | MicroRNA-155 Is Involved in the Pathogenesis of Ulcerative Colitis by Targeting FOXO3 **Min et al 2013** (210) | Colonic Mucosa (Pinch Biopsies) | Active UC (n=20)<br>Healthy Subjects (n=16)<br>HT29 Intestinal cells | Microarray analysis<br>Real Time Q-PCR<br>Cell Culture<br>Luciferase Reporter Assays<br>RNA Interference<br>Western Blot Analysis | • Up-regulation of 33 MicroRNAs**:** Let-7a, let-7g, mir-21, mir-22, mir-148a, mir-29a, mir-152, mir-658, mir-142-3p, mir-452, mir-126*, mir-674, let-7i, mir-182, let-7d, mir-16, let-7f, mir-645, mir-146a, mir-32, mir-142-5p, mir-96, mir-155, mir-505, mir-101, mir-135b, mir-185, mir-126, mir-148b, mir-365, mir-675, mir-614, mir-29b<br>• Up regulation of miR-155 is associated with decreased FOXO3 expression<br>• Over expression of miR-155 and silencing of FOXO3 increases IL-8 |
| 2 | Overexpression of mir-21 in patients with ulcerative colitis impairs intestinal epithelial barrier function through targeting Rho GTPase RhoB **Yang et al 2013** (211) | Colonic Mucosa (Biopsies)<br>Serum | Chronic UC (n=30)<br>Healthy Controls (n=30)<br>Caco-2 cell line | Immunohistochemistry<br>In Situ Hybridisation<br>Cell Culture<br>Cell transfection<br>Real Time Q-PCR<br>Western Blot Analysis | • Significant Up-regulation of miR-21 in UC-affected mucosa and serum<br>• ISH showed miR-21 expression more abundant in UC affected tissue<br>• UC samples showed weaker staining of junctional proteins (occluding)<br>• Increase of epithelial permeability<br>• Decrease of RhoB protein levels |
| 3 | PDCD4/miR-21 dysregulation in inflammatory bowel disease associated carcinogenesis **Ludwig et al 2013** (212) | Colonic Mucosa (Colectomies) | UC (n=30)<br>CD (n=20)<br>Controls / IBS (n=20) | Immunohistochemistry<br>In Situ Hybridisation<br>Real Time Q-PCR | • Decreased PDCD4 expression in UC & CD tissues and in IBD-dysplasia<br>• miR-21 is increased in UC & CD tissues and in IBD-dysplasia |
| 4 | miR-200b inhibits TGF-β1-induced epithelial mesenchymal transition and promotes growth of intestinal epithelial cells **Chen et al 2013** (213) | Colonic Mucosa | UC (n=11)<br>CD (n=11)<br>Adenomas (TVA) (n=5)<br>IEC-6 cell line | Immunohistochemistry<br>Immunofluorescence<br>Cell Culture<br>Real Time Q-PCR<br>Western Blot analysis<br>Luciferase Reporter Assays | • Decreased levels of miR-200b in inflamed UC mucosa vs. adjacent normal |

| | Study | Specimen | Population / Specimens | Techniques | Findings |
|---|---|---|---|---|---|
| 5 | Role of miR-19a targeting TNF-α in mediating ulcerative colitis **Chen at al 2013** (214) | Sigmoid Colonic mucosa (biopsies) | Controls (n=10)<br>Active UC (n=24)<br>HT-29 intestinal cell lines<br>DSS mouse models | Real Time Q-PCR<br>Western Blot analysis<br>Immunohistochemistry | • Decreased expression of miR-19a in UC colon tissues compared to controls<br>• DSS induced experimental colitis shows decreased miR-19a expression<br>• There is up regulation of TNF-α in human colon tissues with ulcerative colitis<br>• There is up regulation of TNF-α in DSS induced experimental colitis<br>• miR-19a regulates TNF-α in HT-29 cells |
| 6 | Up-Regulation of microRNAs-126 May Contribute to Pathogenesis of Ulcerative Colitis via Regulating NF-IκBα **Feng et al 2012** (215) | Colonic mucosa (pinch biopsies)<br>H29 , HCT116 Cell lines | Active UC (n=12)<br>Inactive UC (n=10)<br>IBS (n=15)<br>Normal controls (n=15) | Real time qRT-PCR<br>Immunofluorescence<br>Transfection<br>Luciferase report assay<br>Western Blot analysis | • 18-fold increased of miR-126 (P<0.05) in active UC vs. healthy controls<br>• 14.7 fold increase of miR-21 (P<0.05) vs. healthy controls<br>• In-silico analysis predicted IKBA, PLK2, CRK as miR-126 targets of which IKBA mRNA and miR-126 were inversely correlated<br>• miR-126 may promote UC related inflammation by decreasing IKBA (an important inhibitor of the NF-KB pathway) |
| 7 | Role of miR-150-targeting c-Myb in colonic epithelial disruption during dextran sulphate sodium-induced experimental colitis and human ulcerative colitis **Bian et al 2011** (216) | Human Colonic Mucosa<br>DSS murine mouse model<br>HT-29 Cell Line | Control mice (n=12)<br>DSS mice (n=12) | Microarray analysis<br>Cell Culture<br>Cell Transfection<br>Apoptosis Assays<br>Real time qRT-PCR<br>Western Blot analysis | • Microarray analysis showed up-regulation of miR-612, miR-689, miR-150, miR-142-3p, miR-1, miR-18a<br>• 27 assays validated (including those from the literature) of which miR-146a, miR-150, miR-155, miR-689, miR- 142-3p, miR-18a were found to be significantly up regulated<br>• c-Myb reduced in DSS-induced murine experimental colitis and human active UC<br>• miR-150 may target c-Myb |
| 8 | miR-143 and miR-145 are down regulated in Ulcerative Colitis: putative regulators of Inflammation and Proto-oncogenes **Pekow et al 2011** (217) | Distal human Colonic Mucosa<br><br>HCT-116, HCA-7 cell lines | Cohort 1<br>Quiescent UC (n=8)<br>Normal Control (n=8)<br><br>Cohort 1<br>Quiescent UC (n=11)<br>Normal Control (n=11) | Real Time Q-PCR<br>Western Blot analysis<br>In Situ Hybridization<br>Tissue Culture | • miR-143 was 8.3-fold lower in UC colonic mucosa compared to normal colonic mucosa<br>• miR-145 was 4.3-fold lower UC colonic mucosa compared to normal colonic mucosa<br>• Decrease of miR-143 and miR-145 staining in epithelial cells<br>• IRS-1, KRAS, API5, MEK-2 proteins down regulated in HCT116/HCA-7 cells transfected with miR-143, miR-145 |

### 1.3.4 Summary of MicroRNAs in Ulcerative Colitis and Colitis Associated Neoplasia

- Studies evaluating the role of miRNAs in UC can be divided into those investigating expression profiles and those investigating the role of specific miRNAs in the pathology of UC.

- MiRNAs in UC are difficult to determine due to the massive variation in patient factors and establishing suitable control groups. Many studies have used inactive states of UC or diverticular disease as control cohorts.

- Expression profiles studies have revealed the differential expression of many miRNAs (Table 9), some of which are up regulated in more than one study.

- Functional studies suggested the role of miR-19a, miR-21, miR-126, miR-150, miR-155 and miR-200b in the pathology of UC.

- The complexity of biological mechanisms coupled with marked variation in experimental design mean the results from such miRNA studies should be interpreted with care. Nonetheless, these provide a starting point for future biomarker experiments and highlight miRNAs that have repeatedly found to be important. These could be strong candidates to include in a signature panel of biomarkers.

**Table 9. Summary of MicroRNAs Up-regulated in Ulcerative Colitis, Dysplasia and Colitis Associated Cancer**

| | **Blood**<br>**Over-Expressed MicroRNAs** | **Tissues**<br>**Over-Expressed MicroRNAs** |
|---|---|---|
| **Active UC Versus Control Group** | • **let-7d,** let-7e, **let-7g,** miR-15b, miR-19a, **miR-24,** miR-27a, miR-28-3p, **miR-29a**, miR-30e, **miR-93,** miR-103, miR-142-5p, miR-199a-3p, miR-221, **miR-223,** miR-345, miR-374b, miR-423-5p, miR-532-5p, miR-598, , miR-760, miR-650128, miR-650196b,<br><br>MicroVesicles<br>• miR-221*, miR-455-3p, miR-603, miR-628-5p,<br><br>Platelets /MicroVesicles<br>• miR-423-3p<br><br>Platelets<br>• let-7i*, miR-20b*, miR-22, miR-27a*, **miR-31,** miR-92a-1*, miR-138, miR-140-3p, miR-143*, miR-146b-3p, miR-150*, miR-181b, miR-188, miR-345, miR-330-3p, miR-362-5p, miR-378, miR-378*, miR-422a, miR-500, miR-501-5p, miR-532-5p, miR-550*, miR-720, miR-769-3p, miR-769-5p, miR-874, miR-941, miR-1271, miR-1274b, miR-1296, | • lef-7f, **miR-16, miR-21,** miR-23a, miR-24, **miR-29a, miR-126,** miR-195,<br><br>• **miR-16**, **miR-21, miR-126, miR-203**<br><br>• let7a, let-7c, **let-7g ,let-7d**, **, miR-21, miR-155,** miR-923<br><br>• miR-7, **miR-29a**, miR-29b, **miR-31,** miR-126*, miR-127-3p, **miR-135b, miR-223,** miR-324-3p<br><br>• **miR-28-5p**, **miR-151-5p**, miR-103-2*, **miR-199a-5p**, miR-340*, miR-362-3p, miR-532-3p, miRplus-E1271,<br><br>• **miR-16, miR-21, miR-28-5p, miR-151-5p**, **miR-155, miR-199a-5p**<br><br>• **miR-31,** miR-146a miR-206, MiR-424, |
| **Active UC Versus Inactive UC** | | • miR-548a-3p, miR-650,<br><br>• miR-20b, miR-26b, miR-98, miR-99a, **miR-203,** |
| **Dysplasia or CAC Versus Inflamed Tissue** | | • **miR-31**, miR-31*, miR-96, **miR-135b**, miR-141, miR-183, **miR-192,** miR-192*, **miR-194**, miR-194*, miR-215, miR-200a, miR-200a*, miR-200b, miR-200b*, miR-200c, **miR-203,** miR-224, miR-375, miR-424*, miR-429, miR-552,<br><br>• **miR-31,** miR-133a, **miR-135b,** miR-218, miR-467d, miR-708 |
| **CAC Versus Control Group** | | • miR-20a, **miR-29b,** miR-92a, miR-93, **miR-192, miR-194,** miR-215, |

MicroRNAs that have been elicited by multiple studies are in bold and underlined

## 1.4 Investigation of Biomarkers

### 1.4.1 Definition

A biomarker is any naturally occurring measurable unit that can be used to evaluate a biological process. Biomarkers can therefore diagnose a disease, monitor treatment or determine pathology. Many screening tests including those that ascertain risk and exposure are biomarkers. Biomarkers can also be used to measure a pharmacological response to an administered therapy or discover potential drug targets.

### 1.4.2 Clinical Biomarkers

Medicine today is heavily dependent on a variety of biomarkers to guide clinical decision-making. Biomarkers can be cells, tumour cells, molecules, hormones, enzymes, antibodies, proteins, nucleic acids, or genes (Table 10).

**Table 10. Common Biomarkers in Colorectal Surgery**

| Source | Biomarker | Clinical Use |
|---|---|---|
| **Clinical measurement** | Body mass index (BMI) | Determines ideal weight and hence obese states |
| **Blood Test** | C-Reactive Protein (CRP) | Increased CRP indicates an inflammatory process, its severity and response to treatment |
| | Erythrocyte Sedimentation Rate (ESR) | Increased ESR indicates an inflammatory process and its potential cause |
| | White Cell Count (WCC) | Changes in normal levels may indicate infection, inflammation, immune deficiency or bone marrow disease |
| | Perinuclear antineutrophil cytoplasmic antibodies (p-ANCA) | An auto-antibody increased in autoimmune disorders such as PSC and Rheumatoid arthritis and Wegners Granulomatosis |
| | Carcinoembryonic Antigen (CEA) | A tumour marker used to monitor the treatment of colorectal cancer |
| | Carbohydrate Antigen -19 (CA-19-9) | A tumour marker used to detect pancreatic cancer and monitor its treatment |
| | K-RAS mutation | Identification of mutant KRAS protein may help identify a patients response to cetuximab chemotherapy |
| | Faecal Calprotectin | Derived from neutrophilic cytoplasm, this indicates intestinal inflammation |
| **Stool Test** | Faecal Elastase | Concentration in faeces reflects pancreatic exocrine function and can be used to determine pancreatic insufficiency. |
| | Lactoferrin | Glycoprotien associated with intestinal inflammation |

### 1.4.3 Biomarker Requirements

Biomarkers that can predict disease, response to therapy and prognosis are becoming increasingly sought after, especially within the field of cancer treatment. A biomarker that could predict response to chemotherapy would be extremely valuable. It would allow clinicians to prescribe personalised targeted therapy, eliminating the need for some patients 'non-responders' to undergo chemotherapy and the subsequent side effects. To be of clinical value, an ideal biomarker test needs to fulfil several parameters:

1. Easy to obtain via a swab, biopsy or blood test
2. Acceptable to the patient
3. Short turn around time: results should be available rapidly
4. Results should be reliable and reproducible
5.Results should be accurate: have a high sensitivity and specificity. Importantly the test should have a high positive and negative predictive value when put into the context of disease prevalence in the general population; a large likelihood ratio is desirable.
6. Cost-effective (to perform and ultimately reduce financial burden of mortality and morbidity related to the disease)
7. Easy for the clinical to interpret (with well defined reference ranges if appropriate)
8. Impact upon clinical decision-making and management; it should be relevant (utility).
9. Apply to a well-defined target population (clinical context): a test that detects malignancy may not necessarily be suitable for general screening or monitoring for disease relapse.

### 1.4.4 Biomarker Experiments

#### Discovery

Recent technological advances in molecular biology enable the rapid screening of many potential biomarkers. High output areas include proteomics, genomics and sectretomics. Despite the increase in biomarker discovery, only a fraction (<1%) of these enter the mainstream. As a result the Early Detection Research Network was formed by the National Cancer Institute to formalise and thus raise the standards of biomarker related research (218)

#### Study Phases

Once a potential biomarker candidate has been identified it must be vigorously tested in different stages to ensure its robustness and validity. A comprehensive five-phase model to guide the process has been proposed (219) (Table 11).

**Table 11. Five Stages of Biomarker Development**

| Phase | Description | Primary Aims | Secondary Aims |
|---|---|---|---|
| **I** | **Exploration & Discovery** | 1. Identity a range of biomarkers<br>2. Prioritize potential candidates | |
| **II** | **Validation** | 1. Estimate the TPR, FPR and ROC for subjects with and without cancer | 1. Optimise assays and ensure reproducibility (the assay should also work on stored samples)<br>2. Determine the relationship between different types of specimen in the same patient (tissue vs. plasma)<br>3. Evaluate confounding patient factors (ages, sex)<br>4. Correlate results to disease factors (stage, histology grade, outcome) |
| **III** | **Retrospective Longitudinal** | 1. Determine the period of time in which the biomarker becomes useful (at what point can it detect the disease)<br>2. Delineate range and values of a positive test | 1. Establish the effect of other clinical parameters upon the diagnostic ability of test<br>2. Judge ability of biomarkers to distinguish the above<br>3. Calculate algorithms / panels of markers for best screen positivity<br>4. Calculate the screening interval if applicable. |
| **IV** | **Prospective Screening** | 1. Calculate detection rate and false referral rate in a relevant cohort | 1. Establish the benefits of the test in regards to disease outcome: cancer detected at earlier stage<br>2. Evaluate the logistics of implementing the test (delivery and patient compliance)<br>3. Investigate the initial effects of biomarker test (reduced mortality, morbidity and cost savings)<br>4. Investigate the patients who have disease undetected by the test |
| **V** | **Cancer Control** | 1. Calculate reduction in disease burden | 1. Determine entailed cost savings<br>2. Determine compliance issues in a range of settings<br>3. Evaluate different screening protocols |

Adapted from Pepe et al (219)

A simpler model in which the 4 proposed study phases take the form of diagnostic questions is an alternative guide to designing biomarker studies (220) (Table 12).

**Table 12. Diagnostic Questions to Consider When Designing Study Phases**

| Phase | Question | Practical Importance |
|---|---|---|
| **Phase 1** | Are test results from the disease group different to the normal population? | This phase determines if a marker can differentiate between the disease group (cases of interest) and the normal group (controls). These cases are termed the 'discovery' or test set and aims to generate potential biomarker candidates. |
| **Phase 2** | If the test result is positive is the patient likely to have the disease? | This phase evaluates the biomarkers sensitivity and specificity. The test cases involved are often unequivocal (belong to know, clinically different groups) and the test carried out under perfect conditions, therefore results need to be interpreted with caution. These cases are termed the training set. |
| **Phase 3** | If the test result is positive is the patient likely to have the disease in a clinical setting? | This phase investigates the biomarkers ability to establish a diagnosis in an unknown group or in a group suspected to have the disease 'the real world'. The phase should be carried out in a prospective, independent test set in which blinding occurs. It is also important compare the new test with a 'gold standard of diagnosis'. Subsequently there is often a decrease in the sensitivity and specificity values and likelihood ratios maybe of better clinical value. |
| **Phase 4** | Do the patients who have this test benefit from it? | This phase calculates if there is an improvement in overall health outcomes as a result of the test compared to those who were not tested. |

Adapted from Sacket et al (220)

These guidelines must be noted when designing biomarker experiments in order to eliminate bias and chance findings. During initial screening phases it is not always necessary to establish the mechanism of action of the biomarker, however it is imperative that the biomarker has some biological connection to the disease.

## Study Design

A useful method for conducting prospective biomarker studies has been proposed: the prospective-specimen-collection, retrospective-blinded evaluation (PRoBE) design (221). The PRoBE design is applicable to the latter stages of all diagnostic, screening and prognostic biomarker studies. It concentrates upon four main components; clinical context, performance criteria, the biomarker test itself and the study size. As the scope of this thesis is to focus upon phases 1 and 2, only a brief overview will be given (Table 13).

**Table 13. Summary of PRoBE model for Biomarker Study Design**

| | Component | Factors to be Addressed |
|---|---|---|
| 1. | Clinical Context | Application<br>Outcomes<br>Case-Control Studies<br>Selection |
| 2. | Performance Criteria | True & False Positive rates<br>Minimally acceptable Performance<br>Comparisons |
| 3. | Study Design | Procedures<br>Blinding<br>Combination<br>Other Predictors |
| 4. | Study Size | Null or Alternative Hypothesis<br>Sample Size<br>Early Termination |

Adapted from Pepe et al (221), PRoBE - prospective-specimen-collection, retrospective-blinded evaluation

The introduction of bias into a study is also a major concern. A number of studies have also highlighted the importance of a meaningful sample size and adequate power and moreover, assay performance must be taken into account.

## Reporting

To ensure that biomarker development is conducted in a standardised manner, a number of guidelines have been formulated. The Reporting recommendations for tumour marker prognostic studies (REMARK guidelines) specifically outline a method for reporting the results of biomarker experiments (222). This was a major recommendation of the National Cancer Institute-European Organisation for Research and the Treatment of Cancer (NCI-EORTIC) in order to allow the

assessment of the quality and generality of any given biomarker study. The guidelines highlight the importance of reporting the following study factors (in the study this takes the form of a 20-point checklist):

- Hypothesis: state study objectives
- Patient factors: recruitment protocols, inclusion-exclusion criteria, disease stages, co-morbidities
- Specimen Characteristics
- Study Design: Assay methods and detailed operating procedures including the reagents used should be provided. Quality control, quantification methods and reproducibly assessments must also be included). List case selection method, end points and rationale for sample size.
- Statistics: All methods and models should be transparent with missing data made clear. Methods for determining cut off values should be stated.
- Data: Patient flow and distribution of demographics should be included
- Results: Show relation of marker to outcomes (providing confidence intervals). Present Univariate analysis as a Kaplein-Meier plot (if applicable) and report multivariate analysis with a hazards ratio.
- Discussion: State if results prove or disprove the hypothesis, whilst acknowledging study limitations and recommend future directions.

Other guidelines include Standards for reporting of diagnostic accuracy (STARD) (223), Minimum Information About a Microarray Experiment (MIAME) (224), and Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) (225). Another paper addresses sources of bias correlating to the natural lifecycle of the bio-specimen (patient factors, procedures, acquisition, handling, and processing, storage, distribution, analysis and restocking of unused sample) (226).

**Power Calculations**

In order for a study to generate valid results that prove or disprove the null hypothesis, it must be adequately powered (227,228)This means each study requires sample numbers that are the right size to detect statistically significant differences between the groups being tested. If the sample size is too small, important results can be missed and the null hypothesis rejected. Alternatively if the sample size is too large the validity of the results can be over estimated leading to wrongly accepting the null hypothesis: type. In both cases, the study is not meaningful and resources (time and cost) are ultimately wasted (229)

For all quantitative clinical studies, it is imperative that a sample size calculation is performed and taken into account whilst designing the study protocol. The sample size calculation can either be precision or power based. The following parameters are required for a power-based calculation:

- Type 1 error (α): The probability of determining a difference between two groups when no real difference exists (false positive result). A significance level needs to be set to avoid this (usually set at 5% - 0.05).
- Type 2 error (β): The probability of determining that no difference exists between two groups, when a real difference really does exist (false negative result).
- Power (1-β): To prevent the probability of a type 2 error, the study must have adequate statistical power (this is usually set at 80-90%).
- The effect (δ): the smallest measurement of difference deemed clinically important.
- The standard deviation (s): An estimate of the variability of the outcome measure. This can be difficult to gauge and may require referring to data from a pilot study or similar historical studies.

Once the above values are obtained, they can be entered into a calculation to determine the adequate number of samples or participates required for each group to prevent type 1 & 2 errors (Figure 5).

$$n = f(\alpha, \beta) \cdot \frac{2s^2}{\delta^2}$$

| α | β | | | |
|---|---|---|---|---|
| | 0.05 | 0.1 | 0.2 | 0.5 |
| 0.05 | 13.0 | 10.5 | 7.9 | 3.8 |
| 0.01 | 17.8 | 14.9 | 11.7 | 6.6 |

**Figure 5: Power based sample size calculation.**

An equation to calculate the number of participants or samples required in each arm of a study (n).
α = a significance level, which is set to avoid type 1 error. β = a set level of probability, used to prevent type 2 error. f is a figure calculated from α and β. Common figures for f are also shown alongside. s = the standard deviation of the clinical measure. δ = a meaningful measure of difference (230).

A clinical statistician or statistical computer programme can be used to calculate the sample size. It is also important also account for patient dropout and non-compliance and this can be factored in as an allowance percentage.

**Study Statistics**

Although a large number of microarray studies show promising results their validity and reproducibility has been criticised (231), thereby casting doubt on high output analysis and reporting. These flaws have been summarised following a careful analysis of 90 array studies (232). Potential statistical problems were categorised into 3 groups

1. Finding genes that correlated with outcome (class comparison)
2. Class discovery
3. Supervised prediction (lack of a classifier (test or biomarker being investigated))

A particular problem in the studies was failing to acknowledge the false discovery phenomenon. A microarray experiment may generate thousands of results correlating to gene expression. If a traditional P value of 0.05, is set, it can lead to a large number of false positives, thereby masking truly significant candidates. Methods of controlling for false discovery include reducing the P value ($P<0.001$) when analysing individual genes and the Benjamini-Hochberg method of false discovery rate (FDR) control (233). A 10% rate of false

discovery is generally considered to be acceptable. Many studies analyse data using a student t-test. This must be interpreted with caution, as a t-test requires large amounts of independent data and repeatability. If this is not addressed it can lead to false significant differences. Over fitting of the data must also be avoided by taking into account prediction error estimation and ensuring results are reproducible in an independent set of samples, preferably by a different team (234). Bayesian statistics are sometimes used to overcome this problem. It is essential to ensure results from discovery experiments are legitimate to provide the right direction for the later phases.

To be of clinical value, the biomarker should be found to have a high level of sensitivity and specificity in the later stages of development (although this may change when used in different clinical settings (primary, secondary and tertiary care)). Sensitivity refers to the tests ability to correctly detect a disease (establishing a diagnosis). Specificity refers to the ability of the test to identify those patients without the disease (excluding a diagnosis). Within Medicine the terms positive predictive value and negative predictive value are more relevant. The positive predictive value (PPV) correlates to the certainty of a patient having the disease if tested positive whereas the negative predictive value (NPV) correlates to the certainty that a patient does not have the disease if tested negative. These values take into account the prevalence of the disease.

Receiver operating characteristic (ROC) graphs can be used to assess a tests diagnostic and predictive capacity (235). The ROC curve comprises a plot of 1 - specificity upon the x-axis against sensitivity on the y-axis. The area under the curve (AUC) correlates to the overall accuracy of the classifier, with the perfect test (high sensitivity and specificity) scoring 1.0. The model is commonly used for continuous data, thus an arbitrary ‘cut-off’ value which denotes positive and negative must be decided. Different thresholds result in different values for sensitivity and specificity. Again, sample size is an important factor to consider when carrying out experiments related to biomarkers and diagnostics (236).

### 1.4.5 Summary of Investigation of Biomarkers

- Biomarkers can correlate to disease diagnosis, activity, risk and prognosis and therefore enable clinicians to make informed decisions regarding patient management. In order to be useful in a clinical setting they need to fulfil a series of criteria.

- Proteomics and other technological advances enable high through put screening of a variety of biomarkers. Array profiling is a one such technology.

- Once identified biomarkers need to be tested in different phases to ensure their accuracy suitability for mainstream use. Initial discovery experiments often depend on archived tissues, whereas advanced phases should be carried out upon independent, blinded, prospectively collected samples. Initial discovery experiments may ‘set the scene’ for later phases, thus It is important to design and analyse the study carefully to avoid bias and chance findings. Important aspects include specimen collection, processing, quality, storage, assay performance, uniform reporting, and suitable statistical analysis. Case matching and choosing suitable controls/subjects with the correct features must be considered from the outset. Consequently, it may take many years to transcend through the phases and translate the primary findings into a fully validated, clinically useful test.

## 1.5 Scope and Aims of Thesis

### 1.5.1 Background

Mucosal inflammation is the hallmark of UC. Chronic inflammation and regeneration in the UC affected colon contributes to the development of dysplasia and subsequent progression to CAC. Current surveillance strategies rely upon repeated colonoscopies for the early detection and treatment of dysplasia. However colonoscopy based screening confers several challenges (Table 14). It is therefore necessary to develop new biomarkers to accurately monitor patients with UC. Many miRNA profiling studies have shown dysregulation of miRNAs in a broad range of disease states and suggest that they can act as biomarkers. Biomarker experiments must be carefully planned and carried out in different phases to ensure accuracy and reproducibility and to avoid false discovery.

**Table 14. Summary of Possible Pitfalls of Colonoscopy Based Screening**

| | **Disadvantages of Current Screening Program** |
|---|---|
| **Procedural** | • Colonoscopy has complications (bowel perforation)<br>• Colonoscopy performance depends on users experience, knowledge and availability of up-to-date equipment<br>• Dysplastic lesions are difficult to observe and can also be missed during random biopsies (sampling error)<br>• Inadequate bowel preparation results in a poor quality test, missed lesions and possible re-scheduling of the colonoscopy |
| **Patient Factors** | • Poor patient compliance due to invasive nature of test |
| **Reporting** | • Variation in histology reporting – difficult to standardize reports<br>• Difficult to establish histology (LGD and HGD or sporadic adenomas and DALMS). |
| **Administrative** | • If patients fail to attend a colonoscopy appointment a further colonoscopy will not be arranged resulting in loss of follow-up |

DALMS – Dysplastic Associated Lesions/Mass, HGD – High Grade Dysplasia, LGD – Low-grade dysplasia.

### 1.5.2 Hypothesis

Changes in the mucosa of patients with UC herald a continuum of neoplastic transformation from dysplasia to CAC that are associated with certain miRNAs. Subsequently, miRNA expression profiles can be detected in patients with UC and can act as biomarkers to monitor the progression of disease.

This hypothesis will be tested through the following three aims:

**Aim 1:** Characterise a cohort of patients with CAC to ensure suitability for sequential biomarker discovery

**Objectives for Aim 1:**

- Identify all patients with CAC in the Leicestershire region over a 12-year period.
- Establish the clinico-pathological presentation of CAC by analysing and comparing the variables age, duration of disease, tumour stage and grade of CAC at time of presentation to the known literature.

The results will be described in chapter 3: The clinico-pathological presentation of CAC in UK Population.

**Aim 2:** Discover a panel of deregulated miRNAs in the colonic mucosa of patients with UC and determine their correlation to various disease states: Active UC, Dysplasia and CAC.

**Objectives for Aim 2:**

- Identify ffpe tissue samples suitable for biomarker discovery and allocate them to the groups: UC, dysplasia and CAC (discovery series).
- Extract total RNA from tissues and process samples to enable quantification of gene expression via RT-qPCR
- Screen for differentially expressed miRNAs by conducting high throughput miRNA profiling using Taqman microfludic cards™.
- Validate results in an independent cohort using individual miRNA assays

The results will be described in chapter 4: MiRNA Expression Profiles in the colonic mucosa of patients with Ulcerative Colitis

**Aim 3:** Determine a panel of miRNAS associated with the pathology and progression of disease in the plasma of patients with Ulcerative Colitis and evaluate the potential of these miRNAs to act as blood-based biomarkers for the surveillance of UC patients.

**Objectives for Aim 3.**

- Identify and prospectively collect blood samples from patients undergoing colonoscopic screening for UC.
- Use histological results of biopsies taken during the colonoscopy to allocate the samples to the groups: UC, PSC, Dysplasia and CAC.
- Screen differentially expressed miRNAs by conducting high throughput miRNA profiling using taqman microfludic cards™.
- Validate results using individual miRNA assays
- Investigate if the miRNAs previously identified in colonic mucosa are also detectable in the circulation of patients with UC

The results will be described in chapter 5: miRNA Expression Profiles in the Circulation of Patients with Ulcerative Colitis

The workflow for the aims and objectives are summarised in (Figure 6).

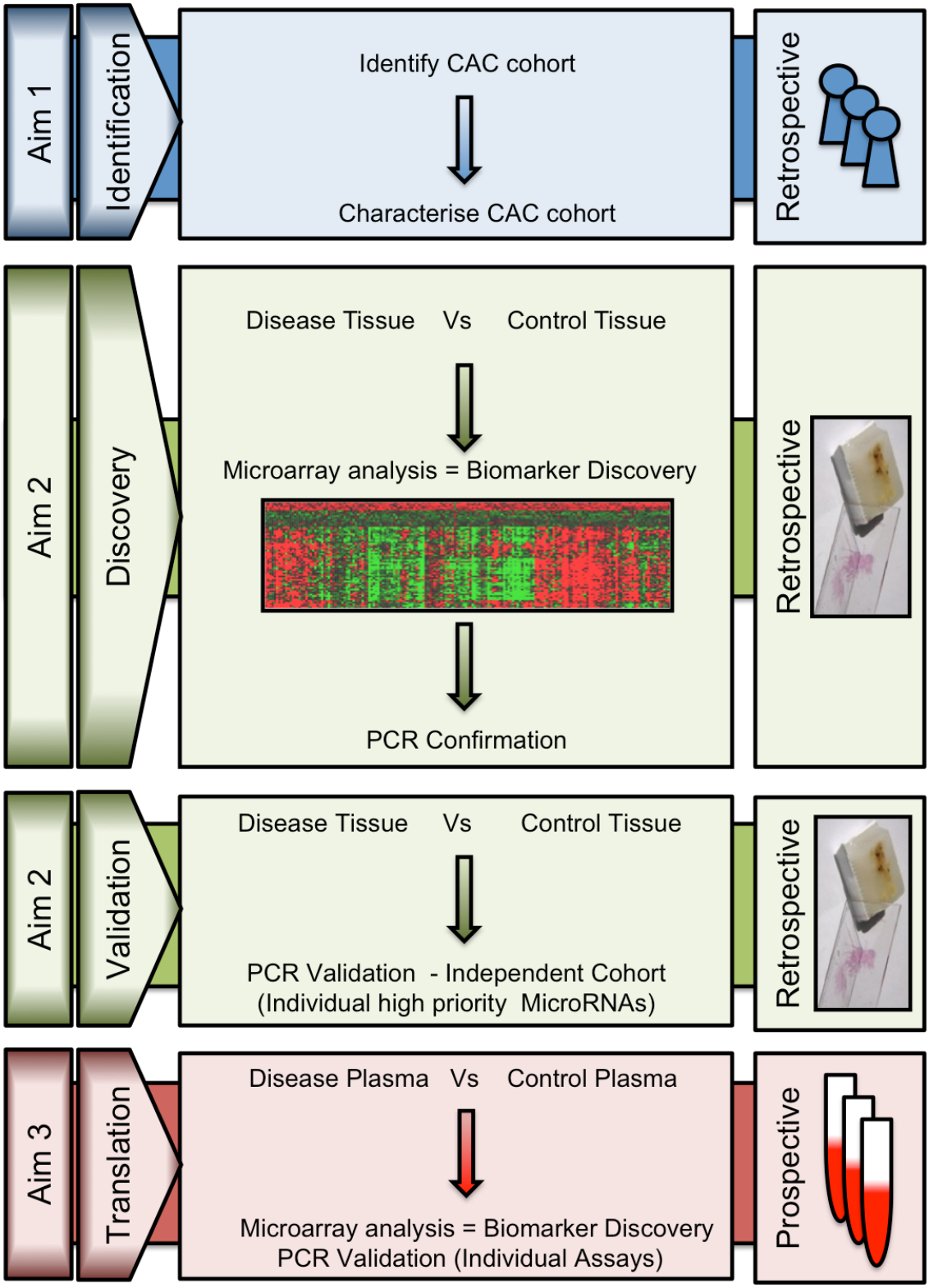



**Figure 6. Workflow of Experiments in this Thesis.**

CAC – colitis associated cancer. PCR – polymerase chain reaction.

Aim one will focus upon characterizing a patient cohort of CAC patients for future biomarker discovery. Aim two will utilise archived tissue to determine differentially expressed microRNAs. Aim 3 will use prospectively collected plasma samples determine differentially expressed microRNAs.

# Chapter 2. Materials and Methods

## 2.1 Ethical Approval

Prior to the commencement of this study, the online Integrated Research Application system (IRAS) was used to complete a mandatory data set required for all social and medical research-taking place in England. Patient materials such as consent forms and information leaflets pertaining to the collection, storage and use of patient's tissues and blood were also submitted. The University of Leicester (UoL) took on the role of the sponsor. The project started once approval was gained from the National Research Ethics committee (NREC) (Nottingham NREC reference: Biomarkers of Bowel Disease - 10/H0408/11) and the University Hospitals of Leicester (UHL) research and development (R&D) department (REGPR11005).

## 2.2 Sample Identification, Collection and Characterisation

### 2.2.1 Identification of Patients With CAC

For the purpose of this study, CAC was defined as any patient who had developed a colorectal cancer and had a concurrent or previous histological diagnosis of UC. The following approaches were used to identify patients in the Leicestershire region who had developed CAC in the last 15 years.

- Surgical treatment for UC refractory to medical treatment or UC related dysplasia or malignancy commonly entails a panproctocolectomy (resection of entire colon and rectum) or a subtotal colectomy (removal of colon without complete resection of the rectum). The UHL information department generated a list of all patients undergoing these procedures during 1999 – 2014. This process also identified a cohort of patients who had undergone surgery for acute severe UC.

- Further cases of CAC were identified from the UHL diagnostic histopathology database (APEX) by conducting several searches using the systematised nomenclature of medical clinical (SNOMED) terms: panproctocoelctomy, subtotal colectomy, dysplasia and ulcerative colitis.

- The UHL regional colorectal database was interrogated by a UHL data analyst to identify cases of CAC.

- Surgical consultants were emailed and asked to search their own records for any known previous cases of CAC.

A comprehensive list of all potential CAC patients was compiled and the corresponding histology reports were obtained and reviewed. Medical records and the relevant clinical details for cases of CAC were extracted and entered into an Excel file. The data was analysed using Excel and SPSS to ensure the cohort was suitable for the next stage (described in Chapter 3).

### 2.2.2 Identification of CAC Tissues For Total RNA Extraction

Formalin fixed paraffin embedded (FFPE) tissue was chosen as a tissue source for this component of the study. Formalin fixation provides excellent preservation of tissues subsequently enabling their use in research after long periods of time. Furthermore the hospital histopathology archive contained a significant number of CAC cases, which may not be possible to acquire if collected prospectively. Histology reports (obtained previously) and paraffin blocks were reviewed in order to identify cases suitable for inclusion (Table 15).

**Table 15. Inclusion and Exclusion Criteria for Archived Tissue Cases**

| Inclusion Criteria | Exclusion Criteria |
|---|---|
| • Clear histological evidence of UC or previous histological diagnosis | • Questionable diagnosis (possibility of CD or IDC) |
| • Dysplasia or CAC lesions arising in areas of UC | • Cases affected by infection (CMV colitis) or ischemic colitis |
| • FFPE tissue blocks contained enough tissue for ≥ 5 slides. | • FFPE tissue block contained insufficient tissue |

UC – Ulcerative Colitis, CAC – Colitis Associated Cancer, IDC – Indeterminate Colitis, CD – Crohns Disease, FFPE – Formalin Fixed Paraffin Embedded, CMV – Cytomegalovirus.

Patient demographics for the discovery and validation cohort are described in Table 16 and Table 17 respectively.

**Table 16. Demographics for Patients in the Discovery Series**

| Group | Specimen Tissue Type | Sex | Age | Operation | Disease Duration (years) | Mucosal Site |
|---|---|---|---|---|---|---|
| **Group** | Adenocarcinoma (T1 N0 M0) | M | 61 | Panproctocolectomy | ? | Rectum |
| **A** | Adenocarcinoma (T1 N0 M0) | F | 52 | Panproctocolectomy | 28 | Ascending Colon |
| | Adenocarcinoma (T3 N1 M0) | M | 61 | Panproctocolectomy | 9 | Left Colon |
| | Adenocarcinoma (T3 N2 M0) | M | 42 | Panproctocolectomys | 25 | Rectum |
| **Group** | Active Chronic UC | M | 61 | Panproctocolectomy | unknown | Caecum |
| **B** | Chronic UC | F | 52 | Panproctocolectomy | 28 | Rectum |
| | Chronic UC | M | 61 | Panproctocolectomy | 9 | Ascending Colon |
| | Chronic UC | M | 42 | Panproctocolectomy | 25 | Large bowel |
| **Group** | LGD | F | 24 | Subtotal Colectomy | 6 | Descending Colon |
| **C** | LGD | M | 61 | Panproctocolectomy | ? | Rectum |
| | LGD | F | 32 | Panproctocolectomy | 14 | Rectum |
| | LGD | M | 60 | Panproctocolectomy | 20 | Rectum |
| **Group** | Chronic UC | F | 24 | Subtotal Colectomy | 6 | Sigmoid/ Rectum |
| **D & F** | Active Chronic UC | M | 61 | Panproctocolectomy | unknown | Caecum |
| | Chronic UC | F | 32 | Panproctocolectomy | 14 | Ascending Colon |
| | Chronic UC | M | 60 | Panproctocolectomy | 20 | Descending Colon |
| | Chronic UC | M | 61 | Panproctocolectomy | 9 | Ascending Colon |
| | Chronic UC | M | 70 | TEMS | 27 | Rectum |
| **Group** | HGD | M | 61 | Panproctocolectomy | 9 | Rectum |
| **E** | HGD | M | 70 | Panproctocolectomy | 30 | Rectum |
| | HGD | M | 79 | Panproctocolectomy | 9 | Rectum |
| | HGD | M | 70 | TEMS | 27 | Rectum |
| **Group** | Severe Acute UC | M | 40 | Subtotal Colectomy | 1 | Ascending Colon |
| **G** | Severe Acute UC | M | 21 | Subtotal Colectomy | 1 | Sigmoid |
| | Severe Acute UC | F | 27 | Subtotal Colectomy | New Diagnosis | Ascending Colon |
| | Severe Acute UC | F | 28 | Subtotal Colectomy | 2 | ? |
| **Group** | Adjacent Normal of UC | M | 40 | Subtotal Colectomy | 1 | Caecum |
| **H** | Adjacent normal of UC | M | 21 | Subtotal Colectomy | 1 | Caecum |
| | Adjacent normal of UC | F | 27 | Subtotal Colectomy | New Diagnosis | Caecum |
| | Adjacent Normal of UC | F | 28 | Subtotal Colectomy | 2 | ? |

CAC – Colitis Associated Cancer, UC – Ulcerative Colitis., TEMS – Transanal endoscopic microsurgery (Surgical technique used for the removal of a small rectal cancer or polyp), TMN – Tumour staging system.

