Plasma microRNAs – novel markers for the detection of colonic polyps and their progression to colorectal cancer.

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 period April 2012 – August 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.

Name: Dr Ajay Verma

Signed: 

Date: 21/09/2017

Abstract

Plasma microRNAs – novel markers for the detection of colonic polyps and their progression to colorectal cancer.
Dr Ajay M Verma

Introduction: Bowel cancer screening programme (BCSP) subjects aged 55 are screened by bowel scope sigmoidoscopy, aged 60-75 by faecal occult blood testing (FOBt - positive subjects undergo colonoscopy). Whilst effective, FOBt lacks high sensitivity, specificity and accuracy, >90% sigmoidoscopies and 50% colonoscopies are normal or non-adenoma diagnosis.

Endoscopy screening is invasive, resource intensive and can cause harm. Uptake of FOBt and bowel scope is <60%. A blood based screening test is an appealing alternative.

Methods: We investigated microRNAs (miRs) – short non-coding RNA molecules as potential biomarkers. 181 FOBt+ subjects and 29 others undergoing endoscopy were recruited – 128 males, 82 females. 117 with polyps (99 adenomas), 12 colorectal cancer, 81 controls.

RNA was extracted from plasma and processed. Pooled groups were analysed using microarray assay cards. Ten candidate miRs 19a, 98, 146b, 186, 331–5p, 452, 625, 222#, 664 and 1247 were identified. Cases were analysed for candidate microRNA expression by quantitative polymerase chain reaction.

Results: Candidate miRs showed significant levels of expression in subjects with adenomas on T-testing. miRs 98 & 19a; p=<0.05, miRs 146b, 625; p=<0.01, miR-186 p=<0.001. The results were more significant for male subjects.

Receiver operated characteristic curves for miR-panels showed: **Polyps in male subjects**, miRs 98, 186, 452; sensitivity 0.600, specificity 0.872. **Adenomas in male subjects**, miRs 98, 186, 452. Sensitivity 0.606/0.591, specificity 0.875/0.900. **Polyps with diverticulosis/haemorrhoids**; miRs 186, 452, 331–5p; sensitivity 0.600/0.633, specificity 0.889/0.867. **Adenomas with diverticulosis/haemorrhoids**; miRs 625, 452, 331–5p; sensitivity 0.714, specificity 0.864.

Conclusion: This study suggest plasma microRNAs are potential screening biomarkers for male subjects with colorectal polyps, adenomas and subjects with adenomas and diverticulosis/haemorrhoids. Further study is needed to validate these exciting findings.

Acknowledgements

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Contents

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List of abbreviations

AD: Anderson-Darling.
APC: Adenomatous polyposis coli.
AUC: Area under the curve.
BCSP: Bowel cancer screening programme.
BDRF: Bowel Disease Research Foundation.
BTN: Benign thyroid nodules.
CAC: Colitis associated cancer.
cDNA: Complimentary DNA.
CI: Confidence interval.
CIMP: CpG island methylator phenotype.
CIN: Chromosomal instability.
CRC: Colorectal cancer.
CT: Cycle threshold.
CT scan: Computed tomography scan.
DNA: Deoxyribonucleic acid.
EDTA: Ethylenediaminetetraacetic acid.
FFPE: Formalin-fixed paraffin embedded.
FIT: Faecal immunochemical testing.
FOBt: Faecal occult blood test.
HNPCC: Hereditary nonpolyposis colorectal cancer.
IBD: Inflammatory bowel disease.
IRAS: Integrated research application system.
IRS-1: Insulin receptor substrate-1.
MDT: Multi-disciplinary team.
miRs: MicroRNAs.
MMR: Mismatch repair.
MRI: Magnetic resonance imaging.
mRNA: Messenger RNA.
MSI+: Microsatellite instability.
NHS: National Health Service.
NREC: National Research Ethics committee.
OSCC: Oesophageal squamous cell cancer.

PDA: Pancreatic ductal adenocarcinoma.

PCR: Polymerase chain reaction.

PhD: Doctorate of Philosophy.

PPV: Positive predictive value.

PSC: Primary sclerosing cholangitis.

PTC: Papillary thyroid carcinoma.

qPCR: Quantitive polymerase chain reaction.

R&D: Research and development.

RNA: Ribonucleic acid.

RNAi: RNA interface.

ROC: Receiver operated characteristic.

RR: Risk ratio.

RT-qPCR: Reverse transcriptase quantitative polymerase chain reaction.

SSA: Sessile serrated adenomas.

TNM: Tumour Node Metastasis (cancer staging classification).

TRIS: Tris (hydroxymethyl)-aminomethane.

UC: Ulcerative colitis.

UHL: University Hospitals of Leicester.

UK: United Kingdom.

UoL: University of Leicester.

UTR: Untranslated region.

INTRODUCTION

1. Colorectal cancer

1.1 Demographics

Colorectal cancer (CRC) is the fourth most common cancer in the UK. Around 41 300 people were diagnosed with CRC in 2014, 22 800 being men making it the third most common cancer in men after prostate and lung cancer. [1]

CRC is the third most common cancer in women after breast and lung cancer, with around 18 400 new cases diagnosed in the UK in 2014. 95% of CRC cases occur in people aged 50 and over. [1]

CRC incidence rates have increased by 5% over the last decade. In Europe, around 477 000 new cases of CRC were estimated to have been diagnosed in 2012. The UK incidence rate is 20th highest in Europe for males and 17th highest for females. Worldwide, an estimated 1.36 million new cases of CRC were diagnosed in 2012, with incidence rates varying across the world. [1]

CRC is the second most common cause of cancer death in the UK after lung cancer (2014). Around 16 200 people died of CRC in 2012 in the UK. CRC death rates have been falling since the 1970s. Over the last decade in the UK (between 2003-2005 and 2012-2014), data from the Office of National Statistics (November 2015) shows bowel cancer age standardised mortality rates have decreased by 12% (decrease in males; 15% and females; 11%). [1]

In Europe around 215 000 people were estimated to have died from CRC in 2012. The UK mortality rate is 10th lowest in Europe for males and 14th lowest for females. Worldwide, around 694 000 people were estimated to have died from CRC in 2012, with mortality rates varying across the world. [1]

A person's risk of developing CRC depends on many factors, including age, genetics, and exposure to risk factors (including some potentially avoidable lifestyle factors).

An estimated 54% of CRC cases in the UK are linked to lifestyle factors including processed / red meat consumption (21%), being overweight / obese (13%), alcohol (12%), smoking (8%) and ionising radiation (2%). [1]

It is well established that 5% of CRC is due to an inherited syndrome, the most common being Lynch syndrome (also known as HNPCC – hereditary nonpolyposis colorectal cancer) due to mutation of one of the many DNA mismatch repair (MMR) genes. Familial adenomatous polyposis is due to a mutation of the adenomatous polyposis coli (APC) gene on chromosome 5 given rise to a carpet of adenomas by the age of 20 requiring colectomy to protect the individual from the inevitable CRC that usually occurs by the age of 30-35. [2]

In October 2015, the World Health Organization released a statement classifying red and processed meat as a carcinogen. [3] This was largely based on a meta-analysis of CRC in ten cohort studies reported a statistically significant dose–response relationship, with a 17% increased colorectal cancer risk (95% CI 1.05 - 1.31) per 100 grams per day of red meat and an 18% increase (95% CI 1.10 – 1.28) per 50 grams per day of processed meat. [4]

Fibre consumption protects against CRC, the summary relative risk of developing colorectal cancer for 10 grams daily of total dietary fibre (based on a meta-analysis of 16 studies) was 0.90 (95% CI 0.86 to 0.94). [5] Physical activity protects against CRC in a meta-analysis of 21 studies, there was a significant 27% reduced risk of proximal colon cancer when comparing the most versus the least active individuals (RR = 0.73, 95% CI = 0.66-0.81), an almost identical result was found for distal colon cancer (RR = 0.74, 95% CI = 0.68-0.80). [6]

CRC risk has been higher in populations with inflammatory bowel disease (IBD) (when it causes a colitis – ulcerative colitis (UC) or the rarer Crohn's colitis) compared with the general population. This is known as colitis associated cancer (CAC). A landmark meta-analysis study from 2001 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. [7]

The CAC risk has decreased in recent times. A large cohort study from Copenhagen County, Denmark published in 2004 followed up 1160 patients with UC over a median follow up period of 19 years, the cumulative incidence of colorectal cancer was 0.4% at 10 years, 1.1% at 20 years, and 2.1% at 30 years of disease. [8] St. Marks Hospital (UK) have a database of patients with UC who undergo surveillance colonoscopy. An analysis of their data reveals a cumulative incidence of colorectal cancer was 0.07% at 10 years, 2.9% at 20 years, and 6.7% at 30 years, 10.0% at 40 years, and 13.6% at 50 years of disease. [9] Another large Danish study looking at the whole population (published in 2012) showed that the relative risk of CAC in UC patients was 1.07 (95% confidence intervals 0.95-1.21). [10]

The factors in inflammatory bowel disease that increase CAC risk are; diagnosis as a child or adolescent, long duration of disease, extent and severity of inflammation, and concomitant primary sclerosing cholangitis (PSC). [7] [10] [11] The St. Marks population are more likely to be complex cases of UC as they are a tertiary centre, despite this the data still shows an improvement, as does the Danish studies, when compared to the 2001 meta-analysis probably due to improved IBD treatment, increased awareness of CAC risk, and endoscopic surveillance of patients with IBD. [9]

1.2 Polyps & adenomas

CRC mostly arises from adenomas (95%), recognised as colonic polyps at endoscopy and the progression of these adenomas to colon cancers is a multi-step process which involves different sequential changes in DNA structure and expression.

This model is likely to be an oversimplification, but it aligns observed clinico-pathological changes with genetic abnormalities in the progression of chromosomally unstable colorectal cancer (the gatekeeper pathway involving genes that regulate cell growth). The initial step in tumour-genesis is that of adenoma formation, associated with loss of APC.

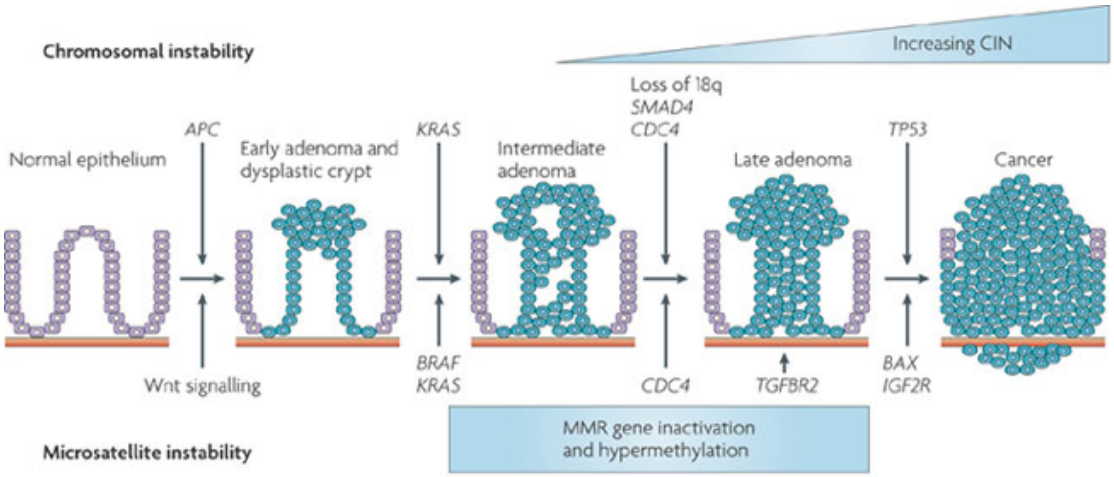


Figure 1 – Adenoma Carcinoma sequence.

The pathway with arrows from the top of the figure shows chromosomal instability which accounts for 80-85% of CRC arising from adenomas. 10-15% of CRCs arising from adenomas is thought to follow the microsatellite instability pathway which is illustrated with arrows from the bottom of the figure.

Larger adenomas and early carcinomas acquire mutations in the small GTPase KRAS gene, followed by loss of chromosome 18q with SMAD4, which is downstream of transforming growth factor-β, and mutations in TP53 in frank carcinoma. This chromosomal instability pathway is likely to account for 80-85% of “sporadic” CRCs arising from adenomas (top pathway in figure 1).

Microsatellite instability (MSI+) CRCs characterised by a deficiency of the mismatch repair system that leads to slippage in microsatellites (the caretaker pathway involving genes that maintain genomic stability), only carry the above changes infrequently; therefore, development of CRC must involve different, but analogous, genetic changes to those described in chromosomal instability (CIN) CRC. Microsatellite instability is uncommon in adenomas (10-15% of sporadic CRCs), and the initial step is thought to involve alteration in Wnt signalling, possibly involving axin.

Mutations in BRAF, common in MSI+ CRC, are likely to occur in the place of KRAS mutations, although the latter do occur in a minority of cases. MMR deficiency in sporadic CRC occurs predominantly by down-regulation of MLH1 through promoter methylation, and MSI+ status is increased by positive selection of tumour cells with mutated microsatellites in MSH3 and MSH6. Further positive selection occurs for mutations affecting microsatellites in TGFβ

receptor 2, insulin-like growth factor 2 receptor and BAX, which in turn provides a TP53-independent mechanism of progression to carcinoma. FBXW7 (F box and WD40 domain protein) inactivation may precede TP53 mutation, leading to increasing CIN, although it is not always associated with CIN and may also have a role in the MSI+ pathway (bottom pathway of figure 1). [12]

The remaining 5-10% of CRCs arising from adenomas is thought to follow an accelerated CpG island methylator phenotype (CIMP), which occurs in approximately 10% sessile serrated adenomas. CRCs demonstrated as being from CIMP pathway have many clinical correlations such as proximal location, poor differentiation, female sex, MSI, high BRAF/KRAS and low APC/p53 mutations.

There are many different types of polyp – adenomas, sessile serrated adenomas, hyperplastic polyps, inflammatory polyps, post-inflammatory pseudopolyps. They are assessed endoscopically and for those that are adenomas, they are classified by surface appearance (Kudo pit pattern), morphology (Paris classification), size and location. Different adenoma types have variable risk of progression to cancer. Molecular analysis of adenomas and have revealed that different polyp types have varying prevalence of KRAS/BRAF mutations and methylation which promote cancer. [13] CRC tissue samples have also undergone molecular analysis and been classified by presence of KRAS/BRAF mutations, methylation and microsatellite instability – suggesting which types of polyps these cancers have arisen from. [14]

Therefore, early detection (and removal) of these adenomas is a cornerstone for screening and managing those individuals who are at a risk of developing CRC. Endoscopic analysis of resected polyps is corroborated and augmented by histopathologic assessment of tissue resected.

Kudo pit classification (figure 2) indicates the type of polyp and represents the different types of polyps from normal colorectal tissue (pit pattern type I), benign hyperplastic polyps (pit pattern type II), adenomas (pit pattern type III and IV, representing tubular and/or villous components) and invasive lesions representing cancer (pit pattern type V). [15]

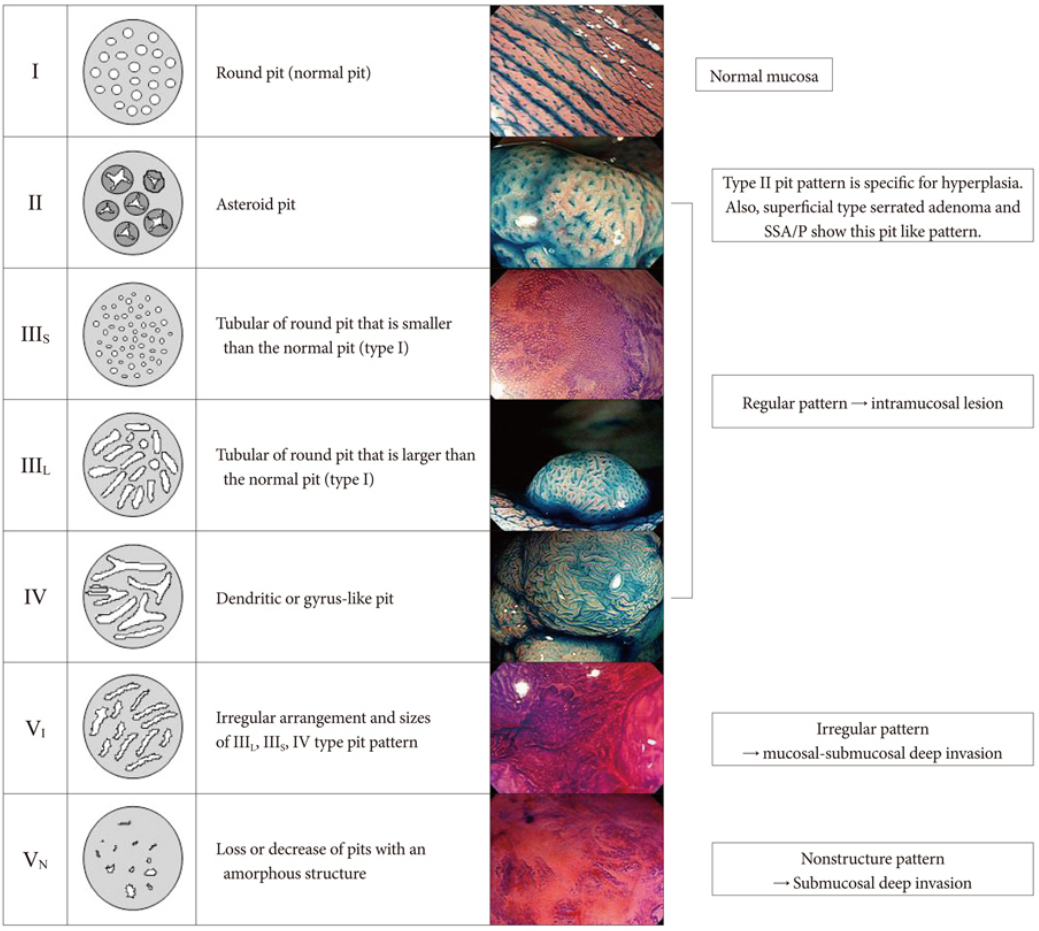


Figure 2 – Kudo classification; pit pattern of polyps

The morphology of the polyp is assessed by the Paris classification (figure 3), whether it is a protruded polyp that is pedunculated (on a stalk - Ip) or sessile (Is). Pedunculated polyps are usually present in the left colon - due to traction on the polyp surface by solid stool causing a stalk to form. As faeces are liquid in the right colon, polyps in this area are usually sessile.