**Table 17. Demographics of the Patients in The Archived Tissue Validation Series**

| Case | Age | Sex | Specimen | Tissue Type | Pathology Summary |
|---|---|---|---|---|---|
| 1 | 54.62 | M | Panproctocolectomy | CAC Rectum | Adenocarcinoma Dukes B (pT3 pN0) Chronic UC |
| 2 | 88.22 | F | Anterior Resection | CAC Left Side | Adenocarcinoma Duke's A (pT2 pN0 pMx) Chronic UC |
| 3 | 76.00 | M | Panproctocolectomy | CAC Rectum | Adenocarcinoma Dukes B (pT3, pN0, pMx) (mucinous) Chronic UC |
| 4 | 53.81 | F | Subtotal colectomy | CAC Rectum | Adenocarcinoma Dukes A Quiescent UC + severe dysplasia |
| 5 | 64.07 | M | Panproctocolectomy | CAC Rectum | Adenocarcinoma (pT4, pN0, pMx) Quiescent UC |
| 6 | 42.28 | F | Panproctocolectomy | CAC Right Side | Adenocarcinoma, Dukes B (pT4, pN0, pMx) Quiescent UC |
| 7 | 55.69 | M | Panproctocolectomy | CAC Right Side | Adenocarcinoma, Dukes B, (pT3/4, pN0) (mucinous) Chronic UC |
| 8 | 75.25 | F | Right Hemicolectomy | CAC Right Side | Adenocarcinoma (pT4, pN2) (Poorly differentiated signet ring type). Active, chronic UC. |
| 9 | 56.05 | M | Panproctocolectomy | CAC Multiple Right Side | 1. Adenocarcinoma, Duke's C1 (pT3 pN1 pMX). 2. Adenocarcinoma, Duke's A (pT2 pN0 pMX) 3. Adenocarcinoma, Duke's B (pT3 pN0 pMX Active, chronic UC + DALMS |
| 10 | 32.08 | M | Palliative resection | CAC Left side | Adenocarcinoma recurrent poorly differentiated (perforation). Active chronic UC and dysplasia |
| 11 | 49.98 | M | Panproctocolectomy | CAC Right side | Adenocarcinoma Dukes' C1, pT3, pN2, pMX (mucinous) Chronic UC and dysplasia |
| 12 | 67.41 | F | Panproctocolectomy | CAC Right-side | Adenocarcinoma, Dukes' A (pT1 pN0 pMX) Active, Chronic UC with multiple DALMS |
| 13 | 55.01 | F | Panproctocolectomy | CAC Rectum | Adenocarcinoma Dukes C1 (C1 pT2, pN1, pMX) (moderate differentiation) Active chronic UC and dysplasia |
| 14 | 76.23 | F | Panproctocolectomy | CAC Rectum | Adenocarcinoma (pT3 pN1) (moderately differentiated) Mild active, chronic UC |
| 15 | 53.30 | M | Panproctocolectomy | CAC Rectum | Adenocarcinoma, Dukes C1 (pT3 pN1 pMX) (moderately differentiated) Active, chronic UC |
| 16 | 50.22 | F | Panproctocolectomy | Dysplasia | DALM with low grade dysplasia and active, chronic UC |
| 17 | 26.05 | M | Subtotal colectomy | UC | florid active ulcerative colitis |
| 18 | 25.76 | M | Subtotal colectomy | UC | active ulcerative colitis with pseudopolyps. |
| 19 | 27.98 | M | Subtotal colectomy | UC | fulminant ulcerative colitis |
| 20 | 17.55 | M | Subtotal Colectomy | UC | severely active ulcerative colitis with multifocal deep ulceration and focal surface peritonitis |
| 21 | 38.93 | M | Subtotal colectomy | UC | severe active distal colitis |
| 22 | 14.86 | F | Subtotal colectomy | UC | fulminant ulcerative pancolitis. |
| 23 | 20.56 | F | Subtotal colectomy | UC | prominent ulcerative colitis more pronounced distally and affecting the distal resection margin |
| 24 | 29.92 | F | Subtotal colectomy | UC | fulminant ulcerative colitis. |
| 25 | 38.16 | F | Subtotal colectomy | UC | proximally quiescent, distally, severe active follicular proctitis |
| 26 | 33.75 | M | Subtotal colectomy | UC | large complex inflammatory polyp against a background of chronic active ulcerative colitis |
| 27 | 34.18 | M | Subtotal colectomy | UC | distal active ulcerative colitis modified by treatment |
| 28 | 53.58 | M | Subtotal colectomy | UC | active chronic ulcerative colitis |
| 29 | 37.18 | F | Subtotal colectomy | UC | active chronic ulcerative colitis |
| 30 | 33.46 | M | Subtotal colectomy | UC | chronic ulcerative colitis with distal active inflammation |
| 31 | 36.07 | M | Subtotal colectomy | UC | mildly active ulcerative colitis with chronic mucosal Changes + dysplasia |
| 32 | 26.23 | M | Subtotal colectomy | UC | active chronic ulcerative colitis |
| 33 | 26.46 | F | Subtotal colectomy | UC | active chronic ulcerative colitis |
| 34 | 24.68 | F | Subtotal colectomy | UC | extensive severe active chronic ulcerative colitis |
| 35 | 27.77 | M | Subtotal colectomy | UC | active chronic ulcerative colitis |

CAC – Colitis Associated Cancer, UC – Ulcerative Colitis. DALM – dysplastic associated lesion/mass. M – Male, F-Female. The pathology summary reflects the information given upon the histology report. There was a huge variation in the report forms, with some tumours staged using the Dukes system and some with the TMN system. Furthermore the earlier report forms contained a very brief description of the tumours, whereas the recent report forms tended to contain detailed information regarding grade and pathological features such as vascular and perilymphatic involvement.

### 2.2.3 Identification and Recruitment of Patients For Blood Sampling

This study used opportunistic sampling. Blood samples were prospectively collected from patients with UC, undergoing colonoscopic screening as part of their routine management. Blood samples were also obtained from range of patients undergoing colonoscopy for investigation of bowel related symptoms. Patients suitable for the study were identified via the Endoscopy department at the University Hospitals of Leicester. All patients were provided with complete study information in advance and fit the inclusion criteria (Table 18).

**Table 18. Inclusion and Exclusion Criteria For Patients Recruited for Phase Three - Blood Sampling**

| Inclusion Criteria | Exclusion Criteria |
|---|---|
| • Patients undergoing colonoscopy for investigation of large bowel symptoms | • Patient below 18 year of age |
| • Patients undergoing colonoscopy for a previous diagnosis of UC | • Patients with known diagnosis of Hepatitis B, HIV or jaundice |
| • Patients must be able to read information leaflet and give valid consent | • Patients unable to provide valid consent |
| | • Patient should not have a current or recent diagnosis of non-CAC related malignancy |

UC – Ulcerative Colitis, CAC – Colitis Associated Cancer, HIV – Human Immunodeficiency Virus

Blood samples and clinical information were obtained prior to the colonoscopy. Colonoscopy and histology reports were obtained following the colonoscopy. Samples were then further excluded from the study if:

- The colonoscopy was incomplete (did not reach the ileo-ceacum or the patient did not tolerate the procedure)
- The endoscopist was unable to visualise the entirety of the colon (poor bowel preparation)
- Biopsies were not taken during the procedure
- Histology reports suggested IDC, infectious colitis or infection complicating existing IBD.
- A sigmoidoscopy was performed instead of a colonoscopy

Patients were designated into a cohort groups (Normal, UC, CD, CAC, Dysplasia, PSC) depending on their biopsy based histological diagnosis. To be designated into the UC group the patient's required histological evidence of UC or had previously been diagnosed with UC (prior histological diagnosis of UC). Patients designated as dysplastic or CAC, had to have their lesions arising in areas of UC. Patients who had 'normal histology' (no diagnosis of bowel disease or evidence of intestinal inflammation) and did not have a previous diagnosis of bowel disease were classed as 'symptomatic normals'. These were generally younger patients who had presented with a short duration of bowel symptoms. It also seemed sensible to deduce that this cohort of 'younger' patients were highly unlikely to harbour undetectable malignant changes. Thus, even though these patients may have presented with bowel symptoms, they were categorised as normal.

In the context of IBD, duration of disease was defined as time elapsed from first histological diagnosis of IBD. It was recognized that the value obtained for duration of disease was not entirely accurate as the patient may have been symptomatic for a long period of time prior to diagnosis. The UC cohort could have been further sub-classified into WHO criteria disease extent (E1 proctitis, E2 Left sided colitis, E2 pancolitis), disease activity (quiescent, mild, moderate, severe) and inflammation type (acute, chronic), however this proved extremely difficult due to the extreme variation in the histology reports.

Demographics for these patients are described in Table 19 and summarised in Table 20.

**Table 19. Demographics of Patients Recruited For Phase 3: Blood Sampling**

| Procedure | Age | Sex | Duration | Colonoscopy | Histology |
|---|---|---|---|---|---|
| Pre-Colonoscopy | 59.59 | M | 11 | Quiescent disease & Pseudopolyps | Normal |
| Pre-Colonoscopy | 49.14 | M | 39 | Normal to insertion | Normal |
| Pre-Colonoscopy | 18.67 | M | 1 | Iletis | Normal |
| Pre-Colonoscopy | 63.28 | M | 29 | Mild active left sided disease | Quiescent disease |
| Pre-Colonoscopy | 72.41 | M | 14 | Quiescent disease & Pseudopolyps | Normal & tubular adenoma (LGD) |
| Pre-Colonoscopy | 43.21 | M | 15 | Mild active proctitis | Mild Proctitis |
| Pre-Colonoscopy | 57.38 | F | 23 | Normal to insertion | Mild Proctitis |
| Pre-Colonoscopy | 53.30 | M | 13 | Mild active pancolitis | Quiescent disease |
| Pre-Colonoscopy | 50.72 | F | 10 | Quiescent disease | Mild Active disease |
| Pre-Colonoscopy | 64.97 | M | 2 | Quiescent disease | Quiescent disease |
| Pre-Colonoscopy | 33.84 | M | 2 | Mild distal Crohns disease | Mild non-specific abnormality |
| Pre-Colonoscopy | 64.26 | M | 25 | Quiescent disease & Polyp | Quiescent disease |
| Pre-Colonoscopy | 67.95 | M | 10 | Normal to insertion + Polyp | Tubular Adenom (LGD) |
| Pre-Colonoscopy | 56.63 | M | 19 | Diverticular disease Normal to insertion | Normal |
| Pre-Colonoscopy | 28.31 | M | 3 | Normal to insertion | Normal |
| Pre-Colonoscopy | 58.45 | F | 12 | Quiescent disease | Quiescent disease |
| Post Colonoscopy | 41.81 | M | 25 | Poly/Dalm rectosigmoid | Adenocarcinoma |
| Pre-Colonoscopy | 45.71 | F | 11 | Mild Active rectosigmoiditis | Mil active and chronic UC |
| Pre-Colonoscopy | 47.98 | F | 20 | Mild active pancolitis | Mildly active chronic disease |
| Pre-Colonoscopy | 62.40 | F | 15 | Mild active pancolitis | Extensive active chronic UC with pseudopolyps |
| Pre-Colonoscopy | 57.15 | M | 23 | Mild Patchy erythema & polypectomy | Chronic quiescent disease |
| Pre-Colonoscopy | 39.04 | F | 12 | Mild Left sided | Left sided Colitis Quiescent disease |
| Pre-Colonoscopy | 44.89 | M | 8 | Moderately Active pancolitis | Severe chronic active pan-UC |
| Pre-Colonoscopy | 32.42 | M | / | Normal to the point of insertion | Normal Mucosa |
| Pre-Colonoscopy | 57.51 | M | 2 | Normal to point of insertion | Normal Mucosa |
| Pre-Colonoscopy | 68.62 | F | 33 | Mild Proctitis & right sided colitis | Normal Mucosa |
| Pre-Colonoscopy | 58.99 | F | 36 | Mild Proctitis & right sided colitis | Mild right sided colitis / Indeterminate colitis |
| Pre-Colonoscopy | 51.61 | M | 7 | Indeterminate colitis, post-inflammatory polyps | Normal Mucosa |
| Pre-Colonoscopy | 47.48 | M | 20 | Moderately active distal colitis | Severe active chronic sigmoid-proctitis |
| Pre-Colonoscopy | 59.96 | F | 23 | Moderately severe pancolitis & polyps | Mild active pancolitis & inflammatory polyps |
| Post Colonoscopy | 64.68 | M | 6 | Moderately active proctosigmoiditis & polyp | Rectal DALM |
| Pre-Colonoscopy | 66.10 | M | 24 | Normal to point of insertion | Quiescent disease |
| Pre-Colonoscopy | 43.30 | M | 13 | Mild pancolitis & inflammatory polyps | Quiescent disease |
| Pre-Colonoscopy | 54.90 | M | 5 | Mild pancolitis & inflammatory polyps | Quiescent disease |
| Pre-Colonoscopy | 75.38 | M | 4 | Mild proctitis | Quiescent disease |
| Pre-Colonoscopy | 60.32 | F | 40 | Left sided mild colitis | Active UC |
| Pre-Colonoscopy | 32.09 | M | 10 | Left sided mild colitis | Chronic quiescent disease |
| Post -Colonoscopy | 51.16 | F | 37 | / | Rectal CAC |
| Pre-Colonoscopy | 27.03 | M | 3 | Severe recto-sigmoid UC | Severe active sigmoid-proctitis |
| Pre-Colonoscopy | 48.64 | F | 6 | Normal to point of insertion | Normal |
| Pre-Colonoscopy | 48.38 | F | 43 | Quiescent disease | Inconclusive |
| Pre-Colonoscopy | 72.68 | M | 16 | Moderately Active pancolitis | Focally Active UC |
| Pre-Colonoscopy | 61.01 | M | 28 | Quiescent disease | Quiescent disease |
| Pre-Colonoscopy | 41.03 | M | 10 | Quiescent disease & Pseudopolyps | Quiescent disease |
| Pre-Colonoscopy | 32.19 | M | 4 | Quiescent disease / mild chronic disease | Diffuse chronic |

| | | | | | |
|---|---|---|---|---|---|
| Pre-Colonoscopy | 53.56 | M | 13 | Severe pancolitis | Active chronic UC |
| Pre-Colonoscopy | 44.95 | F | 10 | Normal to insertion | Normal Mucosa |
| Pre-Colonoscopy | 39.40 | F | 30 | Quiescent disease | Normal / focal paneth cell metaplasia |
| Pre-Colonoscopy | 48.39 | F | 13 | Pancolitis | Quiescent disease |
| Pre-Colonoscopy | 45.32 | F | 20 | Normal to insertion | Chronic quiescent UC |
| Post Colonoscopy | 53.98 | M | 20 | / | Rectal adenocarcinoma / HGD |
| Pre-Colonoscopy | 37.40 | M | 3 | Mild active left sided UC with inflammatory polyps | Quiescent UC |
| Pre-Colonoscopy | 44.08 | M | 25 | Active chronic pancolitis | Active Chronic UC |
| Pre-Colonoscopy | 32.25 | M | 11 | Moderate pancolitis | Active Chronic UC with inflammatory polyp |
| Pre-Colonoscopy | 55.78 | F | 16 | Recto-sigmoid colitis | Quiescent UC |
| Pre-Colonoscopy | 22.73 | F | 4 | Pancolitis | Active Chronic UC |
| Pre-Colonoscopy | 32.85 | M | 9 | Pancolitis | Mild active chronic UC with rectal sparing |
| Pre-Colonoscopy | 39.33 | M | 14 | Quiescent disease | Quiescent disease |
| Pre-Colonoscopy | 51.18 | M | 32 | Quiescent disease | Normal (no UC) |
| Pre-Colonoscopy | 53.08 | M | 11 | Right Sided Disease | Normal (no UC) |
| Pre-Colonoscopy | 63.99 | M | 37 | Normal to point of insertion | No significant pathology |
| Pre-Colonoscopy | 62.15 | M | 8 | Pancolitis | minimally active UC |
| Pre-Colonoscopy | 36.43 | M | 4 | Procto-sigmoiditis | Mild Active chronic UC |
| Pre-Colonoscopy | 61.25 | F | 19 | Quiescent disease | Normal (no UC) |
| Pre-Colonoscopy | 78.32 | F | 15 | Pancolitis mod-severe | Severe Chronic changes |
| Pre-Colonoscopy | 42.92 | F | 10 | Normal to point of insertion | Normal (no UC) |
| Pre-Colonoscopy | 46.15 | F | 16 | Quiescent disease | Patchy left sided inflammation |
| Pre-Colonoscopy | 68.40 | M | 28 | Pancolitis mod-severe | Polyps and mild architectural disturbance |
| Post-Colonoscopy | 67.63 | M | 37 | Pancolitis mild active | Low grade Dysplasia transverse colon |
| Pre-Colonoscopy | 46.56 | M | 19 | Pancolitis mild active | Mild active UC |
| Pre-Colonoscopy | 70.74 | F | 11 | Diverticulosis and pancolitis mild active | Minor non specific inflammation in sigmoid colon |
| Pre-Colonoscopy | 53.40 | F | 11 | Pancolitis mild active | Chronic Quiescent colitis |
| Pre-Colonoscopy | 65.85 | M | 5 | Quiescent disease pancolitis & polyps | Chronic UC with polyps |
| Pre-Colonoscopy | 41.15 | M | 11 | Indertimante Colitis | Active UC |
| Pre-Colonoscopy | 65.23 | M | 31 | Quiescent disease | Quiescent disease |
| Pre-Colonoscopy | 62.10 | M | 35 | Normal to point of insertion | Normal (no UC) |
| Pre-Colonoscopy | 57.22 | F | 30 | Normal to point of insertion | Quiescent disease |
| Pre-Colonoscopy | 25.44 | M | / | Pancolitis | Quiescent disease |
| Pre-Colonoscopy | 49.79 | M | 19 | Colonic polyps | Normal mucosa low grade tubular adenoma |
| Pre-Colonoscopy | 76.50 | F | 11 | Colitis | Active chronic UC |
| Pre-Colonoscopy | 43.27 | F | 6 | Colitis | Normal (no UC) |
| Pre-Colonoscopy | 63.93 | M | 3 | Pancolitis mild active | Mild Active chronic UC |
| Pre-Colonoscopy | 52.90 | F | 29 | Quiescent disease - proctosigmoid | Quiescent disease |
| Pre-Colonoscopy | 56.11 | F | 29 | Normal to point of insertion | Quiescent disease |
| Pre-Colonoscopy | 39.67 | M | 13 | Proctitis | Normal (no UC) |
| Pre-Colonoscopy | 52.53 | M | 8 | Quiescent disease pancolitis & polyps | Normal (no UC) |
| Pre-Colonoscopy | 59.44 | M | 4 | Pancolitis mild active | Mild Active chronic UC |
| Pre-Colonoscopy | 73.50 | M | 15 | Quiescent disease pancolitis & polyps | Quiescent disease |
| Pre-Colonoscopy | 61.63 | M | ?? | Quiescent disease | Quiescent disease |
| Pre-Colonoscopy | 77.22 | M | 18 | Pancolitis | Quiescent disease |
| Pre-Colonoscopy | 72.07 | M | 15 | Mild left sided disease | Non-specific Chronic inflammation |
| Pre-Colonoscopy | 40.64 | F | 5 | Mild Pancolitis | Chronic UC |
| Pre-Colonoscopy | 61.56 | F | 21 | Quiescent disease pancolitis | Quiescent disease |
| Pre-Colonoscopy | 61.28 | F | 41 | Pancolitis mild active | Quiescent disease |
| Pre-Colonoscopy | 77.43 | F | 41 | Quiescent disease pancolitis | Quiescent disease |
| Pre-Colonoscopy | 54.96 | M | 10 | Pancolitis mild active | Non-specific Chronic inflammation |

| | | | | | |
|---|---|---|---|---|---|
| Post-Colonoscopy | 67.98 | M | ?? | LGD | / |
| Pre-Colonoscopy | 56.31 | M | 17 | Normal to point of insertion | Normal (no UC) |
| Pre-Colonoscopy | 42.94 | M | 10 | Quiescent disease | Mild Active chronic UC |
| Pre-Colonoscopy | 73.01 | F | 40 | Pancolitis mild active HAD AP resection in past for rectal cancer | Normal (no UC) |
| Pre-Colonoscopy | 51.13 | M | 12 | Pancolitis- quiescent disease | Quiescent disease |
| Post-Colonoscopy | 59.40 | M | 25 | ?DALM | LGD |
| Pre-Colonoscopy | 76.71 | M | | Normal to point of insertion | Non-specific Chronic inflammation |
| Colonoscopy | 49.58 | M | ?? | pancolitis ulcerated stricture | POST RIGHT HEMI -Dysplasia |
| Pre-Colonoscopy | 57.17 | M | 6 | Active pancolitis with multiple small ulcers | Minimally active UC |
| Pre-Colonoscopy | 30.80 | F | 16 | Moderately active Left sided disease | Active Inflammation but not specific for IBD |
| Pre-Colonoscopy | 41.59 | M | 18 | Indeterminate pancolitis | Limited Inflammation |
| Post-Colonoscopy | 25.12 | F | 17 | Severe pancolitis & inflammatory polyps | Sigmoid DALM |
| Pre-Colonoscopy | 50.29 | M | 26 | Procto-sigmoiditis | Quiescent colitis ? If prior diagnosis |
| Pre-Colonoscopy | 51.61 | F | 8 | Normal to the point of exertion | Quiescent UC (if prior diagnosis made) |
| Pre-Colonoscopy | 38.51 | M | 8 | Stricture | Mild Active chronic inflammation |

UC – Ulcerative Colitis, HGD – high-grade dysplasia, LGD – low-grade dysplasia, DALM – dysplastic associated lesion/mass

**Table 20. Summary of Demographics For The Plasma Samples**

| | | | Females | Males |
|---|---|---|---|---|
| 1 | Number of Patients | | 39 | 72 |
| 2 | Mean Age / Range (years) | | 53.19 ( 22.73-78-32) | 52.47 (18.67-77.22) |
| 3 | Mean Disease Duration (years) | | 20.27 | 14.49 |
| 4 | Disease Duration | 0-9 years | 5 | 24 |
| | | 10-19 years | 17 | 26 |
| | | 20-29 years | 7 | 12 |
| | | >30 Years | 10 | 6 |
| | | Unknown | / | 4 |

## 2.3 Sample Preparation

### 2.3.1 Preparation of Tissue Samples

**Isolation of Total RNA and MicroRNA from FFPE Colonic Mucosa**

Each case correlated to a resected bowel specimen (Figure 7).

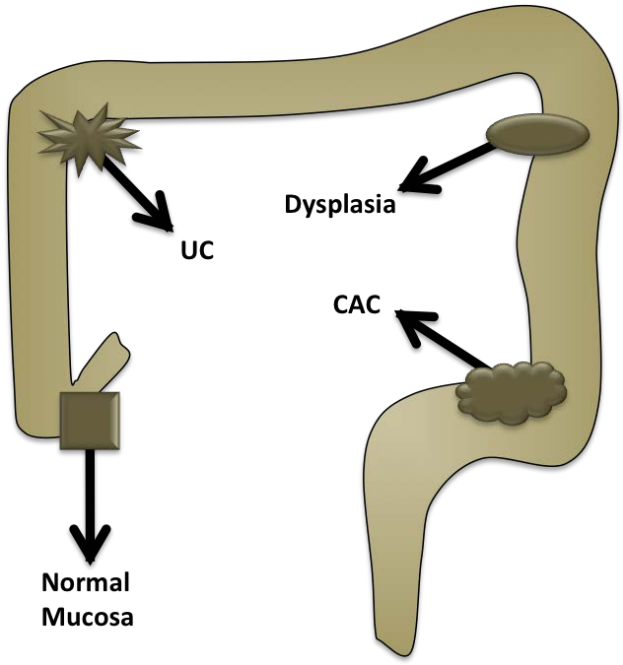



**Figure 7. Resected Bowel Specimen**

Cases were comprised of resected bowel specimens from patients who had undergone a subtotal or panproctocolectomy for Ulcerative Colitis. Therefore, each specimen contained an anatomical range of bowel areas and a possible range of pathology (UC- Ulcerative Colitis, dysplasia, CAC- Colitis Associated Cancer and normal mucosa). Cases were designated a caser number and subsequent tissue blocks a letter. The term 'normal mucosa' was a relative term applied to an area of bowel unaffected by active or chronic UC, dysplasia or CAC. It was acknowledged that the mucosa could have been affected by field change abnormalities and therefore was not 'normal' in the usual sense. Classifying the tissue in such a manner provided an opportunity to pair tissues and increase the number of tissue samples.

For the cases identified, the corresponding Haematoxylin and Eosin (H&E) slides (Figure 8) were requested from the histopathology archive and were examined by a consultant Histopathologist (KW) under a light microscope (E800 Nikon, Japan). The histology was confirmed and the epithelial area of interest marked upon the H&E slides.

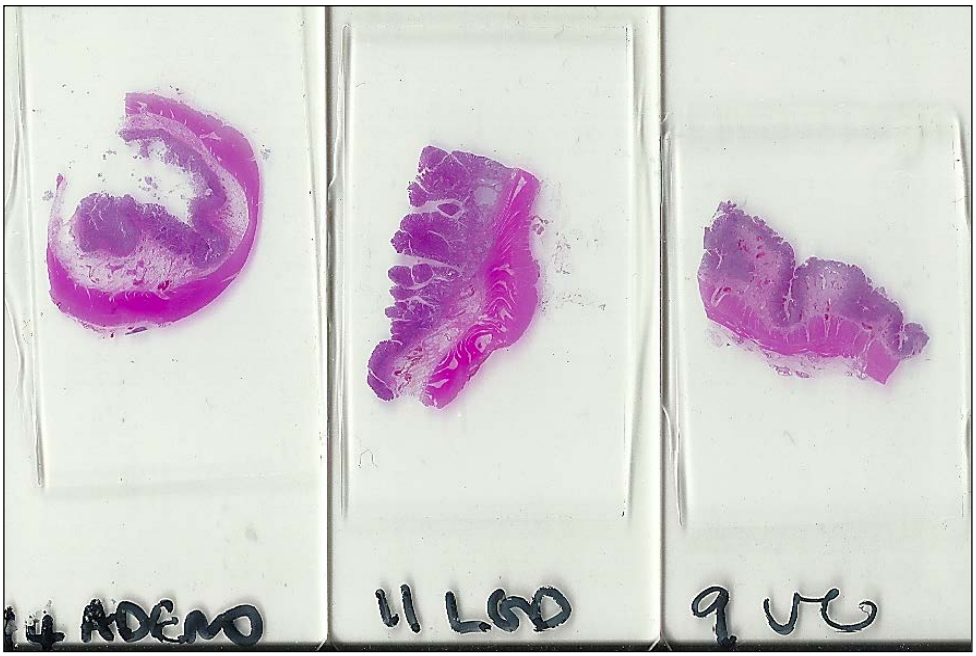



**Figure 8. Histology Slides**

Photographs of 3 different Haematoxyillin and Eosin stained tissue types (Adeno - Colitis Associated Carcinoma, LGD-Low Grade Dysplasia, and UC - Ulcerative Colitis) all from the same patient case.

The FFPE tissue blocks were retrieved from the histopathology department. Blocks containing insufficient quantities of tissue were returned to the archive to avoid the blocks being cut to extinction. The blocks were cut into 5 -10 5µm sections, mounted onto an untreated slide and dried overnight In a 37° incubator. The paraffin was removed from the tissues by immersing the slides in a series of ‘de-waxing’ solutions: xylene (Genta Environmental Ltd) and industrial methylated spirit (IMS) (Genta Medical). Using the original marked H&E slide as a reference, the epithelial tissue was micro-dissected from the slide using a 200µl pipette tip and placed into a 500µl buffer solution: 0.05 M Tris(hydroxymethyl)aminomethane-HCL pH8 / 0.1% sodium dodecyl sulphate (TRIS-SDS solution). Care was taken not to dissect the lamina propria as this layer contained large amounts of inflammatory cells due to the presence of mucosa associated lymphoid tissue (MALT) and may of skewed the results. 5µl of 10mg/ml proteinase-K (Roche) was added to the samples and incubated at 55°C. After 12 hours, the samples were removed, gently mixed and cooled on ice. 500µl of TRI-reagent (Sigma-Aldrich, Pool Dorset, UK) was added to each sample to achieve cell lysis. The lysates (total volume 1ml) were then mixed and stored at -20°C. Several slides were checked by H&E staining (Figure 9).

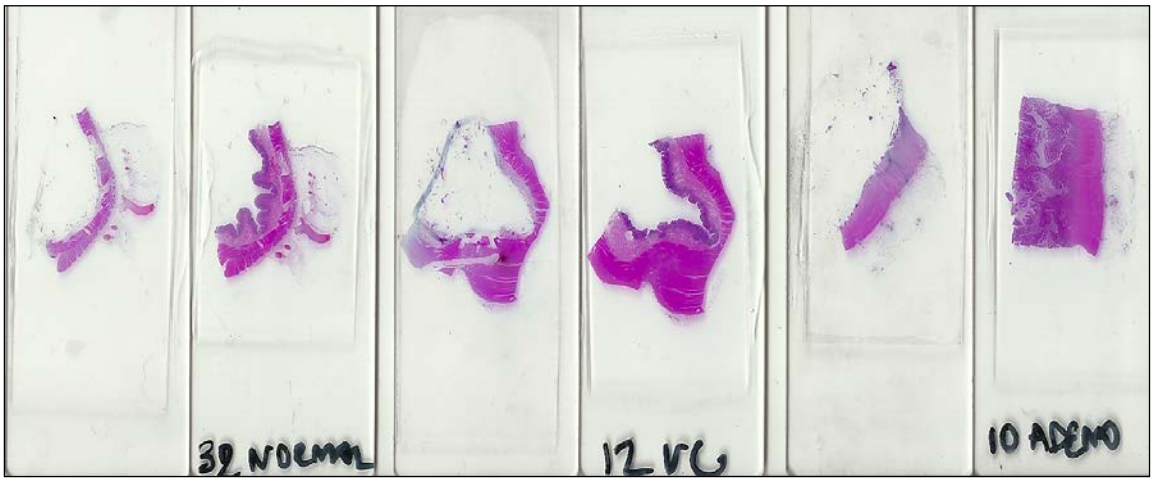



**Figure 9. Haematoxylin and Eosin Slides After Microdissection**

Three different histology slides after microdissection (next to their original reference slide). A comparison was made to ensure the microdissection was accurate.

## RNA Isolation

Lysate samples were thawed on ice and left to stand for five minutes. Phase separation was achieved by adding 100µl of chloroform (Sigma Life Sciences), mixing (Stuart Vortex) and centrifuging at 13000 rpm 4° for 15 minutes. The RNA containing aqueous phase was transferred into a sterile bijoux taking care to avoid the interphase and lower-phase components. Absolute ethanol (Fisher-Scientific) was added (1.25 x the original volume) to the aqueous phase, mixed and stored on ice. The Qiagen RNeasy mini-kit (Qiagen, Valencia CA) was then used to purify the precipitated Total RNA according to manufacturers instructions. The final RNA pellet was recovered, suspended in 30ul of RNAse free water and stored at -80C.

## Nanodrop Spectophometer

RNA was tested using the Nanodrop Spectrophometer (Thermo Scientific, Wilimington, Delaware). Sample concentration and absorbance values at 260nm (A260nm) and 280nm (A280nm), an indication of protein contamination were recorded. The peak and integrity of the absorption curves were also noted for each sample.

### Sample Selection

Only samples, which contained 18.5 ng/ul or more and were deemed to be of sufficient quality (A260/280 ratio above 1.5) were chosen for the subsequent applications. Unfortunately a large proportion did not meet the criteria and were excluded. Matched tissue samples comprised of adjacent normal tissue from the patient witch meant it was perfectly matched for variation in sex age race other genetic differences. A set of UC and normal tissues were also selected to investigate the range of microRNAs associated with inflammation. Ideally factors, which could affect the biomarker other than cancer and inflammation, should be taken into account (sex, age, race, co-morbidities and medications). Patient characteristics for the discovery and validation cohort have been described in Table 16 and Table 17 respectively.

## 2.3.2 Preparation of Blood Samples

### Centrifugation of Whole Blood to Isolate Plasma

Blood samples were drawn from patients prior to their colonoscopy, using a S-Monovette Haematology EDTA sample tube (Sarstedt, Germany) and processed within two hours of venepuncture (Figure 10).

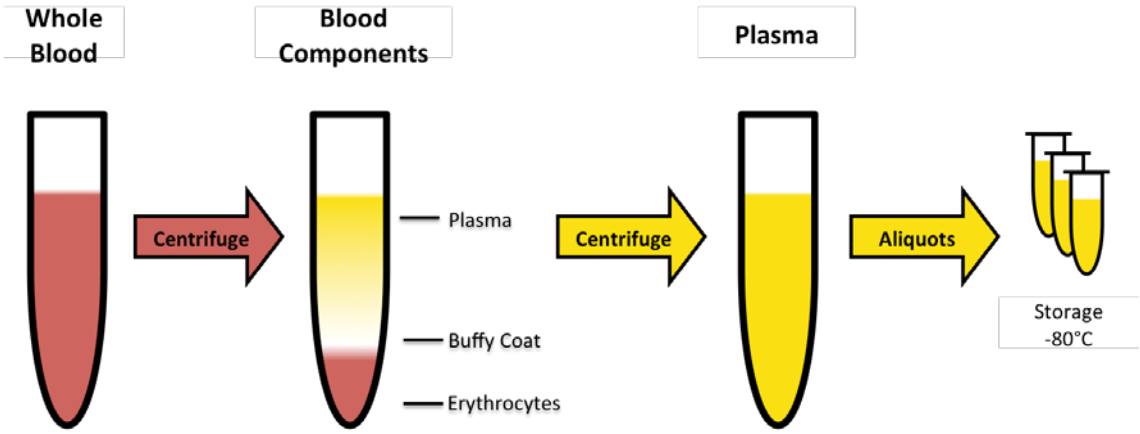



**Figure 10. Processing of Whole Blood Samples**

The whole blood was spun for 10 minutes in a Jouan centrifuge at 2000 rpm/850g, 4°C. The plasma component was decanted into a 15ml falcon tube, avoiding the Buffy coat, erythrocytes and cellular debris layers. The plasma was re-spun at the same settings and then stored in 1ml aliquots in eppdendorf tubes at -80°C for later use

### Isolation of Total RNA and MicroRNA

1ml aliquots of plasma were thawed on ice and 200ul taken for MicroRNA extraction per sample. 1ml of Qiazol Lysis reagent (Qiagen) was added, mixed by vortexting and incubated at room temperature for 5 minutes. 200µl of chloroform (Sigma-Aldrich) was added, mixed vigorously upon the vortex for 15 seconds and left to incubate for 15 minutes. The lysate was centrifuged for 15 minutes at 13000rpm and 600µl of the aqueous phase then transferred into a 1.5ml eppendorf. 900ul of aqueous alcohol was added, mixed and the sample processed using miReasy serum/plasma kit (Qiagen), according to manufacturers instructions. The resultant RNA was eluted in 30ul of RNase free water and stored at -80°C. The nanodrop was used to quantify the amount of RNA obtained from each sample. The concentrations of total RNA ranged from 1.5 – 19.9 ng/µl.

## 2.4 Sample Processing

In order to quantify the microRNAs by microarray or PCR, the total RNA derived from the tissue and plasma samples was processed in a series of reactions (Figure 11).

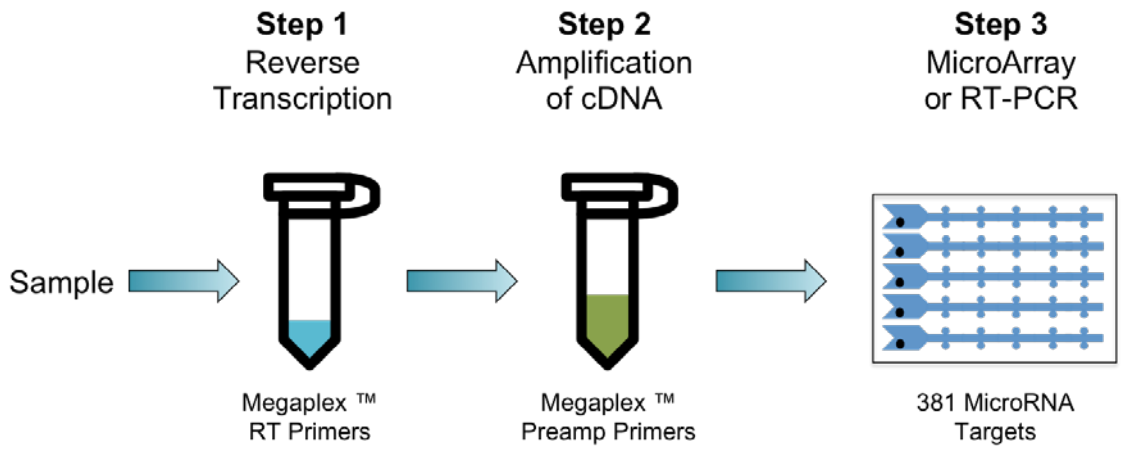



**Figure 11. Workflow for Gene Expression Profiling Using Taqman Megaplex Reagents**

Briefly, small RNAs were converted to complementary DNA (cDNA) and amplified using predefined pools of primers. This allowed for multiple microRNAs to be quantified through microarray cards or single microRNAs to be detected via real time quantitative PCR (RT-q-PCR).

### 2.4.1 Reverse Transcription

Samples were reversed transcribed using the Taqman ® microRNA reverse transcription (RT) kit (Applied Biosystems, Foster City, CA). Total RNA was converted to cDNA in a megaplex reaction using commercially available Taqman ® Human Pool A Megaplex™ RT ™ V2.1 primers. This set contained 380 unique stem-looped RT primers, enabling the simultaneous synthesis of single stranded cDNA for multiple, mature microRNAs transcripts, derived from the Sanger database. A negative plant control and 3 positive controls are included in the set. A small set of reactions devoid of reverse transcriptase enzyme was carried out separately.

The following master mix (MM) was used per sample:

- 0.8µl 10X RT primers
- 0.25µl dNTPS
- 1.5µl Multiscribe™ RT enzyme
- 0.8µl 10X RT buffer
- 1.03µl MgCl2
- 0.125µl RNAse inhibitor

Reactions were prepared in Applied Biosystems micro-AMP® into which, 5.5µl of each sample and 4.5µl of MM was added and then incubated upon ice for 5 minutes. The tissue derived total RNA was diluted to an average concentration of 18.5ng/µl (range 17ng/µl-20ng/µl) ensuring that each RT reaction contained a set concentration of total RNA (100ng/µl). For the reactions containing plasma derived total RNA, a fixed volume of 5.5µl was chosen instead as it was not possible to accurately measure concentrations. The samples were then processed in a Veriti Thermal cycler (Table 21). cDNA Samples (10µl total volume) were stored at -20°C.

**Table 21. Thermal Cyclying Profiles for Reverse Transcriptase and Pre-Amplification Reactions**

| Reverse Transcription Profile | | |
|---|---|---|
| Stage | Temperature | Time |
| Cycle (40 cycles) | 16°C | 5 minutes |
| | 42°C | 1 minute |
| | 50°C | 1 second |
| Hold | 85°C | 5 minutes |
| Hold: | 4°C | ∞ |
| | | |
| | | |

| Pre-amplification Profile | | |
|---|---|---|
| Stage | Temperature | Time |
| Hold | 95° C | 10 minutes |
| Hold | 55° | 2 minutes |
| Hold | 72° C | 2 minutes |
| Cycle (12 Cycles) | 95°C | 15 seconds |
| | 60°C | 4 minutes |
| Hold | 99.9°C | 10 minutes |
| Hold | 4°C | ∞ |

### 2.4.2 Pre-amplification

In order to detect microRNAS expressed at low levels within the sample, the cDNA was amplified using Megaplex™ PreAmp Primers, Human Pool A v2.1 (Applied Biosystems®). Each set contained gene specific forward and reverse primers intended for samples of low concentration or quantity. The following master mix was used per sample

- 2.5µl Pre-amp Primers
- 12.5µl Pre-amplification Master Mix
- 5.0µl Nuclease free water

Briefly, 5µl of cDNA was added to a micro-AMP® tube containing 20µl of MM, sealed, mixed and incubated for 5 minutes. The samples were processed in a Veriti Thermal cycler (Table 21). Once complete, 75µl of 0.1X Tris (hydroxymethyl)aminomethane (TRIS) / ethylenediaminetetraacetic acid (EDTA) (TE) physiological (PCR-compatible) buffer, pH 8.0 was added to each sample and the 1:4 diluted product stored at -20°C.

For the plasma derived samples an additional set of RT and pre-amplification reactions were carried out using Human Pool B v3.0 Megaplex™ RT and PreAmp Primers. Both A and B primer sets contained U6, allowing for batch comparisons later on.

### 2.4.3 Real time Quantitive Reverse Transcriptase PCR

Real-time-qPCR uses a DNA polymerase enzyme and sequence specific primers to amplify target cDNA. Initialisation of the reaction requires activation of the polymerase enzyme. This is followed by cycles of denaturisation, annealing and extension. During formation of the PCR products (amplicons) there is activation of the reporter dye and a subsequent fluorescent signal. The process is described as 'real-time' as the amplicons are quantified at each cycle, resulting in an amplification curve (Figure 12). The point at which the amplification plot intersects a set threshold value is termed the cycle threshold (CT) value. Smaller CT values indicate a greater abundance of the product within the reaction and earlier detection.

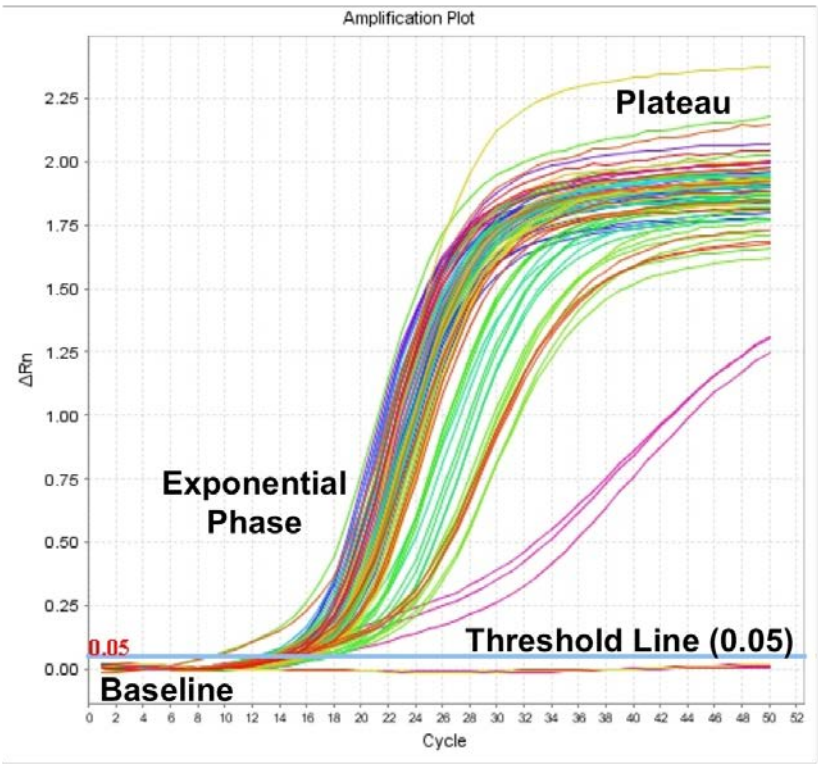



**Figure 12. Phases of the Amplification Curve**

The amplification curve has several phases. The point at which initial fluorescence can be detected (background activity) is termed the baseline. It is important to set the threshold above the baseline, before the plateau and within the exponential phase of the amplification curve .In these experiments the threshold was always set at 0.05.

### 2.4.4 RT-qPCR Set up

Prior to array analysis the cDNA samples were checked by real time quantitative PCR to ensure that the RT and pre-amplification reactions had

worked. A pre-designed Taqman® microRNA specific probe known to be expressed well in tissues was chosen (assay miR-34a). QPCR was performed on MicroAmp ® optical 96 well plates (Applied Biosystems, Foster City, CA) using 4.5µl of dilute cDNA (1:20 dilution), 0.5µl of microRNA-34 assay and 5.0µl of TaqMan® Universal Master Mix, No AmpErase® UNG, (2✕). PCR reactions plates were run upon the Step-One-Plus™ real-time PCR system (Applied Biosystems, Foster City, California, USA) using the following thermal profile:

1. Hold: 95°C (10 minutes)
2. 40 Cycles: 95°C (15 seconds – to allow denaturing), 60°C (60 seconds – to allow annealing /elongation).

Several control measures were employed to ensure reproducibility and accuracy of the PCR reactions (Table 22).

**Table 22. Control Measures Used During PCR**

| | Control Measure | Stage | Description |
|---|---|---|---|
| 1 | Preparation of Mastermix | Pre-PCR | For experiments with large number of samples exceeding the capacity of the 96-well plate, a mastermix containing the PCR components was composed and used for each plate to reduce sample-sample and well-well variation. |
| 2 | Samples run in Triplicate | PCR reaction | Each sample was run three times to confirm reproducibility |
| 3 | Rox-Dye | PCR reaction | A passive reference ROX-dye, which did not take part within the reaction, was included for normalization. This corrected for variations in the reaction secondary to volume and concentration fluctuations. |
| 4 | Endogenous Control | PCR reaction | All samples were tested and normalized with U6 to correct for sample-to-sample variation. For U6 CT values below 26 were considered valid. |
| 5 | Minus RT Reaction | PCR Reaction | A reaction devoid of multiscribe reverse transcriptase enzyme and therefore should not produce an amplification curve. This can be used to determine if the reactions has been contaminated with DNA or amplicons. |
| | Negative Template Control (NTC) | PCR Reaction | A reaction containing all RT-PCR components except the template (RNA) and therefore should not produce an amplification curve. Any amplification indicates cross contamination of the reaction well or mastermix. |
| 6 | Positive Control | PCR Reaction | A well-expressed cell line (SK-Mel-28) was run upon each PCR plate to confirm reproducibility. This particularly useful when the number of samples exceeded the capacity of the 96-well plate. |
| 7 | Variety of Samples Types on Each Plate | PCR Reaction | Each PCR plate contained a variety of samples types (UC, Dysplasia, CAC). Analysing all the samples from the same group on the same plate could lead to bias. |

### 2.4.5 Extraction and Processing of results

The plate was analysed using the Step-One machine and software V2.1. Raw CT values were obtained for each well. Sample with triplicate values with an SD of more than 1.0 were deemed inaccurate and not accepted. As mentioned above, a threshold value of 0.05 was set and CT values for each sample were extracted onto an excel file. Samples that did not produce viable curves or yielded high CT values were deemed unsuitable for the array (Figure 13).

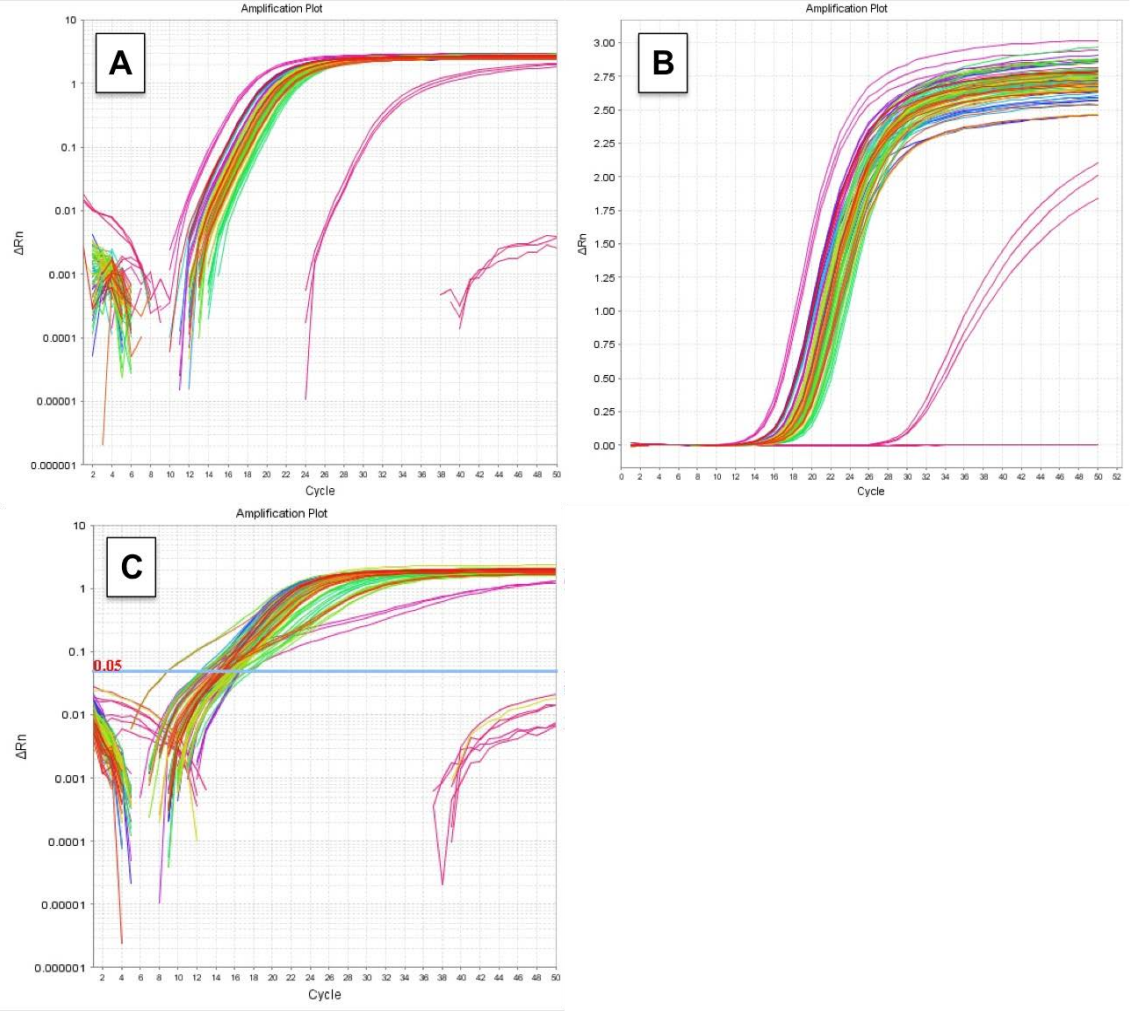



**Figure 13. Good and Bad RT-q-PCR Amplification Plots**

**A.** Logarithmic amplification plot showing 28 samples tested with a well expressed miRNA assay. **B.** Corresponding linear amplification plot for the same plate. **C.** Logarithmic amplification plot showing the same 28 samples tested with a poorly performing miRNA assay (the exponential phases of the reaction curve are flat).

### 2.4.6 Taqman ® microRNA Array

Following total RNA extraction and processing, appropriate samples were chosen and allocated to the above groups. For the tissue derived samples the groups A, C, E and G had a matched normal group comprised of adjacent normal tissue from the same specimen (Table 23). The plasma derived cases, were pooled, however cases of importance were ran on individual microfluidic cards (Table 24).

**Table 23. Pooled Groups of Tissue-Derived pre-amplified cDNA used on the Taqman Cards**

| Card Group | Description | Sample Number |
|---|---|---|
| A | Colitis Associated Adenocarcinoma | 4 |
| B | Chronic Ulcerative Colitis (matched tissue for group A) | 4 |
| C | Low Grade Dysplasia | 4 |
| D & F | Chronic Ulcerative Colitis (matched tissue for groups C and E) | 6 |
| E | High Grade Dysplasia | 4 |
| G | Severe Acute Ulcerative Colitis | 4 |
| H | Normal Non-Inflamed tissue (matched tissue for group G) | 4 |

**Table 24. Pooled Groups of Plasma Derived Pre-Amplified cDNA used for The Taqman Cards**

| Card | Description | Sample Number |
|---|---|---|
| 1 | Colitis Associated Adenocarcinoma | 1 |
| 2 | Colitis Associated Adenocarcinoma | 1 |
| 3 | Colitis Associated Adenocarcinoma | 4 |
| 4 | Dysplasia | 1 |
| 5 | Dysplasia | 1 |
| 6 | Primary Sclerosing Cholangitis | 8 |
| 7 | Ulcerative Colitis (Active) | 8 |
| 8 | Ulcerative Colitis (Chronic) | 8 |
| 9 | Ulcerative Colitis Quiescent | 8 |

A PCR mastermix mix was prepared in a 1.5ml eppendorf using 450µl of TaqMan® Universal Master Mix, No AmpErase® UNG, (2X), 441µl of nuclease free water and 9ul of pooled preamplified cDNA product (end dilution 1:400). Tissue MicroRNA profiling was then performed using Taqman® 384 well, Low Density Human MicroRNA Array Microfluidic cards. Selected groups of the tissue-derived samples were investigated using Pool A cards. Each card contained 381 mature microRNA targets, correlating to the Sanger miRBase v20. Plasma samples were investigated using Pool A and additional B cards, enabling the evaluation a total of 780 microRNA targets. Both sets of cards contained three positive endogenous controls for normalization (Mammalian U6, RNU48, RNU44), of which Mammalian U6 was repeated four times to monitor reproducibility. The Arabidopsis plant microRNA (ath-MiR159a) was present upon the card as a negative control.

Each of the 8 reservoirs upon the microfluidic card was loaded with master mix. The card was then centrifuged (Sorvall® centrifuge), sealed, cut and placed into the Applied Biosystems 7900HT fast real time PCR system. The settings; Relative quantification (ΔΔ CT), 384 well Taqman Low Density Array were chosen and has-human pool A/ B assay information templates were imported. A pre-set thermal cycle profile was used (Table 25).

**Table 25. Thermal Cycling Profiles for Taqman Low Density Array Cards**

| Reverse Transcription Profile | | |
|---|---|---|
| Stage | Temperature | Time |
| Hold | 50°C | 2 minutes |
| Hold: | 94°C | 10 minutes |
| Cycle | 97°C | 30 seconds |
| X40 | 59.7°C | 1 minute |

The process was repeated for each pooled sample group. Final results were analysed with sequence detection software (SDS version 2.2.2 Applied Biosystems). The threshold was set to 0.15 and all plates were added to the analysis. Individual CT values were exported into a Microsoft Excel document.

### 2.4.7 Validation Assays

The microarray cards were used as a large scale-screening tool to identify miRNAs of interest. In order to identify a panel of high-priority miRNAs candidates, an initial analysis of the microarray data was carried out.

A low-stringency criteria was used:

- Chosen miRNAs had to have CT values below 33. MiRNAs that produced a CT value of 33 or more (detection limit) were considered to be poorly expressed and potentially unreliable as biomarkers.

- Several miRNAs that were found to be of importance during the literature review such as those associated with p53 and tumourgenisis and previous work within the laboratory were also considered

- MiRNAS had to be significant (P value <0.05) when analysed with a paired student t-test.

- The groups were ordered according to natural pathological progression of disease. For the tissue based samples: acute UC, LGD, HGD and CAC. For the blood based samples the disease order was as follows: chronic UC, active UC, dysplasia and CAC. A trend correlation value was calculated for each miRNA and ranked. miRNAs with large differential changes or with numerically increasing changes across groups were highlighted.

- Fold changes were calculated for the matched groups and they were ordered according to disease progression. The trends values were ranked and miRNAs with large fold changes and high ranks were flagged.

Selected miRNAs were subsequently chosen for validation in additional cohorts via qRT-PCR using individual Taqman Assays (described in section 2.4.4).

## 2.5 Statistical Analysis

### 2.5.1 Processing PCR Data

The PCR and microarray data was initially processed using the Step-One software V2.1. Triplicate samples that did not produce an amplification curve were classed as undetectable and were assigned a CT value of 40.

- For each sample the average of the triplicate CT values was normalized by subtracting the average of the corresponding U6 values (endogenous control). This resulted in a ΔCT value:

  **Equation 1:** ΔCT = CT of Target - CT of Endogenous Control
  Example: ΔCT = CT of Mir-31 - CT of Mir-U6

- The samples were then compared in their groups, either to a matched normal group or to a different group. This resulted in a ΔΔCT value:

  **Equation 2:** ΔΔCT = ΔCT of Group 1 – ΔCT of Group 2
  Example: ΔΔCT = ΔCT of CAC – ΔCT of Matched Group – Chronic UC

- The $2^{-\Delta\Delta CT}$ method was used to determine relative expression.

  **Equation 3**: $\log 2^{(-\Delta\Delta CT)}$

Assuming the PCR reactions were 100% efficient, each cycle difference equated to a doubling of PCR products, therefore a difference of two cycles equated to a four-fold change ($CT=2^2$) and a three-cycle difference equated to an eight-fold change ($CT=2^3$).

Other well-established methods of normalising data include using a panel of endogenous controls or employing a plate averaging method in which the mean plate average is deducted from the target sample. A variety of the above techniques were used when normalising the data. A good endogenous control

should be abundant, well and consistently expressed across all samples/cells. Microsoft Excel was used to create, plate averages, ΔCT values and ΔΔCT values. It was also used to calculate z-scores, unpaired t-tests, trend values and fold changes for all the array data.

### 2.5.2 Graph Pad Prism

Graph Pad Prism V6.0 was used to carry out Shapiro-Wilks and D'Agostinos & Pearson's Omnibus tests for normalcy. It was also used to perform, paired t-tests, one way-ANOVA and Bland-Altman analysis.

### 2.5.3 Other Statistical Analysis

SPSS statistical package Version 18.0.2 software (SPSS Inc, Chicago, IL) was used to perform binary logistic regression and survival analysis.

A power calculation to determine the number of cases required for each phase of the study was not performed. This was because the study was a pilot study designed to provide proof of concept. Furthermore studies profiling miRNAs in the IBD population have used similar study subject numbers and take into account the difficulty in identifying archived and prospective cases of CAC.

# Chapter 3. Results

## The Clinicopathological Presentation of Colitis Associated Cancer in A UK Population

## 3.1 Background

The molecular basis of CAC differs from sporadic CRC. Repeated bouts of inflammation result in oxidative stress, regeneration and repair, DNA damage p53 loss and genomic instability. This causes CAC to present differently from sporadic CRC. As evidenced in the previous sections CAC arises from flat dysplastic lesions and can progress rapidly presenting at a late stage. It subsequently carries a high mortality and morbidity rate. Patients with UC are required to undergo colonoscopic screening for early detection and treatment of dysplastic lesions. There is a need to find novel biomarkers to address the limitations of the current surveillance program. Biomarker experiments should be conducted carefully to avoid false findings. Therefore, it is imperative to select an appropriate cohort of patients for initial experiments to ensure the findings are relevant and applicable to the population.

## 3.2 Aim 1

Characterise a cohort of patients with CAC to ensure suitability for sequential biomarker discovery experiments.

## 3.3 Objectives

- Identify all patients with CAC in the Leicestershire region over a 12-year period.
- Establish the clinicopathological presentation of CAC by analysing and comparing the variables age, duration of disease, disease extent, tumour distribution and stage at time of presentation to the known literature.

## 3.4 Results

### 3.4.1 Presentation of CAC

The search identified 41 patients diagnosed with CAC in the Leicestershire region from 1999 to 2011 (Table 26). It is unknown how many patients were missed from this list due to incorrect SNOWMED coding. It was not possible to obtain the number of new or existing patients diagnosed with UC diagnosis during this period.

**Table 26. Demographics For Patients With Colitis Associated Cancer**

| | Variable | Subgroup | Data (range) |
|---|---|---|---|
| 1 | Number of Patients | | 41 |
| 2 | Female / Male Ratio | | 11/30 |
| 3 | Age at Presentation of CAC | | 58 (36-92) |
| 4 | Duration of Ulcerative Colitis (years) | | 18.5 (1-38) |
| 5 | Extent of Ulcerative Colitis | E1 – Proctitis | n= 4 (9.76%) |
| | | E2 – Left Sided Disease | n= 12 (29.27%) |
| | | E3 – Extensive Disease (pancolitis) | n= 25 (60.97%) |
| 6 | 5ASA Medication | | 40 (97.6%) |
| 7 | Part of Surveillance Program | | 70% |

There was a female to male ratio of 1:3 and the median age at presentation of CAC was 58 (Figure 14). The median time of duration of UC was 18.5 years.

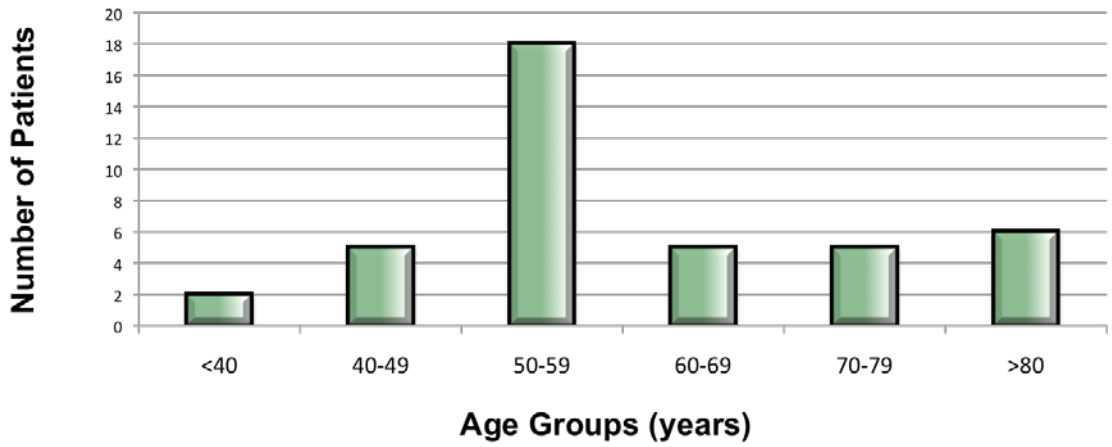



**Figure 14: Age at Diagnosis of Colitis Associated Cancer**

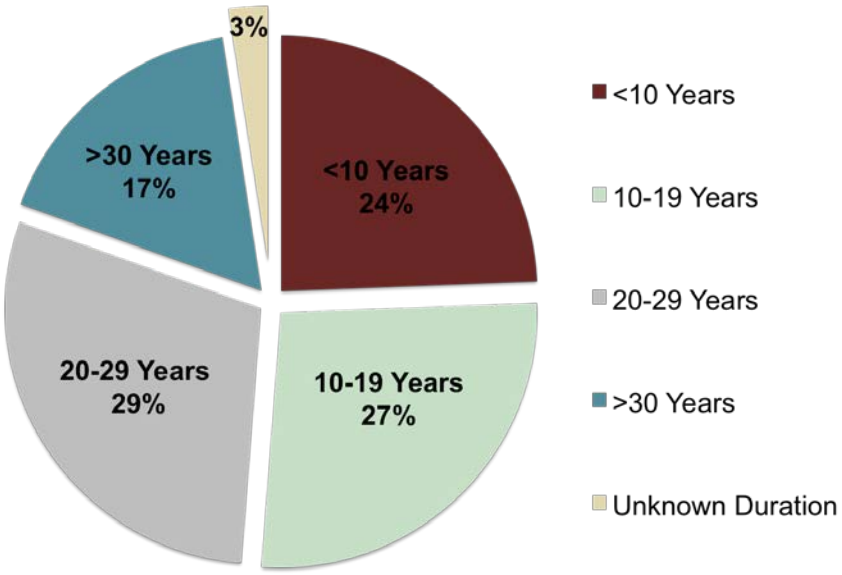



**Figure 15 Duration of Ulcerative Colitis at Time of Presentation With Colitis Associated Cancer**

A quarter of the cases had duration of UC less than ten years (Figure 15). Over 90% of patients with CAC had left sided or extensive disease activity (Figure 16). Over 95% of patients were upon a 5ASA medication and 70% of patients were enrolled upon a surveillance program. Of the 41 patients, 12 – were not suitable for surveillance (duration < 10 years). Of the 29 patients suitable for surveillance there were 20 active participants and 9 not in surveillance (6 no reasons state, 2 lost to follow-up and 1 incompliant). These 9 presented with T1 (1), T2 (2), T3 (4), T4 (2) disease.

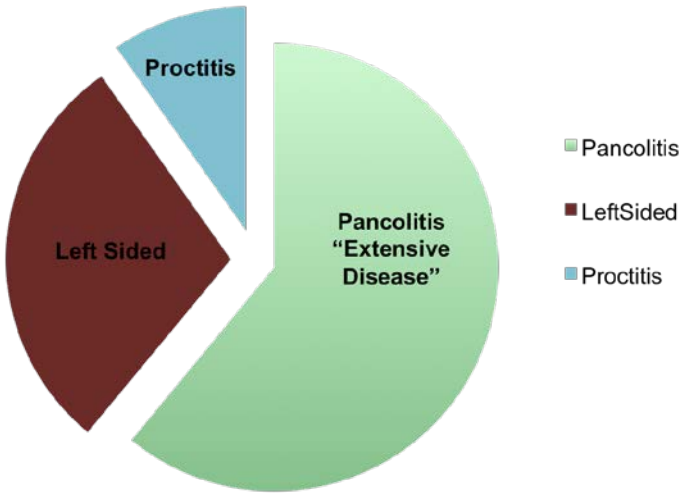



**Figure 16. Extent of Ulcerative Colitis in Patients Presenting with Colitis Associated Cancer**

### 3.4.2 Pathological Features

Synchronous CAC lesions occurred in 12% of cases. Concurrent dysplasia was found in 39% of cases and 16% of tumours were reported as arising from an area of dysplasia. Over 50% of tumours occurred in the rectum or recto-sigmoid area (Figure 17). 28% of tumours were poorly differentiated and 32% had a classification of subtype mucinous / signet cell carcinoma. Staging placed 27% of tumours into stage T4 (Figure 18).

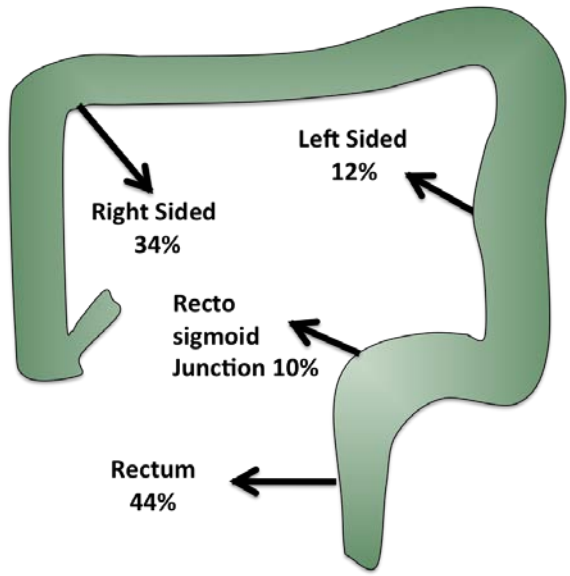



**Figure 17 Anatomical Distribution of Colitis Associated Cancer Amongst Patient Cohort**

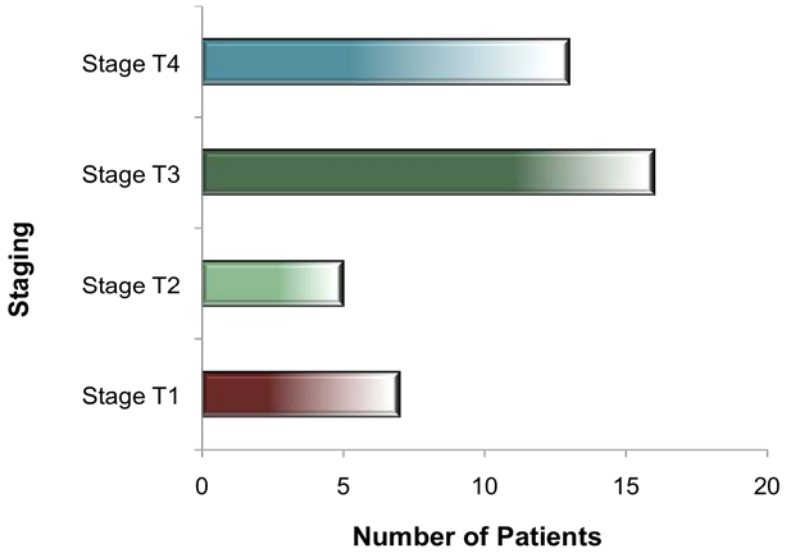



**Figure 18. Staging of Colitis Associated Cancers at Presentation**

The staging is based upon the histological staging that occurs following bowel resection, however 1 patient did not have surgery, and radiological staging was used in this instance.

### 3.4.3 Treatment and Prognosis

Metastatic disease at initial presentation was seen in 10% of cases (Figure 19) and 29% of patients went on to develop recurrent or metastatic disease. The 3-year survival rate was 56%

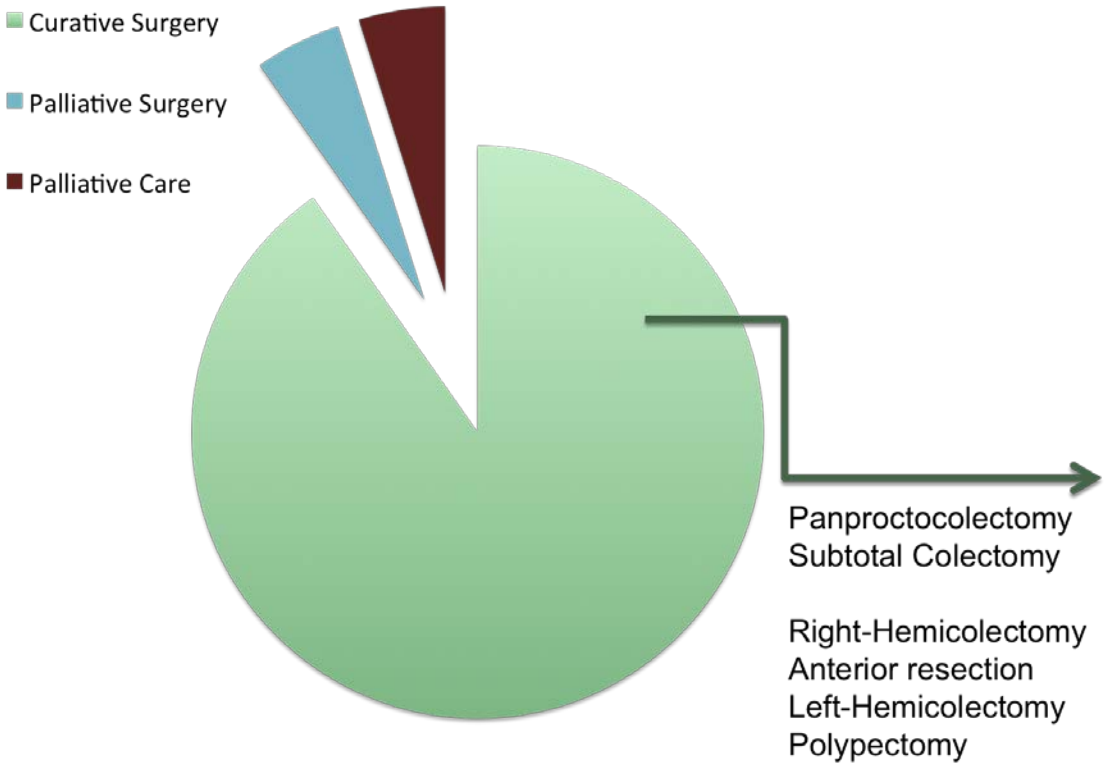



**Figure 19. Treatment Received for Colitis Associated Cancer**

Although the 'recommended treatment for Colitis Associated Cancer is a colectomy, patients underwent a variety of surgeries. In some cases, a segmental bowel resection was performed for what was thought to be a sporadic colorectal carcinoma, however Ulcerative Colitis was found in the specimen. Due to co-morbidities or personal choice, some patients had a hemicolectomy or polypectomy.

**Table 27. Summary of Studies Describing Clinical Features of Colitis Associated Cancer**

| | Experimental Cohort | Inflammatory Bowel Disease Confers A Lower Risk of Colorectal Cancer to Females Than Males Soderlund et al 2010 | Clinicopathological Study of Colorectal Cancer in Patients with UC in Japan Fujita et al 2010 | Clinical Features, Treatment and Survival of Patients With Colorectal Cancer With or Without Inflammatory Bowel Disease Affendi et al 2011 | Clinical Course of Colorectal Cancer in patients with UC Higashi et al 2011 | Ulcerative Colitis-Associated Cancer Shows a Poorer Survival Than SCRC Watanabe et al 2011 | Colorectal Carcinoma in Inflammatory Bowel Disease: A comparison between Crohns and UC Averboukh et al 2011 |
|---|---|---|---|---|---|---|---|
| Location | Leicester - UK | Sweden | Japan | Ireland | Japan | Japan | Israel |
| Duration of Study | 1999-2011 | 1954-1989 | 1998-2006 | 1994-2005 | 1985-2009 | 1978-1998 | 1992-2009 |
| Cases | 41 cases | 196 cases | 6 cases | 170 cases (139 UC) | 22 cases | 169 cases | 40 cases |
| Male: Female Ratio | 3:1 | 123:73 | 1:5 | 104:66 | 14:8 | 1:1 | - |
| Pancolitis (%) | 61% | 31% | 58% | - | 77.3% | - | 60% |
| Distribution of Tumours | Right-sided 34%<br>Left Sided 12%<br>Recto-sigmoid 10%<br>Rectal 44% | Right-sided 37%<br>Left Sided 11%<br>Rectal /Anal 36.6%<br>Unspecified/Multiple 15.5% | Right-sided 28.6%<br>Left Sided 28.6%<br>Recto-sigmoid 14.2%<br>Rectal 28.6% | Right-sided 27%<br>Left Sided 25%<br>Recto-sigmoid 9%<br>Rectal 31%<br>Unspecified 8% | Right-sided 4.5%<br>Left Sided 32%<br>Rectal /Anal 36.3%<br>Multiple 27.3% | Right-sided 24%<br>Left Sided 76% | - |
| Age at CAC Diagnosis | 58 | 54 (Men) 56 (Females) | 44 years | 61.4 | 54.6 years | 56.8 years | 54 years |
| Duration of UC at CAC | 18.5 years | - | 16 years | - | 14.7 years | - | 22.7 |
| Poorly Differentiated | 28% | - | 43% | - | 38.1% | 25% | - |
| Concurrent Dysplasia | 29% | - | 33.% | - | 53.8% | - | - |
| Synchronous Tumours | 12% | - | - | - | 27.3% | 11% | - |
| Risk / Survival Analysis | - | RR: Males Females<br>10 yr. 0.5% 0.7%<br>20 yr. 2% 1.2%<br>40 yr. 8.3% 3.5% | - | Mean age of death – 61.9yrs (7.7 years younger than matched SCRC patients) | - | 43.3% Vs. 57.4% SCRC | 5 year survival rate 61% |
| Comment | UC only | Mixture of UC/CD cases | UC only | Mixture of UC/CD cases | UC only | UC only | UC only |

CAC – Colitis Associated Cancer, UC – Ulcerative Colitis, SCRC – Sporadic Colorectal Cancer, RR – Relative Risk

## 3.5 Discussion

At first glance, it may appear that the value of this data is limited due to the small number of patients, however several other studies contained similar or smaller number of cases (Table 27). Information regarding family history of SCRC and the severity of disease throughout the patient's lifetime would have been extremely useful. This was not possible to obtain as it was not well documented in the notes, and in the majority of cases a scoring criteria such as Truelove and Witt's had not been used.

### Presentation of CAC

There was a high male to female ratio (3:1), in keeping with the literature (Soderlund et al 2010). The mean age at presentation of CAC was 58, however 60% of cases occurred below the age of 60 with the earliest presentation at 36. In contrast in the UK, an average of 43% of SCRC were diagnosed in patient's aged 75 and over and 95% were diagnosed in those aged 50 and over (Cancer Research UK). The age of presentation might of been expected to be earlier, however the slighty delayed presentation could be attributed to general improvements in health care such as increased patient education, access to healthcare, earlier initiation of remission medication and compliance with 5-ASA medication.

The mean time of duration of UC was 18.5 years (literature range 14.7-22.7 years). A quarter of the cases had duration of UC less than ten years, this is important to note, as surveillance traditionally starts a decade following initial diagnosis. Also in keeping with the literature, over 90% of patients with CAC had left sided or extensive disease activity. Of the 4 patients with proctitis, 3 presented with a rectosigmoid cancer and 1 developed a CAC at the hepatic-flexure. This is interesting as patients with proctitis are deemed low risk and not enrolled on a surveillance program. Developing a hepatic-flexure tumour suggests that this patient had a field change throughout their colon, increasing their risk.

**Pathology and Prognosis**

Rates of synchronous tumours (12%) and concurrent dysplasia (29%) are in keeping with the literature and the known mechanism of CAC development. Over-two thirds of tumours presented at stages T3 and T4. The overall 3-year survival rate was 56%. Although this was not a relative or matched survival rate it does crudely illustrate that patients with present with CAC at an earlier age and a later stage. The later stage of presentation is keeping with the aggressive nature of CAC, but could also reflect the inadequacy of surveillance programmes.

The results match what is known in the literature and therefore is a suitbale cohort for further investigation.

**Future Work**

Although there are a large number of population studies investigating risk of UC and CAC, not many studies have described the clinicopathological features of CAC. It would interesting to compare the study cohort to a matched cohort of patients with UC who did not develop CAC in order to calculate a hazard ratios for factors such as gender, duration of disease and age. The cohort could also be matched group of patients with SCRC to investigate the difference in presentation, pathology and prognosis of CAC.

## 3.6 Summary

- The characteristics of this study cohort are in keeping with the literature, making it suitable for biomarker discovery.

- UC seems to confer a lower risk of colitis associated cancer to females then to males

- Findings of synchronous tumours and concurrent dysplasia support the inflammation-dysplasia-adenocarcinoma model and support the identification of dysplastic precursor as the basis of a screening test.

- CAC can present in the period in-between or prior to surveillance. It is important to evaluate patients on an individual basis and stratify their risk according to severity and extent of disease.

# Chapter 4. Results

## MicroRNA Expression Profiles in the Colonic Mucosa of Patients with Ulcerative Colitis

## 4.1 Background

MiRNAs regulate gene function and play a role in pathological mechanisms. Due to technological advances and an increased understanding of the role of miRNAs in disease, many studies have investigated the miRNAs associated with immune mediated disorders and a wide range of cancers. This has led to an attempt to characterise miRNA expression and profiles linked to specific diseases. Only 4 studies to date have investigated tissue-based miRNAs associated with IBD related neoplasia, of which only two studies used human specimens. Therefore, accurate characterisation of miRNA profiles associated with UC neoplasia is required. This could lead to the development of much needed biomarkers for the monitoring of patients with Ulcerative colitis. FFPE archived specimens provide a readily available and accessible source of Ulcerative Colitis tissues collected over a long period of time. For most cases, the relevant clinical information is available within the medical notes.

## 4.2 Aim 2

Discover a panel of deregulated miRNAs in the colonic mucosa of patients with UC and determine their correlation to various disease states: active UC, dysplasia and CAC.