Flatter lesions are still described as polyps (though are less polypoid in appearance) are classified as IIa (slightly raised) or IIb (flat), any depression of the lesion (IIc) is often characteristic of the invasion into deeper muscularis layers representative of cancer. [16]

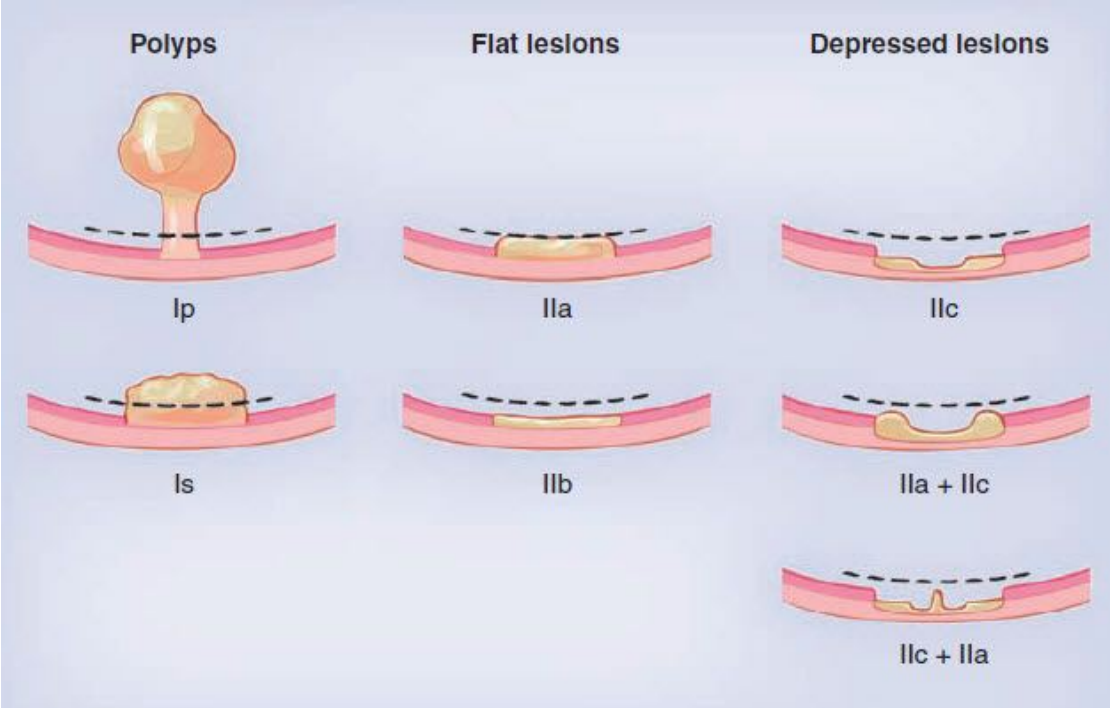


Figure 3 – Paris Classification; morphology of polyps

The size of the adenomas is important too. Larger polyps are much more likely to have advance features such as high grade dysplasia or foci of invasive cancer and have higher risks of endoscopic complications on removal (such as bleeding or colonic perforation). In the UK, the size and number of adenomas at colonoscopy are important as this is used to stratify patients' risk of further adenomas occurring – this guides the recommended interval for repeat surveillance colonoscopy to look for more adenomas (figure 4). [17]

Interestingly the surveillance intervals in the UK do not take into account the histology of adenomas – whether they are tubular, villous or tubulo-villous in nature. Having villous component to adenomas is known to increase your risk to colorectal cancer. [18]

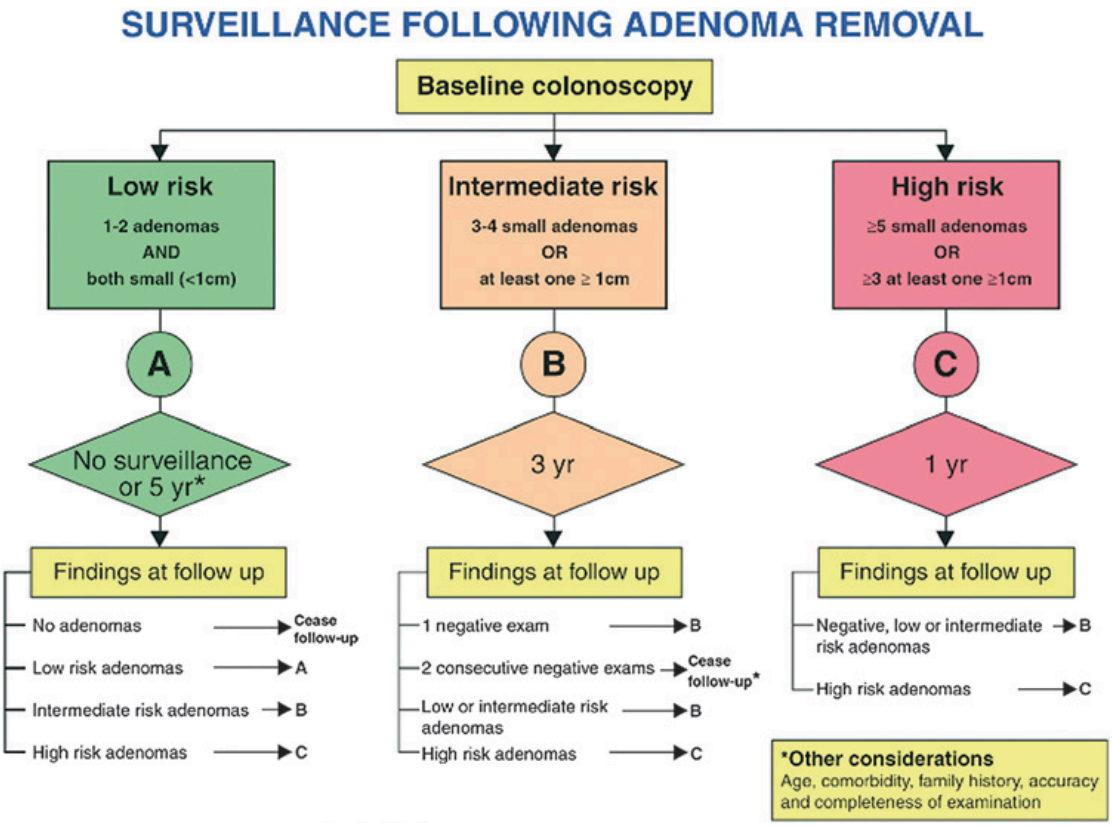


Figure 4 – Adenoma follow up algorithm

There is also a sub-type of sessile adenomas, which were for a long time thought to be hyperplastic lesions. Sessile serrated adenomas (SSA) are a different sub-type of adenomas with appearances similar to hyperplastic polyps but usually larger in size (usually from 5 mm and up to 30 mm in size). They can occur throughout the colon, predominantly in the right colon, as opposed to hyperplastic polyps (usually less than 10 mm in size), which occur throughout the colon but predominate in the rectum. Approximately 10% of SSAs may be in the CIMP pathway which can lead to rapid and aggressive colorectal cancer. [19] Therefore, SSAs are removed when discovered at colonoscopy. [20]

1.3 Colorectal cancer presentation, diagnosis & management

As a large colorectal adenoma progresses to an invasive cancer, it can have classic symptoms or be relatively asymptomatic. Lesions in the left side of colon & rectum can often result in symptoms of rectal bleeding (which can progress to anaemia over time), altered bowel habit and tenesmus. Lesions in the right colon often have vaguer symptoms; altered bowel habit and symptoms of

anaemia. Cancerous lesions overtime will cause weight loss and as the cancer enlarges and invades adjacent tissues it could cause localised pain. If the cancer metastasises to the liver it could cause localised pain or jaundice, if it spreads to the lungs it could cause shortness of breath. Metastatic disease will usually cause or exacerbate weight loss.

Given the broad range of symptoms the colorectal cancer can cause, Doctors are trained to be mindful of certain red flag symptoms and investigate as appropriate. If a General Practitioner has a suspicion that a patient may have colorectal cancer, they are advised to refer on an urgent 2-week cancer pathway to a Gastroenterology or Colorectal Surgery service.

Symptoms	**Investigation if fit**	**Investigation if unfit/frail**
Rectal bleeding	**Sigmoidoscopy** (if fresh rectal bleeding) **Colonoscopy** (if altered rectal bleeding &/or normal sigmoidoscopy) **CT colonogram** (as an alternative to colonoscopy – patient choice or if technically, colonoscopy not possible)	**Sigmoidoscopy**
Iron deficiency anaemia	**Colonoscopy** **CT colonogram** (alternative test)	**CT (scan) of abdomen & pelvis**
Change in bowel habit	**Colonoscopy** **CT colonogram** (alternative test)	**CT abdomen & pelvis**
Gastrointestinal symptoms & weight loss	**CT abdomen & pelvis (+/- chest if suggestive symptoms)**	**CT abdomen & pelvis (+/- chest if suggestive symptoms)**

Table 1 – Investigations suggested for red flag symptoms

Choice of investigation is based on the table. However, patients often have multiple symptoms so choice of investigation is down the discretion of requesting Clinician.

Once a colorectal cancer diagnosis is suspected – it ideally needs a tissue diagnosis via a biopsy of the lesion (via endoscopy) or of a metastatic lesion within the liver. Occasionally a radiological diagnosis is convincing enough to allow a surgeon to operate – (in all parts of the colon except the rectum).

To complete the diagnostic process, a staging CT scan is required of the chest, abdomen & pelvis (ideally with intravenous contrast). If the primary cancerous lesion is in the rectum a staging MRI scan is also required.

Colorectal cancer is staged using two staging system. Dukes' staging which was described in 1932 by British Pathologist Cuthbert Dukes. This has been largely superseded by the TNM staging system (figure 6) but is still in clinical use today. Further modifications to Dukes' staging has led to a Full Dukes' Classification (figure 5). Table 2 shows the relation between Dukes' and TNM staging.

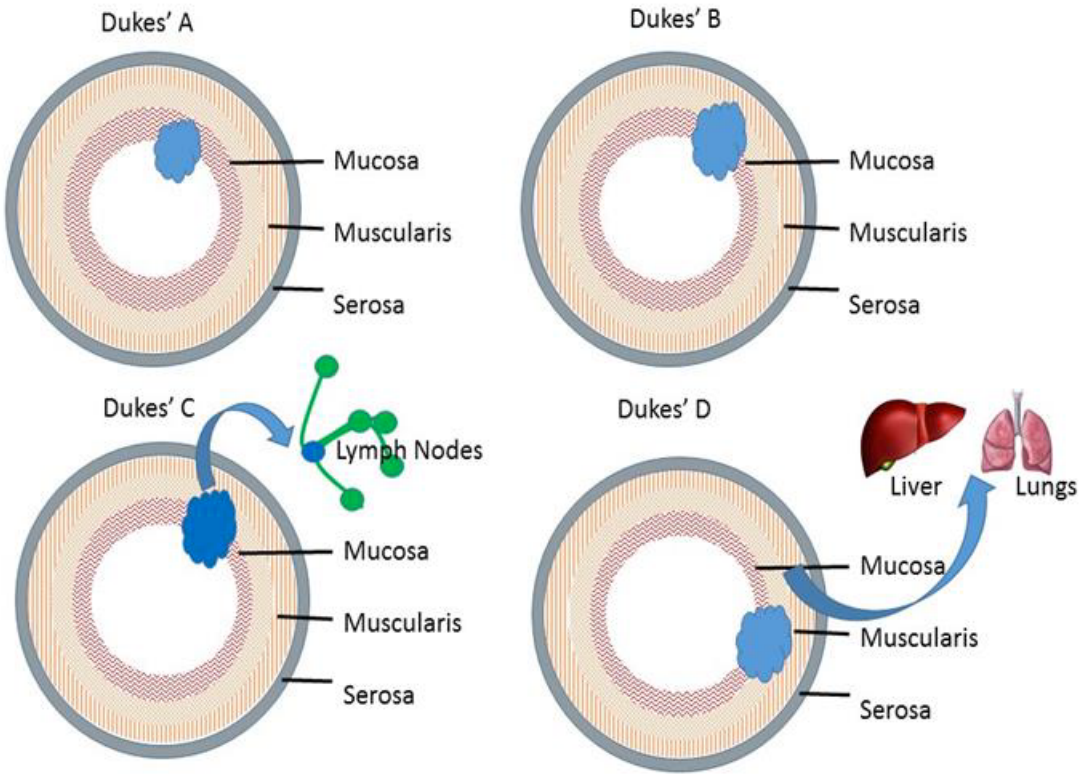
Figure 5 – Dukes' staging of colorectal cancer

Stage A Limited to mucosa

Stage B Extending into (**B1**) or penetrating through (**B2**) muscularis propria, lymph nodes not involved

Stage C Extending into (**C1**) or penetrating through (**C2**) muscularis propria, lymph nodes involved

Stage D Distant metastatic spread (eg to liver, lungs, peritoneum)



The Dukes' staging system - A, B, C and D

Figure 6 – TNM staging of colorectal cancer

Primary tumour (T)

TX Primary tumour cannot be assessed

T1 Tumour invades submucosa

T2 Tumour invades muscularis propria

T3 Tumour invades through muscularis propria into the peri-colonic tissue

T4a Tumour penetrates to the surface of the visceral peritoneum

T4b Tumour directly invades or is adherent to other organs or structures

Regional lymph nodes (N)

N0 No regional lymph node metastasis

N1 Metastasis in 1-3 regional lymph nodes

N2 Metastasis in 4 or more lymph nodes

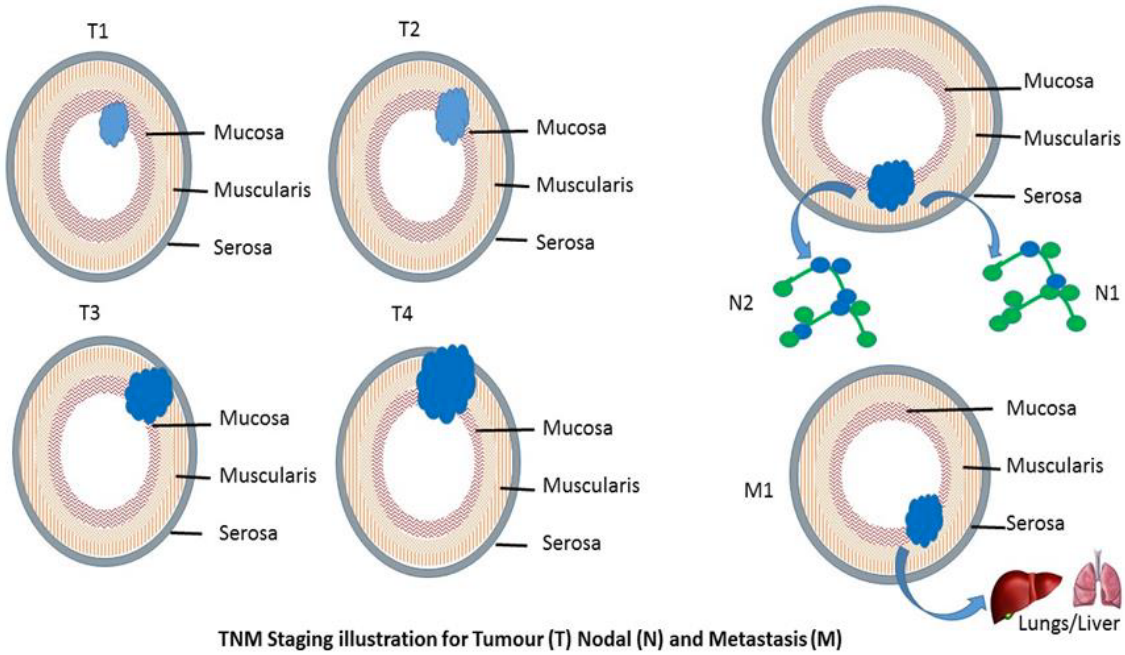
Distant metastasis (M)

M0 No distant metastasis

M1 Distant metastasis

M1a Metastasis confined to 1 organ or site (eg liver, lung, non-regional node)

M1b Metastases in more than 1 organ/ site or the peritoneum



TNM Staging illustration for Tumour (T) Nodal (N) and Metastasis (M)

Dukes' Stage	Spread of CRC	TNM Stage		
A	Submucosa	T1	N0	M0
	Into muscularis propria	T2	N0	M0
B	Beyond muscularis propria	T3	N0	M0
	Into adjacent organs	T4	N0	M0
C	1-3 lymph node metastasis	T1-4	N1	M0
	≥ 4 lymph nodes metastasis	T1-4	N2	M0
D	Distant organ metastasis	T1-4	N0-2	M1

Table 2 – Relation between Dukes' and TNM staging for colorectal cancer

All cancer cases are discussed at specialist cancer multi-disciplinary team (MDT meetings, which has a core membership group of clinicians including Gastroenterologists, Surgeons, Oncologists, Radiologists, Pathologists and Palliative Care specialists).