## 4.3 Objectives

- Identify formalin fixed paraffin embedded tissue samples suitable for biomarker discovery and allocate them to the groups: UC, Dysplasia and CAC.
- Extract total RNA from tissues and process samples to enable quantification of gene expression via RT-qPCR.
- Screen for differentially expressed miRNAs in the discovery series by conducting high throughput miRNA profiling using Taqman microfluidic cards™.
- Validate results in an independent cohort using individual miRNA assays.

## 4.4 Results

### 4.4.1 Quality Control

The concentration of total RNA extracted from the discovery and validation cohort was tested to ensure its suitability for reverse transcription and amplification. Total RNA concentrations varied from 1.35 ng/µl to 625 ng/µl. Yields were especially poor for the acute UC group and all samples of adjacent mucosa. Although RNA extraction from archived tissues is a well-established technique, in these cases the low yield may have resulted from:

- Sample Integrity: The resected bowel may not have been collected and preserved in a standardized manner following surgery. The period of time prior to fixation was unknown; there was a possibility of long ischemic times and autolysis. Failure to wash the bowel specimen following resection could result in inadequate fixation and subsequently a poor quality sample.

- The epithelium of the severe acute and chronic UC samples was thin, secondary to disease processes and contained a large proportion of inflammatory infiltrate.

- Poor RNA extraction techniques.

To overcome this problem, more slides were cut and more tissue dissected. This ensured that there were at least 10 slides per sample for the specimens that contained thin epithelial layers. This worked well with a subsequent increase in yield. Unfortunately a large proportion of the tissue blocks contained insufficient material and no further slides could be cut. To ensure the low yields were not a result of poor extraction techniques, several samples were repeated (with the same number of slides) and the results were similar. Following amplification, the samples were further tested by PCR to check integrity prior to array. As a result, not all cases were included in the final analysis (Figure 20).

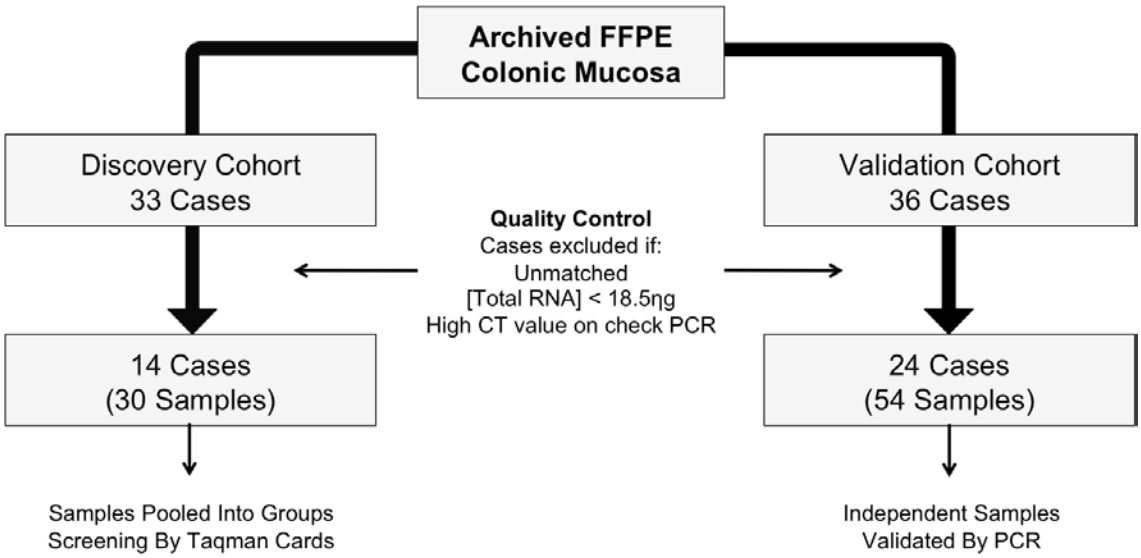



**Figure 20. Archived Tissue Samples Excluded From The Study**

FFPE – Fresh Frozen Paraffin Embedded Tissue, RNA – Ribonucleic Acid, CT- Cycle Threshold, PCR - Polymerase Chain Reaction

### 4.4.2 MiRNA Screening of the Discovery Cohort

In the first set of discovery experiments, a total of 7 Taqman Microfluidic arrays were tested to compare the expression of 381 known human microRNA genes amongst seven disease groups. As this was a screening method, the data was analysed using a relatively low-stringency criterion to identify all possible miRNA candidates of interest. A combination of U6, miR-484 and miR-191 were trialled as normalisers. The data was compared 3 ways.

### 1. Trend Changes

The disease groups were ordered according to natural disease progression and a trend value was calculated for each miRNA based upon ΔΔCT changes and ranked. MiRNAs with large increasing and decreasing trends across groups were noted (a selection is shown in Table 28).

**Table 28. ΔΔ Cycle Threshold Values Across Disease Groups and Trends For Selected MicroRNAs**

| | Acute UC Versus Normal | LGD Versus Chronic UC | HGD Versus Chronic UC | CAC Versus Chronic UC | Trend |
|---|---|---|---|---|---|
| let-7f | -0.32 | 2.49 | 2.66 | 4.98 | 0.96 |
| miR-10a | 1.3 | 0.73 | 0.52 | -0.14 | -0.98 |
| miR-10b | 1.67 | 1.42 | 1.54 | -2.52 | -0.79 |
| miR-17 | 0.75 | 0.03 | 0.74 | -0.95 | -0.71 |
| miR-18a | 5.41 | 1.96 | 0.23 | -2.27 | -0.99 |
| miR-22 | 0.74 | 1.93 | -1.61 | -1.96 | -0.8 |
| miR-31 | 0.12 | 0.4 | -3.82 | -3.17 | -0.83 |
| miR-32 | 6.63 | 0.17 | -0.46 | -2.04 | -0.9 |
| miR-135b | -0.14 | -1.78 | -3.04 | -3.76 | -0.99 |
| miR-190 | 0.94 | -7.04 | -3.1 | 0.32 | 0.07 |
| miR-205 | -2.37 | -8.27 | -7.43 | -7.08 | -0.64 |
| miR-211 | -3.27 | -0.65 | -1.19 | 1.72 | 0.91 |
| miR-215 | 0.6 | 0.37 | -2.75 | -2.64 | -0.9 |
| miR-362-3p | -2.86 | 1.15 | -7.03 | -5.17 | -0.55 |
| miR-374a | -0.29 | 0.05 | -1.37 | -1.55 | -0.85 |
| miR-383 | -2.37 | 4.34 | -4.63 | -7.61 | -0.63 |
| miR-616 | -9.34 | -6.1 | -4.82 | -0.55 | 0.98 |

miR – MicroRNA, UC = Ulcerative Colitis, LGD – Low Grade Dysplasia, HGD - High Grade Dysplasia

## 2. Student T –Test

An average ΔCT value was calculated for each miRNA using the CT values from each group. A SD value and a z-score was calculated and tested using a 2-tailed students t-test in which a P value of <0.05 was considered significant.

## 3. Fold changes

Fold changes were calculated and groups ordered according to disease progression (Table 29). Trend values were calculated and ranked. MiRNAs with large fold changes or with large increasing and decreasing fold change trends were flagged (Figure 20).

**Table 29. Fold Changes and Trend Across Disease Groups**

| | Acute UC Versus Normal | LGD Versus Chronic UC | HGD Versus Chronic UC | CAC Versus Chronic UC | Trend |
|---|---|---|---|---|---|
| let-7f | 1.25 | 0.18 | 0.16 | 0.03 | -0.84 |
| miR-10a | 0.41 | 0.6 | 0.7 | 1.1 | 0.96 |
| miR-10b | 0.31 | 0.37 | 0.34 | 5.75 | 0.78 |
| miR-17 | 0.59 | 0.98 | 0.6 | 1.93 | 0.74 |
| miR-18a | 0.02 | 0.26 | 0.85 | 4.82 | 0.86 |
| miR-22 | 0.6 | 0.26 | 3.05 | 3.88 | 0.91 |
| miR-31 | 0.92 | 0.76 | 14.1 | 8.97 | 0.74 |
| miR-32 | 0.01 | 0.89 | 1.38 | 4.12 | 0.93 |
| miR-135b | 1.1 | 3.44 | 8.25 | 13.58 | 0.99 |
| miR-190 | 0.52 | 131.7 | 8.56 | 0.8 | -0.25 |
| miR-205 | 5.19 | 309.38 | 173.03 | 134.85 | 0.26 |
| miR-211 | 9.66 | 1.57 | 2.28 | 0.3 | -0.84 |
| miR-215 | 0.66 | 0.78 | 6.71 | 6.23 | 0.88 |
| miR-362-3p | 7.25 | 0.45 | 130.96 | 35.93 | 0.46 |
| miR-374a | 1.22 | 0.96 | 2.58 | 2.93 | 0.89 |
| miR-383 | 5.19 | 0.05 | 24.68 | 195.62 | 0.82 |
| miR-616 | 648.83 | 68.7 | 28.3 | 1.46 | -0.83 |

miR – MicroRNA, UC = Ulcerative Colitis, LGD – Low Grade Dysplasia, HGD - High Grade Dysplasia

Fold changes above 2 are denoted in red.

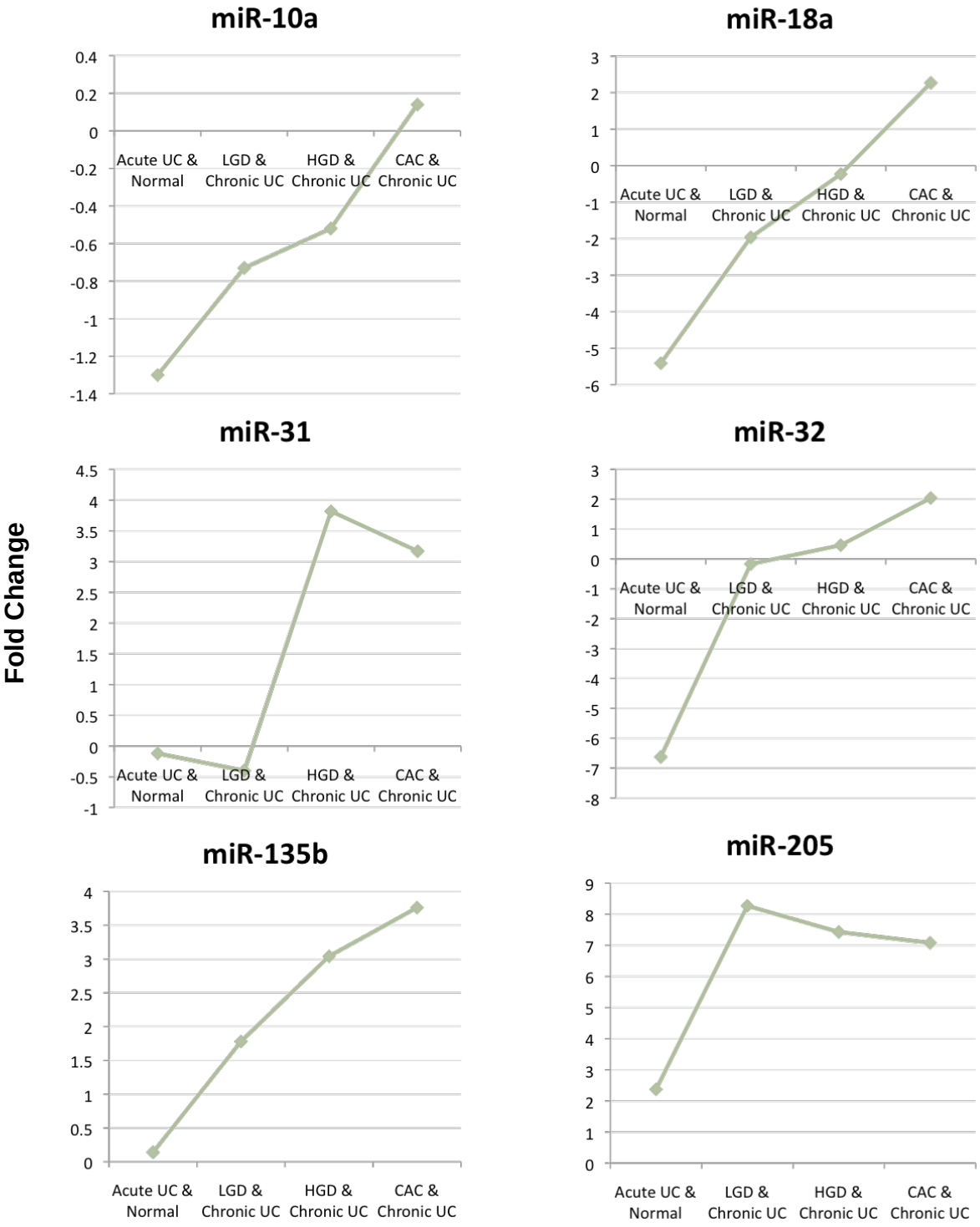



**Figure 20. Diagrammatic Representation of Relative Fold Changes of Selected MicroRNAs Across Disease Groups.**

A false discovery rate was not applied due to the small sample numbers. A list of miRNAs fitting the criteria was generated and miRNAs with CT values consistently above 32 were discarded (Table 30).

**Table 30. Raw Cycle Threshold Values for Selected MicroRNAs**

| Detector | Raw CT Group A | Raw CT Group B | Raw CT Group C | Raw CT Group D&F | Raw CT Group E | Raw CT Group G | Raw CT Group H |
|---|---|---|---|---|---|---|---|
| let-7f | **32.46** | 27.63 | 27.71 | 25.06 | 29.32 | 28.15 | 26.1 |
| miR-10a | 24.84 | 25.14 | 25.33 | 24.44 | 25.69 | 28.28 | 24.61 |
| miR-10b | 25.48 | 28.17 | 27.13 | 25.54 | 27.89 | 29.83 | 25.78 |
| miR-17 | 22.26 | 23.37 | 22.96 | 22.77 | 23.89 | 25.71 | 22.59 |
| miR-18a | 29.34 | 31.77 | **32.85** | 30.72 | **33.01** | **40** | **32.22** |
| miR-22 | 27.06 | 29.18 | 29.85 | 27.76 | 28.29 | **32.45** | 29.33 |
| miR-31 | 23.4 | 26.73 | 26.1 | 25.54 | 23.36 | 29.1 | 26.61 |
| miR-32 | 30.07 | **32.28** | 30.22 | 29.89 | 31.1 | **40** | 30.99 |
| miR-34a- | 24.07 | 24.86 | 25.21 | 24.54 | 25.61 | 27.71 | 26.19 |
| miR-135b | 27.5 | 31.42 | 28.93 | 30.55 | 28.87 | **33.59** | 31.35 |
| miR-190 | **33.5** | **33.34** | **33.12** | **40** | **34.15** | **40** | **36.68** |
| miR-211 | **37.27** | **35.71** | 31.1 | 31.59 | 31.95 | 31.68 | **32.58** |
| miR-215 | 25.12 | 27.93 | 26.54 | 26.01 | 23.53 | 28.69 | 25.71 |
| miR-205 | **32.76** | **40** | 31.89 | **40** | **33.13** | **40** | **40** |
| miR-362-3p | **34.67** | **40** | **34.14** | **32.83** | **32.49** | **38.48** | **38.96** |
| miR-374a | 27.3 | 29.02 | 27.68 | 27.46 | 28.78 | 31.67 | 29.59 |
| miR-383 | **32.22** | **40** | **37.66** | **33.16** | **35.94** | **40** | **40** |
| miR-616 | **34.71** | **35.42** | **32.08** | **38.01** | **35.74** | **33.03** | **40** |

CT – Cycle Threshold, miR – MicroRNA, UC = Ulcerative Colitis, LGD – Low Grade Dysplasia, HGD - High Grade Dysplasia. CT values above 32 are denoted in red.

MiRNAs that were not flagged using the above criteria, but had shown importance in the literature (miR-21) were added to the validation list. Due to restricted resources, it was not possible to validate all the candidates. A final set of 7 high-priority up regulated miRNAs (miR-10b, miR-18a, miR-31, miR-34a, miR-135b, miR-215) and 1 down-regulated microRNA (miR-let-7f) was chosen for initial array validation. RT-qPCR was used to establish miRNA expression in the 14 discovery cases (Figure 21-Figure 22).

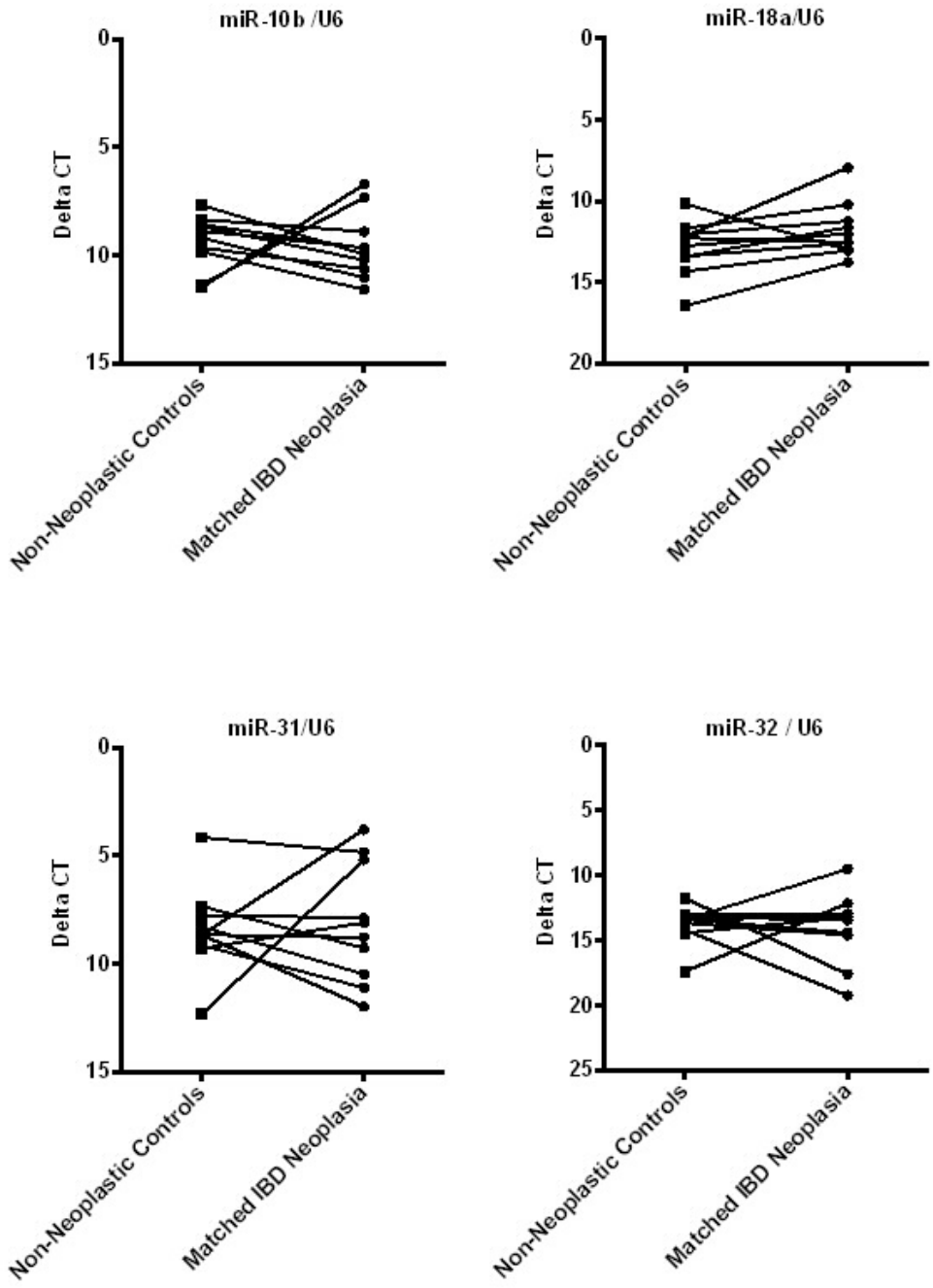



**Figure 21. Before and After Plots for MicroRNAs 10b, 18a, 31 and 32**

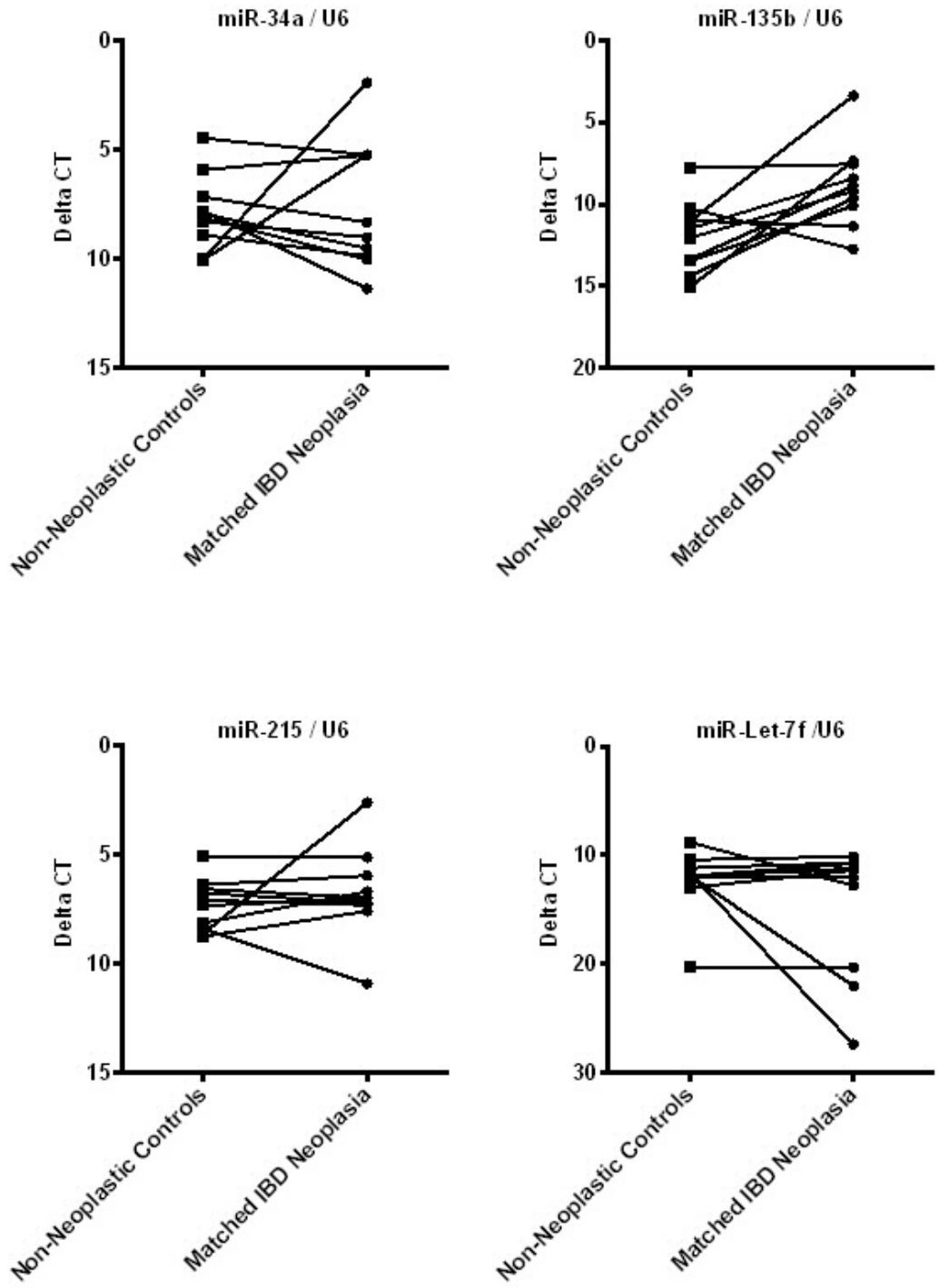



**Figure 22. Before and After Plots of MicroRNAs 34a, 135b, 215 and Let-7f**

### 4.4.3 MiRNA Expression Profiling in the Validation Cohort

To confirm the differential expression of the candidate miRNAs, real-time PCR using individual miRNA assays (8 candidates and U6 as a normaliser) was performed in an additional independent cohort. The cohort comprised 24 cases made up of a complicated set of samples (Table 31). This was due to a limited number of adequate samples and difficulties in obtaining matched controls. Each patient case (which consisted of resected bowel specimen) had several different sample types.

**Table 31. Specimen Breakdown for The Validation Cases**

| Patient Case Number | 'Normal' Tissue For Acute Ulcerative Colitis Cases | Acute Ulcerative Colitis | Unaffected 'Normal' Tissue For Neoplastic Cases | Chronic Ulcerative Colitis For Neoplastic Cases | Dysplasia | Colitis Associated Cancer |
|---|---|---|---|---|---|---|
| 1 | | | ☐ | ☐ | ☐ | ☐ |
| 2 | | | ☐ | | | ☐ |
| 3 | | | ☐ | ☐ | | ☐ |
| 4 | | | ☐ | ☐ | ☐ | |
| 5 | | | ☐ | | | ☐ |
| 7 | | | | ☐ | | ☐ |
| 8 | | | ☐ | ☐ | | ☐ |
| 9 | | | | ☐ | ☐ | ☐ |
| 10 | | | | ☐ | ☐ | ☐ |
| 11 | | | ☐ | ☐ | | ☐ |
| 12 | | | ☐ | | ☐ | ☐ |
| 14 | | | ☐ | ☐ | | ☐ |
| 15 | | | ☐ | ☐ | | ☐ |
| 16 | | | | ☐ | ☐ | |
| 18 | ☐ | ☐ | | | | |
| 19 | ☐ | ☐ | | | | |
| 21 | ☐ | ☐ | | | | |
| 24 | ☐ | ☐ | | | | |
| 25 | ☐ | ☐ | | | | |
| 26 | ☐ | ☐ | | | | |
| 28 | ☐ | ☐ | | | | |
| 30 | ☐ | ☐ | | | | |
| 31 | | | ☐ | ☐ | ☐ | |
| 33 | ☐ | ☐ | | | | |
| 35 | ☐ | ☐ | | | | |
| **Total Samples** | **10** | **10** | **11** | **12** | **7** | **12** |

Normality testing showed most sample groups, had a normal distribution (Table 32). Let-7f was the least consistent. Nonetheless, a parametric test was used for analysis, as miRNA expression is known to follow a normal distribution. It was also acknowledged normalcy would be reduced as a result of the small sample numbers.

**Table 32. Results of Normality Testing For Disease Groups**

| Normalcy Test | Mir | Normal Tissue For Acute UC | Acute UC | Normal Tissue For Neoplasia | Chronic UC Tissue For Neoplasia | Dysplasia | CAC |
|---|---|---|---|---|---|---|---|
| **Shapiro - Wilk Test** | 10b | Yes | Yes | **No** | Yes | Yes | Yes |
| | 18a | Yes | Yes | Yes | Yes | Yes | Yes |
| | 21 | Yes | Yes | Yes | Yes | Yes | Yes |
| | 31 | Yes | Yes | **No** | Yes | Yes | Yes |
| | 34a | Yes | Yes | **No** | Yes | Yes | Yes |
| | 135b | Yes | Yes | Yes | Yes | Yes | Yes |
| | 215 | Yes | Yes | Yes | Yes | Yes | Yes |
| | Let-7f | Yes | **No** | **No** | **No** | Yes | **No** |
| **D'Agostino & Pearson Omnibus Test** | 10b | Yes | No | Yes | Yes | N too small | Yes |
| | 18a | Yes | Yes | Yes | Yes | N too small | Yes |
| | 21 | Yes | Yes | Yes | Yes | N too small | Yes |
| | 31 | Yes | Yes | No | Yes | N too small | Yes |
| | 34a | Yes | Yes | Yes | Yes | N too small | Yes |
| | 135b | Yes | Yes | Yes | Yes | N too small | Yes |
| | 215 | Yes | Yes | Yes | Yes | N too small | Yes |
| | Let-7f | Yes | Yes | Yes | **No** | N too small | **No** |

Mir – MicroRNA, UC – Ulcerative Colitis, CAC – Colitis Associated Cancer
Yes = Passed normalcy test at alpha = 0.05 (the group shows no significant difference). No = group does not show a normal distribution (highlighted in bold). N too small – not enough sample numbers for the test to be performed.

The results were first analysed by 1-way ANOVA with all the specimens in their original groups. There was no statistical difference between the normal for acute UC group and the normal for neoplasia group for any of the candidate miRNAs. Apart from miRNA-18a and 31 there was no difference between the acute UC and chronic UC groups and no difference when comparing CAC to dysplasia apart from miRNA-215. Subsequently the disease groups were merged and a combined group analysis performed (Figure 23 – Figure 30). The results are summarized in Table 33.

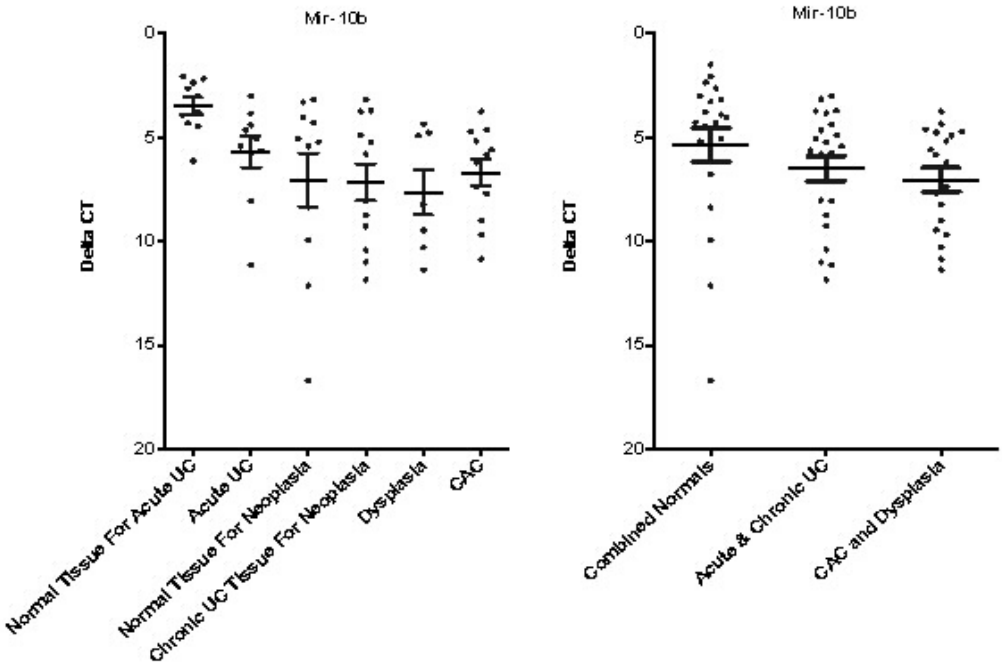



**Figure 23. Scatter Plot of MicroRNa-10b Expression In The Validation Cohort**

CT – Cycle Threshold, UC – Ulcerative Colitis, CAC – Colitis Associated Cancer. Mean and SEM shown.

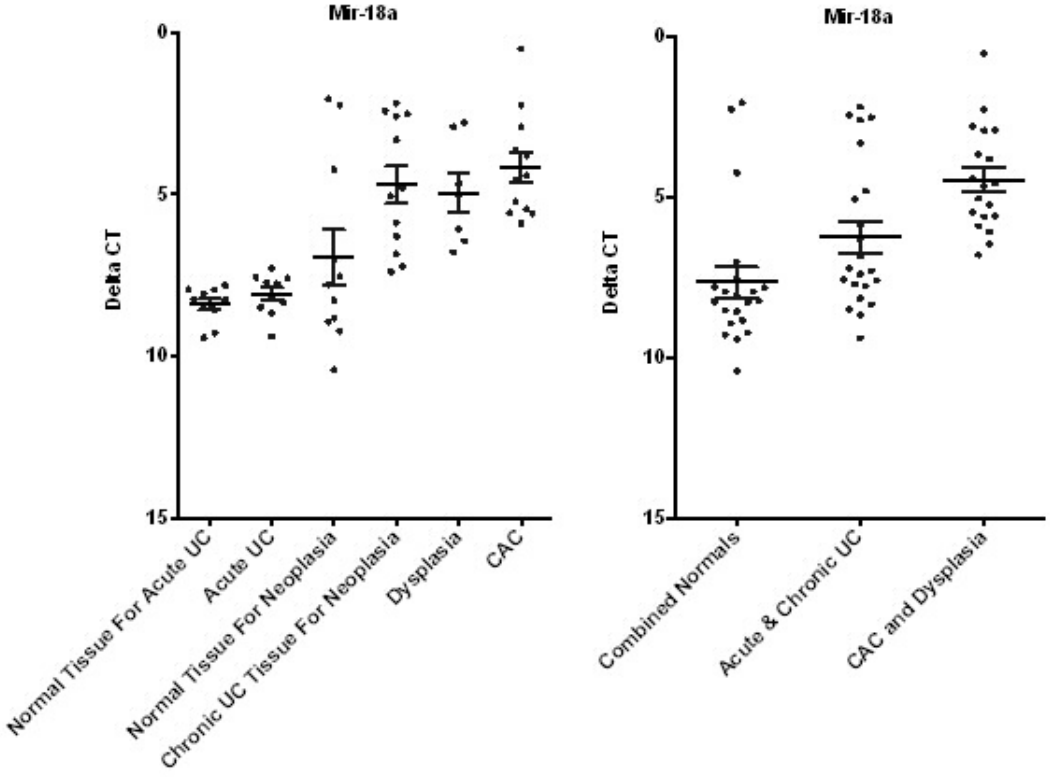



**Figure 24. Scatter Plot of MicroRNA-18a Expression in The Validation Cohort**

CT – Cycle Threshold, UC – Ulcerative Colitis, CAC – Colitis Associated Cancer Mean and SEM shown.

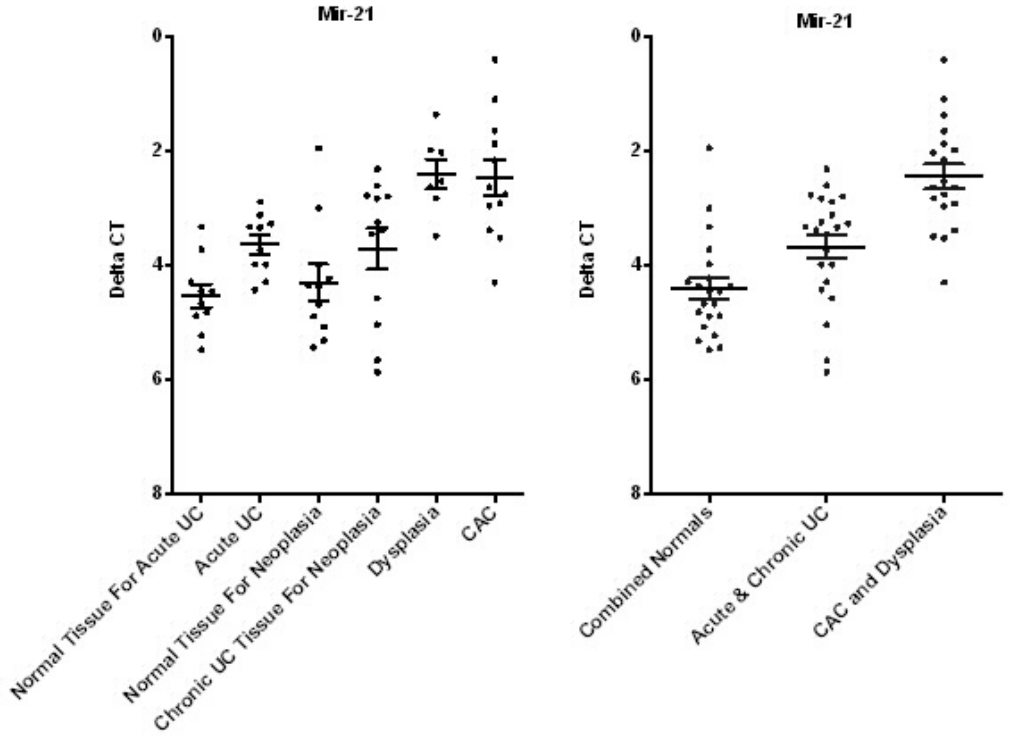



**Figure 25. Scatter Plot of MicroRNA-21 Expression in The Validation Cohort**

CT – Cycle Threshold, UC – Ulcerative Colitis, CAC – Colitis Associated Cancer. Mean and SEM shown.

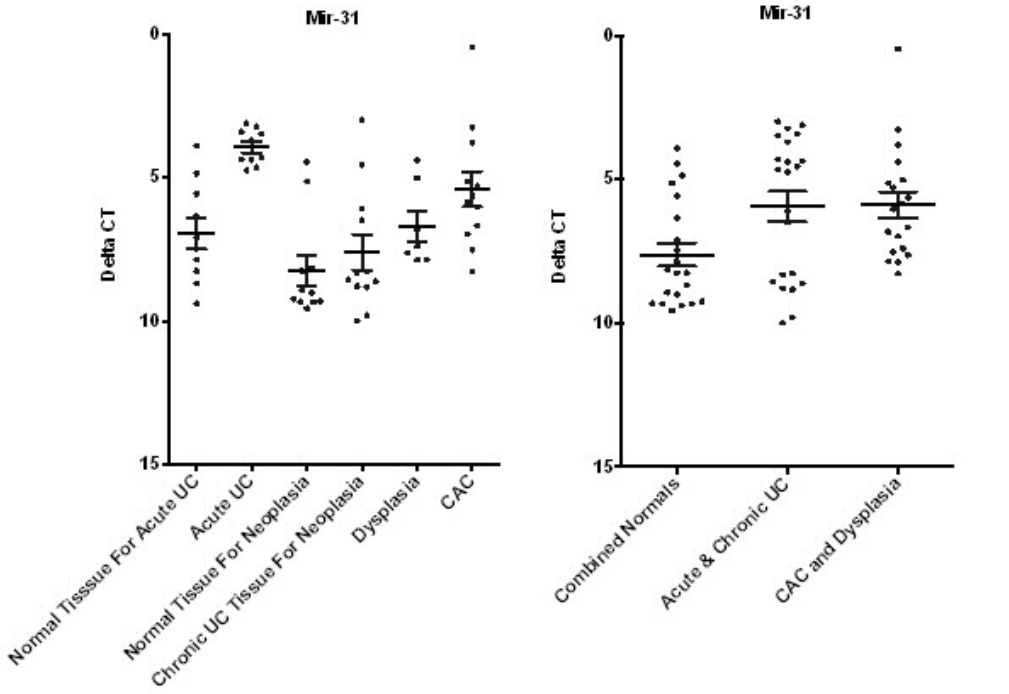



**Figure 26. Scatter Plot of MicroRNA-31 Expression in The Validation Cohort**

CT – Cycle Threshold, UC – Ulcerative Colitis, CAC – Colitis Associated Cancer. Mean and SEM shown.

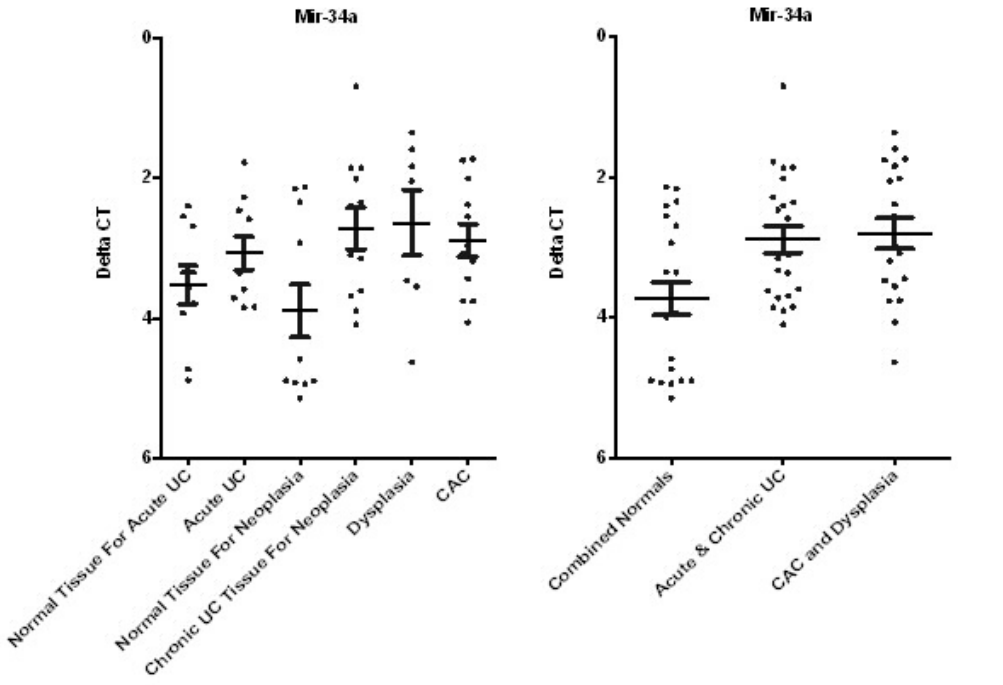



**Figure 27. Scatter Plot of MicroRNA-34a Expression in The Validation Cohort**

CT – Cycle Threshold, UC – Ulcerative Colitis, CAC – Colitis Associated Cancer. Mean and SEM shown.

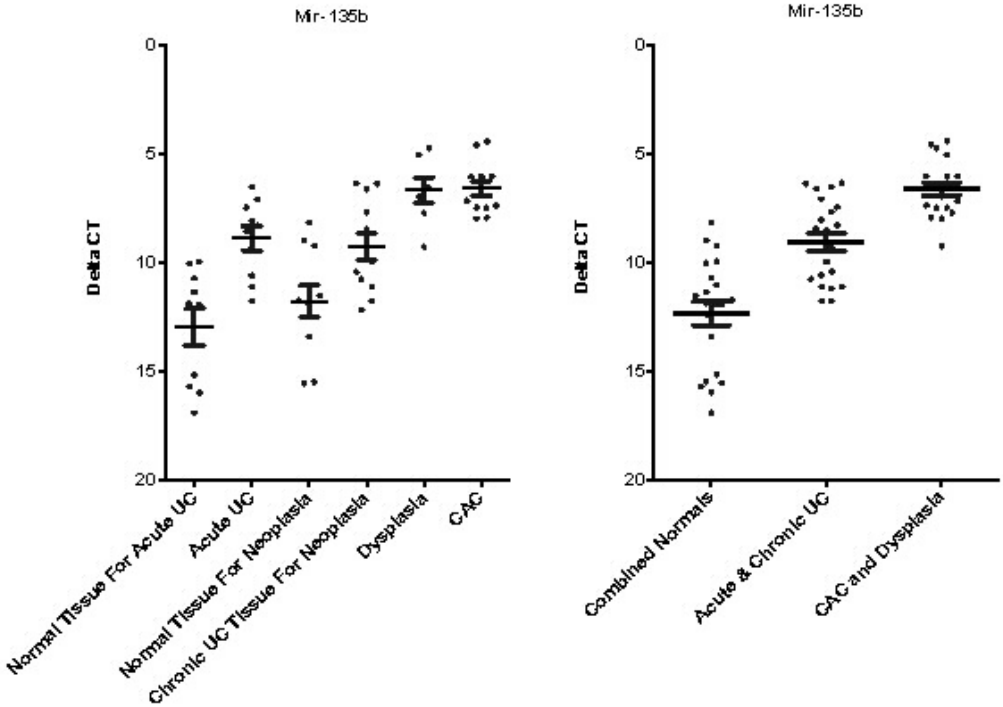



**Figure 28. Scatter Plot of MicroRNa-135b Expression in the Validation Cohort**

CT – Cycle Threshold, UC – Ulcerative Colitis, CAC – Colitis Associated Cancer. Mean and SEM shown.

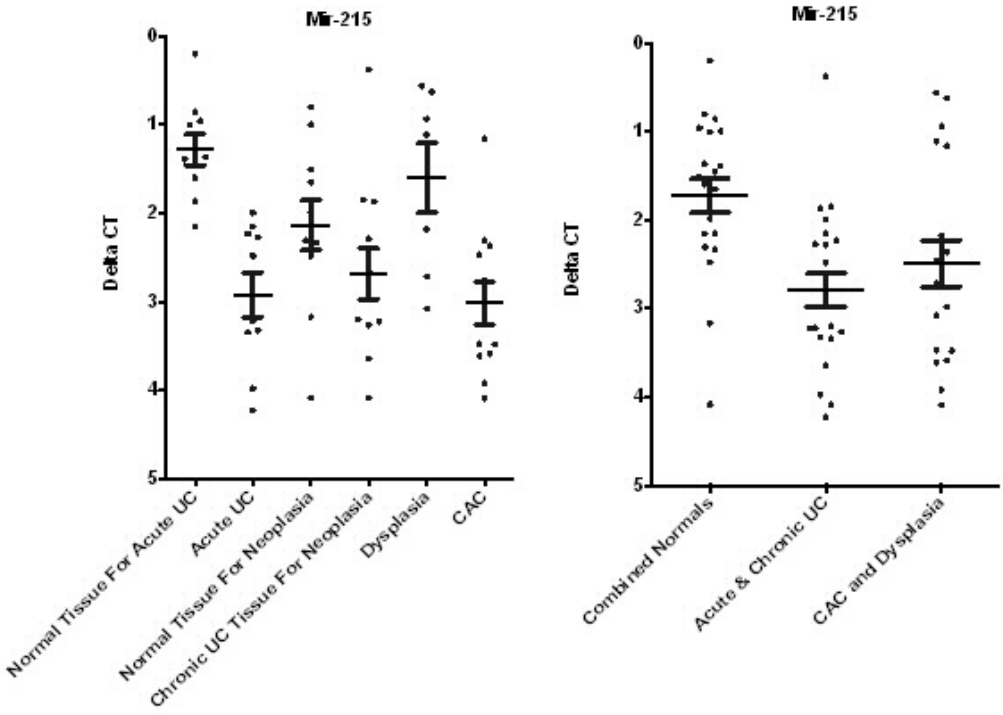



**Figure 29. Scatter Plot of MicroRNA 215 Expression in The Validation Cohort**

CT – Cycle Threshold, UC – Ulcerative Colitis, CAC – Colitis Associated Cancer. Mean and SEM shown.

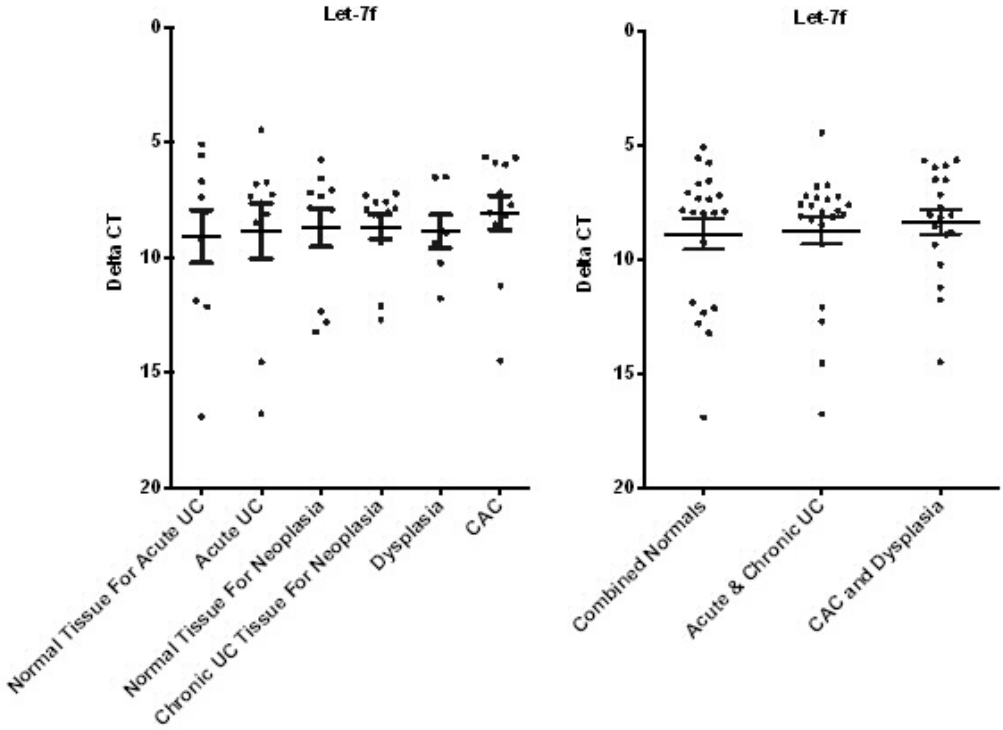



**Figure 30. Scatter Plot of MicroRNA Let-7f Expression in The Validation Cohort**

CT – Cycle Threshold, UC – Ulcerative Colitis, CAC – Colitis Associated Cancer. Mean and SEM shown.

**Table 33. Summary of One-Way ANOVA Results Comparing Separate and Combined Disease Groups**

| | GROUPS | miR-10b | miR-18a | miR-21 | miR-31 | miR-34a | miR-135b | miR-215 | Let-7f |
|---|---|---|---|---|---|---|---|---|---|
| 1. | Normal For Acute UC Versus Normal For Neoplasia | NS | NS | NS | NS | NS | NS | NS | NS |
| 2 | Normal For Acute UC Versus Acute UC | NS | NS | NS | **P = 0.0052** | NS | **P = 0.0005** | **P= 0.0014** | NS |
| 3 | Normal For Neoplasia Versus Dysplasia | NS | NS | **P = 0.0018** | NS | NS | **P <0.0001** | NS | NS |
| 4 | Normal For Neoplasia Versus CAC | NS | **P = 0.005** | **P = 0.0003** | **P = 0.0045** | NS | **P <0.0001** | NS | NS |
| 5 | Chronic UC for Neoplasia Versus Dysplasia | NS | NS | NS | NS | NS | NS | NS | NS |
| 6 | Chronic UC for Neoplasia Versus CAC | NS | NS | **P = 0.0326** | NS | NS | **P = 0.0326** | NS | NS |
| 7 | Dysplasia Versus CAC | NS | NS | NS | NS | NS | NS | **P = 0.0177** | NS |
| | | | | | | | | | |
| 8 | Combined Normals Versus Acute UC + Chronic UC | NS | NS | **P = 0.0358** | **P = 0.0340** | **P = 0.0138** | **P <0.0001** | **P = 0.0019** | NS |
| 9 | Combined Normals Versus Dysplasia + CAC | **P = 0.0277** | **P = <0.0001** | **P <0.0001** | **P = 0.0359** | **P = 0.0125** | **P <0.0001** | **P = 0.0459** | NS |
| 10 | Acute UC + Chronic UC Versus Dysplasia + CAC | NS | **P = 0.0225** | **P <0.0002** | NS | NS | **P = 0.0008** | NS | NS |

UC – Ulcerative Colitis, CAC – Colitis Associated Cancer, miR – MicroRNA, NS – Not Significant P – Adjusted P value (Bonferronis Multiple Testing Correction) Significant results (P<0.05) are in red.

## Combined Panels of MiRNAs

Any potential microRNA biomarker of neoplasia needs to be well expressed (low CT/ ΔCT value) and within a limited range (small SD). Therefore the gross expression profiles for the 8 miRNAs were inspected on a case basis (Figure 31) and correlated with the clinical features (Table 34).

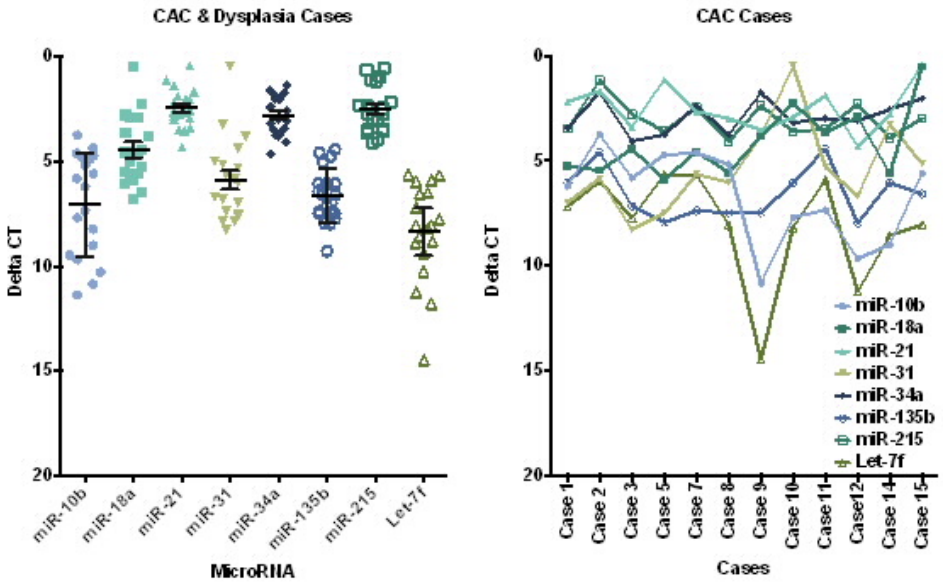



**Figure 31. Gross Expression Profiles for the Colitis Associated Cancer Cases**

CT – Cycle Threshold, CAC – Colitis Associated Cancer. miR – MicroRNA. Mean and Standard Deviation shown.

**Table 34. Summary of Demographics For The Colitis Associated Cancer Cases**

| Case | Age | Sex | Site | Stage and Description |
|---|---|---|---|---|
| 1 | 54.62 | M | Rectum | Dukes B (pT3 pN0) (Chronic UC) |
| 2 | 88.22 | F | Left Side | Duke's A (pT2 pN0 pMx) (Chronic UC) |
| 3 | 76.00 | M | Rectum | Dukes B (pT3, pN0, pMx) (mucinous) (Chronic UC) |
| 5 | 64.07 | M | Rectum | (pT4, pN0, pMx) (Quiescent UC) |
| 7 | 55.69 | M | Right Side | (pT3/4, pN0) (mucinous) (Chronic UC) |
| 8 | 75.25 | F | Right Side | (pT4, pN2) (Poorly differentiated signet ring type). Active, chronic UC. |
| 9 | 56.05 | M | Multiple Right Side | 1. Duke's C1 (pT3 pN1 pMX).<br>2. Duke's A (pT2 pN0 pMX)<br>3. Duke's B (pT3 pN0 pMX)<br>Active, chronic UC + DALMS |
| 10 | 32.08 | M | Left side | Recurrent poorly differentiated (perforation). Active chronic UC and dysplasia |
| 11 | 49.98 | M | Right side | Dukes' C1, pT3, pN2, pMX (mucinous) Chronic UC and dysplasia |
| 12 | 67.41 | F | Right-side | Dukes' A (pT1 pN0 pMX) Active, Chronic UC with multiple DALMS |
| 14 | 76.23 | F | Rectum | (pT3 pN1) (moderately differentiated) Mild active, chronic UC |
| 15 | 53.30 | M | Rectum | Dukes C1 (pT3 pN1 pMX) (moderately differentiated) Active, chronic UC |

There were 12 CAC cases (average age of 62.4) of which male n=8 and female n=4. There was a good anatomical distribution )42% rectum, 42% right-sided and 16% left-sided. The majority of cases were T3 stage.

The above information was used to combine different miRNAs in order to evaluate if this increased the capacity of the panel to distinguish between disease groups. (Figure 32 - Figure 34). The results are summarized in Table 35.

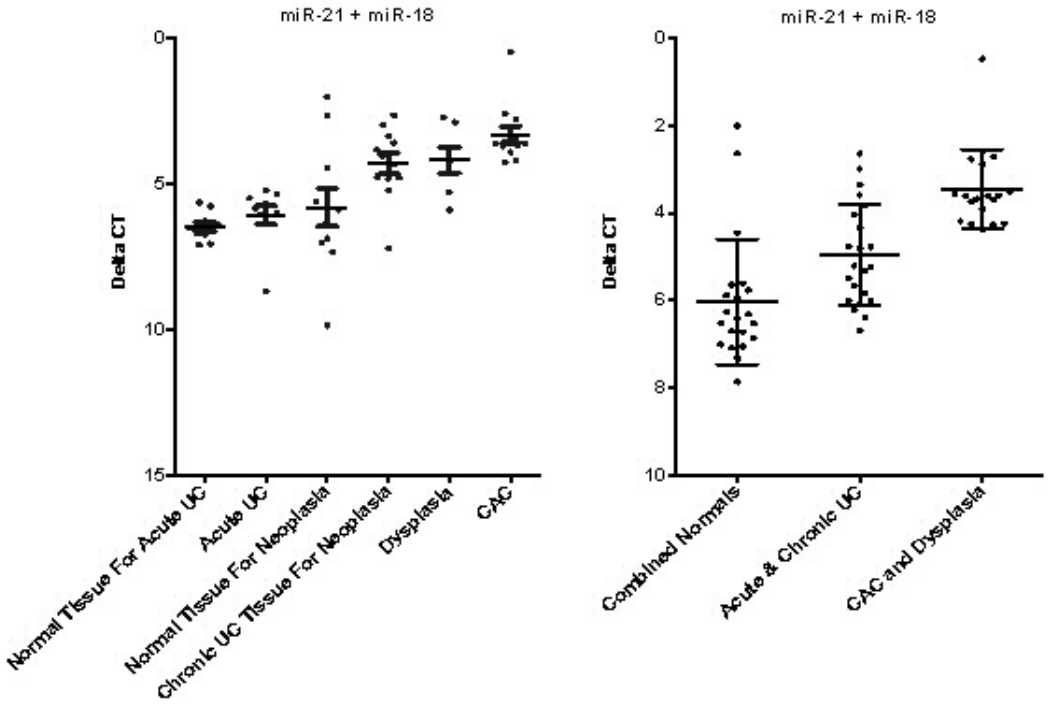



**Figure 32. Scatter Plot for Combined Expression of MicroRNA 18a and 21**

CT – Cycle Threshold, UC – Ulcerative Colitis, CAC – Colitis Associated Cancer, miR – MicroRNA, Mean and Standard Error of mean shown

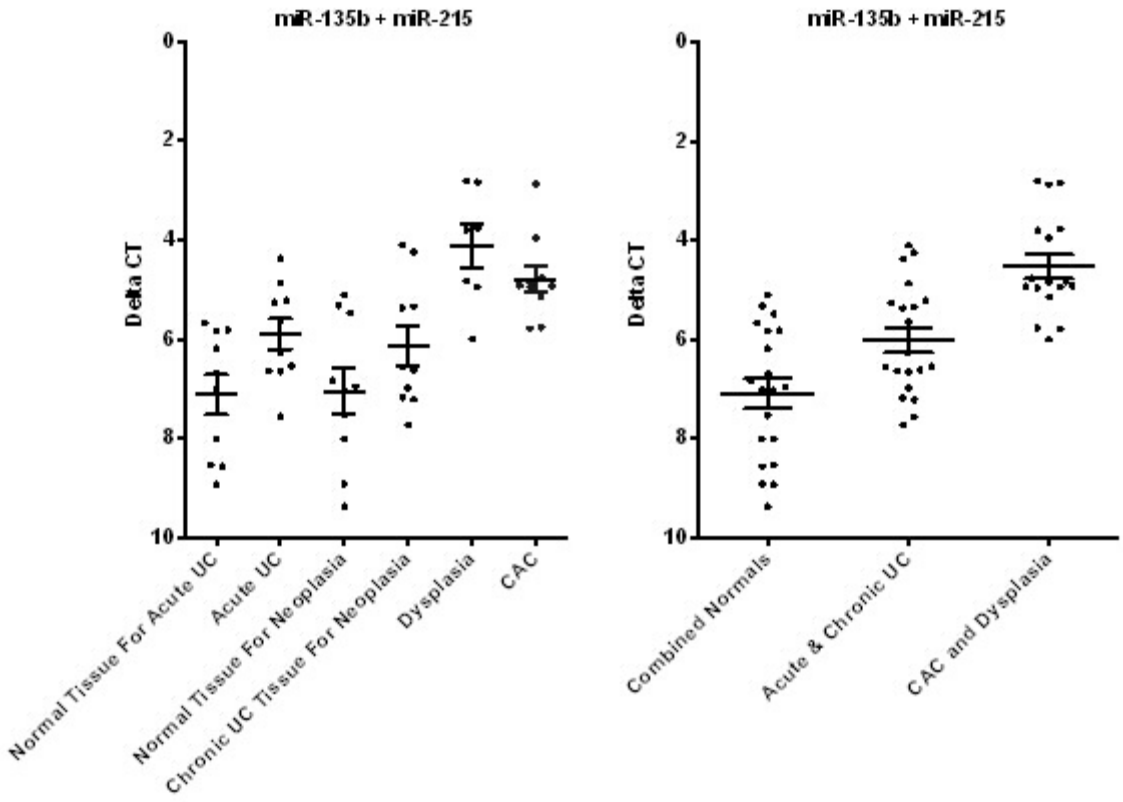



**Figure 33. Scatter Plot for Combined Expression of MicroRNA 135b and 215**

CT – Cycle Threshold, UC – Ulcerative Colitis, CAC – Colitis Associated Cancer, miR – MicroRNA, Mean and Standard Error of mean shown

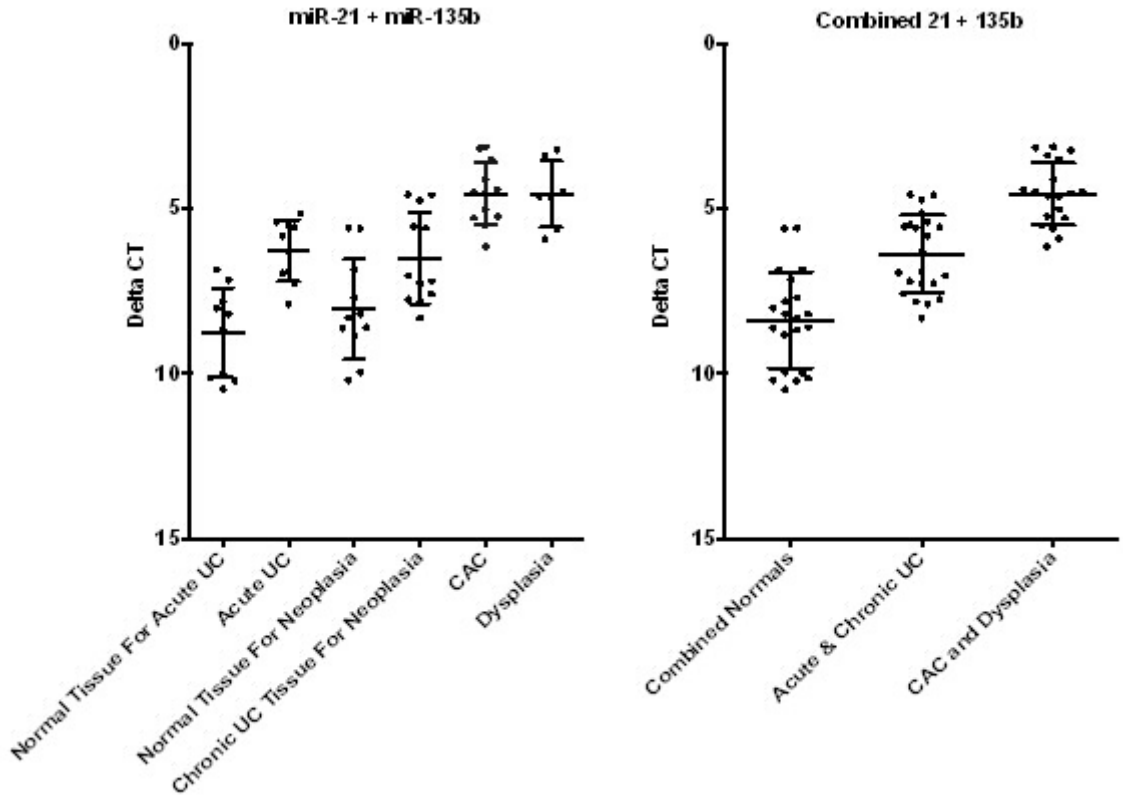



**Figure 34. Scatter Plot For Combined Expression of MicroRNA 21 and 135b**

CT – Cycle Threshold, UC – Ulcerative Colitis, CAC – Colitis Associated Cancer, miR – MicroRNA, Mean and Standard Error of mean shown

**Table 35. Summary of ANOVA results for The Combined MicroRNAs**

| | GROUPS | miR-18a + 21 | miR-135b + 215 | miR-21+ 135b |
|---|---|---|---|---|
| 1. | **Normal For Acute UC Versus Normal For Neoplasia** | NS | NS | NS |
| 2 | **Normal For Acute UC Versus Acute UC** | NS | NS | **P=0.0005** |
| 3 | **Normal For Neoplasia Versus Dysplasia** | NS | **P = 0.001** | **P<0.0001** |
| 4 | **Normal For Neoplasia Versus CAC** | **P = 0.004** | **P = 0.014** | **P<0.0001** |
| 5 | **Chronic UC for Neoplasia Versus Dysplasia** | NS | **P=0.0202** | **P=0.0240** |
| 6 | **Chronic UC for Neoplasia Versus CAC** | NS | NS | **P=0.0041** |
| 7 | **Dysplasia Versus CAC** | NS | NS | NS |
| 8 | **Combined Normals Versus Acute UC + Chronic UC** | P = 0.005 | P=0.0165 | P<0.0001 |
| 9 | **Combined Normals Versus Dysplasia + CAC** | **P <0.001** | **P<0.0001** | **P<0.0001** |
| 10 | **Acute UC + Chronic UC Versus Dysplasia + CAC** | NS | **P=0.0009** | **P<0.0001** |

UC – Ulcerative Colitis, CAC – Colitis Associated Cancer, miR – MicroRNA, NS – Not Significant P – Adjusted P value. Groups and results of clinical importance are highlighted in bold. Results of statistical significance are shown in green.

## Paired Analysis

Finally a paired analysis was carried out in which the ΔCT of CAC and dysplasia samples were compared to the ΔCTs of the equivalent adjacent normal tissue (Table 36).

**Table 36. Summary of Paired T-Tests for Neoplastic Tissue Versus Normal Control Tissue**

| MicroRNA | 10b | 18a | 21 | 31 | 34a | 135b | 215 | Let-7f |
|---|---|---|---|---|---|---|---|---|
| **P Value** | 0.1336 | 0.0612 | 0.0041 | 0.0155 | 0.0524 | 0.0001 | 0.2079 | 0.1141 |
| **Summary** | NS | NS | **Yes** | **Yes** | NS | **Yes** | NS | NS |
| **T** | 1.609 | 2.065 | 3.538 | 2.820 | 2.153 | 6.840 | 1.331 | 1.704 |
| **SD** | 3.270 | 3.304 | 2.046 | 2.007 | 1.457 | 2.678 | 1.212 | 2.191 |
| **SEM** | 0.9070 | 0.9165 | 0.5674 | 0.5566 | 0.4042 | 0.7429 | 0.3361 | 0.0078 |
| **95% CI** | -0.5170 to 3.436 | -0.1043 to 3.889 | 0.7709 to 3.243 | 0.3569 to 2.782 | -0.7709 to1.751 | 3.463 to 6.70 | -1.180 to 0.2850 | -0.2885 to 2.360 |
| **R** | 0.8452 | 0.1074 | -0.6951 | 0.2639 | 0.1716 | 0.07247 | 0.4260 | 0.5738 |

NS – Not Significant, P – P Value (α significance set at 0.05), SD – Standard Deviation, SEM – Standard Error of the Mean, CI – Confidence Interval, R – Correlation co-efficient

## 4.5 Discussion

### 4.5.1 Findings

This chapter aimed to discover dysregulated miRNAs in the colonic mucosa of patients with UC and determine their correlation to the following disease states: active UC, dysplasia and CAC. In order to do so, archived ffpe samples belonging to a representative population were identified, processed and allocated to the aforementioned groups. Within the discovery cohort, Taqman microfluidic array cards were used to screen for the differential expression of 381 miRNAs. A low stringency criterion was employed to highlight all miRNAs of interest. Poorly expressed miRNAs were eliminated. 6 upregulated miRNAs (miR-10b, miR-18a, miR-31, miR-34a, miR-135b, miR-215) and 1 down-regulated miRNA (miR-let-7f) were chosen for further validation. MiRNA-21 was added to the list as it has been found to be upregulated in many human cancers and UC (Chapter 1). The array data was subsequently validated. This would have been expected to show an increased expression of the upregulated miRNAs in the neoplastic cases. However the results were variable with some cases exhibiting high ΔCT values, when compared to the control cases, an indication of down-regulation. Nonetheless, as this was based on a small number of cases, it was decided that it was appropriate to continue testing the chosen assays in the larger, independent validation cohort (Figure 23 - Figure 30). In general, the dysplasia and CAC expression profiles appeared to be very similar with a great deal of overlap. On inspection, of the scatterplots, miRNA 10b and 215 became down-regulated as disease pathology progressed. MiRNAs 18a, 21, and 135b became up-regulated as disease progressed. Interestingly, miRNA-31 appeared to be linked to active inflammation, as it was upregulated in the active UC group and appeared to be quite distinct from chronic UC (Figure 26). Let-7f which was initially thought to be down-regulated was uniformly expressed in all disease groups. True down-regulation of a miRNA is difficult to calculate as a comparatively low CT or ΔCT value may arise due to number of reasons and therefore low expression within the samples may not equate to down-regulation.

Each miRNA was analysed as an independent variable using ANOVA (Table 33 rows 1-7). This showed that miRNAs 10b, 34a and let-7f could not significantly differentiate between any of the disease groups. MiRNA-215 expression was significantly decreased in active UC (compared to normal) and CAC (compared to dysplasia), neither of which is clinically helpful.

The most useful miRNAs are 21 and 135b as they are significantly increased in both dysplastic and CAC tissues when compared to their corresponding normal. These two miRNAs are also upregulated in the CAC cases when compared to chronic UC. MiRNA 18a and 31 appear to distinguish the CAC cases from normal, but as mentioned before miRNA-31 is also highly expressed in acute UC in these results. Therefore could be related to tumours that have invoked a large inflammatory response or a tumour arising on a background of severe inflammation.

**Combining Disease Groups**

The results revealed that there was no statistical difference between the two 'normal' groups ('normal for UC' and normal for neoplasia). Furthermore, no difference was noted between the dysplasia and CAC group (except for miRNA-215). Subsequently, the sub-groups were combined (Table 33 rows 8-10). ANOVA analysis revealed a significant ($P<0.05$) upregulation of miRs 18a, 21,31, 34a and 135b in the neoplasia group compared to the combined normal samples. MiRNA-34a had a similar distribution in the combined UC and neoplasia groups, which may correlate to its associated role with p53 or an increased level of mutated p53. The microRNAs 10b and 215 appeared to be down regulated in the UC and UC-neoplasia groups when compared against their corresponding normal tissue. The samples in both these groups had a very similar distribution. Lef-7f, originally thought to be downregulated appeared to be uniformly expressed within disease groups in the validation cohort. Interestingly, for most miRNAS, the combining the samples seemed to further define the disease groups. However for some miRNAs (18a and 31), this highlighted the difference between acute UC and chronic UC.

**MicroRNA Panels**

Dysplasia and CAC occurs as the result of multistep molecular pathological mechanisms such as oxidative stress, p53 loss, anueploidy, loss of cell cycle control and various genetic mutations. As range of miRNAs have been associated with these mechanisms, it would therefore seem logical that a panel of pathological miRNAs would reflect the CAC group more accurately than a single miRNA. Thus, the ΔCT values of different miRNAs were combined in order to evaluate if this affected their discriminatory power (Table 35). Combining miRNAS improved their ability to distinguish between the groups but in a complicated manner, depending upon which microRNAs were chosen. All combinations (MiRs-18a+21 / MiRs-135b + 215 / MiRs-21+135b) were able to significantly distinguish between normal tissue and CAC and combined normal tissue and the combined neoplasia group. In a clinical context the ideal biomarker would pick out patients with dysplasia or CAC on a back ground of acute or chronic UC and identify those patients with UC who are at high risk of developing CAC and thus require close monitoring. Therefore, in this situation the ideal combination would pick out the CAC, dysplasia or combined neoplasia groups when compared to normal or colitic tissues. Combinations that distinguish between normal tissues or colitic tissues are not helpful as diagnostic or prognostic test in patients with diagnosed UC undergoing surveillance for CAC. Unfortunately all combinations were also significant when comparing the UC groups to the normal groups.

**Paired Analysis**

Finally a paired t-test was carried out to assess the differences arising in neoplastic tissue compared to adjacent normal tissue. This showed that miRNA 21, 31 and 135b were the only microRNAs that could detect a statistically significant difference between the tissue types.

### 4.5.2 Comparison To The Current Literature

In section 1.3, the studies investing miRNA expression in UC and in CAC were reviewed. MiRNAs which were repeatedly found to be overexpressed in active UC tissues compared to control tissues were miRNAs let-7g, let-7d, 16, 21, 28-5p, 29a, 31, 126, 135b, 151-5p, 155, 199a-5p, 203, 223 (Table 6). MiRNA 203 was also increased in active UC tissue compared to inactive UC tissue.

MiRNAs that were increased in dysplastic or CAC tissues (when compared to UC tissues or control groups) were miRNAs: 31, 135b, 192, 194 (Table 7). Of note, miRNAs 31 and 203 was upregulated in dysplastic tissue in one of the studies (206) and miRNAs 31 and 135b in another (207). The functional studies also highlighted the upregulation of miRNA 21,126 and 155 in the pathogenesis of UC. In comparison, this study found 18a, 21,31, 34a and 135b to be upregulated, all of which are in keeping with the literature review in section 1.3.

The results revealed a mixed picture for miRNA 215. The initial results showed increased expression of 215 in the context of disease progression. However this was not substantiated in validation, which showed underexpression with disease progression. This is interesting as the literature has contradictory results where studies have reported both over expression of miRNA 215 in CAC tissue (209) and under expression (207).

When comparing results to the literature it is important to note that all studies were carried out using different tissue sources, species, models and used different experimental techniques and normalisation methods.

### 4.5.3 Study Strengths

The design of this study employed biomarker discovery strategy as described in section 1.4 (Table 11). The miRNAs of interest were first identified in a discovery cohort followed by validation in a separate cohort. Although phase 1 of study contained a small number of CAC cases, it was on par with other published studies (Table 6). Furthermore not all of these published studies conducted their experiments in phases with separate discovery and validation stages.

The methodology utilised within this thesis was based upon well-established standard operating procedures that were validated within the study laboratory and cited within the literature. Only ffpe samples of sufficient concentration and quality were included in the reverse transcription and pre-amplification steps. The MIQE guidelines were also followed when carrying out the RT-qPCR experiments with multiple controls built in to ensure accuracy and reproducibility (Table 22).

The process of normalisation is an important issue in miRNA studies as it corrects for inherent biological discrepancies arising from sample variation and induced discrepancies arsing from technical, experimental error (reaction efficiency and inaccurate handling). A stable reference gene known as an endogenous control is used for this purpose. It can be calculated via a variety of methods including freely available web based software such as geNorm or NormFinder. This study utilized U6 and 484 for normalisation as both have been widely used in the literature. The array data was normalized using a well-established plate averaging method.

The experiments generated from this chapter are novel experiments; no prior study to date has used ffpe specimens as a source of dysplastic and CAC tissue. The study shows that there is differential expression of miRNAs in CAC, dysplastic, UC and adjacent normal tissues. In particular the microRNAs 18a, 21, 31 and 135b are of interest and may be associated with pathological processes that drive the inflammation-dysplasia-adenocarcinoma pathway.

Furthermore the upregulation of miRNAs 21, 31 and 135b has been substantiated in the literature. These results replicate the literature and provide proof of concept that miRNAs can act as biomarkers to distinguish between the different tissue types.

### 4.5.4 Study Limitations and Difficulties Encountered

RNA extraction from archived tissues is a well established method, however the age and poor fixation of the paraffin embedded tissues could have contributed to low RNA concentrations and poor quality total RNA. Subsequently a large number of samples were eliminated, reducing the power of the study.

Due to the limited number of samples and biological variation, the study was unable to match the samples for age, gender, medication or duration of disease. It was also difficult to match all the CAC and dysplasia cases with adjacent normal tissue. In some cases chronic UC tissue constituted the 'normal' tissue for that particular case. The study did not contain any truly 'normal' normal cases. It may have been a useful exercise to compare the 'normal adjacent tissue from the neoplastic cases to healthy, non-diseased colonic mucosa to investigate the effects of a field change and to identify the miRNAs associated with the development of dysplasia. This category of tissue was not available and thereby classing chronic UC tissue as 'normal' may have diminished the pick up rate of miRNAs truly related to carcinogenesis.

When a patient undergoes a bowel resection, multiple sections from the specimen are embedded into paraffin and processed onto an H&E slide for diagnostic purposes. This process only samples a small proportion of the entire bowel specimen. It is possible that flat areas of dysplasia or CAC may have been missed, as they are difficult to detect. Furthermore, some of the dysplastic samples came from specimen's harbouring CAC. It is also known that the finding of dysplasia within a sample correlates with concurrent dysplasia or CAC. This may account for the overlap of delta CT values across different groups or non-liner trends across the disease groups.

The tumours in the validation series mainly constituted T3/T4 tumours. Therefore the identified, miRNAs of interest might be related to advanced pathological processes such as invasion and metastasis. It would have been ideal to use as many cases as possible with early events (dysplasia and T1 tumours) as a clinically useful test needs to identify patients at risk. However

due to the nature of these tumours (late presentation) and potential poor compliance with the surveillance programme, such cases were not available. Furthermore, if a larger number of cases were available, it would allow the study to investigate if variables such as CAC location (right sided, tumours, left sided tumour and rectal tumours) and gender have an effect upon miRNA expression.

The microarray results were used to choose MiRNAs for further analysis. The validation results did not entirely reflect the expression results found in the microarrays. Low correlation co-efficient between the two modalities has been reported before and serves to prove the importance of rechecking screening data with RT-qPCR.

A power based sample size calculation was not carried out during the study design and protocol, as this was a pilot study. The study aimed at providing proof of concept that tissue based miRNAs could logistically be used as biomarker molecules to detect differences between 'normal' UC, dysplastic and CAC tissues. None of the studies appraised in Table 6, 7, or 8 had used a power calculation. It is acknowledged that the sample numbers are low and this could lead to both type 1 and 2 errors and potentially undermines confidence in the results obtained. As discussed before, an inadequately powered study has less ability to detect a true result and decreases the probability of any significant results being true (unless the outcome being measured has a massive effect). In order to minimise this, the study attempted to adhere to a robust study design in all other aspects. An alternative would be to increase the threshold for statistical analysis (decrease α) to avoid a type 1 error.

It was genuinely impossible to gain a larger number of cases due to costs, time, identification difficulties and limited amount of dysplastic and CAC tissues. To overcome this, future studies will need recruit over multiple sites in order to gain adequate sample numbers. Furthermore due to the limited number of cases, a false discovery rate was not applied to the array analysis, potentially increasing the rate of false findings. Now that this study has been completed, its shortcomings can be used to improve future studies.

### 4.5.5 Future Work

The aim of this chapter was to discover novel tissue based miRNAs associated with disease progression in UC and the development of dysplasia and CAC. With further resources it would have been possible to validate a larger range of miRNAs such as those that had been repeatedly found in the literature and those related to the disease processes that occur in UC such as p53 mutation. It would also be interesting to further explore the role of miRNA 31 to investigate whether it is related to inflammation or true neoplastic transformation and investigate its end targets.

This study showed proof of concept that miRNAs can be extracted and profiled in archived UC, dysplastic and CAC tissues in a reproducible manner. The next step in biomarker development would be validation of the results in a larger cohort of adequate power. If this study were to be repeated on a larger scale, a power based sample size calculation would need to be carried out, therefore requiring a figure for both s (standard deviation of the clinical measure) and δ (a measure of the smallest difference that needs to be detected). Thus further work would need to be done in this area to determine what measurements are appropriate: fold change, the magnitude of fold change or the presence of absence of one or several miRNAs.