The ultimate treatment aim is to operate and to resect cancerous lesions (T1-3). If the lesion is too locally advanced (T4), or had metastatic spread (M1) it may not be resectable. Neo-adjuvant radiotherapy and chemotherapy may shrink tumours down to make surgery possible, also patients with a resectable primary lesion and isolated metastases in liver and/or lungs may also be able to undergo surgery with a curative intent. [21]

Postoperatively patients undergo further discussion at the MDT meeting and if the histology of the cancer has adverse prognostic markers such as lymph node metastasis, vascular invasion or poor differentiation, then a patient would be offered adjuvant chemotherapy to reduce the risk of recurrence in the future (as long as they are fit to receive chemotherapy). [21]

If patients have a disease that is classified as inoperable due to metastases, patients can be offered palliative chemotherapy (if fit) or best supportive care for advanced cancer symptoms as disease progresses. Occasionally that may be the case if surgery was not deemed to have cleared the cancerous tissue. [21]

1.4 Bowel Cancer Screening Programmes

The NHS CRC bowel cancer screening programme (BCSP) was rolled out 2006-10 with individuals aged 60-69 receiving a 2 yearly invite. The program has age-expanded in 2011-2 to cover ages 60-75, individuals older than this have the option to opt in. Patients are sent a faecal occult blood test (FOBt) kits and patients with positive (abnormal) tests are invited for a screening colonoscopy.

The evidence for the effectiveness of FOBt screening four large trials which took place in Nottingham (UK), Funen (Denmark), Göteburg (Sweden) and Minnesota (USA).

The Nottingham trial (published in 1996) compared 75 253 participants in the screening cohort (by way of biennial FOBt) compared to 74 998 controls. During median follow-up of 7.8 years (range 4.5-14.5), 360 people died from CRC in the screening group compared with 420 in the control group, a 15% reduction in cumulative CRC mortality in the screening group (odds ratio = 0.85 [95% CI 0.74-0.98], p = 0.026). [22]

The Funen trial (also published in 1996) compared 30 967 participants in the screening cohort (by way of biennial FOBt) compared to 39 966 controls. During follow-up of 10 years, 205 people died from CRC in the screening group compared with 249 in the control group, an 18% reduction in cumulative CRC mortality (odds ratio = 0.82 [95% CI 0.68-0.99], p = 0.03). [23]

The Minnesota trial (published in 1993) randomly assigned 46 551 participants to an annual FOBt screening cohort, a biennial FOBt screening cohort and a control cohort. During follow-up of 13 years, the cumulative CRC mortality rate was 5.88 per 1000 when annually FOBt screened, 8.33 per 1000 when biennially screened and 8.83 per 1000 in the control group. The trial group strongly advocated annual FOBt screening in its study conclusion. [24]

The Göteburg trial (also published in 1993) randomly assigned 68 308 to a screening group or a control group. The screening group were invited to complete FOBt on 3 days and to repeat the test after 16 to 24 months. 21 347 performed the test (prevalence round), 19 991 repeated the test (rescreening round). Investigation of the 942 (4.4%) with positive tests in the prevalence round showed 47 CRCs and 129 subjects with large adenomas (≥ 1.0 cm). In the rescreening round, 5.1% were positive; 34 CRCs and 122 with large adenomas were found. CRCs were also diagnosed in 34 subjects in-between tests or in those who didn't rescreen. 44 CRCs were diagnosed in the control group and were at a more advanced stage than the screening group. This trial illustrated the diagnostic yield of FOBt testing. [25]

A meta-analysis in 2007 published by the Cochrane library analysed the combined results from the 4 randomised controlled trials (detailed above), and the subsequent publications showing the extended follow up data. This neatly showed that participants allocated to FOBt screening had a statistically significant 16% reduction in the relative risk of colorectal cancer mortality (RR 0.84; CI: 0.78-0.90). In the 3 studies that used biennial screening (Funen, Minnesota, Nottingham) there was a 15% relative risk reduction (RR 0.85, CI: 0.78-0.92) in colorectal cancer mortality. [26]

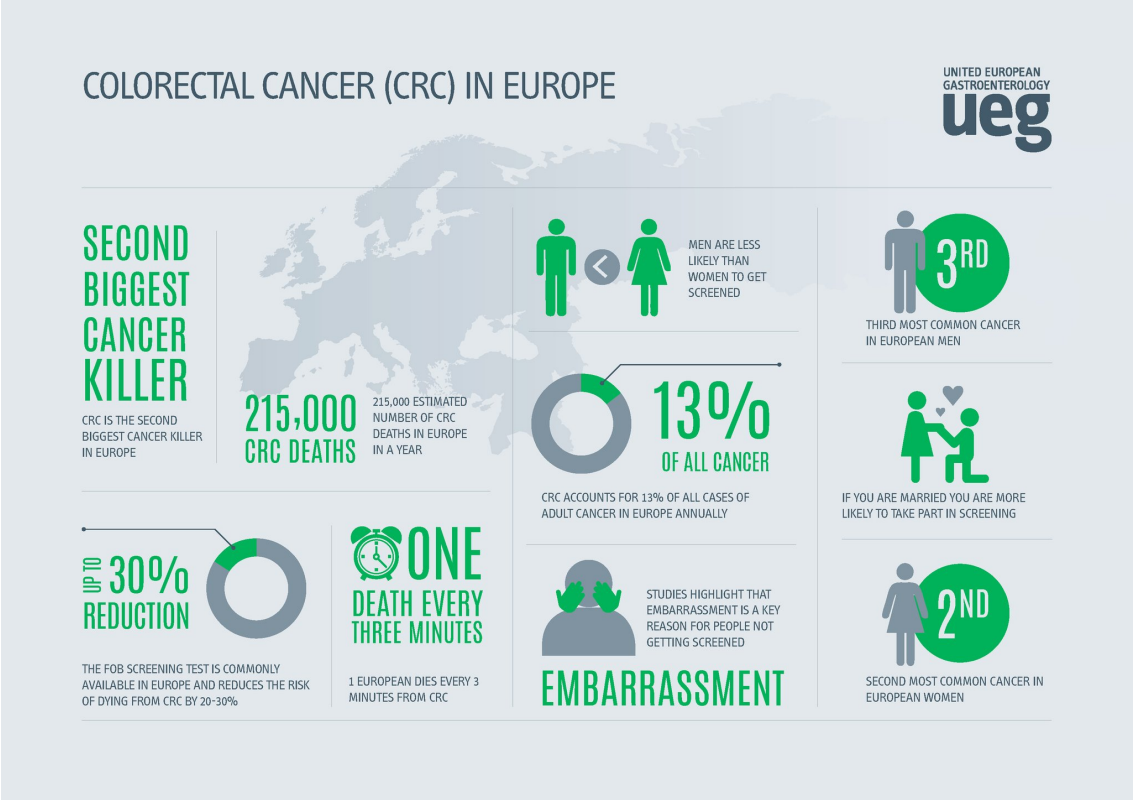


Figure 7 – Infographic of European colorectal cancer facts and statistics

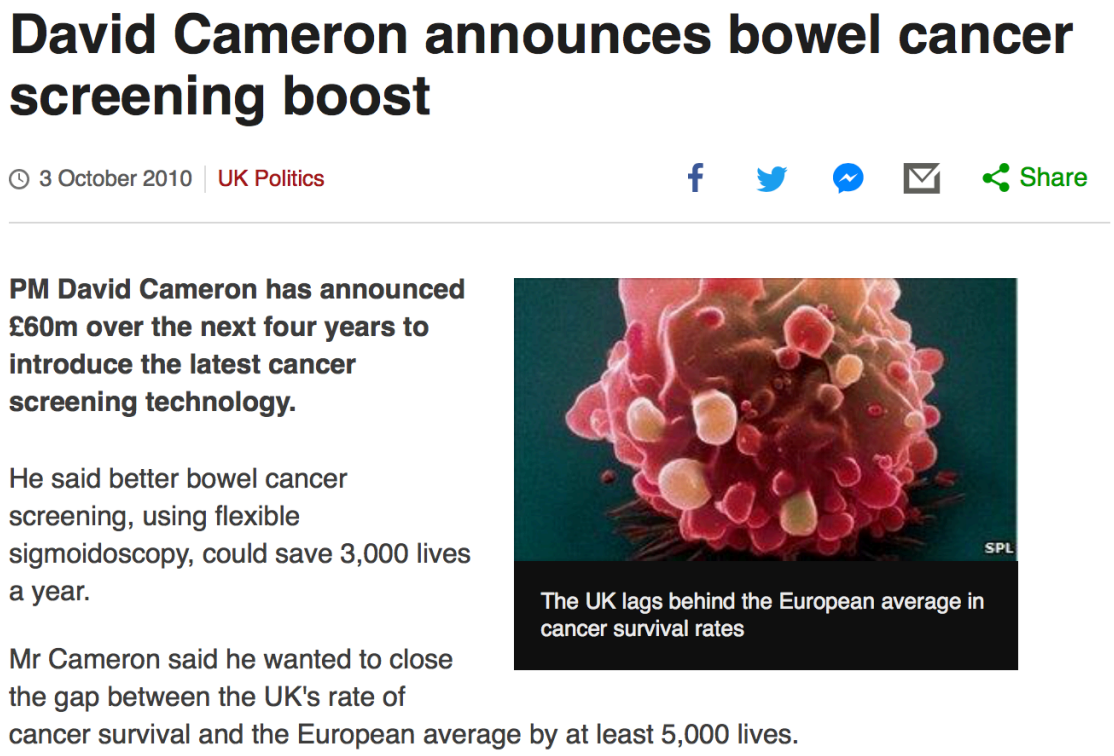
Since commencement of the BCSP, over 18 million FOBt kits have been sent out with approximately 10 million kits returned, of which approximately 2% were positive (FOBt screening positivity). Overall, approximately 56% of patients return kits (FOBt screening uptake). FOBt+ episodes undergoing diagnostic tests, have led to detect CRC in approximately 8%, adenomas in 45%, other pathology in 23% and normal colons in 24%. [27] Higher rates are noted in white Caucasian population (compared to South Asian populations) and in males (compared to females). [28]

In 2013, a parallel programme of one-off flexible sigmoidoscopy screening (known as “bowel scope”) offered to all 55 year olds was introduced after a large randomised controlled trial showed a significant mortality benefit.

A landmark trial (Atkin et al) published in 2010 (Lancet) screened 40 674 out of 57 099 individuals with flexible sigmoidoscopy in an intervention group between 1995 and 1999 (The UK Flexible Sigmoidoscopy Screening Trial). When compared to the control group (112 939), during a median follow up period of 11.2 years, incidence of colorectal cancer in the intervention group was reduced

by 33% (0.67, 0.60-0.76) and mortality by 43% (0.57, 0.45-0.72) when compared to the control group. Incidence of distal colorectal cancer (rectum and sigmoid colon) was reduced by 50% (0.50, 0.42-0.59). The numbers needed to be screened to prevent one colorectal cancer diagnosis or death, by the end of the study period, were 191 (95% CI 145-277) and 489 (343-852), respectively. [29]

On the background of this, the government announced that they would introduce bowel scope screening and following a successful pilot study; this is now being rolled out across the country (see figure 8). [30]



David Cameron announces bowel cancer screening boost

3 October 2010 | UK Politics

Share

PM David Cameron has announced £60m over the next four years to introduce the latest cancer screening technology.

He said better bowel cancer screening, using flexible sigmoidoscopy, could save 3,000 lives a year.

Mr Cameron said he wanted to close the gap between the UK's rate of cancer survival and the European average by at least 5,000 lives.

Figure 8 – BBC news article on funding for Bowel Scope screening

A follow up study looked at the two groups again after a median of 17.1 years' follow-up. Colorectal cancer was diagnosed in 1230 individuals in the intervention group versus 3253 in control group, with 353 individuals in the intervention group versus 996 individuals in the control group died from colorectal cancer. In intention-to-treat analyses, colorectal cancer incidence was reduced by 26% (RR 0.74 [95% CI 0-70-0.80], p=<0.0001) in the

intervention group versus the control group and colorectal cancer mortality was by 30% (0.70 [0.62-0.79] p<0·0001) in the intervention group versus the control group. In per-protocol analyses, adjusted for non-compliance, colorectal cancer incidence and mortality were 35% (RR 0.65 (95% CI 0.59-0.71)) and 41% (0.59 (0.49-0.70)) lower in the screened group. This shows that a single flexible sigmoidoscopy continues to provide substantial protection from colorectal cancer diagnosis and death, with protection lasting at least 17 years. [31]

1.5 Protocols

The bowel scope protocol involves subjects aged 55 being invited by a regional hub (separate from primary and secondary care services) for a screening sigmoidoscopy at a screening centre. Individuals book their procedure and are sent an enema to their home to administer pre-procedure before they attend their appointment. Individuals undergo screening procedure. Those who have a clear sigmoidoscopy are reassured and discharged. If 1-2 small (less than 10mm) tubular adenomas are identified and resected, the patient is reassured & discharged, if either have a villous component the patient is offered a colonoscopy and any further adenomas results in being followed up in endoscopy polyp surveillance programmes as per national polyp follow up guidance. If 3 or more small adenomas or any larger (10mm or more) adenomas are identified at bowel scope, patients are offered a colonoscopy and are followed up as per national polyp surveillance guidelines.

The FOBt testing protocol involves a 6-windowed card sent (in the post) to individuals aged 60, identified and managed by the one of the five regional hubs in England (figure 9).

Subjects are asked to smear faecal samples – two from a bowel motion on 3 consecutive days. After each paired sample a cardboard flap is closed to seal. After 6 samples are obtained, the card is returned to the hub (in the post) for analysis. If 5 or 6 windows are positive for faecal occult blood then the patient is invited to a screening centre for a colonoscopy. If 1-4 windows are positive then repeat test(s) are sent – if windows are positive on repeat card(s) then the

patient is invited to a screening centre for a colonoscopy. If all 6 windows are negative, the individual is informed and will be sent a further kit in 2 years' time (up to and including age 75, individuals older than this who wish to receive a screening kit have to contact the hub to opt in).

The protocol for FOBt testing does require a 3-day commitment to stool sampling with a possibility of a further 3 days sampling for if the first card has 1-4 out of 6 windows positive for faecal occult blood. Dealing with one's stools over 3 days or more and sending cards away may be perceived as embarrassing and cumbersome, this may explain why the uptake of screening is less than 60%. Despite the success of the BCSP, FOBt lacks high sensitivity and accuracy.

Figure 9 – FOBt 3 day test card used for screening

There have been trials comparing FOBt with semi-quantitative faecal immunochemical testing (FIT). Using these kits require only one stool sample and give a value for the amount of human blood within each sample (FOBt kits give a positive or negative result to the presence of any blood – which can be influenced by consumption of meat). A study in Spain of 2288 individuals compared 3 paired sampled FOBt against single sample FIT (taken from the same patient cohort using day 3 sample), patients with positive FOBt (3.5%)

and/or positive FIT results (8.1% using a cut-off on 50 ng/ml) underwent complete colonoscopy (158), as did a cohort of patients with negative FOBt/FIT (244). Sensitivity for FIT for CRC or advanced adenomas (defined as ≥ 10mm in size & 20% villous component or high grade dysplasia) was 61.0% (95% CI 47.8 – 72.9) compared to 23.8% (95% CI 10.8 – 43.5) for FOBt (P = <0.001). Specificity was 95.1% for FIT and 97.7% for FOBt. Positive predictive value (PPV) for CRC or advanced adenomas was 43.4% for FIT and 39% for FOBt. [32]

A Dutch study revealed that FOBt positivity was 2.4% (117 positives) from 4836 individuals (10 301 received FOBt kit, uptake of 46.9%). FIT positivity was 5.5% (339 positives from a different cohort of 6157 – using a cut-off of 100 ng/ml, 10 332 individuals received FIT kit, uptake of 59.6%). PPV for CRC or advanced adenomas was 51.8% for FOBt and 55.3% for FIT. Specificity was 97.8% for FOBt and 99.0% for FIT. [33]

The Spanish study show that FIT is much more sensitive than FOBt. Depending which cut-off level you use for FIT affects the PPV and specificity. The conclusion after looking at both studies is that FIT is going to identify many more individuals with advanced adenomas or CRC, however careful consideration of cut-off is required to ensure the specificity isn't worse than FOBt. A lower specificity results in more individuals requiring a colonoscopy unnecessarily.

On the back of these studies the BCSP is switching from FOBt to FIT. Whilst FIT is expensive, it will probably increase uptake of screening (due to single sample requirement). However, there are concerns that using FIT could overwhelm the capacity of screening centres due to high positivity rates hence the cut off for a positive test will be adjusted to avoid this.

Stool testing kits, sigmoidoscopy and colonoscopy can be embarrassing and inconvenient to patients, contributing to the relatively low uptake of screening of 56%. Also, there are small but serious risks of endoscopy – the worst outcome being perforation if the colon which can be fatal (risk of less than 1 in a 1000 procedures). Endoscopy is also an expensive procedure costing £200-500+ each procedure.

The development of a minimally invasive blood test for detection, diagnosis and monitoring of colorectal adenomas and cancers would be of potentially massive benefit which would be more acceptable to patients and possibly improve screening uptake, it also would be safer. This could be an adjunct to the current screening strategies and further lower cancer related morbidity and mortality.

2. MicroRNAs

2.1. The evidence for microRNA expression in cancer

MicroRNAs (miRs) are evolutionarily conserved, small (18-24 nucleotides), noncoding RNA molecules that regulate gene expression at the level of translation by binding to a target messenger RNA (mRNA). [34]

MicroRNAs repress gene expression by binding to complementary sequences in the 3´ untranslated region (3´ UTR) of mRNAs to target them for degradation and thereby prevent their translation. Considering that hundreds of individual microRNAs have been identified (representing approximately 1% of all predicted genes in most species), that an individual microRNAs can target hundreds or thousands of different mRNAs, and that an individual mRNA can be co-ordinately suppressed by multiple different microRNAs, the microRNA biogenesis pathway therefore has an important role in gene regulatory networks. Over the past decade, it has emerged that more than half of the microRNAs are mapped within cancer-associated genomic regions or fragile sites.

Searching for ‘microRNA” on Pubmed shows greater than 6000 articles/papers in the last 5 years on the role in pathophysiology of many disorders. Framed quite simplistically, microRNA expression profiles reveal a biological signature which may be unique for certain disorders. When narrowing the search for “microRNA” & “cancer”, this shows greater than 1200 abstracts/papers (in the last 5 years) showing an association with several cancers. This suggests that microRNAs have a role in carcinogenesis.

MicroRNAs are formed from larger transcripts that fold to produce hairpin structures and serve as substrates for the Dicer family of RNase III enzymes. They share this process with an experimental system, RNA interference (RNAi), which is used to silence the expression of endogenous genes in eukaryotic cells. The products of Dicer cleavage are short double-stranded RNA molecules, one strand of which is retained in a ribonucleoprotein complex called the RNA-induced silencing complex. The retained RNA acts as a guide to target

this complex to a complementary mRNA sequence which is inactivated either by cleavage or translational interference, depending on the degree of complementarity between the microRNA and its target (figure 10).

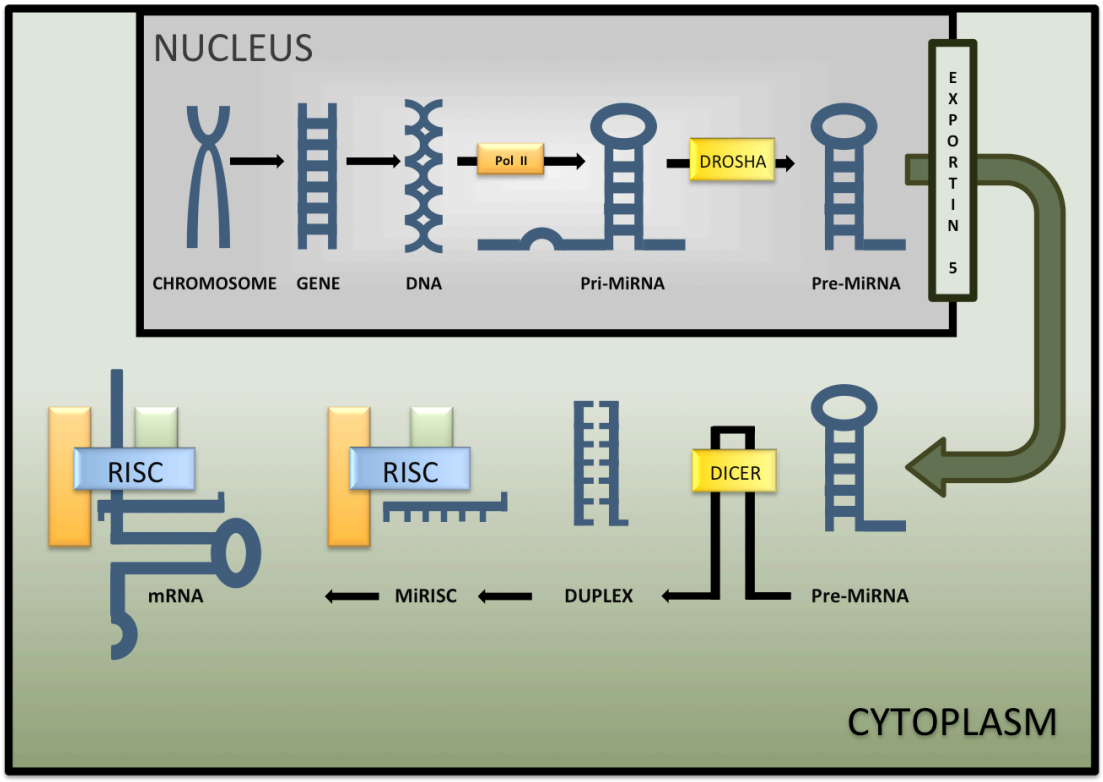


Figure 10 – Simplified summary of microRNA biogenesis

RNA polymerase II action in the nucleus creates a primary microRNA transcript, which undergoes processing by the microprocessor RNaseIII enzyme drosha and its co-factor Pasha. [35] [36] ***A short stem 60-70 nucleotide loop structure known as pre-miR-is formed and leaves the nucleus via the guanosine triphosphate (GTP) dependent transporter exportin 5. Once in the cytoplasm, the Pre-miR-interacts with the RNase enzyme Dicer producing a miR/miR-double stranded duplex.*** [37] [38] ***Further processing results in a mature miR-strand, which is combined with Argonaute proteins to form the RNA-inducing silencing complex (RISC). The other miR-strand is degraded. The RISC complex and its incorporated mature miR-subsequently cleaves, inhibits or degrades target mRNAs thereby negatively regulating gene expression and inhibiting protein translation.*** [39] [40]

There are several cancers linked with expression of microRNAs including lung cancer,[41] ovarian cancer, [42] glioblastoma, [43] renal cancer, [44] bladder cancer, [45] prostate cancer, [46] melanoma [47] [48] and breast cancer. [49]

2.2 Circulating Plasma microRNAs

Mature microRNAs are released into the intercellular and extracellular environment via secreted membrane vesicles known as exosomes. Exosomes transport circulating microRNAs in plasma, [50] allowing them to exist and function in blood, in a stable form protected from endogenous RNase activity. MicroRNAs are very stable resisting degradation from RNase activity, high temperatures and changes in pH. [41 46]

MicroRNAs are present in a range of human tissues and bodily fluids. Validated techniques have been developed to extract, detect and analyse microRNA from blood (plasma), saliva and urine.[45 46 51 52] Advances have led to processes that allows microRNA extraction from plasma stored at room temperatures up to 48 hours. This potentially allows for microRNAs to act as a plasma based test for the detection of disease. MicroRNAs can also be extracted from fresh frozen tissue specimens and from formalin fixed paraffin embedded samples (FFPE). [53 54]

Many established methods exist for the screening and analysis of microRNAs, including microarray analysis, next generation sequencing and polymerase chain reaction (PCR). [55 56] Microarray analysis enables the detection of hundreds of microRNAs from a single sample, albeit without pre-amplification which limit sensitivity. [57] More recently stem-loop quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) is a validated method for quantitative and qualitative analysis allowing for the sensitive of minute amounts of microRNA. [58 59 60 61 62 63]

2.3. MicroRNA expression in colorectal cancer

Many studies have profiled microRNA expression from colorectal cancer tissue and blood, using RT-qPCR, micro-array and mirage technologies. [64 65 66 67 68] There are many papers and review articles on this topic. [69 70 71 72]

Michael et al was the first to establish the role of microRNAs in colorectal cancer. The microRNAs; miR-143 and miR-145 were found to be dysregulated and acting as potential tumour suppressors. [73] MiR-145 may act as a tumour suppressor through the down-regulation of TGFR-II and the insulin receptor substrate 1 (IRS-1). [74] [75] Many further papers have been published on microRNA expression profile in colorectal cancer.

MicroRNAs have been implicated at each stage of the adenoma-carcinoma pathway for CRC. 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. [76] High expression of miR-21 in adenomas and CRC could be a factor in CRC progression. [77] [78]

Ng et al (2009) published in Gut describes a study split into 3 phases. The 1st phase "marker discovery", pre-operative plasma samples, primary tissue biopsies from colorectal cancer lesions and their adjacent non-cancerous colonic tissues from five patients were collected. Plasma from five age-matched healthy subjects was collected as the control. MicroRNA profiles were generated from CRC plasma, normal control plasma, CRC tissue and adjacent normal tissue. By comparing microRNA profiles from CRC plasma versus normal plasma and CRC tissues versus adjacent normal tissues, two differential microRNA expression patterns were established and then compared. Upregulated microRNAs in both plasma and tissues were identified for further analysis in the 2nd phase.

In the 2nd phase "marker selection and validation", plasma samples were collected from 25 patients with CRC before undergoing endoscopy. Plasma from 20 healthy subjects was collected as normal control. MicroRNA markers identified in the 1st phase were verified on these plasma samples from 25 patients with CRC and 20 controls. Plasma from another 10 patients with CRC was collected prior to and 7 days post-surgical resection. Markers proceeding to 3rd phase validation had to be significantly elevated in the 25 patients with CRC and reduced after tumour resection.

In the 3rd phase “large-scale validation”, plasma was collected from an independent group of 90 patients with CRC before endoscopy. Plasma from a set of 50 healthy subjects was collected as the control. MicroRNAs markers identified from the 2nd phase were verified on these independent sets of plasma samples. Plasma from 20 patients with inflammatory bowel disease (IBD) and 20 patients with gastric cancer was also included to examine the specificity of the makers.

Analysis of the results showed significantly elevated levels of miR-92 and miR-17-3p in the plasma of patients with CRC patients when compared to controls (non-diseased, those with IBD & those with gastric cancer).

Plasma miR-92 alone demonstrated as the best marker for CRC prediction and yielded an area under receiver operated characteristic (ROC) curve of 0.885%. With a sensitivity of 89% and specificity of 70% in discriminating CRC from controls. [79]

Mr Imran Aslam, a surgical research fellow at the University of Leicester ran a pilot study for his PhD degree project. This study aimed to identify which circulating plasma miRs could be used for the detection of CRC and to assess the utility of tissue miRs combined with common gene mutations, to predict the development of metastasis in patients with Dukes’ B CRC.

MicroRNA expression profiling was performed from plasma samples (colonoscopy negative controls = 11, adenomas = 9, carcinomas = 12) and formalin-fixed paraffin embedded (FFPE) matched paired cancerous with adjacent normal tissue (n = 20, 5 cases from each group; Dukes’ A, Dukes’ B with metastasis during 5 year follow up, Dukes’ B without metastasis during 5 year follow up, and Dukes’ C). MicroRNAs identified from plasma and tissue expression profiles were validated further on cohorts of plasma (n=190) and FFPE tissues (n=72). Three common gene mutations (KRAS, BRAF and PIK3CA) were analysed in DNA extracted from FFPE cancer tissue. microRNA expression analysis was applied to circulating exosomes to quantify CRC-related exosomal microRNAs.

ROC curve analysis showed miR-135b was associated with an area under the curve (AUC) value of 0.82 (95% CI: 0.71-0.92), with 80% sensitivity and 84% specificity for the detection of adenomas and carcinomas. MiR-135b was also detectable in immunoaffinity-isolated plasma exosomes from patients with CRC. No significant differences were noted for mutation status and the development of metastasis. Expression levels of miR-135b and miR-15b were significantly associated with Dukes' B cancers tissue and the development of metastasis. [80] [81]

Looking for similar studies for patients with colorectal adenomas, one study was published in the Annals of Surgery in 2013, Kanaan et al. In their study, they screened for microRNA markers using an initial cohort of 12 healthy controls, 9 patients with adenomas, and 20 patients with CRC. A panel of the most dysregulated microRNAs was then validated in a blinded cohort of 26 healthy controls, 16 patients with large adenomas, and 45 patients with CRC.

A panel of 8 plasma microRNAs (miR-532-3p, miR-331, miR-195, miR-17, miR-142-3p, miR-15b, miR-532, and miR-652) distinguished polyps from controls with high accuracy; area under curve = 0.868. In addition, a panel of 3 plasma microRNAs (miR-431, miR-15b, and miR-139-3p) distinguished Stage IV CRC from controls with an AUC = 0.896. ROC curves of microRNA panels for all CRC versus controls and polyps versus all CRC showed AUC values of 0.829 and 0.856 respectively. [82]

3. Project aims and study plan

3.1. Project aims

3.1.1 Hypothesis

I hypothesised that blood test (plasma) screening of individuals for candidate microRNAs may be able to pick out those with colorectal adenomas when compared to a control cohort.

To test the hypothesis, I would need to:

1. **Recruit patients to a trial and collect blood samples**
   The aim of our study was to recruit more than two hundred patients – with at least one hundred patients with endoscopy detected polyps (with the remaining patients with non-polyp/CRC acting as controls). This sample size was chosen as it was thought to be the highest number of subjects recruitable in the patient recruitment phase of the study.

2. **Run discovery experiments to identify candidate microRNA targets**
   We would then identify microRNA targets which we would identify using microRNA assay array cards.

3. **Run larger validation experiments to test candidate microRNAs**
   Once we had suitable microRNA targets we would then test for these targets in the plasma from recruited patients and analyse the data to see if there are microRNA targets that could identify patients with adenomas when compared to controls.

4. **To analyse the data to test hypothesis and to look at sub-analysis.**
   We planned to sub-analyse to see if the expression of the microRNA targets could differentiate between different sub-types of polyps.

3.1.2 Project background

Mr Imran Aslam had completed a PhD project looking at microRNA expression in colorectal cancer tissue in affected patients as well as their blood samples. Given my training as a Gastroenterologist and my interest in polyps discovered at endoscopy, it was a natural progression to design a project around endoscopy and polyps – given the pre-malignant nature of these lesions. In Leicester, the University Hospitals Leicester NHS trust has an endoscopy unit at Glenfield Hospital that offers Bowel Cancer Screening Programme (BCSP) colonoscopy procedures.

Ethics permission had been obtained for the use of blood samples obtained from patients undergoing BCSP screening colonoscopy. It was agreed that patients could be recruited from Dr Peter Wurm's (Consultant Gastroenterologist) endoscopy lists at Glenfield Hospital. Dr Wurm had patients undergoing BCSP colonoscopies and patients undergoing polypectomy which had been discovered (but not removed) on a previous diagnostic procedure.

There are usually 4-5 patients on a typical endoscopy list which would facilitate recruitment of a larger number of patients compared to other published studies on microRNA expression in patients with colorectal polyps.

3.2. Study plan

I was undertaking an out of programme endoscopy fellowship training post which gave me two days a week for this project. The project started in April 2012 and all the project work had to be completed by August 2014 (29 months in total).

3.2.1. Phase 1 – Patient recruitment

This involved recruiting patients and processing their blood samples so that plasma could be frozen and stored. We envisaged this phase would take 12-15 months to recruit the required number of patients.

3.2.2. Phase 2 – Laboratory project work

This involved processing the plasma to extract RNA and convert to complimentary DNA (cDNA). Then by pooling cases together, microRNA array cards would be utilised to identify suitable microRNA targets. Once these targets were identified – all the cases would be analysed for these microRNA targets. We envisaged this phase would also take 12-15 months.