As UC related dysplasia and carcinoma is not as common as SCRC this would require collaboration with multiple institutions. If this could be achieved it would then be possible to conduct the middle stages of biomarker development (phase II-IV) such as retrospective longitudinal and prospective screening (Table 11). During these stages it would be possible to determine the effect of clinical parameters upon the test, calculate statically algorithms and receiver operating curves and evaluate the logistics of implementing the test (219).

A further direction of investigation involve comparing the results from this study to the same tissue types collected prospectively from an alternative source such as fresh frozen tissue. This would eliminate the problems associated with archived tissue and would determine if the results from a fresh tissue source were more accurate, reproducible and logistically viable. In a clinical context,

this could be very useful test as it could utilise a minimum amount of mucosal pinch biopsy tissue that is taken during a colonoscopy to ascertain if the patient is at risk of developing dysplasia or CAC. Furthermore if small amounts of colonic tissue taken from any part of the colon were sufficient to detect neoplastic transformation (in the context of a field change), it would mean that flexible or rigid sigmoidoscopy based screening would be feasible instead of a colonoscopy.

This chapter focused upon the miRNAs that showed increased expression when correlated to disease progression in UC. It would be interesting to look at stand-alone miRNAs specifically increased in dysplasia compared to truly 'normal' colonic mucosa. This is because the ideal biomarker would want to highlight high-risk patients as opposed to detecting later stage disease eg CAC. A very large number of dysplastic cases would be required, once again necessitating the need for institutional collaboration.

A potentially useful direction would be to establish the prevalence and type of mutations present in the dysplastic and CAC tissues. This could be done using immunohistochemistry to detect p53 mutations or by using mutation specific assays for Kras, and BRAF. This could help direct the search towards miRNAs that were known to be associated with these mutations.

## 4.6 Summary

- Array analysis of the discovery cohort revealed the differential expression of over 60 miRNAs of which miRNAs 18a, 21, 31, 34a, 135b, 215 and let-7f were specifically chosen to be validated in an independent cohort.

- Validation showed significantly increased expression of miRNA 18a, 21, 31 and 135b in CAC tissues compared to adjacent normal tissues. MiRNAs 31 and 135b were also significantly increased in dysplastic tissue compared to normal tissues.

- A combined miRNA panel improved the ability of the test to distinguish between different disease states.

- Provides proof of concept that miRNA expression is altered in UC associated neoplasia.

- MiRNA expression profiling in UC is challenging due to patient and biological variation, limited specimen availability and quality. The lack of a dedicated IBD patient database within the hospital trust made it difficult to identify retrospective cases of CAC. For some cases there was a lack of clinical information.

# Chapter 5. Results

## MicroRNA Expression Profiles in the Circulation of Patients with Ulcerative Colitis

## 5.1 Background

MiRNAs expression is dysregulated in UC tissues as exemplified in the literature (Chapter 1) and by the results in Chapter 4. These results have largely been based on experiments carried out on colonic mucosa, mouse models and cell lines. At the time of writing date, no studies had investigated miRNA profiles in the blood of patients with UC related dysplasia or CAC and there are no studies exploring the correlation between miRNAs found in the colonic mucosa tissue and those in the circulation. If miRNA profiles associated with UC related neoplastic transformation could be characterised, it could form the basis of a blood based biomarker test. This could be used in lieu of surveillance colonoscopy or as a screening tool to highlight the high-risk patients who need regular or urgent colonoscopic surveillance. This could be cost effective and increase patient compliance with surveillance.

## 5.2 Aim 3

Determine a panel of plasma miRNAS associated with the pathology and progression of disease in patients with Ulcerative Colitis and evaluate the potential of these microRNAs to act as blood-based biomarkers for the surveillance of UC patients.

## 5.3 Objectives

- Identify and prospectively collect blood samples from patients undergoing colonoscopic screening for UC.
- Use histological results of biopsies taken during the colonoscopy to allocate the samples to the groups: UC, PSC, Dysplasia and CAC.
- Screen for differentially expressed miRNAs by conducting high throughput microRNA profiling using taqman microfludic cards™.
- Validate results using individual miRNA assays.
- Investigate if the miRNAs previously identified in colonic mucosa are also detectable in the circulation of patients with UC.

## 5.4 Results

Several issues were encountered during the study, mainly pertaining to recruitment and the way in which the endoscopies were performed. This will be described further in the discussion. There was also a lack of standardization within the histology reports, which used a wide variety of terms to describe UC tissue (Figure 35). Therefore during the array analysis the samples were split into: active, chronic and quiescent groups. They were subsequently analysed separately and in a combined group.

During the recruitment process, a cohort of patients with PSC and UC were encountered and samples procured. As PSC is a risk factor for CAC these were analysed upon a separate array card.

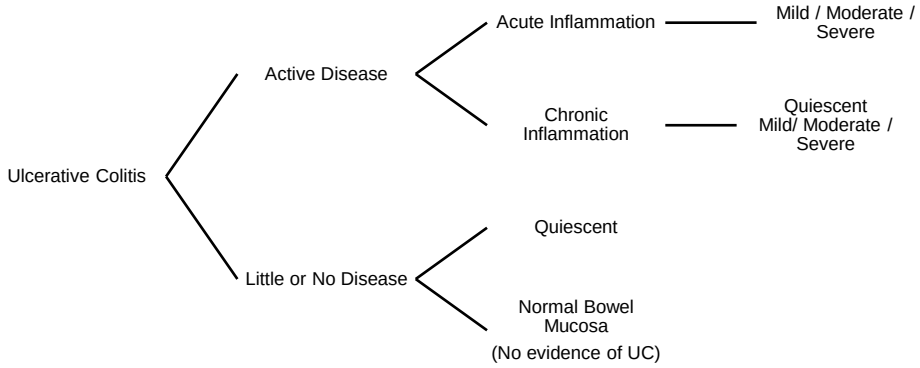



**Figure 35. The Terms Used to Describe Ulcerative Colitis in Histology Reports**

### 5.4.1 Quality Control and Sample Overview

Several quality control measures were employed to detect and reduce the error and bias that could arise throughout the different steps of sample processing (Figure 36).

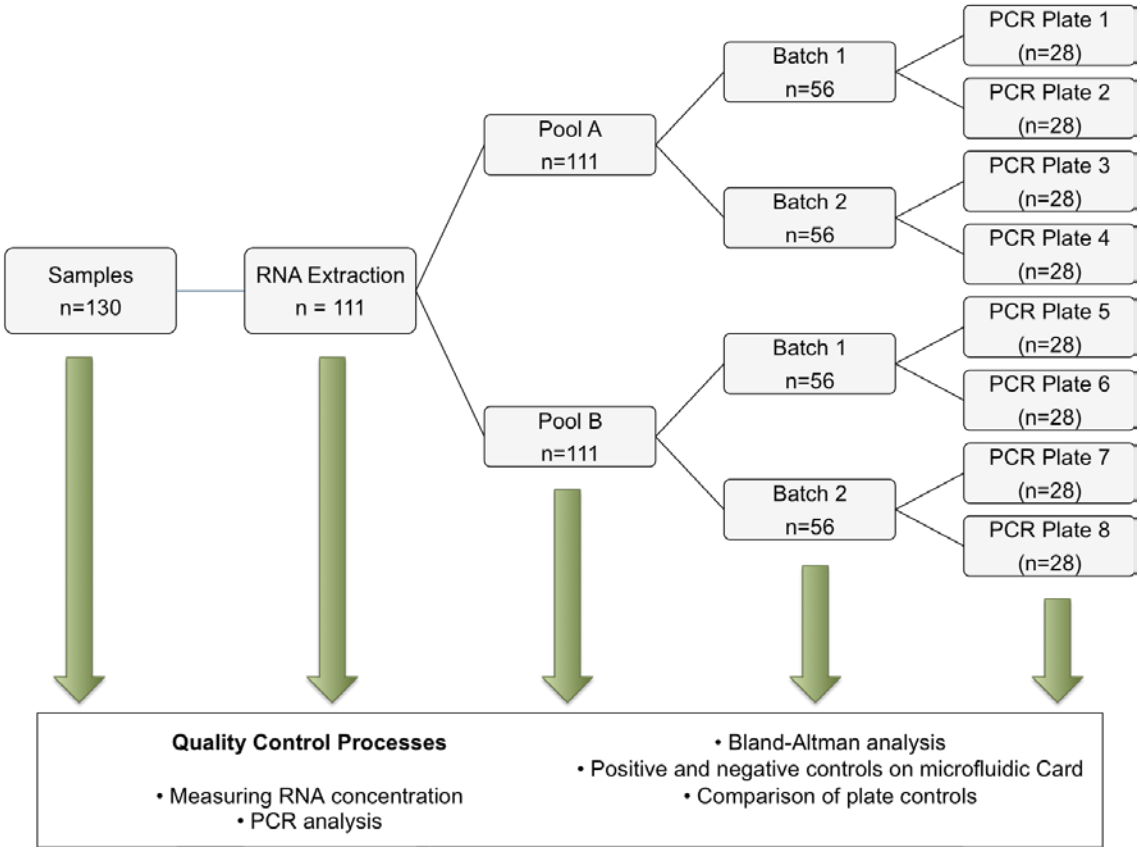



**Figure 36. Quality Control Processes Employed During The Different Steps of Sample Processing.**

Samples (n=130) were collected from patients over a 18-month period. Once sample collection was complete, the total RNA was extracted and processed with pool A & B primers as described in chapter 2. The samples were processed in two batches per pool (4 batches in total). Running each individual assay in triplicate entailed using 4 plates (8 plates for the U6 assay which is included in both sets of primers for normalisation).

**Evaluation of Total RNA Concentration**

The median concentration value of plasma extracted total RNA was 7.366 (range 1.7-19.1 for 112 samples). The 260/280 absorbance ratios ranged from 0.94 – 2.03. Low ratios were attributed to a combination of low nucleic acid concentration and possible phenol and/or guanidine contamination from the extraction kits. Following comparison to similar work in the laboratory, the range was deemed suitable for RT and pre-amplification reactions.

**Check PCR**

Prior to array analysis, both sets of samples were tested with 3 well-expressed microRNAs. The pool A samples were tested with U6 and miR-191. Pool B samples were tested with U6 and miR-30d. This showed that many samples had underperformed, resulting in CT values of above 26 for both pool A and pool B U6 samples. These samples were deemed unsuitable for array analysis and PCR validation. Resources were available for RNA extraction from 56 plasma samples. Testing of the 56 samples showed that the CT values were on average 5-6 cycles lower than the previous set, within the expected range and therefore appropriate for subsequent applications.

**Technical Replicate Analysis**

A Bland-Altman analysis for the newly extracted samples (Figure 37) showed a small bias and narrow limits, suggesting little batch variation between the pool A and B RT and pre-amplification reactions. Three outliers were found, which were retained in the sample set due to small sample numbers. Both groups were statistically similar (paired t-test P=0.4389).

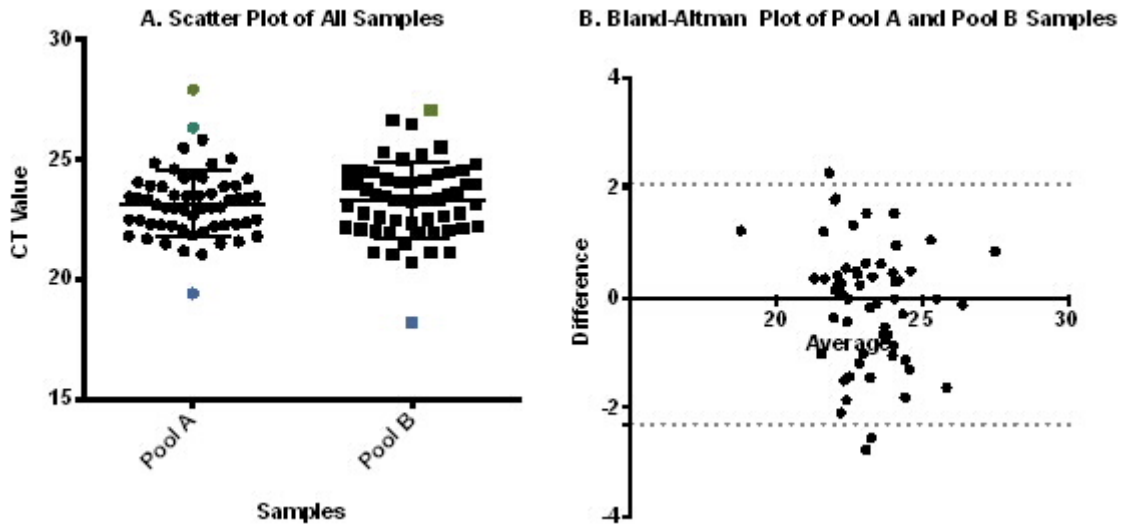



**Figure 37. Scatter Plot and Bland-Altman Plot for Pool A and Pool B Plasma Samples.**

A. Scatter plot of U6 average CT values for all pool A and pool B samples. Results are presented with mean and standard deviation. Pool A mean = 23.15 (SD 1.408). Pool B mean – 23.56 (SD 1.56). Outliers (samples that lie 2 SD from the mean) are highlighted (cases matched by colour). B.Bland-Altman plot showing 95% limits of agreement (from -2.300 to 2.072). Bias -0.1142 (SD of bias 1.115).

The samples included in the study analysis are summarised in Figure 38.

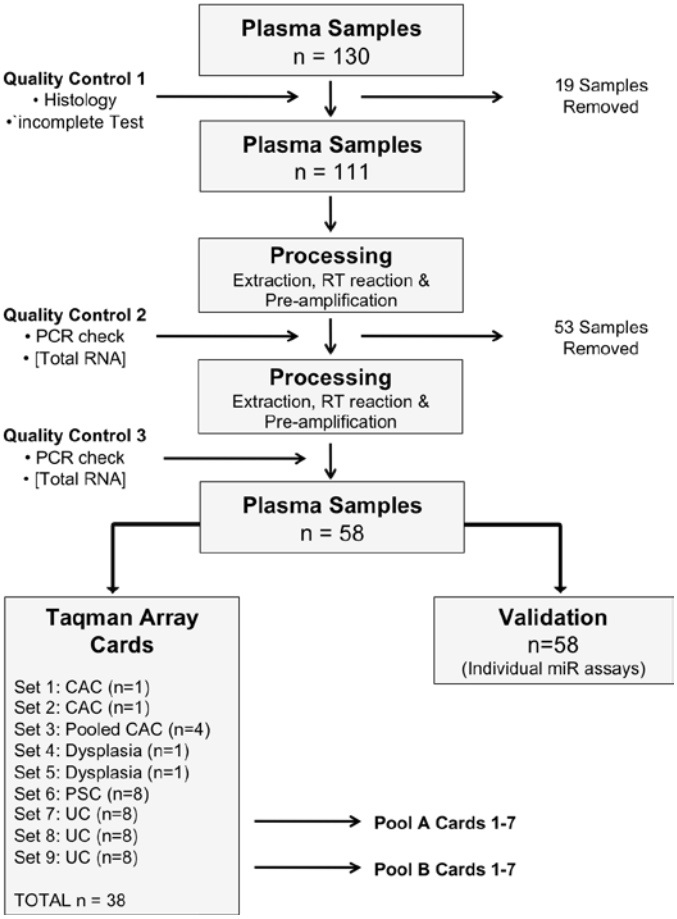



**Figure 38. Summary of Sample Numbers**

UC – Ulcerative Colitis, PSC – Primary Sclerosing Cholangitis, CAC – Colitis Associated Cancer, n- number of patients. 130 samples were collected form patients under going colonoscopy for bowel related symptoms or UC-related screening. 19 Samples were excluded as patients due to an incomplete colonoscopy or a histological diagnosis of Crohns Disease. Check PCR, following the first stage of processing revealed poor quality samples. Resources were available for total RNA to be extracted from 58 samples, of which 38 samples were run upon the array cards. All 58 samples were validated with independent microRNA assays.

As a large number of samples were eliminated from the analysis, the demographics for the patients analysed upon the microarray cards and validation series are re-presented in Table 37 and Table 38.

**Table 37. Summary Demographics Of Samples Tested Upon the Microarray Cards**

| Card | Code | Histology | Sex | Disease Duration (years) | Other |
|---|---|---|---|---|---|
| **Card 1** | | | | | |
| Sample 17 | CAC | CAC – Rectal | M | 25 | |
| **Card 2** | | | | | |
| Sample 31 | CAC | CAC – Rectal | M | 6 | Multiple co-morbidities |
| **Card 3** | | | | | |
| Sample 17 | Pooled CAC | CAC – Rectal | M | 25 | |
| Sample 31 | Pooled CAC | CAC – Rectal | M | 6 | Multiple co-morbidities |
| Sample 38 | Pooled CAC | CAC – rectal polyp cancer | F | 37 | |
| Sample 112 | Pooled CAC | CAC – rectal polyp cancer | F | ? | |
| **Card 4** | | | | | |
| Sample 73 | LGD | LGD | M | 36 | |
| **Card 5** | | | | | |
| Sample 122 | LGD | LGD | F | 35 | |
| **Card 6** | | | | | |
| Sample 10 | PSC | PSC + UC Quiescent | M | 1 | |
| Sample 45 | PSC | PSC + UC Chronic - mild | M | 2 | |
| Sample 48 | PSC | PSC + UC inconclusive | F | 30 | |
| Sample 55 | PSC | PSC + UC Quiescent | F | 16 | |
| Sample 61 | PSC | PSC + UC Active – mild | M | 10 | |
| Sample 72 | PSC | PSC + UC inconclusive | M | 27 | |
| Sample 79 | PSC | PSC + UC Quiescent | M | 30 | |
| Sample 109 | PSC | PSC+ UC Active – mild | M | 5 | |
| **Card 7** | | | | | |
| Sample 53 | UC | UC – Active - mild | M | 25 | |
| Sample 67 | UC | UC – Active - mild | M | 4 | |
| Sample 103 | UC | UC – Active - mild | M | 10 | |
| Sample 23 | UC | UC – Active – severe | M | 8 | |
| Sample 29 | UC | UC – Active – severe | M | 20 | |
| Sample 36 | UC | UC – Active – severe | F | 36 | |
| Sample 39 | UC | UC – Active – severe | M | 3 | |
| Sample 56 | UC | UC – Active – severe | F | 3 | |
| **Card 8** | | | | | |
| Sample 6 | UC | UC – Chronic - mild | M | 15 | |
| Sample 18 | UC | UC – Chronic - mild | M | 10 | |
| Sample 19 | UC | UC – Chronic - mild | F | 20 | |
| Sample 25 | UC | UC – Chronic - mild | M | 2 | |
| Sample 42 | UC | UC – Chronic - mild | M | 15 | |
| Sample 46 | UC | UC – Chronic - mild | M | 13 | |
| Sample 54 | UC | UC – Chronic - mild | M | 11 | |
| Sample 74 | UC | UC – Chronic - mild | M | 18 | |
| **Card 9** | | | | | |
| Sample 7 | UC | UC – Quiescent | F | 23 | |
| Sample 16 | UC | UC – Quiescent | F | 22 | |
| Sample 21 | UC | UC – Quiescent | M | 33 | |
| Sample 33 | UC | UC – Quiescent | M | 13 | |
| Sample 43 | UC | UC – Quiescent | M | 28 | |
| Sample 76 | UC | UC – Quiescent | F | 20 | |
| Sample 88 | UC | UC – Quiescent | F | 22 | |
| Sample 92 | UC | UC – Quiescent | M | 14 | |

UC – Ulcerative Colitis, PSC – Primary Sclerosing Cholangitis, CAC – Colitis Associated Cancer, LGD – Low Grade dysplasia. M – Male, F – Female.

**Table 38. Summary Demographics Of Extra Validation Samples**

| Sample | Code | Histology | Sex | Disease Duration | Other |
|---|---|---|---|---|---|
| **Extra Cases** | | | | | |
| Sample 8 | UC | UC – Quiescent | M | 12 | |
| Sample 22 | UC | UC – Quiescent | M | 11 | |
| Sample 34 | UC | UC – Quiescent | F | 5 | |
| Sample 44 | UC | UC – Quiescent | M | 10 | |
| Sample 49 | UC | UC – Quiescent | F | 13 | |
| Sample 51 | CAC | CAC – Rectal | M | 20 | 2 rounds of chemo/radio |
| Sample 52 | UC | UC – Quiescent | M | 3 | |
| Sample 62 | PSC | PSC UC - Quiescent | M | 14 | Autoimmune Hepatitis |
| Sample 66 | PSC | PSC UC - Active - Mild | M | 8 | Liver Transplant + PSC |
| Sample 77 | UC | UC – Quiescent | M | 4 | |
| Sample 91 | UC | UC - Active - Mild | M | 4 | |
| Sample 96 | UC | UC – Quiescent | F | 5 | |
| Sample 97 | UC | UC – Quiescent | F | 21 | |
| Sample 99 | UC | UC – Quiescent | F | 42 | |
| Sample 101 | LGD-prior | | M | 36 | LGD in past |
| Sample 106 | LGD-prior | | M | 25 | LGD in past |
| Sample 108 | CAC-post op | H-700-12 CAC – (post R hemi) | M | ? | Right Hemi / PSC (DALM) |
| Sample 121 | CAC | CAC - Need Histology | M | ? | 2 rounds of chemo/radio |
| Sample 123 | UC | UC – Active - Mild | F | 11 | |
| Sample 124 | UC | UC – Quiescent | M | 11 | |

UC – Ulcerative Colitis, PSC – Primary Sclerosing Cholangitis, CAC – Colitis Associated Cancer, LGD – Low Grade dysplasia. M – Male, F – Female. For two patients, the disease duration was unknown by the patient and not recorded in the notes

### 5.4.2 Microarray Profiling

Pool A and Pool B Microfluidic arrays were used to quantify the expression of 754 known human microRNA genes amongst seven disease groups. When checking the endogenous card controls, it was noticed that some of the array cards had inappropriately high CT values for the endogenous controls, including CT values of over 26 for U6 (Table 39). As a result, the data was normalized via a card averaging method. This yielded a similar average CT across the cards (mean 24.66, range 23.85 – 25.67).

**Table 39. Comparison of Pool A and Pool B Cycle Threshold Values of The Endogenous Controls From The Array Cards**

| MicroRNA | Case 17 | Case 31 CAC | Grouped CAC | Case 73 LGD | Case 122 LGD | Grouped PSC | UC Active | UC Chronic | UC Chronic |
|---|---|---|---|---|---|---|---|---|---|
| **U6 A** | 29.97 | 28.67 | 27.63 | 25.10 | 24.68 | 29.88 | 28.08 | 25.55 | 24.69 |
| **U6 A** | 31.34 | 30.69 | 27.28 | 24.15 | 25.12 | 32.74 | 29.30 | 25.18 | 24.81 |
| **U6 A** | 30.52 | 30.08 | 26.32 | 24.20 | 24.97 | 30.83 | 29.22 | 25.83 | 25.36 |
| **U6 A** | 31.24 | 31.32 | 27.62 | 25.11 | 24.65 | 31.19 | 28.57 | 25.69 | 25.17 |
| **U6 B** | 29.30 | 30.34 | 29.42 | 34.03 | 32.26 | 23.43 | 33.40 | 25.70 | 25.03 |
| **U6 B** | 27.67 | 31.45 | 28.72 | 32.07 | 31.55 | 27.36 | 33.20 | 26.49 | 25.82 |
| **U6 B** | 27.82 | 30.73 | 28.40 | 33.50 | 32.45 | 27.38 | 33.51 | 24.75 | 26.31 |
| **U6 B** | 28.73 | 31.04 | 28.18 | 33.11 | 32.89 | 27.69 | 34.39 | 25.06 | 26.74 |
| **RNU44 A** | 35.59 | 34.53 | 29.06 | 37.59 | 33.43 | 37.45 | - | - | 25.81 |
| **RNU44 B** | 37.27 | 36.79 | - | - | - | - | - | 25.62 | 34.70 |
| **RNU48 A** | - | 34.14 | 32.27 | 29.87 | 30.90 | 30.59 | 30.61 | 29.98 | 29.27 |
| **RNU48 B** | 31.89 | 33.02 | 31.79 | 32.98 | 34.84 | 32.35 | - | 30.55 | 30.89 |

UC – Ulcerative Colitis, PSC – Primary Sclerosing Cholangitis, CAC – Colitis Associated Cancer, LGD – Low Grade dysplasia. Blocks - = undetermined.

Sample recruitment did not yield enough ‘normal’ samples therefore ΔΔCT values for the disease groups were created using a separate set of control data generated in the laboratory. In this cohort, ‘normal cases’ constituted blood samples obtained from male and female patients aged 60-69 who took part in the national bowel-screening programme (Appendix 2). All patients had tested positive for faecal occult blood test but were subsequently found to have macroscopically and microscopically normal colonoscopy. An additional

‘normal’ cohort consisted of patients enrolled upon the bowel screening programme, in which colorectal cancer or polyps were excluded but had haemorrhoids or diverticular disease. A combination of the above patients was thought to clinically represent a good control cohort. This data was kindly supplied by AV and was suitable for analysis as samples had been collected and processed using the same standard operating procedures.

It was noted that a large proportion of assays upon the Pool B cards produced CT values that were consistently below 30. This could have been a result of low plasma RNA yield, assay inefficiency or Pool B assays representing poorly expressed miRNAs. The amplification curves for these assays were assessed on the SDS software which revealed flat curves for the majority of the assays. Subsequently, these assays were not considered for validation. As this was primarily a screening method to identify potential plasma based miRNA biomarkers, once again a low-stringency criterion was of trend change, fold-change and T-tests were employed. A selection of examples are given:

- Trend changes (Figure 39-Figure 40)
- Fold Changes (Figure 41- Figure 42)
- T-Tests (Table 40)

This led to the following miRNAs being chosen:
Pool A: 125b, 139-3p, 375, 501-3p
Pool B: 409-3p, 483-3p, 720, 1274B.

Further assays were added to the validation set, based upon literature findings, tissue-based profiling results (miR-10b, miR-21, miR-31, miR-135b, miR-215) and other work carried our within the laboratory (miR-10a, miR-30e, miR-32, miR-98, miR-146b, miR-205, miR-331-5p, miR-452, miR-486 and miR-625). A total of 28 miR assays were tested in total, of which 4 were performed for normalization purposes (miR-U6, 30d, 191 and 484).

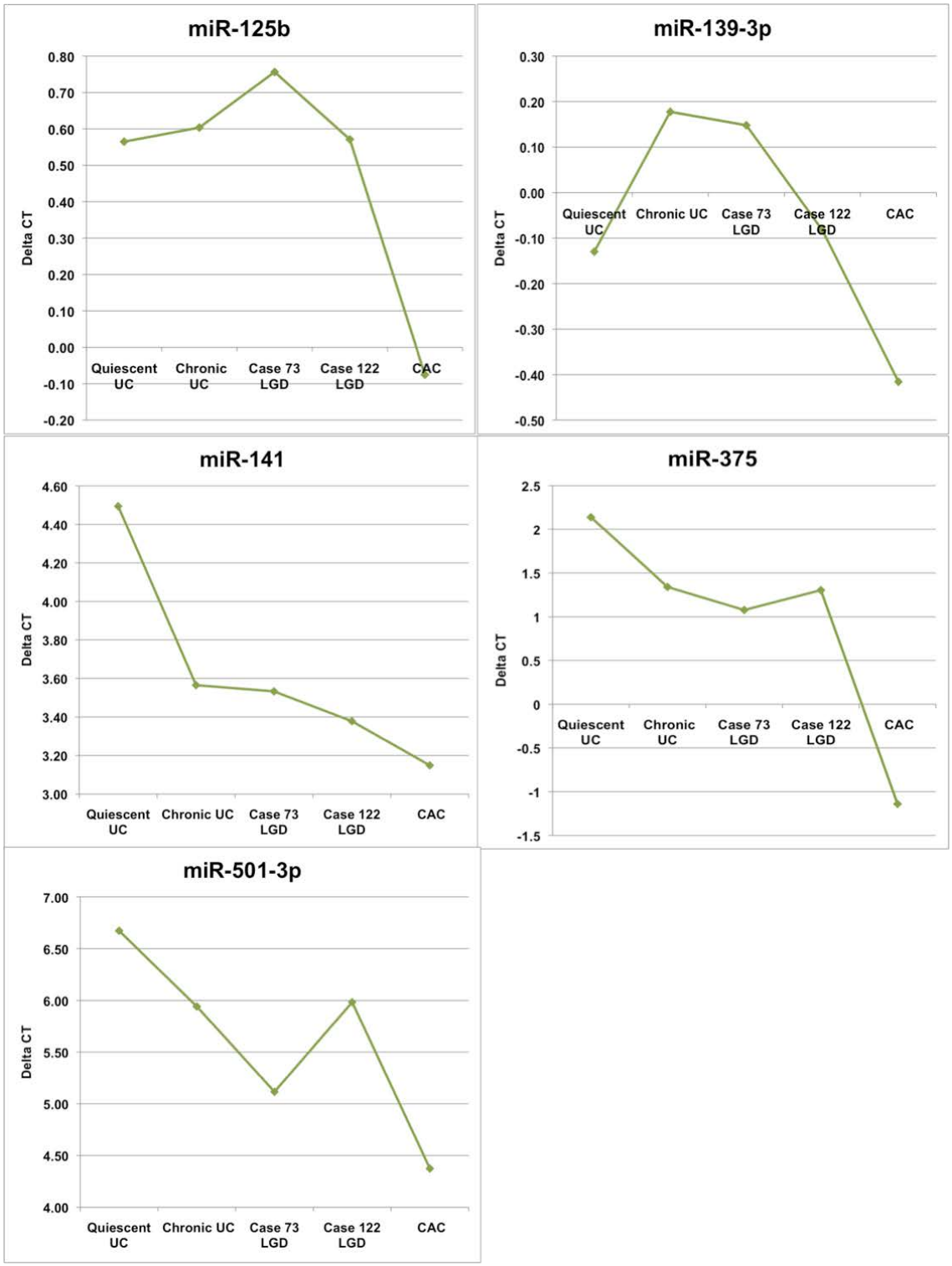



**Figure 39. Trend Changes for Selected Pool A MicroRNAs Across Selected Cases**

UC – Ulcerative Colitis, CAC – Colitis Associated Cancer, LGD – Low Grade dysplasia.

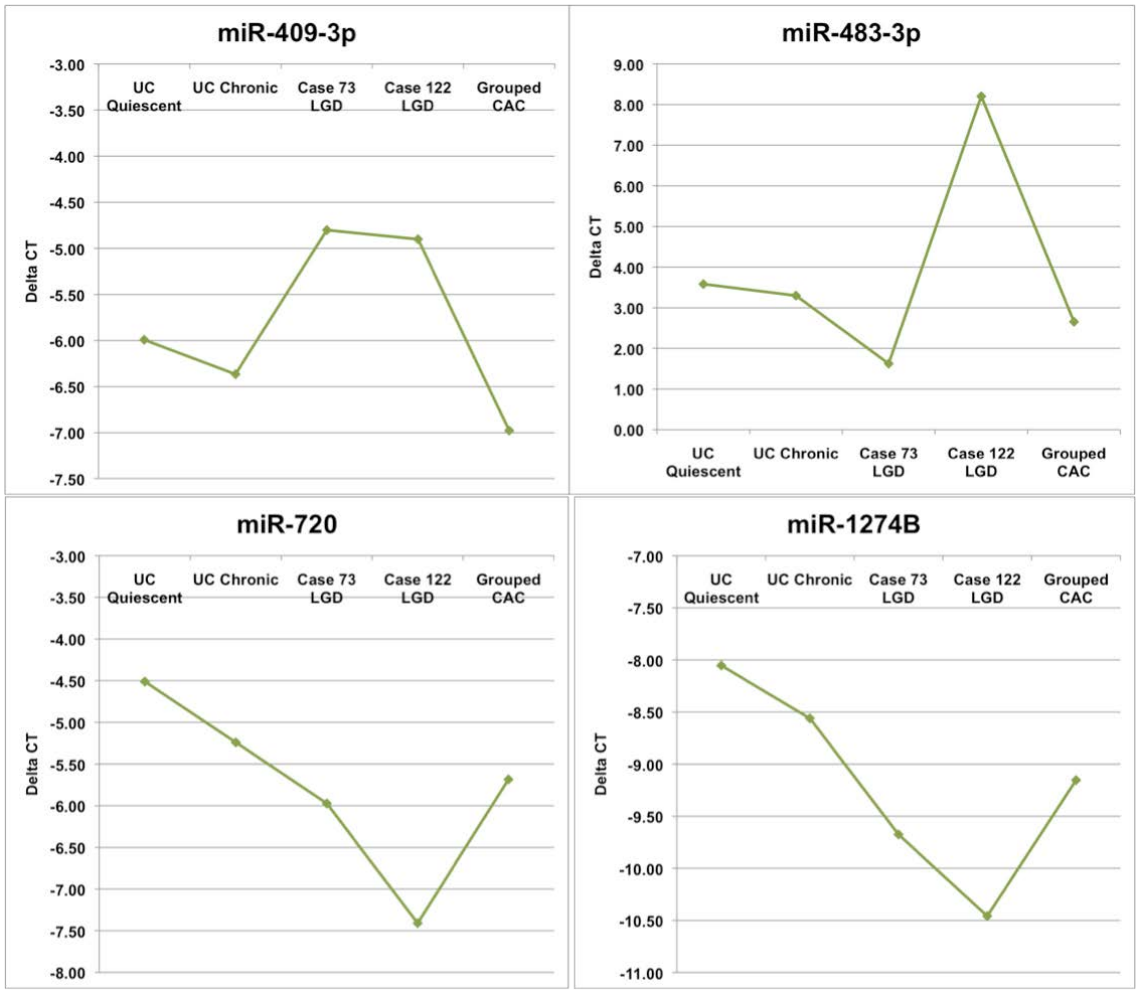



**Figure 40. Trend Changes for Selected Pool B MicroRNAs Across Selected Cases**

UC – Ulcerative Colitis, CAC – Colitis Associated Cancer, LGD – Low Grade dysplasia.

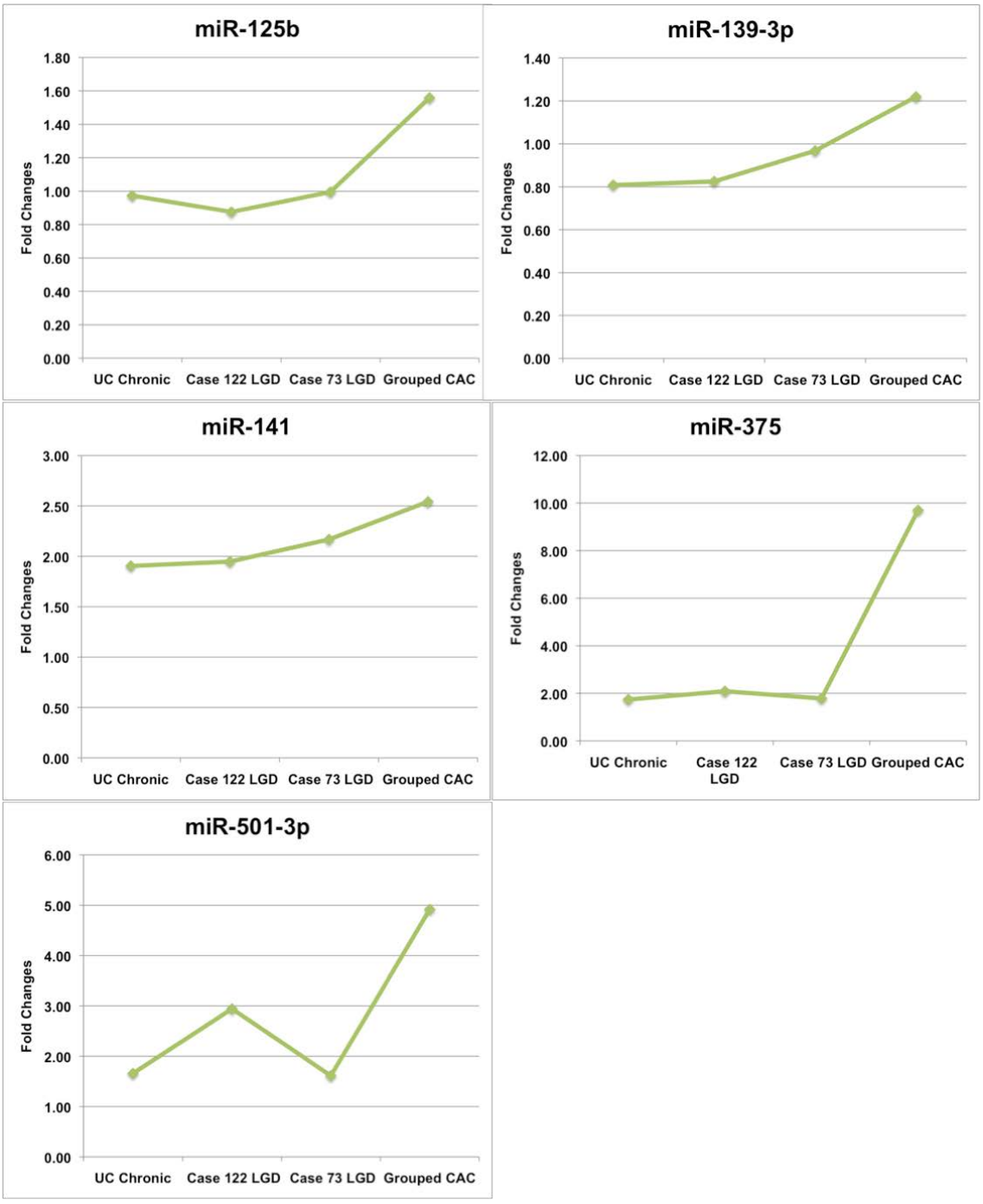



**Figure 41. Fold Changes for Selected Pool A MicroRNAs Across Selected Cases**

UC – Ulcerative Colitis, CAC – Colitis Associated Cancer, LGD – Low Grade dysplasia.

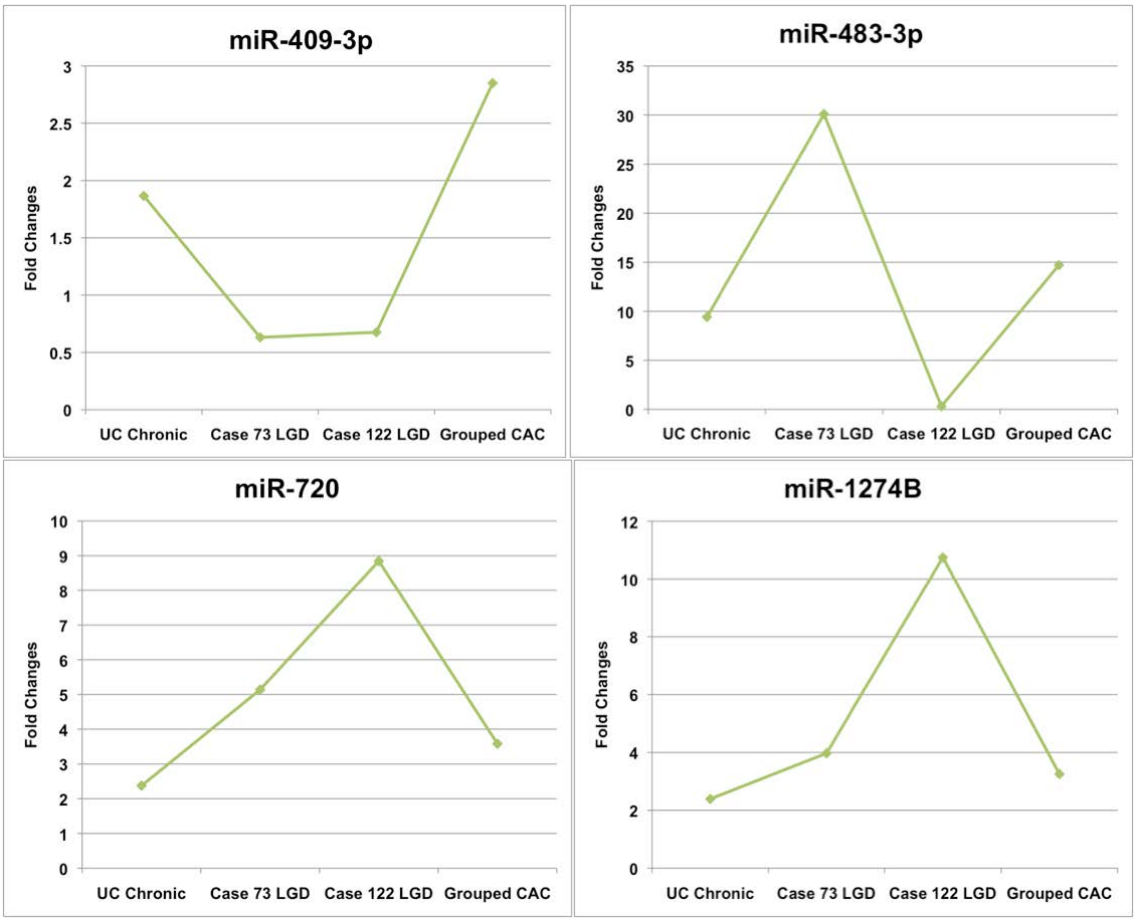



**Figure 42. Fold Changes for Selected Pool B MicroRNAs Across Selected Cases**

UC – Ulcerative Colitis, CAC – Colitis Associated Cancer, LGD – Low Grade dysplasia.

**Table 40. Summary of Significant Array MicroRNAs Found on T-Test**

| | Neoplasia Vs Controls | | Controls Versus UC | | UC Versus CAC | |
|---|---|---|---|---|---|---|
| | P ≤ 0.05 | P ≤ 0.01 | P ≤ 0.05 | P ≤ 0.01 | P ≤ 0.05 | P ≤ 0.01 |
| **Pool A** | 40 | 10 | 39 | 13 | 10 | 4 |
| **Pool B** | 114 | 25 | 16 | 10 | 75 | 3 |

UC – Ulcerative Colitis, CAC – Colitis Associated Cancer. The neoplasia refers to a combined group of CAC and dysplasia. The table shows how many microRNAs were found to be significant during unpaired t-testing, comparing the different groups.

### 5.4.3 Independent Validation of Candidate MicroRNAs

To confirm the differential expression of the identified candidate miRNAs, real-time PCR was performed on 58 cases using individual miRNA assays.

### Quality Control Processes

As the PCR was carried out over several plates, quality control processes had to be employed. Bland-Altman analysis (Figure 43), and plate control comparison (Table 41) confirmed the reproducibility of the PCR reactions across plates.

For several samples, a range of CT values produced by the individual assays was compared to the equivalent CT values generated upon the array cards (Table 42). When the data was plotted (Figure 44) it showed that for most samples the individual assays consistently produced a lower CT value than the array. This was because a slightly higher concentration of dilute pre-amplified product was used per sample reaction. Furthermore, it has already been noted that some of the array assays did not work as efficiently as they should have. The data was then tested for normality to ensure it was suitable for the intended parametric tests (Table 43).

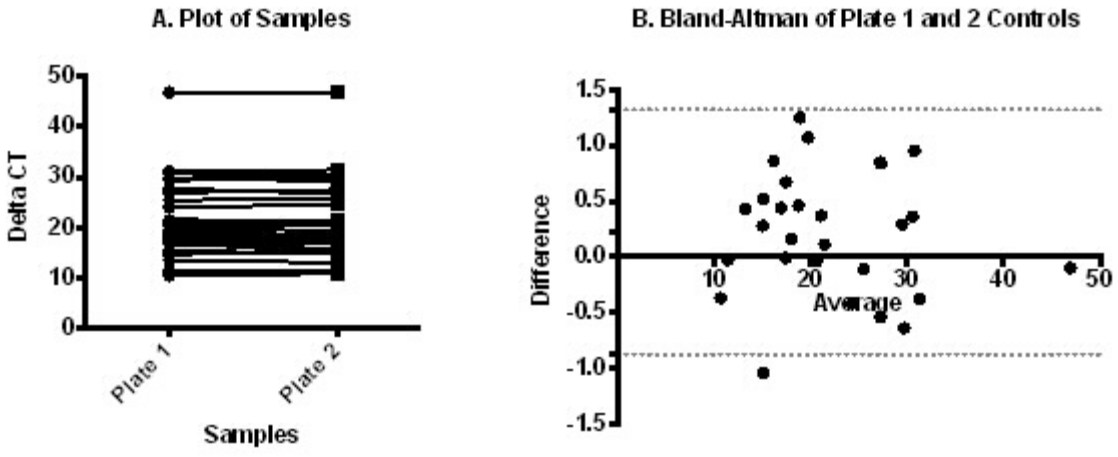



**Figure 43. Before-After Plot and Bland Altman Plot of Plate 1 and 2 Controls**

The cell line SK-MEL-28 was included upon every PCR plate. A. Before-After plot of 'raw' CT values for plate 1 and 2 cell line control samples. This shows that the PCR reactions were reproducible. B.Bland-Altman plot showing 95% limits of agreement (from -0.8729 to 1.318). Bias = 0.2225 (SD of bias 0.5589). All sample except one lie within the limits of agreement.

**Table 41. Comparisons of PCR Plate Controls**

| MicroRNA | Cell Line Sk-Mel28 | | Minus RT Reaction | | No Template Control | |
|---|---|---|---|---|---|---|
| | Plate 1 | Plate 2 | Plate 1 | Plate 2 | Plate 1 | Plate 2 |
| **10a** | 18.09 | 17.93 | Undetermined | Undetermined | Undetermined | Undetermined |
| **10b** | 20.59 | 20.63 | Undetermined | Undetermined | Undetermined | Undetermined |
| **21** | 16.64 | 15.78 | Undetermined | Undetermined | Undetermined | Undetermined |
| **31** | 17.79 | 17.12 | Undetermined | Undetermined | Undetermined | Undetermined |
| **32** | 25.47 | 25.58 | Undetermined | Undetermined | Undetermined | Undetermined |
| **98** | 19.51 | 18.26 | Undetermined | Undetermined | Undetermined | Undetermined |
| **122** | 31.23 | 30.28 | Undetermined | Undetermined | Undetermined | Undetermined |
| **125b** | 18.98 | 18.52 | Undetermined | Undetermined | Undetermined | Undetermined |
| **135b** | 21.29 | 20.92 | Undetermined | Undetermined | Undetermined | Undetermined |
| **139-5p** | 30.71 | 30.35 | Undetermined | Undetermined | Undetermined | Undetermined |
| **141** | 29.63 | 29.34 | Undetermined | Undetermined | Undetermined | Undetermined |
| **146b** | 17.40 | 17.41 | Undetermined | Undetermined | Undetermined | Undetermined |
| **191** | 15.34 | 14.82 | 33 | 33 | 34 | 33 |
| **205** | 29.37 | 30.01 | Undetermined | Undetermined | Undetermined | Undetermined |
| **215** | 27.70 | 26.86 | Undetermined | 41.66 | Undetermined | Undetermined |
| **331-5p** | 20.25 | 19.18 | Undetermined | Undetermined | Undetermined | Undetermined |
| **375** | 27.62 | 26.77 | Undetermined | Undetermined | Undetermined | Undetermined |
| **452** | Undetermined | Undetermined | Undetermined | Undetermined | Undetermined | Undetermined |
| **484** | 17.18 | 16.74 | 32.87 | 34.67 | Undetermined | Undetermined |
| **486** | 26.96 | 27.50 | 36.9 | 36.12 | Undetermined | Undetermined |
| **625** | 31.09 | 31.47 | Undetermined | Undetermined | Undetermined | Undetermined |
| **U6 (A)** | 10.49 | 10.86 | 30.9 | 28.66 | Undetermined | Undetermined |
| **30d** | 21.51 | 21.40 | Undetermined | 39.84 | Undetermined | Undetermined |
| **30e** | 24.06 | 24.48 | 34.5 | 33.11 | Undetermined | Undetermined |
| **409-3p** | 14.61 | 15.65 | Undetermined | Undetermined | Undetermined | Undetermined |
| **483-3p** | 46.86 | 46.96 | Undetermined | Undetermined | Undetermined | Undetermined |
| **720** | 15.19 | 14.91 | Undetermined | 35.84 | Undetermined | Undetermined |
| **1274B** | 13.47 | 13.04 | 28.98 | 30.41 | Undetermined | Undetermined |
| **U6 (B)** | 11.39 | 11.42 | 24.87 | 24.66 | Undetermined | Undetermined |

Raw CT values for cell line-SK-Mel-28 tested with each microRNA across plates. Results of the Minus RT and NTC reaction are also shown. The minus RT are devoid of RT enzyme and should always be undetermined. Occasionally, a non-specific reaction takes place and is detected by the step-one PCR machine. The 'no template control' samples contain enzyme but are devoid of sample and should always be undetermined. All the non-template control reactions were negative apart from the miR-191 reaction, which was repeated.

**Table 42. Comparison of Cycle Threshold Values From Array and Validation Samples**

| MicroRNA | Case 17 | | Case 31 | | Case 73 | | Case 122 | |
|---|---|---|---|---|---|---|---|---|
| | Array | Assay | Array | Assay | Array | Assay | Array | Assay |
| **Pool A** | | | | | | | | |
| miR-98 | 26.24 | 23.02 | 27.35 | 23.18 | 25.20 | 23.15 | 24.15 | 22.00 |
| miR-122 | 21.79 | 18.44 | 23.40 | 18.81 | 21.72 | 20.44 | 21.88 | 20.41 |
| miR-125b | 26.07 | 23.23 | 27.09 | 24.28 | 24.94 | 24.25 | 25.19 | 24.91 |
| miR-139-5p | 23.67 | 24.37 | 24.78 | 22.98 | 22.13 | 24.77 | 21.77 | 23.22 |
| miR-146b | 22.38 | 20.13 | 24.33 | 19.92 | 21.19 | 19.82 | 21.00 | 19.11 |
| miR-331-5p | 29.08 | 23.23 | 29.33 | 22.59 | 28.33 | 22.65 | 27.78 | 22.60 |
| miR-375 | 26.37 | 23.88 | 24.76 | 22.02 | 25.67 | 25.65 | 25.51 | 25.65 |
| U6 Pool A | 29.97 | 24.57 | 28.67 | 24.21 | 25.10 | 23.86 | 24.68 | 21.47 |
| **Pool B** | | | | | | | | |
| U6 Pool B | 29.29 | 23.62 | 30.33 | 23.75 | 34.02 | 22.32 | 32.26 | 21.12 |
| miR-409-3p | 19.74 | 16.06 | 19.29 | 14.77 | 22.17 | 17.40 | 22.07 | 16.16 |
| miR-483-3p | 28.73 | 26.47 | 27.82 | 25.98 | 28.59 | 26.08 | 35.18 | 36.43 |
| miR-1274B | 17.71 | 20.16 | 17.70 | 21.33 | 17.29 | 20.37 | 16.52 | 20.27 |
| miR-720 | 21.08 | 17.55 | 21.07 | 18.17 | 21.00 | 16.89 | 19.56 | 17.71 |
| miR-30d | 25.43 | 22.38 | 28.48 | 21.14 | 28.89 | 20.36 | 31.55 | 20.90 |
| miR-30e-3p | 26.93 | 25.59 | 29.71 | 22.15 | 29.05 | 23.48 | 32.98 | 24.26 |

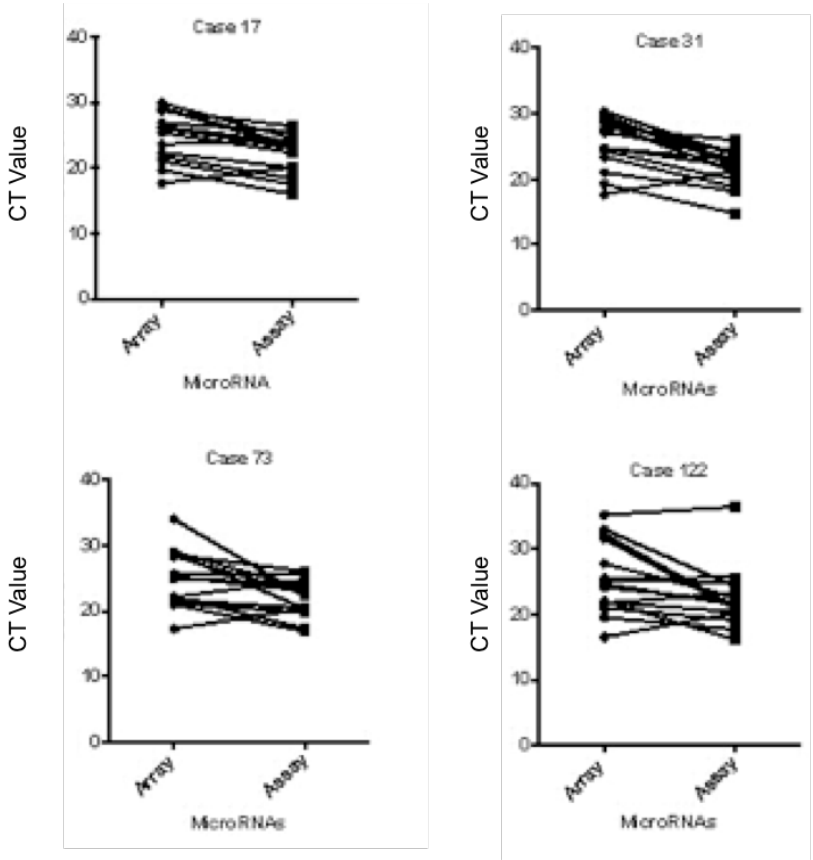



**Figure 44. Before-After Plots Showing Correlation Between Array and Individual MicroRNAs Assays**

**Table 43. Sharpiro-WIlk Normality Tests of Validation Data**

| MiR | All UC | Quiescent UC | Chronic UC | Active UC | PSC | Dysplasia | CAC | All Neoplasia |
|---|---|---|---|---|---|---|---|---|
| **10a** | **No** | Yes | Yes | Yes | Yes | n too small | n too small | Yes |
| **10b** | **No** | **No** | Yes | Yes | Yes | n too small | n too small | Yes |
| **21** | **No** | Yes | Yes | **No** | Yes | n too small | n too small | Yes |
| **31** | Yes | Yes | Yes | **No** | Yes | n too small | n too small | Yes |
| **32** | **No** | **No** | Yes | Yes | Yes | n too small | n too small | Yes |
| **98** | **No** | Yes | Yes | **No** | Yes | n too small | n too small | Yes |
| **122** | Yes | Yes | Yes | Yes | Yes | n too small | n too small | Yes |
| **125b** | Yes | Yes | Yes | Yes | Yes | n too small | n too small | Yes |
| **135b** | **No** | Yes | **No** | Yes | **No** | n too small | n too small | Yes |
| **139-5p** | Yes | Yes | Yes | Yes | Yes | n too small | n too small | Yes |
| **141** | **No** | **No** | Yes | Yes | Yes | n too small | n too small | Yes |
| **146b** | **No** | **No** | Yes | Yes | Yes | n too small | n too small | Yes |
| **191** | **No** | **No** | Yes | **No** | Yes | n too small | n too small | Yes |
| **205** | **No** | Yes | Yes | Yes | Yes | n too small | n too small | **No** |
| **215** | **No** | Yes | Yes | Yes | Yes | n too small | n too small | Yes |
| **331** | **No** | **No** | Yes | Yes | Yes | n too small | n too small | Yes |
| **375** | Yes | Yes | Yes | Yes | Yes | n too small | n too small | Yes |
| **452** | Yes | Yes | Yes | Yes | Yes | n too small | n too small | Yes |
| **484** | Yes | **No** | Yes | Yes | Yes | n too small | n too small | Yes |
| **486** | **No** | Yes | Yes | Yes | Yes | n too small | n too small | Yes |
| **625** | Yes | Yes | Yes | Yes | Yes | n too small | n too small | Yes |
| **30d** | Yes | Yes | Yes | Yes | Yes | n too small | n too small | Yes |
| **30e** | Yes | Yes | Yes | Yes | Yes | n too small | n too small | Yes |
| **409** | Yes | Yes | Yes | Yes | No | n too small | n too small | Yes |
| **483** | **No** | Yes | Yes | **No** | Yes | n too small | n too small | Yes |
| **720** | Yes | Yes | Yes | Yes | Yes | n too small | n too small | Yes |
| **1274B** | Yes | Yes | Yes | Yes | Yes | n too small | n too small | Yes |

UC – Ulcerative Colitis, PSC – Primary Sclerosing Cholangitis, CAC – Colitis Associated Cancer, n-number of samples

## Scatter Plots

The PCR data was plotted for general inspection (Figure 46). As before, a low ΔCT value correlated to early detection in the PCR reaction as a result of the microRNA being expressed at a relatively higher level. The UC samples were plotted in separate groups and as a combined group. The combined group was thought to be a good clinical representation of a patient with UC as many patients can harbour a combination of both active and chronic disease.

**Figure 46. Scatter Plots of Pool A + Pool B MicroRNA Validation Assays**

CT – Cycle Threshold, UC – Ulcerative Colitis, PSC – Primary Sclerosing Cholangitis, CAC – Colitis Associated Cancer, miR – MicroRNA, All neoplasia = Dysplasia and CAC samples. Mean and Standard Error of mean shown

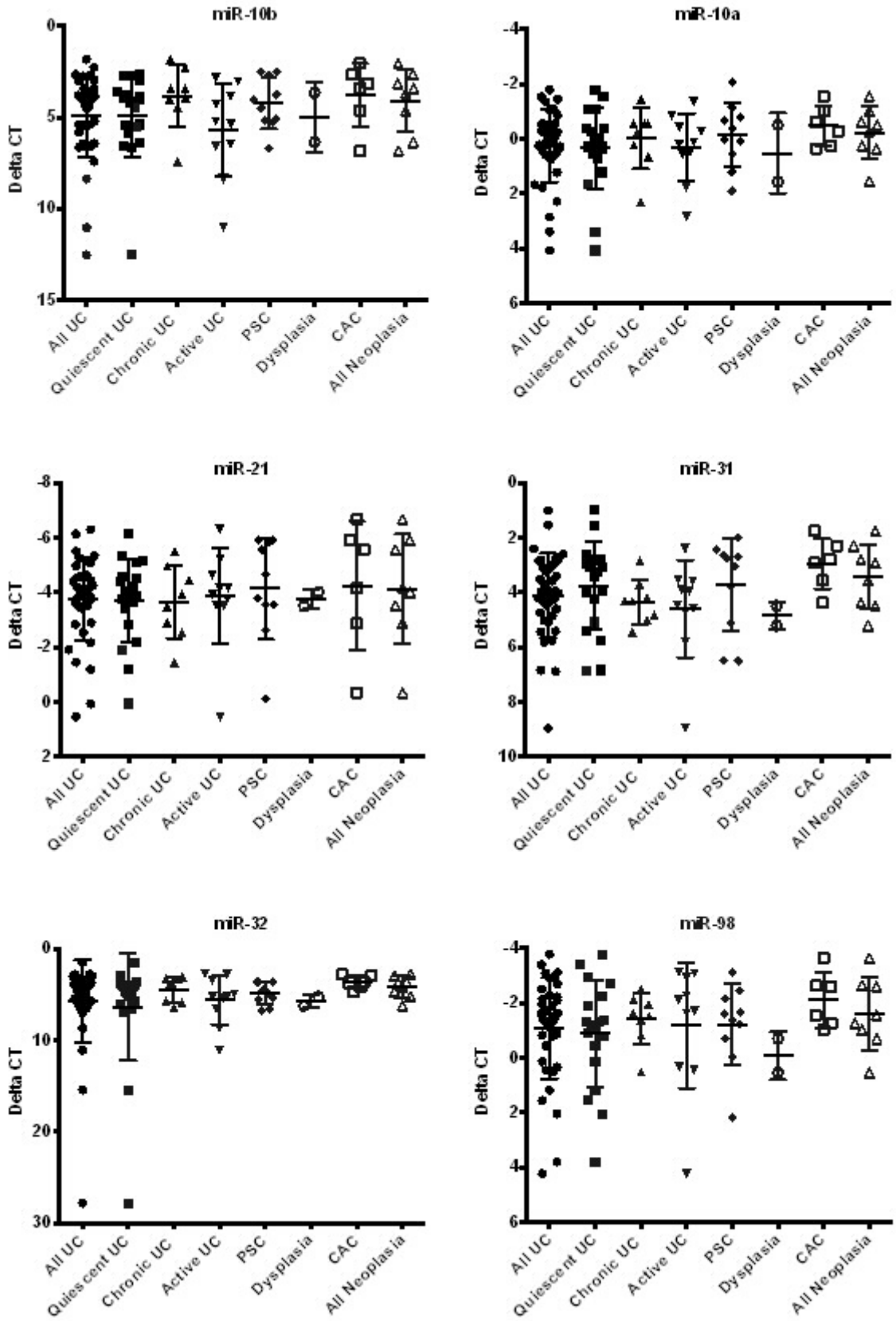


**Figure 46. Scatter Plots of Pool A + Pool B MicroRNA Validation Assays**

CT – Cycle Threshold, UC – Ulcerative Colitis, PSC – Primary Sclerosing Cholangitis, CAC – Colitis Associated Cancer, miR – MicroRNA, All neoplasia = Dysplasia and CAC samples. Mean and Standard Error of mean shown

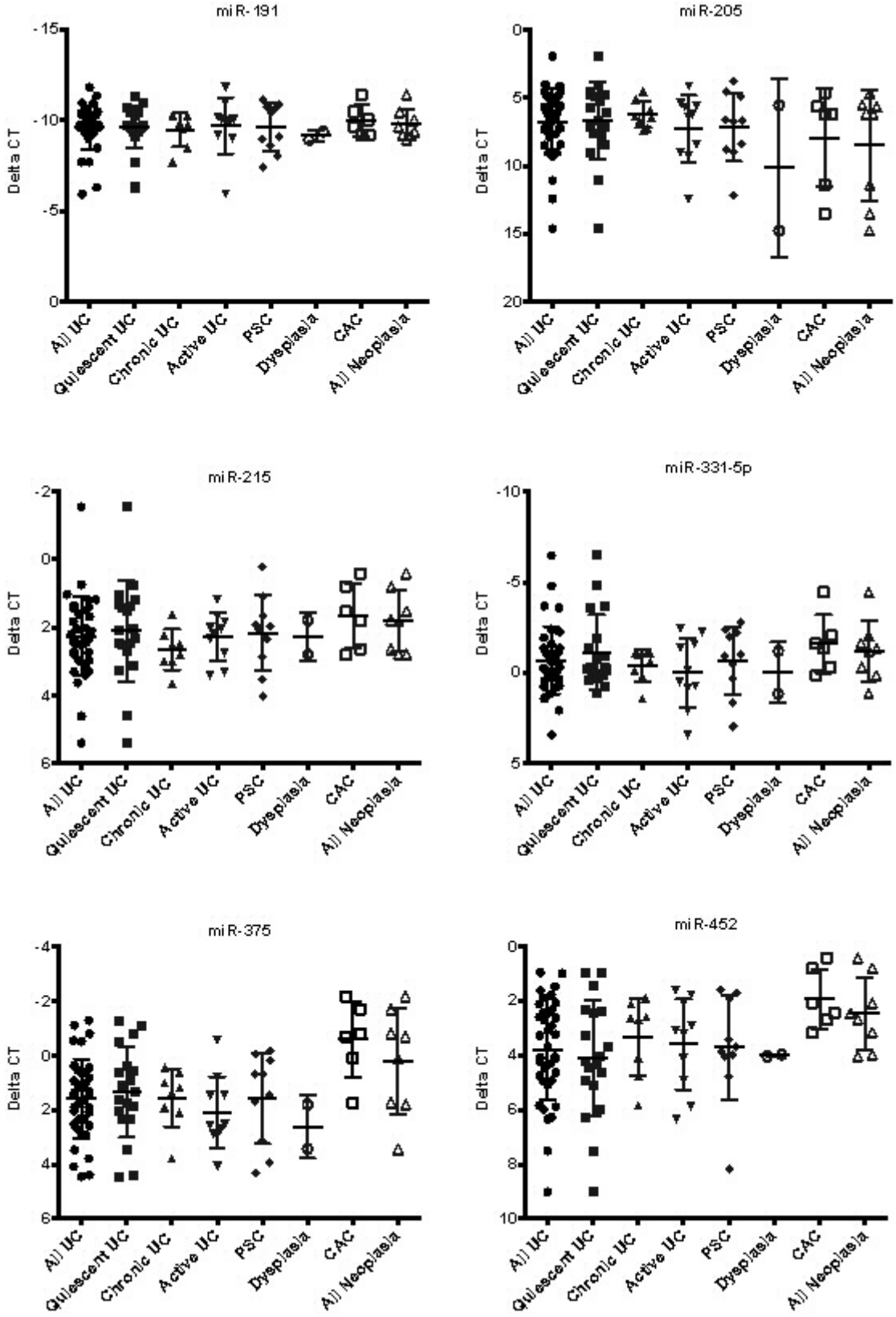
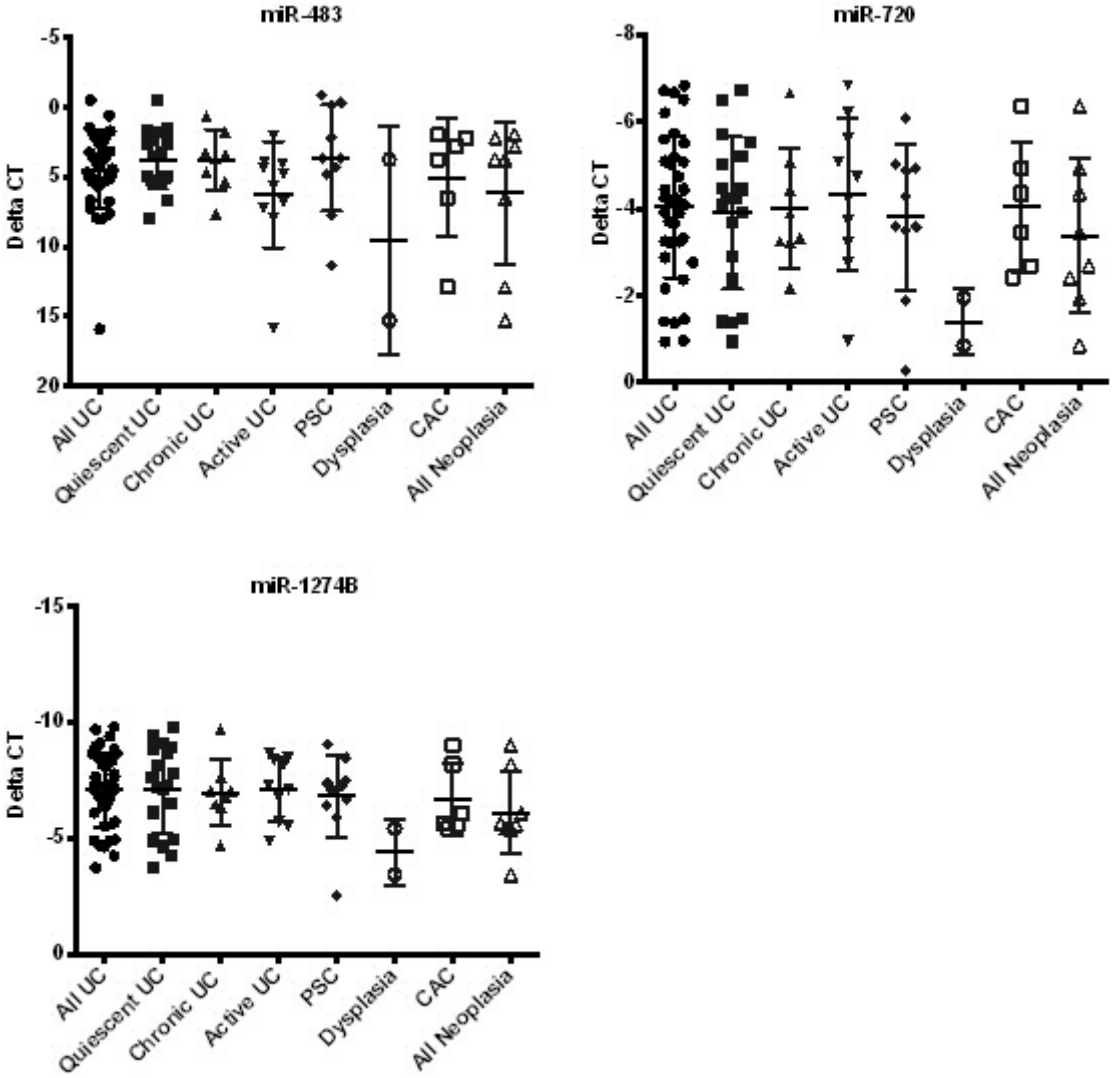


**Figure 46. Scatter Plots of Pool A + Pool B MicroRNA Validation Assays**

CT – Cycle Threshold, UC – Ulcerative Colitis, PSC – Primary Sclerosing Cholangitis, CAC – Colitis Associated Cancer, miR – MicroRNA, All neoplasia = Dysplasia and CAC samples. Mean and Standard Error of mean shown




## Analysis of One Way Variance

On general inspection of the scatter plots, it was obvious that the dysplasia samples on average had a low or a wide spread of ΔCT values when compared to the other groups. According to the previous chapter, these samples would have been expected to lie between the UC and CAC samples for most of the miRNA assays. Due to this abnormality and the low number of dysplastic samples it was felt that his group did not accurately represent dysplasia and was subsequently omitted from the ANOVA analysis. Only the CAC and UC groups were entered into the analysis (Table 44).

**Table 44. Summary of Analysis of One Way Variance Results Comparing MicroRNA Expression in The CAC Group To Different Types Of Ulcerative Colitis**

| MicroRNA | CAC Vs. Quiescent UC | CAC Vs. Chronic UC | CAC Vs. Active UC |
|---|---|---|---|
| **10a** | No | No | No |
| **10b** | No | No | No |
| **21** | No | No | No |
| **31** | No | No | No |
| **32** | No | No | No |
| **98** | No | No | No |
| **122** | No | No | No |
| **125b** | No | No | No |
| **135b** | No | No | No |
| **139-5p** | No | No | No |
| **141** | No | No | No |
| **146b** | No | No | No |
| **191** | No | No | No |
| **205** | No | No | No |
| **215** | No | No | No |
| **331** | No | No | No |
| **375** | **Yes (P = 0.0449)** | No | **Yes (P = 0.0061)** |
| **452** | No | No | No |
| **484** | No | No | No |
| **486** | No | No | No |
| **625** | No | No | No |
| **30d** | No | No | No |
| **30e** | No | No | No |
| **409** | No | No | No |
| **483** | No | No | No |
| **720** | No | No | No |
| **1274B** | No | No | No |

UC – Ulcerative Colitis, CAC – Colitis Associated Cancer, No – non-significant result, Yes = significance. P=α value – significance set at 0.05.

## Combined Analysis

As before, based upon the scatter plots, the ΔCT values of selected miRNAs were combined and then plotted (Figure 47). The analysis is shown in Table 45 and depicted in (Figure 48).

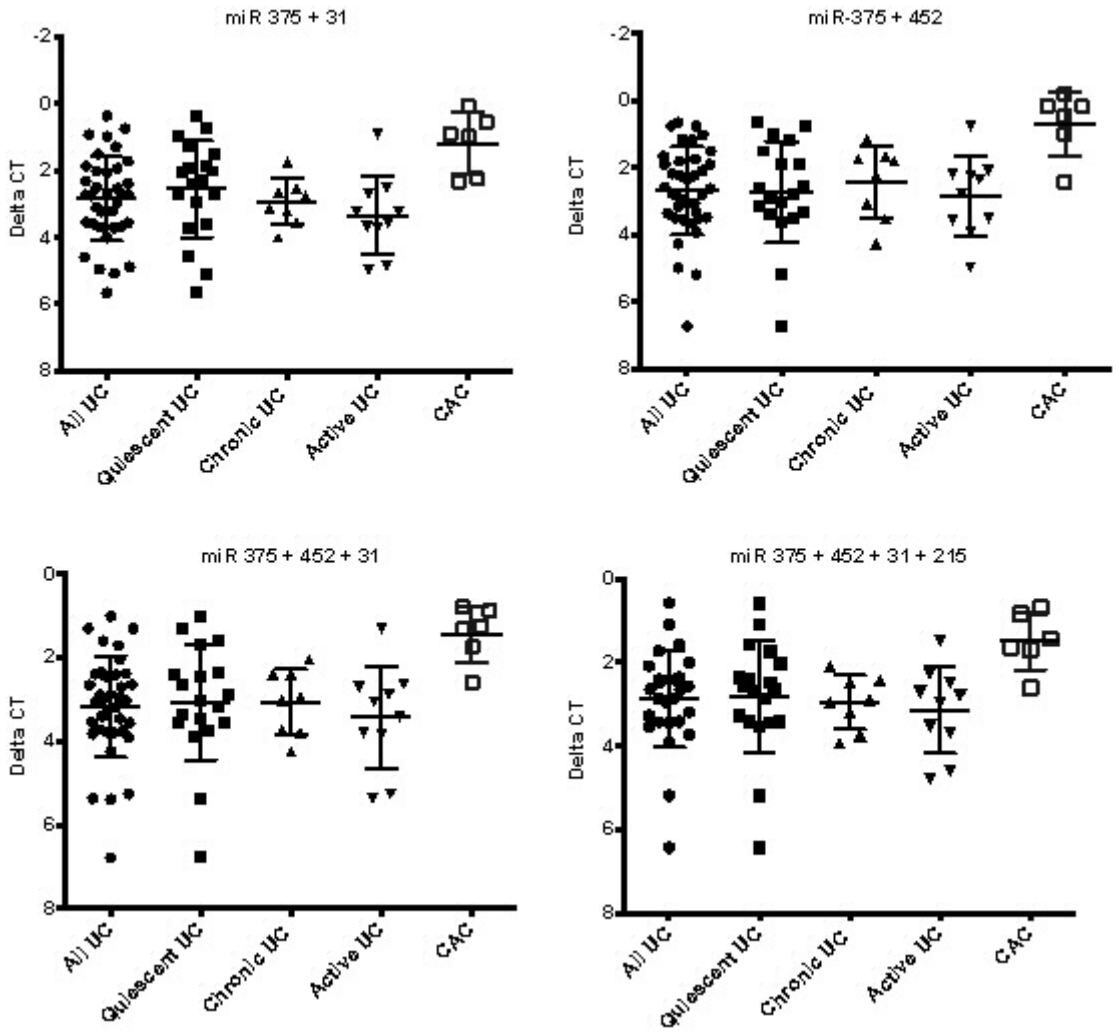



**Figure 47. Scatter Plots For Combined Plasma MicroRNAs**

CT – Cycle Threshold, UC – Ulcerative Colitis, CAC – Colitis Associated Cancer. Mean and SEM shown.

**Table 45. Summary of Analysis of One Way Variance Results Comparing Combinations of MicroRNAs.**

| MicroRNAs | 375 + 31 | 375 + 452 | 375 + 452 + 31 | 375 + 452 + 31+ 215 |
|---|---|---|---|---|
| **All UC Vs. CAC** | **Yes P = 0.0300** | **Yes P = 0.008** | **Yes P = 0.0147** | NS |
| **Active UC Vs. CAC** | **Yes P = 0.0113** | **Yes P = 0.0202** | **Yes P = 0.0179** | NS |
| **Quiescent UC Vs. CAC** | NS | **Yes P = 0.0133** | **Yes P = 0.0444** | NS |
| **Chronic UC Vs. CAC** | NS | NS | NS | NS |

UC – Ulcerative Colitis, CAC – Colitis Associated Cancer, NS – non-significant result, Yes = significance. P=α value – significance set at 0.05.

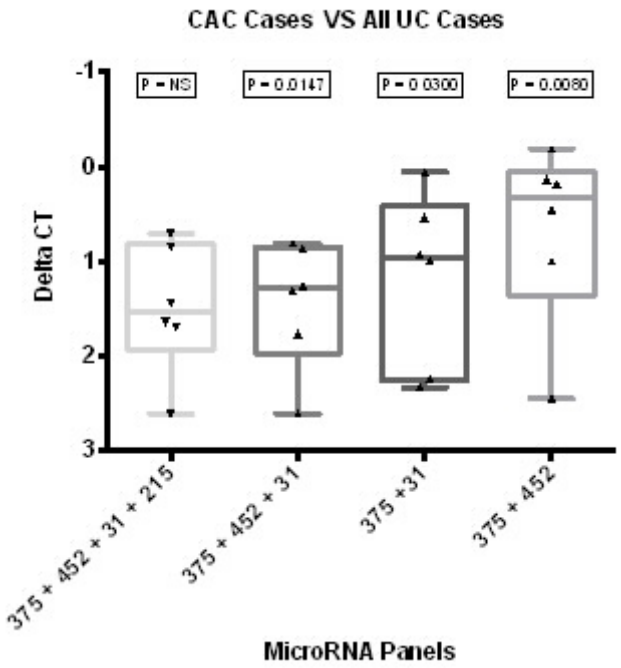



**Figure 48. Box and Whiskers Plots Showing The Capacity of MicroRNA Panels To Show A Difference Between Colitis Associated Cancer and The Combined Ulcerative Colitis Group.**

CT – Cycle Threshold, UC – Ulcerative Colitis, CAC – Colitis Associated Cancer. Mean and SEM shown. NS – non-significant result, P=α value – significance set at 0.05.

## 5.5 Discussion

### 5.5.1 Findings

The aim of this chapter was to determine a panel of plasma miRNAS associated with the pathology and progression of disease in patients with Ulcerative Colitis and evaluate the potential of these microRNAs to act as blood-based biomarkers for the surveillance of UC patients. In order to establish this, blood samples were prospectively collected from a cohort of UC patients enrolled on a colonoscopic surveillance program and from patients who presented with CAC via other routes. Patients were allocated to different disease groups based upon biopsy based histology results. Within the discovery cohort, Pool A and Pool B Taqman microfluidic array cards were used to screen for the differential expression of 754 microRNAs within the disease groups. A low stringency criterion was employed to highlight all microRNAs of interest. Poorly expressed microRNAs were eliminated. 28 microRNAs were validated upon 58 samples using RT-qPCR.

**Validation Findings**

The validation results were analysed using ANOVA. Only the CAC group was included in the analysis as there were only two dysplastic samples, which had abnormal CT values. MiRNA 375 was found to be statistically significant when compared to the quiescent and active UC groups. This is clinically useful as it suggests an ability of miRNA 375 to identify CAC on a background of UC, regardless of whether it is active or quiescent.

Unfortunately, levels of miR-375 were not elevated in the array work carried out on dysplastic and CAC tissue in chapter 4. Subsequently it was not chosen for further validation in the independent tissue cohort. This leads to the question of it was a real finding in the plasma or a false discovery, as it would have been expected to show a step wise increase from dysplastic tissue to CAC tissue and have significant levels in the tissue overall compared to plasma.

Unfortunately no other miRNA showed significance. It was surprising that miRNAs 21, 31 and 135b were not over expressed. This could be for a number of reasons, including the small number of samples and the heterogeneity of the samples (patients might harbour multiple co-morbidities). The miRNAs added to the validation cohort on the basis of prior tissue profiling results and the literature search (10b, 21, 31, 135b, and 215) were not significant. This suggests that overall the circulatory profiles are different from the tissue profiles: perhaps not all the miRNAs from the tissues are released into the circulation, or confounded by miRNAs related to other co-morbidities. Subsequently, it might be more accurate to use a tissue-based profile to highlight high-risk patients who are in need of regular surveillance.

Further miRNAs were added to the validation cohort based upon work conducted in the laboratory investigating miRNA profiles in other colorectal disease states such as SCRC and polyps (10a, 30e, 32, 98, 146b, 205, 331-5p, 452, and 625). None of these proved to be significantly expressed in CAC. Once again, this might be the result of small sample numbers or those specific miRNA may reflect pathological processes, which do not occur in UC-based neoplasia.