3.2.3. Phase 3 – Data analysis

With the large amount of data from the laboratory analysis, the data would be analysed carefully to see if microRNAs can be used as a diagnostic test for patients with colorectal polyps when compared to non-polyp/non-cancer controls. We envisaged this phase would take 3 months.

METHODS

4. Recruitment

4.1. 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), - see appendices.

4.2. Recruitment

I worked closely with Dr Peter Wurm (Consultant Gastroenterologist at University Hospitals Leicester) attending his endoscopy lists at Glenfield Hospital. I occasionally attended endoscopy lists of Dr Richard Robinson, Dr John de Caestecker (Consultant Gastroenterologists) and Mr John Jameson (Consultant Surgeon).

Subjects undergoing BCSP screening colonoscopies and undergoing planned polypectomies at sigmoidoscopy/colonoscopy were recruited to the trial. They signed consent forms pre-colonoscopy and had blood samples taken – this was usually done on insertion of venous cannulas, which are a requirement for patients undergoing colonoscopy to give intravenous sedation, analgesia and anti-spasmodic (as well as having intravenous access in case of complication).

I typically attended 2 lists per week to recruit subjects, recruitment was generally successful with less than a handful of patients refusing to enter the trial. I identified and consented patients, took blood samples and processed the blood so that we could have stored plasma.

4.3 Sample collection & processing

Blood samples were drawn using a S-Monovette Haematology EDTA sample tube (Sarstedt, Germany) and processed within two hours of venepuncture. Typically, 2-4 patients were recruited per endoscopy list.

Blood samples were spun in a balanced manner in a high-speed centrifuge for 10 minutes. (4°C, 750 G). This separated the blood samples into 3 layers. A lower opaque layer of red blood cells, a thin middle layer containing white blood cells (buffy coat) and a top clear layer of plasma (
Figure 11).

The plasma was carefully aspirated off using a Gilson pipette from the two blood tubes and transferred into a single centrifuge tube. This was spun again for a further 10 minutes.

This 2nd centrifuge cycle caused any remaining red and white blood cells to lie at the bottom of the centrifuge tubes. The plasma above was aspirated off and stored into 1ml Eppendorf and then stored at -80 °C in a freezer. Samples could be stored for many months.

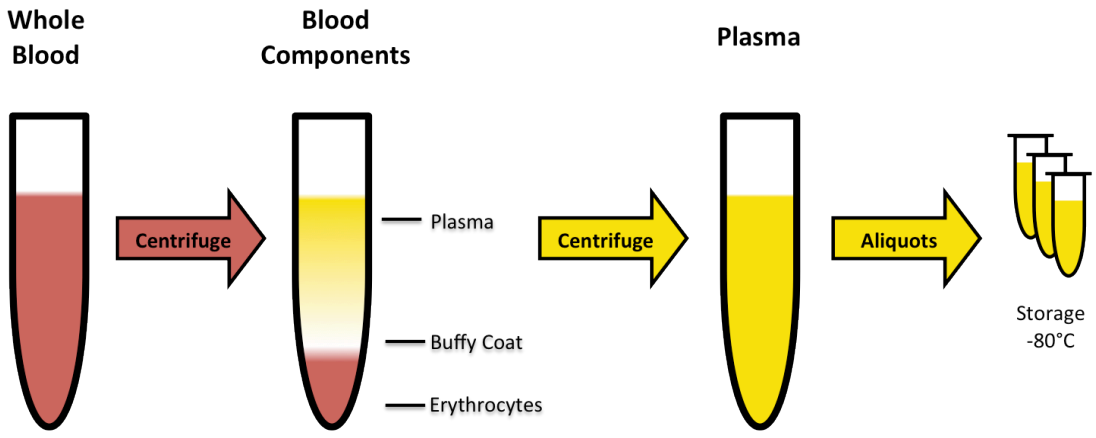


Figure 11 – Processing of Whole Blood Samples

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 Eppendorf tubes at -80°C for later use

Patient details were recorded into a database. Post endoscopy, details of the endoscopy report describing the findings and also the histology report detailing the results of biopsies/polypectomy were entered onto this database – the cut off time for data collection was at the end phase 1 – patient recruitment phase of the study (15 months).

5. Laboratory Methods

5.1. Extraction of MicroRNAs using Qiagen miRNeasy® serum/plasma kit

An Eppendorf containing 1 ml of frozen plasma (stored at -80°C) was thawed on ice. When fully thawed, 200 µL of plasma was aspirated – the remaining plasma is returned to the -80°C freezer. 1 ml of QIAzol® reagent was added to the plasma and vortexed. This was incubated at room temperature for 5 minutes. 200 µL of chloroform was added and mixed well and incubated for 3 minutes. Samples were then centrifuged for 15 minutes at 13000 RPM at 4°C.

After centrifuging, samples separated into three phases. An upper colourless aqueous phase containing RNA, a white interphase and a lower red organic phase.

600 µL of the upper aqueous phase was carefully aspirated off into a sterile bijou container – to which 900 µL of absolute ethanol was added and vortexed.

Half the sample (approximately 700 µL) was then transferred into an RNeasy® mini spin column and centrifuged at 13000 RPM for 30 seconds (room temperature), The follow through was discarded and the remaining half of the sample was processed this way using the same mini spin column.

700 µL of RWT buffer was added followed by 500 µL of RPE buffer and a further 500 µL of RPE buffer. Between each step the mini spin column was centrifuged (13000 RPM for 30 seconds) and the follow through was discarded.

The mini spin column was then transferred into a clean 2 mL collection tube and centrifuged for 3 minutes (13000 RPM). Finally, the mini spin column was placed into a clean Eppendorf and 50 µL of RNase free water was added to the membrane.

The column was incubated for 1 minute and then centrifuged for 13000 RPM for 1 minute. The eluted RNA was then stored at -80°C in a freezer.

5.2 Sample Processes

In order to quantify the microRNAs by microarray or PCR, the total RNA in the samples was processed in a series of reactions (figure 12).

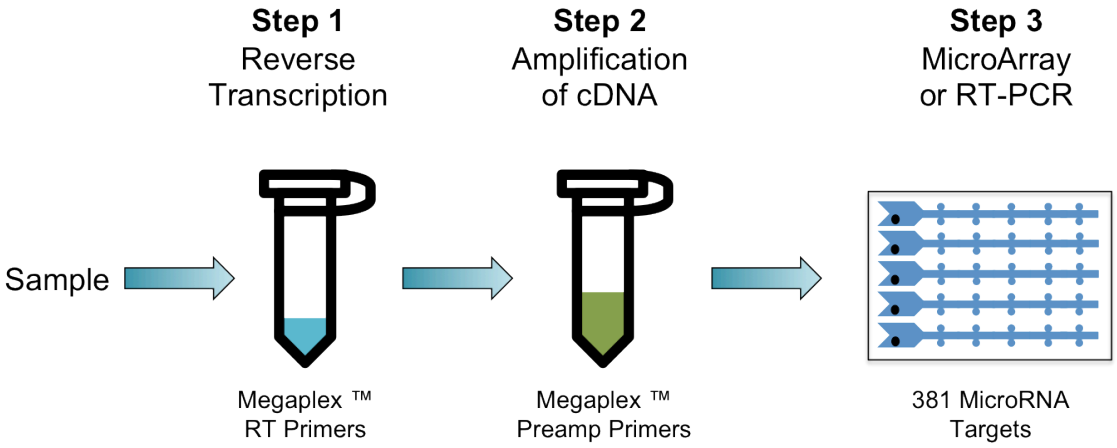


Figure 12 – Workflow for gene expression profiling

Briefly, small RNAs were converted to 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 PCR.

5.3 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 and Human Pool B v3.0 Megaplex™ RT. These sets each 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:

- 0.8 µl 10X RT primers
- 0.2 µl dNTPS
- 0.8 µl 10X RT buffer
- 0.9 µl Magnesium Chloride
- 0.1 µl RNAse inhibitor
- 0.2 µl RNAse free water (1.7 µl in the –RT control reactions)
- 1.5 µl Multiscribe™ reverse transcriptase enzyme

Reactions were prepared in Applied Biosystems micro-AMP® into which, 3 µl of each sample and 4.5 µl of MM was added to make a reaction volume of 7.5 µl.

The sample was incubated upon ice for 5 minutes. The samples were then processed in a Veriti Thermal cycler (Table 3). Final cDNA Samples (7.5 µl total volume) were stored at -20°C.

Table 3 – Thermal cycling profiles for reverse transcriptase

Reverse Transcription Profile		
Stage	**Temperature**	**Time**
Cycle (40 cycles)	16°C	2 minutes
	42°C	1 minute
	50°C	1 second
Hold	85°C	5 minutes
Hold:	4°C	∞

5.4. 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 & B 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:

- 6.25 µl Pre-amplification Master Mix
- 1.25 µl Pre-amp Primers
- 3.75 µl Nuclease free water

Briefly, 1.25 µl of cDNA was added to a micro-AMP® tube containing 11.25 µl of MM, sealed, mixed and incubated on ice for 5 minutes. The samples were processed in a Veriti Thermal cycler (table 4).

Table 4 – Thermal cycling profiles for pre-amplification

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

Once complete, 37.5 µl of 0.1X Tris (hydroxymethyl)-aminomethane (TRIS) / ethylenediaminetetraacetic acid (EDTA) (TE) physiological (PCR-compatible) buffer, pH 8.0 was added to each sample (to bring up the volume to 50 µl, diluted 1:4) and stored at -20°C.

5.5. Selection of pooled groups for PCR testing & microRNA array

The patient database contained demographic, endoscopic and disease data from 215 cases I had recruited and 45 cases previously recruited by Mr Aslam.

To aid discovery of candidate microRNAs, plasma samples would need to be pooled into disease groups and run on microRNA array cards to identify suitable microRNA targets. Based on clinical judgement of colorectal disease, 13 groups were identified with typically 3-7 cases pooled in each group. Care

was taken in choosing the cases with typically the largest adenomas chosen to maximise the yield of microRNA detection.

5.6. Real time quantitative 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 and is recorded as an amplification plot. During formation of the PCR products (amplicons) there is cleavage of xx, resulting in 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. The point at which initial fluorescence can be detected (background activity) is termed the baseline. 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. It is important to set the threshold above the baseline and within the exponential phase of the amplification curve. In these experiments the threshold was always set at 0.05.

5.7. Reverse transcriptase qPCR set up

Before proceeding to array analysis, the cDNA samples were checked by quantitative PCR to ensure the that the RT and pre-amplification reactions had worked. Cases were chosen based on the disease groups and also from across the RT & pre-amplification runs to ensure that there had been no major errors in the previous laboratory stages of sample processing. Also, we wanted to test the cases from the disease groups to ensure they were worthy of being pooled.

Taqman® microRNA targets were chosen based on previous experience of well-expressed microRNAs in normal and diseased cases. Namely miRs 135b, 223, 184, 19, 484 and U6 (pool A) and miRs 30d and U6 (pool B). QPCR was performed on MicroAmp ® optical 96 well plates (Applied Biosystems, Foster

City, CA) using 3 µl of dilute cDNA (1:20 dilution), 0.5 µl of miR-probe assay and 6.5 µl 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:

- Hold: 95°C (10 minutes)
- 45 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 5).

Table 5 – 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 Duplicate	PCR reaction	Each sample was in duplicate 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 normalised 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 have 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		Each PCR plate contained a variety of case types (polyp & non-polyp). Analysing all the samples from the same group on the same plate could lead to bias.

5.8. 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 duplicate 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.

5.9. Taqman® microRNA array cards

Following total RNA extraction and processing, pooled samples were chosen from the 13 disease groups.

A PCR mastermix mix was prepared in a 1.5ml Eppendorf using 450 µl of TaqMan® Universal Master Mix, No AmpErase® UNG, (2✕), 441 µl of nuclease free water and 9ul of pooled pre-amplified cDNA product (end dilution 1:400).

Plasma samples were investigated using pool A and pool B cards (in total 26 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-miR-159a) was also included 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 6).

Table 6 – Thermal cycling profiles for Taqman low density microarray cards

Array cards Profile		
Stage	**Temperature**	**Time**
Hold	50°C	2 minutes
Hold	94°C	10 minutes
Cycle x40	97°C	30 seconds
	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, were analysed and target microRNAs were selected.

A considerable effort to test 225 patient samples for 14 microRNA targets took place over many weeks. This required five 96 well plates of PCR for each microRNA target, each case being run in duplicate.

6. Statistics

6.1 Pooled discovery

6.1.1. Selection disease groups

Before embarking on microarray assay card analysis, significant time and effort was spent in pooling cases together appropriately taking into account clinical relevance and practicalities. The database was reviewed carefully to ensure that the potential cases for pooled testing were appropriate and did not have too many confounding variables, this involved reviewing additional information held about the patient such as previous cancer or significant co-morbidity.

Given the polyp cohort, we decided to have 13 pooled groups with between 3 and 7 cases (a total of 61 cases), this was thought to be the best balance between identifying target microRNAs and limiting confounding factors. The 13 groups were made up of 9 disease groups and 4 control groups (table 13). As each group would be tested for pool A and pool B microRNAs, 26 microRNA microarray assay cards would be tested.

Table 7 – Pooled disease groups

Disease groups	Number of pooled cases
Large (≥10 mm) left colon sessile adenomas	4
Large left colon pedunculated tubulo-villous adenomas	4
Large right colon sessile adenomas	5
Multiple small (<10 mm) adenomas, both sides of colon	4
Malignant polyps	3
Sessile serrated adenomas	3
Villous adenomas	5
High grade adenomas	5
Colorectal cancer	6
BCSP patients, controls with diverticulosis	4
BCSP patients, controls with haemorrhoids	5
BCSP, controls with normal colonoscopy, female	7
BCSP, controls with normal colonoscopy, male	6

6.1.2. Selection of candidate target MicroRNAs

Significant time and effort was required to prepare, run and process 26 microRNA assay cards. The CT values for the candidate microRNAs were collated in a database for analysis.

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

The low-stringency criteria were used:

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

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

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

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

After careful consideration, 10 microRNA targets were chosen. MiRs 98, 19a, 146b, 186, 331-5p, 452 and 625 from pool A, miRs 222#, 664 and 1247 from pool B (the table showing the microRNA targets is in appendix 3).

6.1.3. Selection of normalising microRNAs

Normalisation microRNA were selected based on the experience of our research group in looking at microRNA expression in colorectal cancer. In previous studies, array data were analysed using two freely-available programs, the geNorm (version 3.5) [83] and NormFinder, [84] in order to obtain stability

comparisons of candidate reference genes. Both validation programs ranked all tested microRNAs according to their expression stability across samples. Best combination of two endogenous genes was also calculated. Based on the expression stability demonstrated by this analysis, miR-484 and miR-U6 were used as normalisation microRNAs for pool A targets and miR-U6 for pool B targets.

6.2. MicroRNA targets

After running all the cases on real time qPCR, against all 14 microRNA targets, 210 cases provided viable data providing 2940 CT values. Each of the target pool A microRNA (miRs 98, 19a, 146b, 186, 331-5p, 452 and 625) were looked at individually and also as a delta value against microRNAs 484 and pool A U6 (normalised values). The target pool B microRNAs (miRs 222#, 664 and 1247) were also looked at individually and as a normalised delta against pool B microRNA U6.

In addition, miR-191 was also tested as a target based on the positive results for colorectal cancer in previous studies conducted by our research group.

6.2.1. Mean across plates

With each microRNA target being tested, it involved raw CT values from 5 platers. This led to concerns over variability across the plates for each target microRNA. To combat this, the CT values for cases (run in duplicate) were averaged and the mean CT values for all the cases on each plate were calculated and all the CT values were adjusted across five plates to ensure that any variability between real time qPCR reactions / master-mixes were mitigated for. After the qPCR for all the cases and target miRs had been done. The data was collated in an excel spreadsheet database.

6.2.2. Categorising disease groups

As discussed in the introduction there are many ways to describe the features of a vast array of polyps. The dataset included size of polyp(s), location in

colon, morphology (Paris classification) and the histology including the presence of villous component (tubular adenoma, tubulovillous adenoma or villous adenoma) and grade of dysplasia (low grade or high grade). Villous component and high grade dysplasia are markers of high CRC risk.

Another way to classify adenomas is by using the adenoma follow up algorithm (see figure 4). This classifies adenomas as high, intermediate and low risk based on size (small <10 mm, large ≥10 mm) and number of adenomas.

With the control groups, the majority of recruited patients were undergoing BCSP endoscopy, hence were FOBt positive. Potential causes of FOBt positivity were noted, mainly haemorrhoids and diverticular disease. This was all recorded in the dataset.

Taking all the relevant factors and differing ways to classify adenomas in combination with the number of cases available for analysis, we used 16 disease groups as listed in Table 8.

Table 8 – Classification of disease and control groups

All polyps	Controls
Adenomas (excl. sessile serrated adenomas)	Controls with haemorrhoids *
Left colon adenomas	Controls with diverticular disease *
Left colon large (≥10 mm) pedunculated adenomas	**Not used in T-testing*
Left colon large sessile adenomas	‡*Not used in T-testing – male subjects*
Left colon small (<10 mm) adenomas	§*Not used in T-testing – female subjects*
Multiple adenomas on both sides of colon	
Multiple large adenomas both sides of colon §	
Multiple small adenomas both sides of colon §	
Right colon adenomas	
Right colon large adenomas §	
Right colon small adenomas	
Sessile serrated adenomas ‡ §	
Colorectal cancer §	
All cancer (CRC & malignant polyps) §	
Disease (all polyps excl. hyperplastic, & CRC)	

6.2.3. T-testing

T-tests were calculated comparing the 16 disease groups (listed on the left side of Table 8) in comparison to controls as a combined group. This was done using Microsoft Excel T-testing function (two-tailed, type 3). Testing the ten target miRs using raw CT values, testing miR-191 and the miRs used for normalising (miR-484, pool A miR-U6, pool B miR-U6), and testing normalised miRs meant in total, 608 T-tests were initially performed.

6.2.4. T-testing by gender

When reviewing the T-test results, we wondered if sub-analysing by gender would show more significant results. Using groups only with ≥ 5 cases (see table 8), 15 disease groups in male subjects and 10 disease groups in female subjects were compared to controls. This involved 950 T-test calculations.

6.2.5. Normal distribution Anderson-Darling testing

Anderson-Darling (AD) test is used to prove normal distribution of a set of values. Whilst there isn't a test that definitively proves normality, a positive AD test (result < 0.05) proves that the values are *not* normally distributed. Hence an AD test result > 0.05 would suggest the set of values are normally distributed, the confidence that the values are normally distributed increases the higher the AD test result (up to a maximum result of 1). This calculation was performed on the larger disease groups (all polyps, adenomas & controls) using a freely available online excel based Anderson-Darling testing calculator.

6.3. Regression modelling approach.

Regression modelling was done with the assistance of Dr J H Pringle. We used; backward conditional logistical regression using IBM SPSS software (version 18.0.2). Data was transferred from the excel database to a SPSS database containing information on age, gender, polyp sub-types, (non-adenoma / non-CRC) control cases and other endoscopic diagnosis such as haemorrhoids or diverticulosis. The raw and normalised CT values for the disease and control groups were added to the SPSS database also.

Some initial modelling using smaller disease groups (left sided adenomas, right sided adenomas, multiple pan-colonic adenomas) revealed that these groups did not have enough cases for modelling. Hence modelling was concentrated on using larger disease groups with more data (adenomas, all polyps), this produced two viable models of panels of microRNAs to distinguish subjects with polyps and subjects with adenomas from controls. The addition of the data of other endoscopic diagnosis when added into the dataset provided two further models of a panel of microRNAs. ROC curves were generated from these models with SPSS software functionality.

RESULTS

7. Patient database

7.1. Recruits breakdown

Recruitment took place over a 15-month period (April 2012 till July 2013) with 215 patients recruited, 185 of which were BCSP subjects. Of the 215 recruits, 130 were male (60.47%) and 85 were female (39.53%). 101 had classic adenomas (46.98%), 14 had CRC (6.51%), 16 had non-classical adenoma polyps (including hyperplastic and sessile serrated lesions), 84 were classed as controls (39.07%) with normal colonoscopy or with non-cancer non-polyp pathology (see table 9). In addition, plasma samples from 44 patients recruited by Mr Imran Aslam were also available to add to the database.

Table 9 – Recruitment breakdown

Recruits	Mean age	Male	Adenoma	CRC
215	66.25	130	101	14
		60.47%	46.98%	6.51%
	Median	Female	Other	Control
	66.41	85	16	84
		39.53%	7.44%	39.07%

BCSP	Mean age	Male	Adenoma	CRC
185	65.85	114	80	12
86.05%		61.62%	43.24%	6.49%
	Median	Female	Other	Control
	65.23	71	13	80
		38.38%	7.03%	43.24%

Non-BCSP	Mean age	Male	Adenoma	CRC
30	68.69	16	21	2
13.95%		53.33%	70.00%	6.67%
	Median	Female	Other	Control
	69.38	14	3	4
		46.67%	10.00%	13.33%

A majority of 185 out of the 215 patients recruited were from the bowel cancer screening programme (86.0% of total recruited), of which 114 were male (61.6%) and 71 were female (38.4%) – see table 10.

Gender	Numbers	Adenomas	CRC	Other polyp	Control
Male	114 (61.6%)	57 (50.0%)	10 (8.8%)	8 (7.0%)	39 (34.2%)
Female	71 (38.4%)	23 (32.4%)	2 (2.8%)	5 (7.0%)	41 (57.8%)
Total	**185**	**80 (43.2%)**	**12 (6.5%)**	**13 (7.0%)**	**80 (43.2%)**

Table 10 – BCSP recruits

A further 30 patients (14.0% of total recruited) were from the symptomatic service, mainly made up of patients with known polyps undergoing polypectomy – see table 11.

Gender	Numbers	Adenomas	CRC	Other polyp	Control
Male	16 (53.3%)	11 (68.7%)	1 (6.3%)	2 (12.5%)	2 (12.5%)
Female	14 (46.7%)	10 (71.4%)	1 (7.1%)	1 (7.1%)	2 (14.3%)
Total	**30**	**21 (70.0%)**	**2 (6.7%)**	**3 (10.0%)**	**4 (13.3%)**

Table 11 – Symptomatic patients recruited

7.2. Further cases available

Mr Imran Aslam during his PhD project has recruited cases with polyps and cancers. After analysing his database, 44 patients had plasma samples available which would be suitable to be considered for my study, all patients had been recruited from the BCSP – see table 12.

Gender	Numbers	Adenomas	CRC	Other polyp	Control
Male	34 (77.3%)	15 (44.1%)	2 (5.9%)	3 (8.8%)	14 (41.2%)
Female	10 (22.7%)	4 (40.0%)	1 (10.0%)	0 (0.0%)	5 (50%)
Total	**44**	**19 (43.2%)**	**3 (6.8%)**	**3 (6.8%)**	**19 (43.2%)**

Table 12 – Patients recruited by Mr Aslam

7.3. Total cases available

In total, we had 259 cases available with stored plasma which underwent RNA extraction from plasma and converted to cDNA. However, to enable microRNA target analysis across 5 plates, only 225 cases were used made up of 215 cases I recruited and 10 selected cases from Mr Imran Aslam's study cohort. After analysing 225 cases, data from 15 cases were discarded due to problems with the samples and/or incomplete data sets to allow analysis. The final breakdown of cases is shown in table 13.

Gender	Numbers	Adenomas	CRC	Other polyp	Control
Male	128 (61.0%)	66 (51.6%)	11 (8.6%)	11 (8.6%)	40 (31.2%)
Female	82 (39.0%)	33 (40.2%)	1 (1.2%)	7 (8.6%)	41 (50.0%)
Total	**210**	**99 (47.1%)**	**12 (5.7%)**	**18 (8.6%)**	**81 (38.6%)**

Table 13 – Final breakdown on recruits used in the study

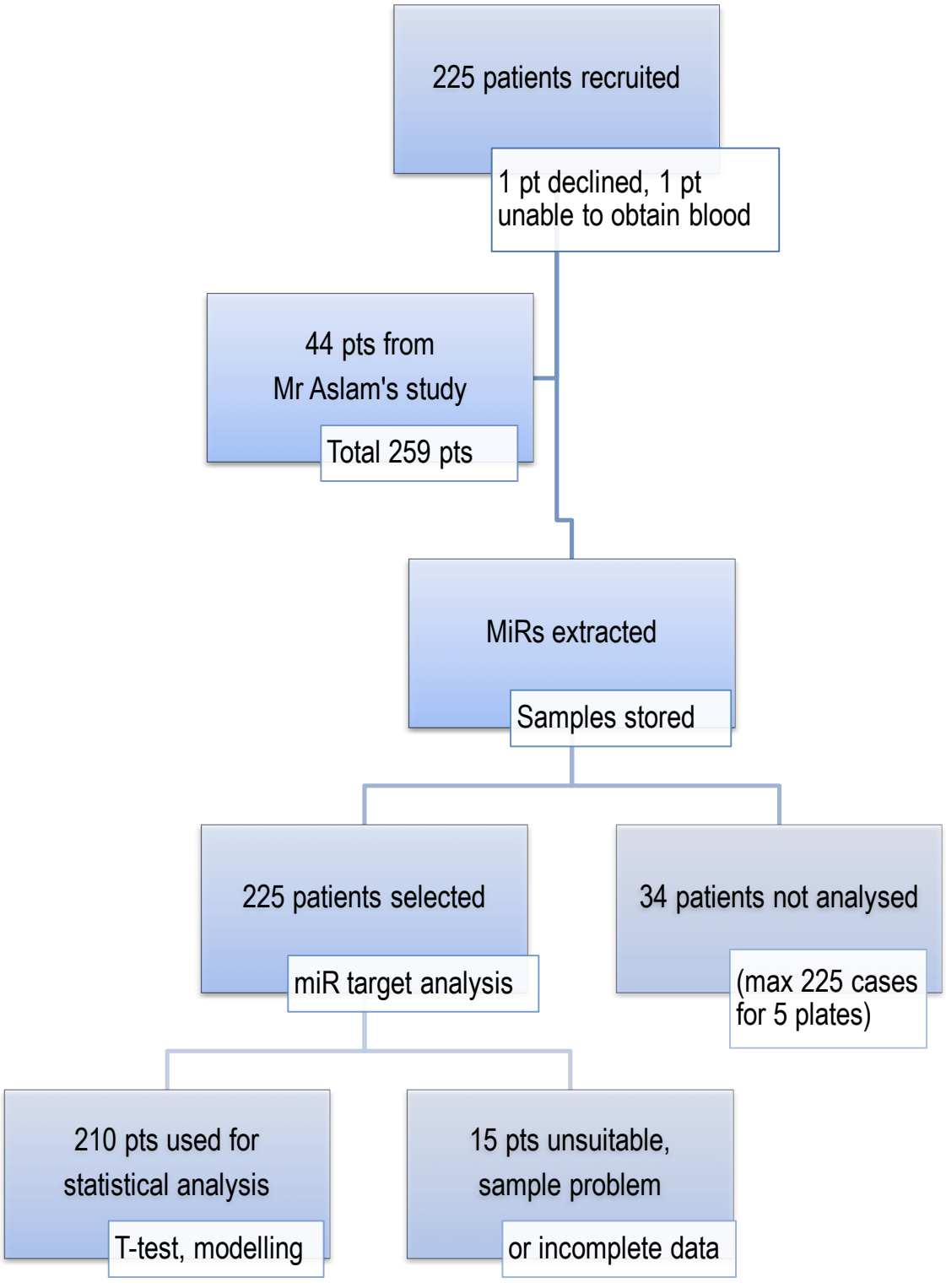
7.4. REMARK guidelines

The REporting recommendations for tumour MARKer prognostic studies (REMARK Guidelines) are detailed in appendix 4. This study is fully adherent to these guidelines.

7.5. Patient flow through the study

The patient selection and flow through the study has been detailed above within the results section. Figure 13 is a visual flow chart to represent patient flow.

Figure 13 – Patient flow through the study



8. Pool A MicroRNA T-test results

8.1. T-testing – disease groups versus control groups.

The analysis of the data initially centred around T-testing by comparing the CT and delta CT values for disease groups versus controls. The groups were selected on the basis of being clinically relevant to endoscopy findings. The control group is for any patient who had a non-polyp and non-cancer diagnosis or a normal colonoscopy (table 14). It is established that colorectal adenomas and cancer is more prevalent in men than women. Hence the data was also sub-analysed by gender (excluding all groups with less than 5 cases – too small to give meaningful results).

Table 14 – Disease groups

Disease group	*n*, male	*n*, female	*n*, total
All polyps	77	40	117
Adenomas (excluding sessile serrated adenomas)	66	33	99
Left colon adenomas	34	18	52
Left colon large (≥10 mm) pedunculated adenomas	17	7	24
Left colon large sessile adenomas	9	5	14
Left colon small (<10 mm) adenomas	8	6	14
Multiple adenomas on both sides of the colon	18	7	25
Multiple large adenomas on both sides of the colon	10	4*	14
Multiple small adenomas on both sides of the colon	8	3*	11
Right colon adenomas	14	8	22
Right colon large adenomas	5	2*	7
Right colon small adenomas	9	6	15
Sessile serrated adenomas	3*	2*	5
Colorectal cancer	11	1*	12
All cancer (colorectal cancer and malignant polyps)	12	4*	16
Significant disease (all polyps excl. hyperplastic, & colorectal cancer)	81	39	120

*Not used for T-testing – groups too small

8.2. Overall T-test results

When calculating T-test results and collating them in a table, highlighting results with a value <0.05 reveals a table that acts as a *heat map* for disease groups and when sub-analysed by gender (table 15). Looking at this busy table overall (each column represents a target microRNA), it is striking at how the larger disease groups (polyps, adenomas and all disease) are significant for many target microRNAs. The significance of the T-tests is strengthened by sub-analysing by gender for male subjects, but not for female subjects.

KEY: MALE FEMALE P=<0.05	miR-98	miR-19a	miR-146b	miR-186	miR-331-5p	miR-452	miR-625	miR-222#	miR-664	miR-1247	miR-191
Polyp versus control	0.0224	0.0233	0.0030	0.0004	0.0630	0.1008	0.0010	0.7257	0.8382	0.3848	0.8367
Adenoma versus control	0.0266	0.0339	0.0045	0.0008	0.1222	0.2029	0.0049	0.4548	0.9466	0.3734	0.5809
Left sided adenomas versus control	0.1989	0.2761	0.1083	0.0381	0.3074	0.5219	0.1435	0.3815	0.5105	0.8667	0.1951
Left pedunculated >10mm vs control	0.2147	0.4293	0.1208	0.1051	0.3062	0.6150	0.1468	0.4598	0.0634	0.3712	0.6627
Left sessile >10mm vs control	0.3926	0.3362	0.2211	0.2572	0.2850	0.7891	0.4067	0.5464	0.3891	0.2163	0.1045
Left sub 10mm vs control	0.5860	0.6205	0.7010	0.2477	0.9330	0.6095	0.5488	0.0496	0.9884	0.7016	0.1301
Multiple adenomas vs control	0.0616	0.0590	0.0194	0.0226	0.7211	0.2517	0.0130	0.5931	0.7836	0.1369	0.1143
Multiple adenomas >10mm vs control	0.7115	0.6597	0.3943	0.3540	0.5726	0.8176	0.1616	0.8445	0.4705	0.1491	0.1216
Mult adenomas sub 10mm vs control	0.0029	0.0150	0.0023	0.0047	0.0774	0.0251	0.0205	0.2724	0.8015	0.6195	0.4986
Right sided adenomas vs control	0.0310	0.0716	0.0076	0.0041	0.0142	0.2577	0.0049	0.9967	0.2753	0.8245	0.0173
Right >10mm vs control	0.0704	0.2275	0.0568	0.0140	0.0692	0.2271	0.0117	0.3178	0.3292	0.0389	0.6798
Right sub 10mm vs control	0.1512	0.1811	0.0502	0.0684	0.0722	0.7397	0.0712	0.4594	0.4977	0.3800	0.0046
Sess serrated adenoma versus control	0.1860	0.4784	0.4101	0.2330	0.0625	0.5432	0.1835	0.3429	0.2437	0.6279	0.7545
Colorectal cancer versus control	0.3022	0.1659	0.1936	0.5714	0.2257	0.6901	0.1494	0.7014	0.8197	0.8853	0.0075
All cancer versus control	0.4336	0.4342	0.3011	0.4450	0.2793	0.6371	0.0649	0.4258	0.9198	0.6894	0.1607
Significant disease versus control	0.0233	0.0324	0.0050	0.0008	0.0612	0.1893	0.0018	0.7975	0.8749	0.3446	0.4432
Polyp versus control	0.0008	0.0009	0.0002	0.0005	0.0194	0.1050	0.0060	0.2317	0.6262	0.3641	0.2205
Adenoma versus control	0.0005	0.0010	0.0002	0.0002	0.0193	0.1192	0.0083	0.1877	0.7679	0.2355	0.3198
Left sided adenomas versus control	0.0191	0.0544	0.0242	0.0150	0.0664	0.4093	0.1239	0.1354	0.3229	0.6296	0.0163
Left pedunculated >10mm vs control	0.1309	0.3690	0.1347	0.0487	0.1251	0.7762	0.1213	0.2761	0.0571	0.6453	0.3242
Left sessile >10mm vs control	0.0370	0.0886	0.0443	0.1748	0.1470	0.4799	0.5144	0.9748	0.7104	0.5011	0.0155
Left sub 10mm vs control	0.2834	0.2328	0.3922	0.2684	0.6052	0.5044	0.4587	0.0021	0.9382	0.0769	0.1680
Multiple adenomas vs control	0.0015	0.0005	0.0007	0.0078	0.2972	0.0688	0.0111	0.5249	0.8248	0.1662	0.8556
Multiple adenomas >10mm vs control	0.1717	0.0748	0.1109	0.1979	0.8343	0.6613	0.1987	0.7126	0.5236	0.2595	0.9003
Mult adenomas sub 10mm vs control	0.0000	0.0003	0.0000	0.0033	0.0883	0.0160	0.0041	0.5428	0.8129	0.3736	0.6222
Right sided adenomas vs control	0.0520	0.0746	0.0146	0.0053	0.0032	0.4608	0.0369	0.5778	0.3522	0.6277	0.1284
Right >10mm vs control	0.1616	0.5697	0.1646	0.0769	0.0971	0.6015	0.0680	0.6169	0.1816	0.0875	0.9868
Right sub 10mm vs control	0.1768	0.0898	0.0540	0.0383	0.0099	0.6235	0.1714	0.3446	0.8347	0.6107	0.0273
All cancer versus control	0.3662	0.1289	0.0896	0.3601	0.2876	0.7280	0.1546	0.5814	0.9292	0.4417	0.1983
Colorectal cancer versus control	0.1496	0.0371	0.0589	0.3656	0.0464	0.4611	0.1526	0.9391	0.8280	0.7504	0.0836
Significant disease versus control	0.0008	0.0011	0.0003	0.0006	0.0151	0.1566	0.0065	0.3397	0.8646	0.2193	0.5269
Polyp versus control	0.7680	0.8250	0.3963	0.1178	0.5187	0.3199	0.0464	0.3966	0.3697	0.5538	0.0779
Adenoma versus control	0.9655	0.9709	0.5739	0.3528	0.9737	0.6588	0.1880	0.7221	0.8070	0.8536	0.0385
Left sided adenomas versus control	0.8727	0.9384	0.6931	0.6906	0.7319	0.7869	0.6115	0.6470	0.8707	0.9646	0.2922
Left pedunculated >10mm vs control	0.3405	0.3569	0.2218	0.9185	0.9096	0.4825	0.6535	0.7795	0.6074	0.5736	0.1301
Left sessile >10mm vs control	0.2137	0.5208	0.4104	0.8652	0.9880	0.7082	0.6067	0.5758	0.4615	0.1984	0.4168
Left sub 10mm vs control	0.5768	0.5682	0.8455	0.6751	0.4676	0.9929	0.9967	0.8850	0.9259	0.4429	0.4676
Multiple adenomas vs control	0.2245	0.3863	0.5868	0.7058	0.6585	0.7579	0.4688	0.7393	0.8077	0.3902	0.1065
Right sided adenomas vs control	0.1055	0.3835	0.1515	0.2525	0.3612	0.3171	0.0546	0.7282	0.6239	0.8316	0.1722
Right sub 10mm vs control	0.3742	0.9319	0.4990	0.7990	0.8044	0.9857	0.2479	0.8858	0.4672	0.5039	0.1408
Significant disease versus control	0.7601	0.9300	0.4649	0.2223	0.5719	0.4711	0.0808	0.5085	0.6394	0.7865	0.0772