**MicroRNA Combination Panels**

Combining miRNA 375 with other miRNAs increased the statistical significance when comparing CAC to different UC groups. MiRNA 31 was chosen based upon its prominence in the literature and miRNA 452 based upon the scatter plots. MiRNA 375 in combination with miRNA 31 could differentiate between active UC and the combined UC group. Combining miRNA 375 with 452 alone or with miRNA 452 and 31 allowed for differentiation between CAC and quiescent, active and combined UC groups. Ideally a biomarker should be able to differentiate between all types of UC states and highlight high-risk patients in the presence of inflammation. Interestingly none of these combinations were significant when compared to the chronic UC group.

### 5.5.2 Comparison to The Literature

The over expression of miRNA 375 in the plasma of patients with CAC is a novel finding. Review of literature (as summarised in Table 6 and Table 7) shows contradictory results. MiRNA 375 was both down regulated in human UC colonic mucosa (194) and up regulated in human dysplastic colonic mucosa (206).

### 5.5.3 Study Strengths

As with the experiments in Chapter 4, the experiments in this Chapter were conducted in accordance with biomarker experiment guidelines (219,226). All samples used in this study (including those collected by AJ) were collected on a prospective basis (excluding the CAC samples), in standardised manner and processed within 2 hours, using the same standard operating procedure. All samples were stored in the same place within a University monitored freezer. This is an extremely important issue as the processing of plasma samples can have a consequence on upon microRNA expression.

A discovery and validation cohort was utilised, as was the same robust methodology and multiple controls in order to avoid experimental bias. As a result, standardisation of these steps allow for a meaningful comparison to be made between the miRNAs profiles derived from the colonic mucosa and from the plasma.

The REMARK guidelines pertain to the reporting of prognostic biomarker studies. Although this was not a study into prognostic biomarkers, the guidelines were used to direct result reporting, including the incorporation of an inclusion-exclusion criterion and a representation of specimen flow and characteristics. An attempt was made to collect as much clinical data as possible and an outline of patient demographics has been included.

As with Chapter 4, number of samples and in particular the CAC samples are low but again, on par with other studies in the literature.

The experiments generated from this chapter are novel experiments; no prior study to date has used blood samples from patients with CAC or conducted miRNA profiling using both pool A&B microfluidic cards.

### 5.5.4 Study Limitations and Difficulties Encountered

Despite the prevalence of UC in the UK, a relatively low number of UC cases were collected over an 18-month period. This reflected the inherent difficulties of recruiting patients with UC, within the Leicester Hospitals. A database of all IBD patients within the Leicestershire region did not exist, making it extremely demanding to identify and track patients with UC. Patients were detected by manually searching paper-based colonoscopy surveillance request forms on a monthly basis. An attempt was made to recruit as many surveillance patients as possible that were available at the time of the study.

Another barrier to recruitment was the lack of designated colonoscopy lists solely dedicated to IBD surveillance. UC patients were screened sporadically, on different lists throughout the week. Doctors of varying grades carried out the UC surveillance colonoscopies, in different ways. Only two consultants utilised the recommended dye spray procedure and the alternative 4-quadrant biopsy regimen was performed infrequently. This meant less than 1% of the total colonic mucosa was sampled in these patients resulting in potential missed lesions. Therefore it was not possible to completely eliminate the diagnosis of dysplasia or CAC in these patients and this needs to be considered during the analysis. It was also noted that patients who were in remission of their disease and thus, feeling well within themselves, often failed to attend their colonoscopy (poor compliance). They were subsequently removed from the waiting list and not offered another appointment. Thus a cohort of UC patients that have been lost to follow-up may exist. Furthermore, prospective blood sampling failed to enlist patients with dysplasia and CAC. These patients were often symptomatic and had presented though other routes and were identified via the colorectal cancer MDT (a potential source of selection bias as these were collected retrospectively). In total only 2 cases of dysplasia and 4 cases of CAC were collected (of which two were polyp cancers). Subsequently it was difficult to test for normality and perform a meaningful analysis with this number of samples. As with the prior chapter, this experiments in this chapter were not subject to a power based sample size calculation. This again raises the question of false discovery.

Despite an effort to collect as much clinical information as possible by using a standardised proforma, not all the necessary clinical information was available. Patients themselves were unsure of their clinical details such as date of diagnosis of UC. It was also difficult to collect and standardise past medical history, particularly information regarding medication. This was because patients themselves did not keep an accurate record of the medications they had previously been on, or the dosage or duration of treatment. The information was not always accurately recorded in the medical records. As expected, there was also huge variation in medication regimens, with some patients having been on repeated courses of steroids for acute flair ups. Also several patients admitted to being non-compliant with their medication especially during times of remission when their symptoms were well controlled.

The samples were collected from a wide range of surveillance patients and were not clinically homogeneous. Due to the limited number of samples and biological variation, the study was unable to match the samples for age, gender, medication or duration of disease. Variables such as gender, comorbidities, medication, and the range and severity of inflammation in the colon mucosa could act as confounding factors and alter the profiling results. Matched controls could have been collected from symptomatic patients undergoing investigation for bowel disease who had macroscopic and microscopic normal colonoscopies. However, the resources did not exist to collect and process these additional samples.

Not all the patients undergoing colonoscopic surveillance had underwent the gold standard dye-spray procedure or the recommended 4-quadrant biopsy regimen. Therefore a number of potential missed diagnoses could have arisen and thus samples that were included in the UC cohort might of contained dysplasia or CAC. This would subsequently affect the CT value of the sample. Allotting patients to clinical groups based upon histology results also proved problematic due to a lack of standardized histology reporting.

The microarray data was analysed using a low stringency criteria to identify miRNAs of interest. Perhaps a stricter threshold combined with a false

discovery rate would identify statistically valid miRNAs of interest. This would also prevent resources from being wasted during validation.

There was an element of experimental error resulting from the underperformance of one of the array cards. Furthermore as miRNAs entail several processing steps before the final result, there will always be a potential for experimental error and bias. During the analysis it was assumed that relatively higher expression when compared to a control equates directly to up-regulation and this may be somewhat over simplified.

### 5.5.5 Recent Developments

A further literature search was conducted using a combination of search terms to identify any new research pertaining to miRNAs and UC. This was in order to establish if any of the findings from chapter 4 or 5 had subsequently been corroborated or refuted. This search resulted in a combination of 74 primary research and review articles. Interestingly, over a three-year period, only six papers were found specific to miRNAs and the development of CAC. This strongly reflects the challenges in conducting miRNA and CAC profiling studies CAC: namely that of sample collection, sample size, biological heterogeneity within the samples, varying methodology and overall costs. The most relevant profiling studies will be briefly discussed.

**Study 1: Lewis et al** (237)

This small study is the most important one published since the original literature search. It used tissue arrays to undertake comprehensive miRNA profiling (1899 miRNAs) upon colonic mucosal biopsies taken from patients undergoing surveillance colonoscopy for UC. The samples included dysplasia (n=7), UC controls (pancolitis) (n=10), inflammatory polyps (n=7). The results were validated by both qPCR and in situ hybridization and correlated to serum samples carried out at the same time as colonoscopy. Principal component analysis showed some separation of dysplastic samples when compared to the UC controls. Significance analysis of microarrays software was used to identify differentially expressed miRNAs. The analysis identified 22 up-regulated miRNAs of which 6 have been previously reported in literature (miR-15a, 200a, 192, 194, 21, 552). A further 2 miRNAs correlated with plasma work in this chapter (miR-21 and 30e) and only miR-21 correlated with the dysplastic tissue miRNAs from chapter 4. Validation did no show any significant difference between dysplastic and UC tissue. MiR-21 showed a stronger associated with disease activity than with dysplasia. Surprisingly there was no upregulation of miR-31, which has previously been reported to correlate to progressive neoplastic change in UC patients, and serum profiling did not identify significant miRNAs.

This is a very interesting study as it showed that miRNA serum profiles do not necessarily mirror the tissue profiles as would have been expected. It would also be expected that the dysplastic would have similar profiles to that of CAC tissue but perhaps at lesser levels; this was not the case. MiRNA-21 seems to have a significant role in UC, but this may be related to inflammation and more work needs to be done on this. Furthermore the authors recruited samples from ten NHS trusts with established IBD surveillance programs. Despite this only ten dysplastic samples were recruited, suggesting that the incidence of dysplasia is low, decreasing or not well identified. This also strengthens the case for multisite studies and the need to maintain databases of IBD patients.

**Study 2: Benderska et al** (238)

Part of this study investigated the expression of miR-26b in CAC in two human cohorts. The first cohort was comprised of 38 retrospectively collected ffpe colonic mucosa punch biopsies from 17 patients with UC and CAC. In the second cohort, fresh frozen colonic punch biopsies and corresponding serum samples were prospectively collected from 6 UC and 15 non-UC patients. In situ hybridisation was performed on cohort 1 and showed significant increased in staining intensity as disease progressed from inactive UC, to active UC, to severe UC, to the CAC group. Furthermore, the expression of miR-26b was significantly increased in the biopsies and serum of patients with UC, when compared to patients with healthy bowel tissue.

**Study 3: Shi et al** (239)

In this international study, miR-21 expression was found to be upregulated in 62 cases of SCRC and in 22 out of 37 cases of CAC. Mir-21 levels were also upregulated in a dss mouse model. It was interesting that not all the cases of CAC expressed significant levels of miR-21 suggesting that like SCRC, different pathological mechanisms and miRNAS may contribute to the development of CAC.

**Study 4: Polytarchou et al** (240)

In this study, results from in-vitro human colonocyte profiling experiments were extrapolated to human colonic tissue from patients with CAC and UC. This showed that miR-21 and miR-146a was increased in IBD compared to health controls. MiR-214 showed a 8-fold increased expression in UC tissues compared to CD. Further validation, substantiated a significant increase of miR-214 in UC tissues, with a correlation to active disease. Further experiments, demonstrated a link of miR-214 to chronic inflammation in mouse models and increased levels of miR-214 in CAC tissues. This lead the author to suggest that miR-214 is increased during UC and plays a role in the development of CAC.

Another very small-scale study insinuated DNA methylation of miR-214 as a risk marker for CAC based on four samples only (241). If the experiments from this chapter were to be taken forward, it would be worth determining the expression of miR-26, miR-214 and miR-200b across different UC tissues and exploring the role of miR-21 in greater depth. No further studies have elicited raised levels of miR-375.

### 5.5.5 Future Work

Once again, the next step in biomarker development would be validation of the results in a larger cohort of adequate power with detailed investigation of the latter phases of biomarker development. This would require collaboration with other institutes in order to generate the large sample numbers required for establishing clinically useful test characteristics (NPV, PPV, ROCs and cox hazard modelling).

The work in this thesis could be extended in several ways. The work conducted so far was based solely on samples from patients with UC and focused upon aberrant miRNA expression in CAC. Patients with CD are also at risk of developing IBD cancer and the work could be replicated in patients with Crohns disease and Crohns associated neoplasia. It would be interesting to identify the presence of circulating mutations within the plasma samples and establish any correlation to specific miRNAs. The role of MiRNA 375 in UC and UC neoplasia also needs to be explored. Pathway analysis may be useful to study the effects of dysregulated miRNAs on different biological processes and target prediction tools could be used to investigate the functional significance of aberrant expression. It would be worthwhile carrying out qPCR on the original tissues from chapter 4 to establish miR-375 levels.

MiRNAs that showed relatively high expression (up-regulation) in the array data were chosen for further validation. Thus the aforementioned work focused on over expressed miRNAs. However, the literature has repeatedly reported the under expression of miRNA with tumour suppressor functions such as miRNA 143 and 145; it would be interesting to investigate if this finding was replicated within the plasma samples of UC patients particularly those with dysplasia and CAC.

Lastly, by means of an additional literature review, the profiling results could be compared to miRNA profiles found in other cancers (especially that of SCRC). There is a need to elicit if the over expressed miRNAs are attributable to cancers in general (such as the increased expression of miRNA 21) as a result

of common pathological processes. A surveillance biomarker test would need to need to specific for CAC and not pick up ‘any type of malignancy’.

## 5.6 Summary

- Array analysis of the discovery cohort revealed the differential expression of multiple miRNAs of which miRNAs 125b, 139-3p, 375, 501-3p (Pool A) and 409-3p, 483-3p, 720, 1274B (Pool B) were chosen to be validated in an independent cohort. The miRNAs miR-10b, miR-21, miR-31, miR-135b, miR-215 and miR-10a, miR-30e, miR-32, miR-98, miR-146b, miR-205, miR-331-5p, miR-452, miR-486 and miR-625 were also validated.

- Validation showed significantly increased expression of miRNA 375 in CAC tissues compared to UC tissue.

- A combined miRNA panel improved the ability of the test to distinguish between different disease states.

- Provides proof of concept that miRNA expression is altered in the circulation of UC associated neoplasia.

- As with issue profiling, MiRNA expression profiling in the circulation of patients with UC is challenging due to patient and biological variation, limited specimen availability and quality. Once again, the lack of a dedicated IBD patient database within the hospital trust made it difficult to identify patients undergoing surveillance colonoscopy. For some cases there was a lack of clinical information.

- Further work needs to be conducted in a larger cohort with more cases of CAC in order to validate miRNA 375 and elucidate additional miRNAs of interest.

# Chapter 6. Final Conclusion

This study hypothesed that changes in the mucosa of patients with UC herald a continuum of neoplastic transformation from dysplasia to CAC and these changes are associated with specific miRNAs. Subsequently, miRNA expression profiles can be detected in patients with UC and can act as biomarkers to monitor the progression of disease. The hypothesis was tested through three aims.

**Aim 1: Characterise a cohort of patients with CAC to ensure suitability for sequential biomarker discovery.**

This was achieved in chapter 3: The Clinicopathological Presentation of Colitis Associated Cancer in UK Population. It showed that the cohort of patients used for sequential biomarker study were similar to cohorts described in the literature. Although the cohort compromised a relatively small number of patients, it did illustrate that patients with UC tend to develop CAC at an earlier age when crudely compared to the SCRC patients. Patients presented with late T3/T4 tumours increasing their morbidity and mortality. Interesting there were 4 patients with proctitis who presented with rectal cancers. This suggests that patients with proctitis should also be included in the surveillance programme based upon their risk. The chapter highlights the both importance of the UC surveillance programme and its potential pitfalls such as a missed diagnosis. Furthermore, patients with UC can present with interval cancers and compliance with surveillance will always be an issue. This necessitates an on-going search for accurate biomarkers.

**Aim 2: Discover a panel of deregulated miRNAs in the colonic mucosa of patients with UC and determine their correlation to various disease states: Active UC, Dysplasia and CAC.**

This aim was achieved in Chapter 4: MiRNA Expression Profiles in the Colonic Mucosa of Patients with Ulcerative Colitis. Validation showed that miRNAs are differentially expressed in tissues with UC, dysplasia and CAC. In particular miRNAs 18a, 21 and 135b showed increased expression in UC related neoplasia. MiRNA 31 was also upregulated in UC neoplasia but also in acute UC. A combination of miRNAs 21 + 215 was effective at distinguishing between normal tissue and dysplasia and CAC and was also able to distinguish between chronic UC and neoplasia.

**Aim 3: Determine a panel of miRNAS associated with the pathology and progression of disease in the Plasma of patients with Ulcerative Colitis and evaluate the potential of these miRNAs to act as blood-based biomarkers for the surveillance of UC patients.**

This was partly achieved in Chapter 5: MiRNA Expression Profiles in the Circulation of Patients with Ulcerative Colitis. Validation showed that miRNA 375 was overexpressed in the circulation of patents with CAC.

## Summary of Thesis

1. Patients with UC are at risk of CAC and require an accurate screening test to diagnose the changes that herald neoplastic transformation.

2. MicroRNAs are differentially expressed in the colonic mucosa of patients with UC. The microRNAs 18a, 21 and 135b show a significant step-wise increase in the tissue as disease pathology progress from dysplasia to CAC.

3. The differential expression of MicroRNAs can be detected in the circulation of patients with CAC when compared to UC. MicroRNA-375 was significantly increased in the circulation of patients with CAC and showed potential as a prognostic biomarker to indicate the patients that are increased risk of developing CAC.

4. Combining different microRNAs in a panel increases their capacity to differentiate between disease states.

5. This feasibility study serves to increase the limited amount of knowledge that is currently held on miRNAs in UC and CAC and provides proof of concept that the aberrant expression of miRNAs has biomarker ability.

# Appendices

## Appendix 1: Presentations Arising From Thesis

### Oral Presentations

Screening of novel plasma microrna associated with disease progression in Ulcerative Colitis

**Date:** October 2014

**Forum:** European Union of Gastroenterology Week, Vienna, Austria

**Authors:** Maleene Patel, Ajay Verma, Muhammad Imran Aslam, Kevin West, John Stuart Jameson, James Howard Pringle, Baljit Singh.

Biomarkers of Bowel Disease – A Research Update

**Date:** April 2014

**Forum:** Illeostomy & Pouch Support Association Conference. Newcastle, UK.

**Authors:** Miss Maleene Patel, James Howard Pringle, Baljit Singh

Colitis associated cancer: a UK institutions 12-year experience

**Date:** May 2012

**Forum:** Royal Society of Medicine. Geneva, Switzerland

**Authors:** Maleene Patel, John Stuart Jameson, James Howard Pringle, Mike Norwood, Baljit Singh.

Identification Of Novel MicroRNAs In Development And Progression Of Dysplasia And Carcinoma In The Ulcerative Colitis Affected Colon

**Date:** January 2012

**Forum:** Society of Academic Research in Surgery (SARS). Nottingham, UK

**Authors:** Maleene Patel, Muhammad Imran Aslam, Kevin West, John Stuart Jameson, James Howard Pringle, Mr Baljit Singh.

**Poster Presentations**

MicroRNA expression profiling in the plasma of patients with Ulcerative Colitis and Colitis associated cancer

**Date:** June 2015

**Forum:** Digestive Disorder Federation, London, UK

**Authors:** Maleene Patel, Ajay Verma, Imran Aslam, Kevin West, John Jameson, Howard Pringle, Baljit Singh

Novel plasma microRNA biomarkers for the identification of CAC

**Date:** February 2015

**Forum:** Academy of Medical Sciences Spring Meeting, London, UK

**Authors:** Maleene Patel, Ajay Verma, Imran Aslam, Kevin West, John Jameson, Howard Pringle, Baljit Singh

Screening of novel plasma microRNAs associated with disease progression in Ulcerative Colitis

**Date:** October 2014

**Forum:** European Union of Gastroenterology Week, Vienna, Austria.

**Authors:** Maleene Patel, Ajay Verma, Imran Aslam, Kevin West, John Jameson, Howard Pringle, Baljit Singh

Differential expression of microRNAs is associated with dysplasia and adenocarcinoma in patients with Ulcerative Colitis

**Date:** September 2012

**Forum:** European Society of Coloproctology, Vienna, Austria.

**Authors:** Maleene Patel, Imran Aslam, Kevin West, John Jameson, Howard Pringle, Baljit Singh

Clinico-pathological Study of Colitis Associated Cancer in a UK Cohort

**Date:** July 2012

**Forum:** Association of Coloproctology Great Britain & Ireland, Dublin, Ireland

**Authors:** Authors: Miss Maleene Patel, Mr John Stuart Jameson, Dr James Howard Pringle, Mr Mike Norwood, Mr Baljit Singh.

# Appendix 2: Study Documentation

## 2.1 National Research Ethics Service Letter of Approval

**National Research Ethics Service**
**Nottingham Research Ethics Committee 2**
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Telephone: 0115 8839440
Facsimile: 0115 9123300

31 January 2011

Dr James Howard Pringle
Reader in Molecular Pathology
University of Leicester
Rm 340 Department of Cancer Studies and Molecular Medicine
L3 Robert Kilpatrick Building
PO BOX 65 Leicester Royal Infirmary
LE2 7LX

Dear Dr Pringle

**Study Title:** **Tissue and blood biomarkers of bowel disease progression and response to therapy**
**REC reference number:** **10/H0408/116**

Thank you for your letter of 13 January 2011, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered at the meeting of the Committee held on 04 February 2011. A list of the members who were present at the meeting is attached.

**Confirmation of ethical opinion**

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

**Ethical review of research sites**

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

**Conditions of the favourable opinion**

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

10/H0408/116 Further information favourable opinion letter to insert missing document 29/02/2011

*Where the only involvement of the NHS organisation is as a Participant Identification Centre (PIC), management permission for research is not required but the R&D office should be notified of the study and agree to the organisation's involvement. Guidance on procedures for PICs is available in IRAS. Further advice should be sought from the R&D office where necessary.*

*Sponsors are not required to notify the Committee of approvals from host organisations.*

**It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).**

**Approved documents**

The final list of documents reviewed and approved by the Committee is as follows:

| Document | Version | Date |
|---|---|---|
| Protocol | 2.0 | 03 December 2010 |
| Response to Request for Further Information | | |
| Participant Information Sheet: Biomarkers of bowel disease | 3 | 13 January 2011 |
| Covering Letter | | 30 November 2010 |
| Letter from Sponsor | | 24 November 2010 |
| Letter from funder | | 23 September 2010 |
| CV Mr Aslam | | |
| Participant Information Sheet: Colorectal tissue bank | 3 | 13 January 2011 |
| CV Miss Patel | | |
| Letter of invitation | 2.0 | 03 December 2011 |
| REC application | | 06 December 2010 |
| CV Mr Jameson | | |
| CV Dr West | | |
| CV Mr Singh | | |
| Investigator CV | | 24 November 2010 |
| Participant Consent Form: Colorectal Tissue Bank | 4 | 13 January 2011 |
| Participant Consent Form: Biomarkers of bowel disease | 4 | 13 January 2011 |

**Statement of compliance**

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

**After ethical review**

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

| **10/H0408/116** | **Please quote this number on all correspondence** |
|---|---|

With the Committee's best wishes for the success of this project

Yours sincerely

pp

**Dr Martin Hewitt**
**Chair**

Email: jennifer.lee@nottspct.nhs.uk

*Enclosures:* "After ethical review – guidance for researchers"
*Copy to:* *Graham Hewitt, Miss Maleena Patel*

## 2.2 Research and Development Approval Letter

University Hospitals of Leicester NHS
NHS Trust

DIRECTORATE OF RESEARCH & DEVELOPMENT

**Director:** **Professor D Rowbotham**
**Assistant Director:** **Dr David Hetmanski**
**R&D Manager:** **Carolyn Maloney**

Research & Development Office
Leicester General Hospital
Gwendolen Road
Leicester
LE5 4PW

Direct Dial: (0116) 258 8351
Fax No: (0116) 258 4226

03/03/2011

Dr James Howard Pringle
University of Leicester
Rm 340 Department of Cancer Studies and Molecular Medicine
L3 Robert Kilpatrick Building
PO BOX 65 Leicester Royal Infirmary
Leicester Royal Infirmary
LE2 7LX

Dear Dr James Howard Pringle

**Ref:** **UHL 11005**
**Title:** **Tissue and blood biomarkers of bowel disease progression and response to therapy**
**Project Status:** **Project Approved**
**End Date:** **01/01/2016**

I am pleased to confirm that with effect from the date of this letter, the above study now has Trust Research & Development permission to commence at University Hospitals of Leicester NHS Trust.

All documents received by this office have been reviewed and form part of the approval. The documents received and approved are as follows:

| Document Name | Version Number | Date |
|---|---|---|
| Protocol | 2.0 | 03/12/2010 |
| Participant Information Sheet: Biomarkers of bowel disease | 3 | 13/01/2011 |
| Participant Information Sheet: Colorectal tissue bank | 3 | 13/01/2011 |
| Letter of invitation | 2.0 | 03/12/2010 |
| Participant Consent Form: Colorectal Tissue Bank | 4 | 13/01/2011 |
| Participant Consent Form: Biomarkers of bowel disease | 4 | 13/01/2011 |

***Please be aware that any changes to these documents after approval may constitute an amendment. The process of approval for amendments should be followed. Failure to do so may invalidate the approval of the study at this trust.***

Version 5, 20.04.10

We are aware that undertaking research in the NHS comes with a range of regulatory responsibilities. Attached to this letter is a reminder of your responsibilities during the course of the research. Please ensure that you and the research team are familiar with and understand the roles and responsibilities both collectively and individually.

You are required to submit an annual progress report to the R&D Office and to the Research Ethics Committee. We will remind you when this is due.

The R&D Office is keen to support research, researchers and to facilitate approval. If you have any questions regarding this or other research you wish to undertake in the Trust, please contact this office.

We wish you every success with your research.

Yours sincerely

Carolyn Maloney
**R&D Manager**

## 2.3 Consent Form: Biomarkers for Bowel Disease Progression

Patient Name, Address, DOB *(or ID label)*

University Hospitals of Leicester NHS
NHS Trust

**Study Number:** .....

**Study Site Number:** .....

**Patient Study Number:** .....

**Study: Biomarkers for bowel disease progression**

# PATIENT CONSENT FORM

**Researchers: Miss Patel, Mr Aslam, Mr Singh, Mr Jameson. Principle Investigator: Dr Howard Pringle.**

**This form should be read with the Biomarkers for Bowel Disease Progression Leaflet Version 3.0 13.01.2011**

| | Terms and Conditions | Please Initial |
|---|---|---|
| 1. | I (the patient) confirm that I have had time to read and understand the information sheet for the above study and have had the opportunity to ask questions. | |
| 2. | I agree to donate tissue from my procedure and blood samples and allow their use in medical research as described in the Patient Information Leaflet. | |
| 3. | I understand that my tissue and blood samples are donated by free will and that I will not benefit from any intellectual property that results from its use or be offered any financial incentive. | |
| 4. | I understand that the tissue or blood samples will not be used to undertake any genetic tests whose results may have adverse consequences on me or my families insurance or employment. | |
| 5. | I understand that if research carried out on my tissue or blood sample produces information, which has immediate clinical relevance to me, I will be contacted by my hospital consultant or GP to discuss how this may affect my treatment or follow up. | |
| 6. | I understand that blood samples and associated clinical data may be transferred to commercial / non-commercial research partners of the University Hospitals of Leicester NHS Trust, but that the information will be coded prior to transfer. | |
| 7. | I understand that I may withdraw my consent for my tissue and blood samples being used at any time without justifying my decision and it will not affect my normal care and medical management. | |
| 8. | I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from The University of Leicester and/or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. | |
| 9. | I agree to take part in the above study. | |

I have read the patient information leaflet relating to the Colorectal Tissue Bank and have had the opportunity to ask any questions.

Patient Name:.....Patient Signature: .....Date:.....

I confirm I have explained the purpose of the tissue bank, as detailed in the Patient Information Sheet, in terms, which in my judgment are suited to the understanding of the patient.

Researcher Name:.....Researcher Signature: .....Date: .....

## 2.4 Patient Information Leaflet: Biomarkers for Bowel Disease Progression

# Participant Information Sheet

University Hospitals of Leicester NHS
NHS Trust

**STUDY TITLE: BIOMARKERS FOR BOWEL DISEASE PROGRESSION**

University of Leicester - Department of Molecular Medicine
Robert Kilpatrick Building Level 3 Leicester Royal Infirmary

**Researchers: Miss Patel, Mr Aslam, Mr Singh, Mr Jameson.**
**Principle Investigator: Dr Pringle**

You are being invited to take part in a research study. Before you decide if you would like to take part, it is important for you to understand why the research is being done and what it will involve. Please take some time to carefully read the following information and discuss it with others if you wish. If there are any points that are not clear to you or if you would like more information, please do not hesitate to ask further questions.

1. **Why have I been chosen?**

You have been chosen because you are going to have a procedure to investigate or treat bowel disease. We are requesting your agreement to let us study a portion of your bowel that will be removed as part of the procedure.
We will also study a sample of your blood, which will be taken around the time of your procedure.

2. **What is the purpose of the study?**

This study will investigate the changes that occur in the lining of the bowel in a range of diseases including inflammatory bowel conditions and bowel cancer (colorectal cancer). We will compare these changes to normal bowel tissue to help us understand the mechanisms involved in the development and progression of bowel disease. Our research will be used towards developing a test, which in the future may help diagnose and monitor bowel disease. This test may reduce the need for other tests such a colonoscopy, barium enema or CT scan.

3. **Do I have to take part?**

No. This study is independent of your medical treatment. It is entirely your decision as to whether or not you wish to take part in the study. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw from the study at any time without giving a reason. In practice, withdrawal would mean destruction of any donated tissue samples or blood samples and, should you also wish, any associated data. The decision you make will never affect your management or any of the treatment you may receive.

4. **What will happen to me if I take part?**

Colon tissue removed during your procedure is always sent to a pathologist for examination. If you agree to take part in the study we will take an additional small sample from the tissue being removed. This tissue would otherwise be discarded, so its selection will not alter the routine assessment of your tissue. Since not all of the sample will be used in this study we also request that we can store the sample for further similar studies (see attached 'Tissue Bank Information Sheet'). Storing or 'archiving' samples in this way is extremely useful to scientists as it allows us to gather data and monitor changes over a time period. The blood samples will be collected in small tubes in the usual way that a blood test is performed and will be destroyed when the study is complete.

5. **What are the possible disadvantages and risks of taking part?**

If you chose to take part in the study we will collect samples from the bowel tissue that has routinely been removed as part of your procedure. This will take place following the examination that is always carried out on surgically removed tissue and will in no way alter how your tissue will be treated. We will also require a blood sample; the risks of which are limited to discomfort at the site of the blood test.

6. **What are the possible benefits of taking part?**

There is no benefit to you personally from taking part in this study. However, we hope that our results may allow us to develop new tests to detect and monitor bowel disease. We will not give you any financial compensation for taking part in the study.

7. **What if new information becomes available?**

We will not be performing any tests that have an influence on your care. It is therefore unlikely that the study will yield any new information that will affect you personally.

8. **What if something goes wrong?**

The chance of any problems arising because of your inclusion in the study is extremely small. If you do feel that taking part in this research project has harmed you, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action. If you wish to complain, or have any concerns regarding the way you have been approached or treated throughout the study, you may contact National Health Service complaints department in the normal way.

9. **Will my taking part in this study be kept confidential?**

All personal or medical information collected about you during the study will always remain strictly confidential. Any information regarding you and your sample, which may leave the hospital, will have your name and address removed so that you cannot be identified from it.

10. **What will happen to the results of the research study?**

The results from this study will be presented at scientific meetings and published in scientific journals. You will not be identified in any report or publication.

11. **Who is organising and funding the research?**

This study is a small-scale study that is being financed by Leicester University, University Hospitals Leicester and a scientific fund. The researchers will not receive extra payments for performing this study.

12. **Who has reviewed the study?**

All research that involves NHS patients, staff, information from NHS medical records or uses NHS premises or facilities must be approved by an NHS Research Ethics Committee before it goes ahead. Approval does not guarantee that you will not come to any harm if you take part. However, approval does mean that the committee is satisfied that your rights will be respected, that any risks have been reduced to a minimum and balanced against possible benefits and that you have been given sufficient information on which to make an informed decision.

13. **Contacts for Further Information**


Dr. Howard Pringle
Department of Molecular Medicine
Robert Kilpatrick Building Level 3
Leicester Royal Infirmary
Leicester LE2 7LX
E-mail: JHP@le.ac.uk
Phone: 0116 252 3227

Miss Maleene Patel
Department of Molecular Medicine
Robert Kilpatrick Building Level 3
Leicester Royal Infirmary
Leicester LE2 7LX
Email: mp364@le.ac.uk
Phone: 07912570253


14. **Thank you for reading this.**

Please keep this copy of this Information Sheet to refer to in future. If you agree to take part in the study, you will also receive a copy of the signed consent form to keep.

## 2.5 Consent Form: Colorectal Tissue Bank

Patient Name, Address, DOB *(or ID label)*

University Hospitals of Leicester NHS Trust

**Study Number:** .....

**Study Site Number:** .....

**Patient Study Number:** .....

**Colorectal Tissue Bank**

# PATIENT CONSENT FORM

**Researchers: Miss Patel, Mr Aslam, Mr Singh, Mr Jameson. Principle Investigator: Dr Pringle**
**Tissue Bank Custodians: Dr Richards and Mr Jameson**

**This form should be read in conjunction with The Colorectal Tissue Bank Leaflet, Version 3.0 (13.01.2011)**

| | Terms and Conditions | Please Initial |
|---|---|---|
| 1. | I (the patient) agree to donate the tissue samples as identified to the Colorectal Tissue Bank and allow their use in medical research as described in the Patient Information Sheet entitled Colorectal Tissue Bank, Version 3.0 dated 13.01.2011 | |
| 2. | I understand that I may withdraw my consent to my tissue and blood sample being used at any time without justifying my decision and without affecting my normal care and medical management. | |
| 3. | I understand that members of University Hospitals of Leicester NHS Trust and Leicester University research teams may wish to view relevant sections of my medical records, but that all the information will be treated as confidential. | |
| 4. | I understand that samples from the tissue bank and associated clinical data may be transferred to non-commercial research partners of the University Hospitals of Leicester NHS Trust and Leicester University, but that the information will be coded and hence anonymous, prior to transfer. | |
| 5. | I understand that medical research is covered for mishaps in the same way; as for patients undergoing treatment in the NHS i.e. compensation is only available if negligence occurs. | |
| 6. | I understand that samples from the tissue bank will not be used to undertake any genetic tests whose results may have adverse consequences on my or my families insurance or employment. | |
| 7. | I understand that if research using my tissues produces information, which has immediate clinical relevance to me, I will be informed by my hospital consultant or GP and be given an opportunity to discuss the results. | |
| 8. | I understand that my tissue is being donated by free will and that I will not benefit from any intellectual property that result from the use of the tissue or receive any financial compensation. | |
| 9. | I would be willing to be contacted again regarding future use of this tissue for purposes not foreseen at the present time. | |
| 10. | I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from The University of Leicester and/or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. | |

**I have read the patient information leaflet relating to the Colorectal Tissue Bank and have had the opportunity to ask any questions.**

**Patient Name:**.....**Patient Signature:** .....**Date:**.....

**I confirm I have explained the purpose of the tissue bank, as detailed in the Patient Information Sheet, in terms, which in my judgement are suited to the understanding of the patient.**

**Researcher Name:**.....**Researcher Signature:** .....**Date:** .....

## 2.6 Patient Information Leaflet: Colorectal Tissue Bank

# Participant Information Sheet

University Hospitals of Leicester NHS
NHS Trust

**COLORECTAL TISSUE BANK**
University of Leicester - Department of Molecular Medicine
Robert Kilpatrick Building Level 3 Leicester Royal Infirmary

**Researchers: Miss Patel, Mr Aslam, Mr Singh, Mr Jameson**
**Principle Investigator: Dr Pringle**

Dear Patient – you are being invited to take part in a research study. Before you decide if you would like to participate, it is important for you to understand why this research is being done and what it will involve. Please take some time to carefully read the following information and discuss it with others if you wish. If there are any points that are not clear to you or if you would like more information, please do not hesitate to ask further questions.

**1. Why have I been chosen?**
You have been asked to read this information because you are due to undergo a procedure (test or treatment) for bowel disease. This procedure will be part of the management recommended by the consultant surgeon responsible for your care. As part of the procedure you will routinely have some bowel removed or bowel samples taken. This will be sent to a pathologist for analysis. We would like to take some of this tissue and a blood sample for our research; these samples will be stored in a tissue bank.

**2. What is a tissue bank?**
A tissue bank is a collection of tissue and blood samples being stored over a period of time. The tissue bank is a valuable research resource and will allow us to carry out future research into a specific disease or group of diseases or investigate disease processes and their treatment. Tissue banks are increasingly being established at local, regional and national level.

**3. What will the tissues in the tissue bank be used for?**
The tissues will be used for research into bowel diseases such as inflammatory bowel disease and bowel cancer (colorectal cancer). We hope to investigate ways of detecting and monitoring different bowel diseases. This research will also increase our understanding of how bowel disease develops, progresses and the effects of current treatment.

The NHS Research Ethics Committee must approve any research that is being carried out within the NHS before it goes ahead. Approval means that the Committee is satisfied that by participating in the study, your rights would be respected and that any risks to you are reduced to a minimum. It will also ensure that you have been given sufficient information on which to make an informed decision to take part or not. Approval, however does not guarantee that you will not come to any harm if you take part.

**4. How much of my tissue will be taken?**
During your bowel procedure pieces of bowel tissue will be taken and kept so that a pathologist can analyse them for disease presence. After the routine sampling of your tissue, we will take further small samples from the tissue specimen to be stored for our research. This tissue would otherwise be discarded, so its selection will not alter the routine assessment of your tissue. We will also obtain a blood sample from you in the same way that a routine blood test would be carried out. This will also be stored in the tissue bank.

**5. Will I be contacted again in the future?**

Maybe. If any of the research carried out on your tissue reveals new information that impacts upon your care, we will contact your GP or Consultant and this information will be discussed with you. We would also contact you again to seek permission to use your tissue samples, for any future research, which is not described in this information sheet.

**6. Who will have access to my tissue and how will confidentiality be maintained?**

Access to your tissue samples will be only available through the Colorectal Tissue Bank, controlled by the University Hospitals of Leicester NHS Trust. Your tissue samples will be handled in a confidential manner in accordance with the data protection act. Any samples being transferred to other research partners will remain anonymised and you will not be identified in any way from your tissue and blood sample. Basic clinical details regarding your procedure, age, sex and the pathology results will be linked to your sample(s) but will not include your name or address.

**7. Will I receive payment for the tissue that I donate to the tissue bank?**

No. Your tissues are being donated by free will and you will be not offered any financial incentive or payment. Neither yourself nor your relatives will benefit from any inventions or intellectual property that result from the use of the tissue

**8. What happens if I wish to have my tissue removed from the tissue bank?**

If you do not wish your tissues and blood to be held in the tissue bank you may withdraw your consent at any time without having to justify your decision. Your future treatment will not be affected. If you wish to have your tissue removed from the tissue bank please inform us (contact details below).

**9. Location of Colorectal Tissue Bank**

University of Leicester - Department of Molecular Medicine
Robert Kilpatrick Clinical Science Building (level 3)
Leicester Royal Infirmary
Infirmary Square
Leicester LE2 7LX

**10. Contact Details**


Miss Maleene Patel or Dr J.H. Pringle
Department of Molecular Medicine
Robert Kilpatrick Clinical Sciences Building
Leicester Royal Infirmary
Infirmary Square
Leicester LE2 7LX
Tel: +44 116 2523227

OR

Research Office
Directorate of Research & Development
University Hospitals of Leicester NHS Trust
Leicester General Hospital
Gwendolen Road
Leicester LE5 4PW
Tel: 0116 258 4109


11. **Thank you for reading this.**

Please keep this copy of this Information Sheet to refer to in future. If you agree to take part in the study, you will also receive a copy of the signed consent form to keep.

## 2.7 University Sponsorship Letter

University of Leicester

School of Medicine
Medical School Office
Maurice Shock Building
PO Box 138
University Road
Leicester LE1 9HN
UK

T +44 (0)116 252 2969/2985/2966/3015
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Project :Tissue and blood biomarkers of bowel disease progression and response to therapy

CI: Howard Pringle

24/11/10

Dear Sir/Madam

I can confirm that the University of Leicester will act as Sponsor of the above project as outlined by the Department of Health's Research Governance Framework (2005).

Yours

Graham Hewitt BA(Hons) PGDip MICR

Research Governance Manager

College Research and Enterprise Office

College of Medicine, Biological Sciences and Psychology

University of Leicester

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Tel: 0116 223 1262 or 0116 258 8241

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## 2.8 National Bowel Cancer Screening Program Letter

NHS
Bowel Cancer Screening Programme
Research Committee

**John Scholefield**
Chair

C/o NHS Cancer Screening Programmes
Fulwood House
Old Fulwood Road
Sheffield
S10 3TH

Tel: 0114 2711060

mia7@leicester.ac.uk

6 November 2013

Re: Circulating MicroRNAs are Novel Biomarker for Colorectal Cancer Screening

Dear Mr Aslam

Thank you for your call and subsequent email today. I'm sorry we were unable to find the original confirmation letter to you regarding the extension to the above project that you requested in 2012.

I can confirm that the extension you requested was approved via Chairman's Action following the Research Committee meeting in September 2012. The current end date for the project stands at 01 August 2014.

Kind regards,

TJ Day MSc, MA
On behalf of the NHS Bowel Cancer Screening Programme Research Committee

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