Table 15 – Heat map of microRNA T-test results

This is a snapshot, we looked at each target microRNA in detail and also checked if the disease group and control cohorts' CT values are likely to be normally distributed using the Anderson-Darling normality test calculator.

Table 16 is a summary table which shows the strongest performing target microRNAs. In addition to listing p-values for the t-test comparing all adenomas to controls, it lists the mean CT value for the target microRNA. The delta of the mean CT values of the adenoma cases and the mean CT values for all cases is also listed, as is the delta with mean CT values for control cases and the mean CT values for all cases. The difference between these two delta values is listed and from this fold change values were calculated & recorded.

	miR 98	miR 19a	miR 146b	miR 186	miR 625
Mean CT	22.7458	19.3393	19.5930	17.9700	27.5378
Δ Mean for adenomas	-0.1825	-0.1666	-0.2585	-0.3581	-0.3246
Δ Mean for controls	0.2912	0.3047	0.4059	0.5158	0.7260
Δ-Δ	-0.4736	-0.4713	-0.6643	-0.8739	-1.0505
Fold change	1.3886	1.3863	1.5848	1.8326	2.0713
T-test p value	**0.0266**	**0.0233**	**0.0045**	**0.0008**	**0.0049**
Mean Δ for male adenomas	-0.2623	-0.2493	-0.3400	-0.5269	-0.3719
Mean Δ for male controls	0.6090	0.5952	0.6836	0.6384	0.8319
Δ-Δ	-0.8712	-0.8445	-1.0236	-1.1653	-1.2038
Fold change	1.8292	1.7957	2.0330	2.2427	2.3034
T-test p value	**0.0005**	**0.0010**	**0.0002**	**0.0002**	**0.0083**

Table 16 – Summary table of strongest performing target microRNAs

8.3. MicroRNA 98 T-test results

MicroRNA 98 shows great promise. Target T-testing has shown that the different expression levels in plasma of miR-98 significantly picks out subjects with polyps, adenomas (multiple and right colonic) and all disease (polyps and cancer) when compared to controls.

Sub analysis shows that miR-98 picks out subjects with adenomas and all disease with a much higher statistical significance in men predominantly. Apart from picking out female subjects with large right colon adenomas, most T-test values are non-statistically significant in woman.

Table shows the T-test values for all polyps, and adenoma disease groups when compared to controls, when looking at male subjects the T-test values are much more significant (table 17).

Disease group	MiR-98	*n*
All polyps	0.0224	117
Adenomas (excl. sessile serrated adenomas)	0.0266	99
All polyps – male patients	0.0008	77
Adenomas (excl. sessile serrated adenomas) - male patients	0.0005	66

Table 17 – T-test results for miR-98

Table 18 shows the Anderson-Darling (AD) normality test values for all polyps and adenoma disease groups, given the AD test values we have assumed the CT values for the disease groups are normally distributed and T-testing is valid.

Disease group	MiR-98	*n*
All polyps	0.2199	117
Adenomas	0.6736	99
Control	0.4600	81
All polyps- male patients	0.2736	77
Adenomas- male patients	0.4015	66
Control- male patients	0.3169	40

Table 18 – Normality testing for miR-98 disease groups

Figure 14 shows the box and whiskers plots for the CT values when comparing subjects with adenomas and polyps when compared with controls. The significance of the results is obviously higher when analysing male subjects.

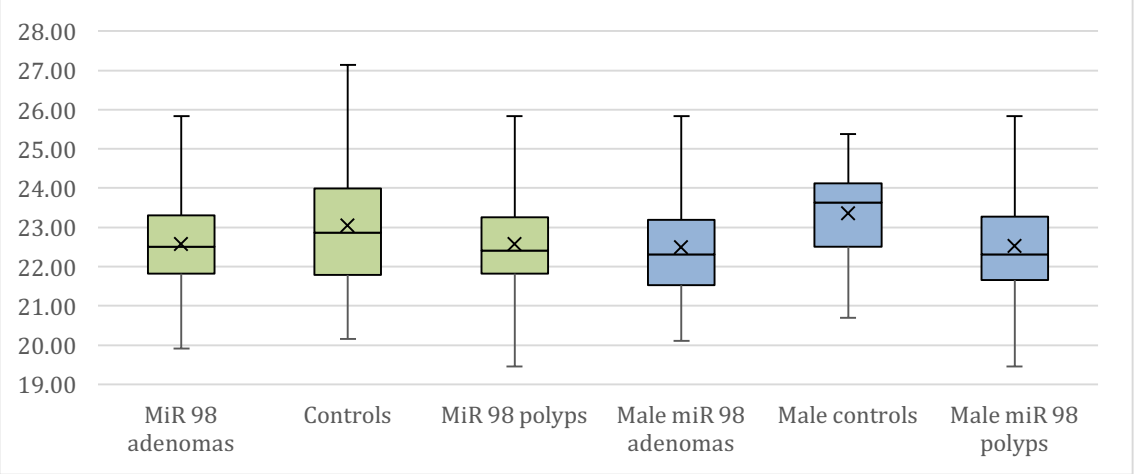


Figure 14 – Box and whiskers plots for miR-98

8.4. MicroRNA 19a T-test results

MicroRNA 19a also shows great promise. Target T-testing has shown that the different expression levels in plasma of miR-19a significantly picks out subjects with polyps, adenomas (including multiple) and all disease (polyps and cancer) when compared to controls.

Sub analysis shows that miR-19a picks out subjects with adenomas and all disease with a much higher statistical significance in men predominantly. T-test values are non-statistically significant in woman.

Table 19 shows the T-test values for all polyps, and adenoma disease groups when compared to controls, when looking at male subjects the T-test values are much more significant.

Disease group	MiR-19a	*n*
All polyps	**0.0233**	117
Adenomas (excl. sessile serrated adenomas)	**0.0339**	99
All polyps - male patients	**0.0009**	77
Adenomas (excl. sessile serrated adenomas) - male patients	**0.0010**	66

Table 19 – T-test results for miR-19a

Table 20 shows the Anderson-Darling (AD) normality test values for all polyps and adenoma disease groups, given the AD test values we have assumed the CT values for the disease groups are normally distributed and T-testing is valid.

Disease group	MiR-19a	*n*
All polyps	0.8168	117
Adenomas	0.5162	99
Control	0.8984	81
All polyps - male patients	0.7201	77
Adenomas - male patients	0.3509	66
Control - male patients	0.9428	40

Table 20 – Normality testing for miR-19a disease groups

Figure 15 shows the box and whiskers plots for the CT values when comparing subjects with adenomas and polyps when compared with controls. The significance of the results is higher when analysing male subjects.

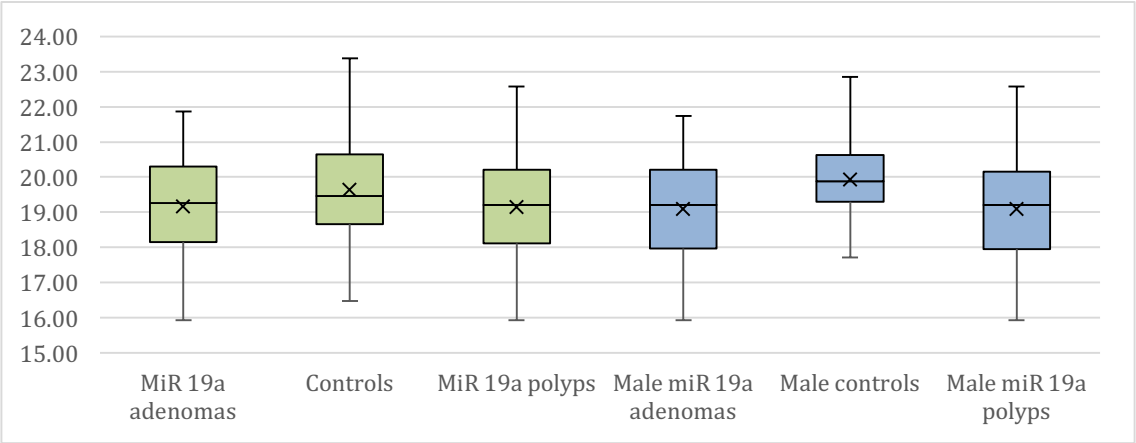


Figure 15 – Box and whiskers plots for miR-19a

8.5. MicroRNA 146b T-test results

MicroRNA 146b is another strong target with T-testing showing that the different expression levels in plasma of miR-146b highly significantly picks out subjects with polyps, adenomas (including multiple and right colonic) and all disease (polyps and cancer) when compared to controls.

Sub analysis shows that miR-146b picks out subjects with adenomas and all disease with a much higher statistical significance in men predominantly. T-test values are non-statistically significant in woman.

Table 21 shows the T-test values for all polyps, and adenoma disease groups when compared to controls, when looking at male subjects the T-test values are much more significant. The table also shows the results when normalising the results with miR-484.

Disease group	MiR-146b	Δ MiR-146b – miR-484	*n*
All polyps	0.0030	0.0146	117
Adenomas (excl. sessile serrated adenomas)	0.0045	0.0146	99
All polyps - male patients	0.0002	0.0003	77
Adenomas (excl. sessile serrated adenomas) - male patients	0.0002	0.0002	66

Table 21 – T-test results for miR-146b

Table 22 shows the Anderson-Darling (AD) normality test values for all polyps and adenoma disease groups, given the AD test values we have assumed the CT values of miR-146b for the disease groups are *not* normally distributed. With the normalised analysis using a delta of miR-146b and miR-484, the AD test values are such that we have assumed the CT values for the disease groups are normally distributed and T-testing is valid.

Disease group	MiR-146b	Δ MiR-146b – miR-484	*n*
All polyps	0.0384	0.6705	117
Adenomas	0.0162	0.8143	99
Control	0.2145	0.7364	81
All polyps - male patients	0.1219	0.5238	77
Adenomas - male patients	0.0286	0.6052	66
Control - male patients	0.8240	0.4022	40

Table 22 – Normality testing for miR-146b disease groups

Figure 16 shows the box and whiskers plots for the CT values when comparing subjects with adenomas and polyps when compared with controls. The significance of the results is higher when analysing male subjects.

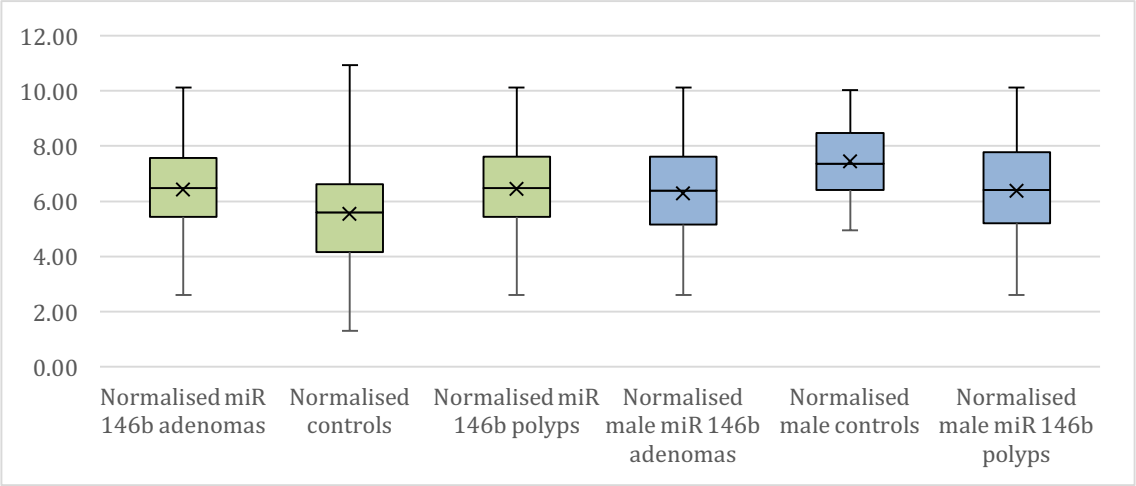


Figure 16 – Box and whiskers plots for miR-146b

8.6. MicroRNA 186 T-test results

T-testing has shown that the different expression levels in plasma of microRNA 186 highly significantly picks out subjects with polyps, adenomas (including multiple left colonic and right colonic) and all disease (polyps and cancer) when compared to controls. It is the best performing microRNA for picking out subjects with polyps / adenomas / all disease.

Sub analysis shows that miR-186 picks out subjects with adenomas and all disease with a much higher statistical significance in men predominantly. T-test values are non-statistically significant in woman.

Table 23 shows the T-test values for all polyps, and adenoma disease groups when compared to controls, when looking at male subjects the T-test values are much more significant. The table also shows the results when normalising the results with miR-484.

Disease group	MiR-186	Δ MiR-186 – miR-484	*n*
All polyps	0.0004	0.0023	117
Adenomas (excl. sessile serrated adenomas)	0.0008	0.0029	99
All polyps - male patients	0.0005	0.0009	77
Adenomas (excl. sessile serrated adenomas) - male patients	0.0002	0.0004	66

Table 23 – T-test results for miR-186

Table 24 shows the Anderson-Darling (AD) normality test values for all polyps and adenoma disease groups, given the AD test values we have assumed the CT values of miR-186 for the disease groups are *not* normally distributed. With the normalised analysis using a delta of miR-186 and miR-484, the AD test values are such that we have assumed the CT values for the disease groups are normally distributed and T-testing is valid.

Disease group	MiR-186	Δ MiR-186 – miR-484	*n*
All polyps	0.0023	0.0990	117
Adenomas	0.0030	0.2161	99
Control	0.7349	0.8916	81
All polyps - male patients	0.0390	0.5305	77
Adenomas - male patients	0.0465	0.7543	66
Control - male patients	0.5455	0.8794	40

Table 24 – Normality testing for miR-186 disease groups

Figure 17 shows the box and whiskers plots for the CT values when comparing subjects with adenomas and polyps when compared with controls. The significance of the results is higher when analysing male subjects.

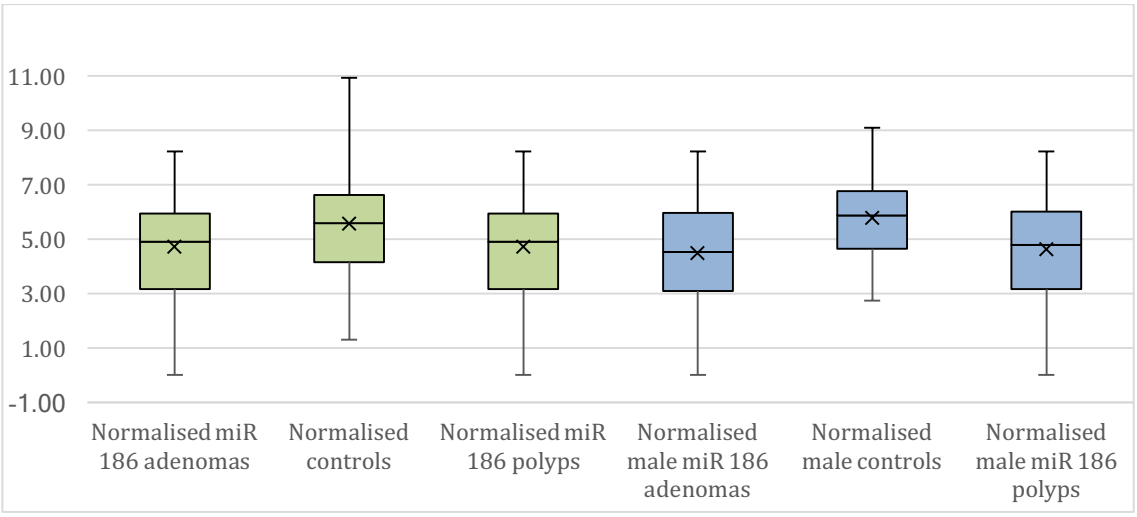


Figure 17 – Box and whiskers plots for miR-186

8.7. MicroRNA 625 T-test results

T-testing has shown that the different expression levels in plasma of microRNA 625 significantly picks out subjects with adenomas (multiple and right colon) and subjects with all disease (adenomas and cancers) when compared to controls.

Sub analysis shows that miR-625 picks out subjects with adenomas (left colon, right colon and multiple) with a statistical significance in men predominantly. T-test values are non-statistically significant in woman.

Table 25 shows the T-test values for all polyps, and adenoma disease groups when compared to controls, when looking at male subjects the T-test values are much more significant. The table also shows the results when normalising the results with miR-484.

Disease group	**MiR-625**	**Δ MiR-625 – miR-484**	***n***
All polyps	0.0010	0.0030	117
Adenomas (excl. sessile serrated adenomas)	0.0049	0.0092	99
All polyps - male patients	0.0060	0.0056	77
Adenomas (excl. sessile serrated adenomas) - male patients	0.0083	0.0066	66

Table 25 – T-test results for miR-625

Table 26 shows the Anderson-Darling (AD) normality test values for all polyps and adenoma disease groups, given the AD test values we have assumed the CT values of miR-625 for the disease groups are *not* normally distributed. With the normalised analysis using a delta of miR-625 and miR-484, the AD test values are such that we have assumed the CT values for the disease groups are normally distributed and T-testing is valid.

Disease group	**MiR-625**	**Δ MiR-625 – miR-484**	***n***
All polyps	0.0103	0.3004	117
Adenomas	0.0242	0.2729	99

Control	0.7504	0.4919	81
All polyps - male patients	0.0855	0.3887	77
Adenomas - male patients	0.1402	0.2734	66
Control - male patients	0.6579	0.5350	40

Table 26 – Normality testing for miR-625 disease groups

Figure 18 shows the box and whiskers plots for the CT values when comparing subjects with adenomas and polyps when compared with controls. The significance of the results is higher when analysing male subjects.

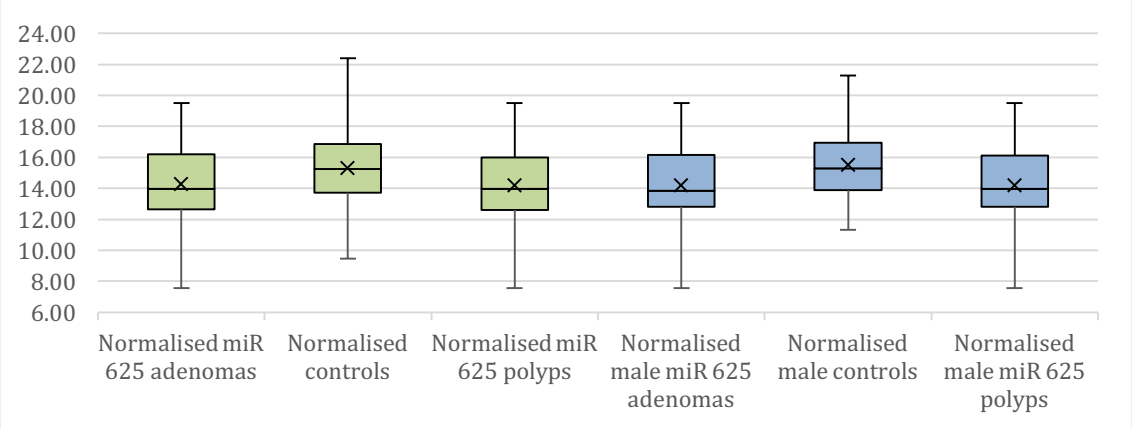


Figure 18 – Box and whiskers plots for miR-625

8.8. MicroRNA 191 T-test results

MicroRNA 191 was used as a control to normalise CT values for the identified pool A target microRNAs. However, miR-191 CT values in itself showed promising T-test results for different expression levels in plasma significantly picking out subjects with colorectal cancer compared to controls (table 27).

Disease group	MiR-191	*n*
Colorectal cancer	0.0010	12

Table 27 – T-test result for miR-191

Table 28 shows the Anderson-Darling (AD) normality test value for colorectal cancer disease group, given the AD test values we have assumed the CT values of miR-191 for the disease groups are normally distributed.

Disease group	MiR-191	*n*
Colorectal cancer	0.4919	12
Controls	0.1470	81

Table 28 – Normality testing for miR-191 disease groups

Figure 19 shows the box and whiskers plots for the CT values when comparing subjects with colorectal cancer when compared with controls.

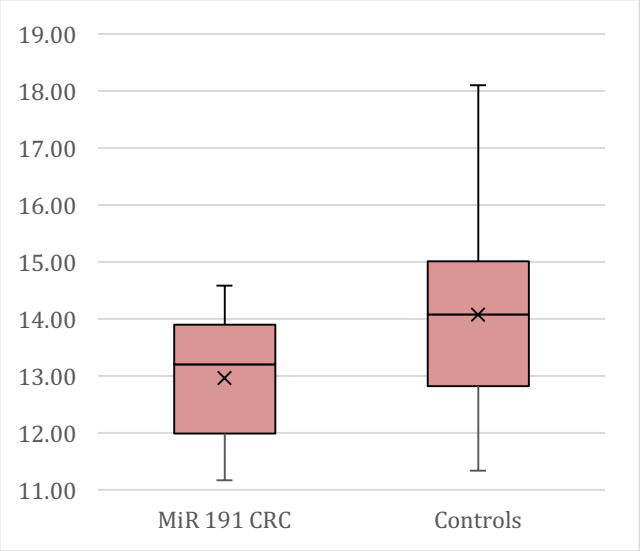


Figure 19 – Box and whiskers plots for miR-191

8.9. The other target microRNAs

T-testing for pool A target microRNAs 331-5p, 452 and for all of pool B target microRNAs 222#, 664, 1247 have not shown meaningful results for different expression levels in plasma to significantly picking out subjects with disease when compared to controls. MicroRNA 331-5p did show some significant T-test results for male patients but at a much lower significance level then microRNAs 98, 19a, 146b, 186 and 625. Considering the amount of work and time taken to identify pool B target microRNAs, it was disappointing to see that the three pool B microRNA targets (222#, 664, 1247) did not show any meaningful results.

9. Summary of microRNA targets for disease groups and potential screening tests.

9.1. Summary of microRNA targets

The analysis of the T-tests shows that there are several target microRNAs that picks out subjects within the disease groups when compared to controls. The best target microRNAs are 98, 19a, 146b, 186 and 625.

When sub-analysing by gender, for male subjects, the microRNA targets are more statistically significant. For female subjects, the microRNA targets can't significantly pick out subjects with within disease groups when compared to controls.

T-testing alone is not sufficient to prove microRNA targets are appropriate screening tests. We need to do logistical regression analysis to model panels of microRNAs as a screening test to pick out certain disease groups.

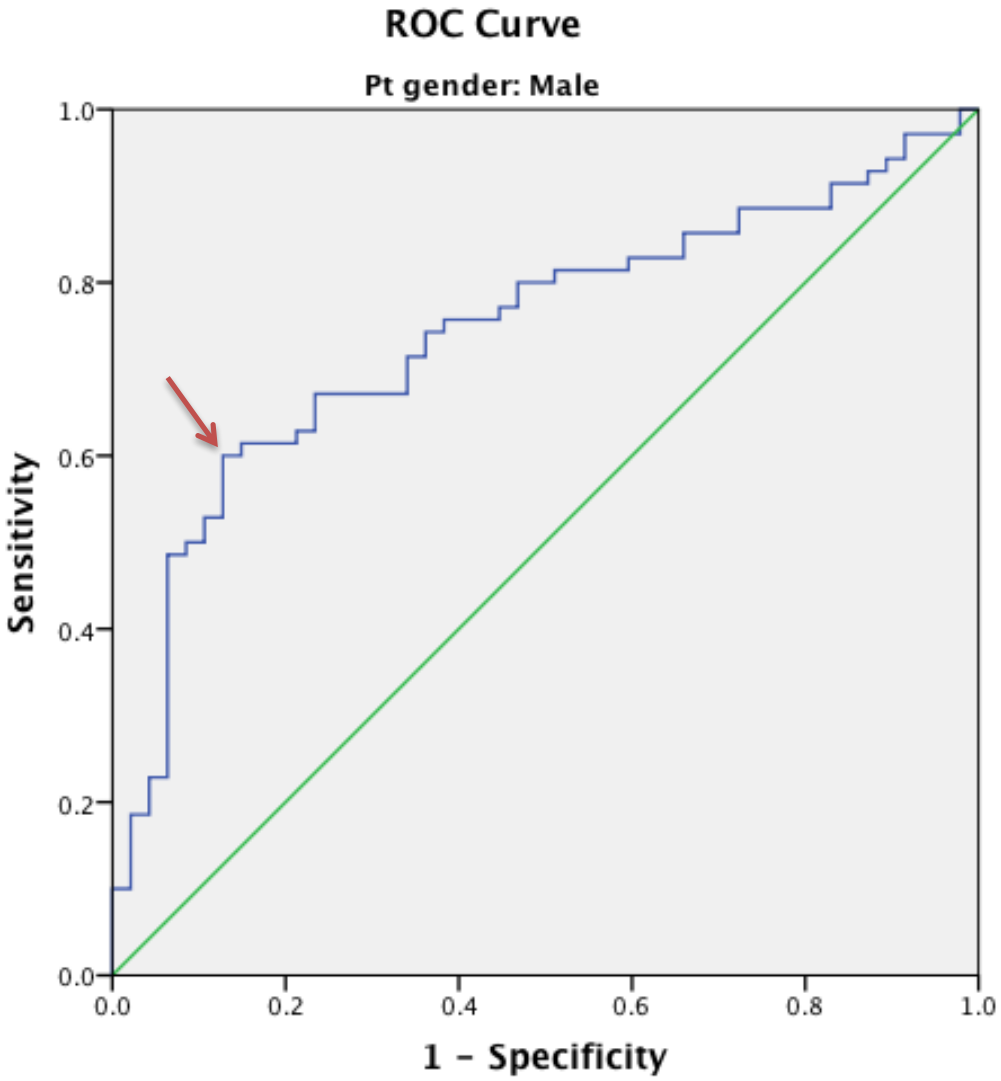
9.2. Modelling of microRNA panels as a screening test

T-testing of the CT values from the analysed cases has revealed several strong miR-target candidates. We ran all the results and the complete variables dataset through a multivariate analysis.

9.2.1. Panel: MiRs 98, 186, 452, detection of polyps (excl. hyperplastic polyps) in male subjects

The ROC curve shows the output of the regression analysis for the panel of miRs 98, 186 and 452 – indicating this model to be a good screening test in detecting polyps (excluding hyperplastic polyps) in male subjects; with a sensitivity of 0.600 and a specificity of 0.872 – this cut point is indicated by the red arrow on figure 20.

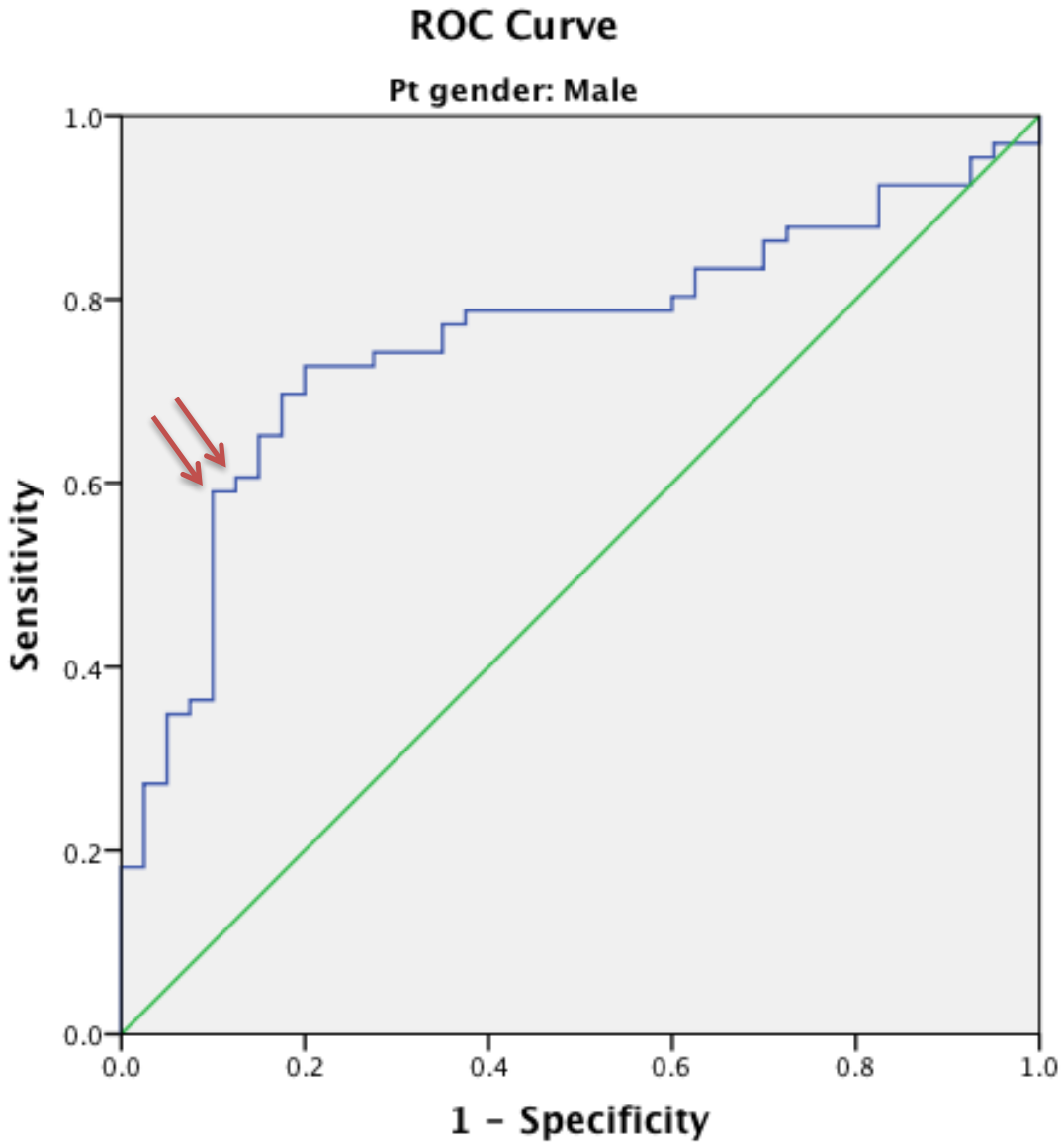
Figure 20 – MiRs 98, 186, 452, polyps (excluding hyperplastic polyps) in male subjects



9.2.2. Panel: MiRs 98, 186, 452, detection of adenomas in male subjects

The ROC curve shows the output of the regression analysis for the panel of miRs 98, 186 and 452 – indicating this model to be a good screening test in detecting adenomas in male subjects; with a sensitivity of 0.606 and a specificity of 0.875 or sensitivity 0.591 and a specificity of 0.900 – these cut points are indicated by the red arrows on figure 21.

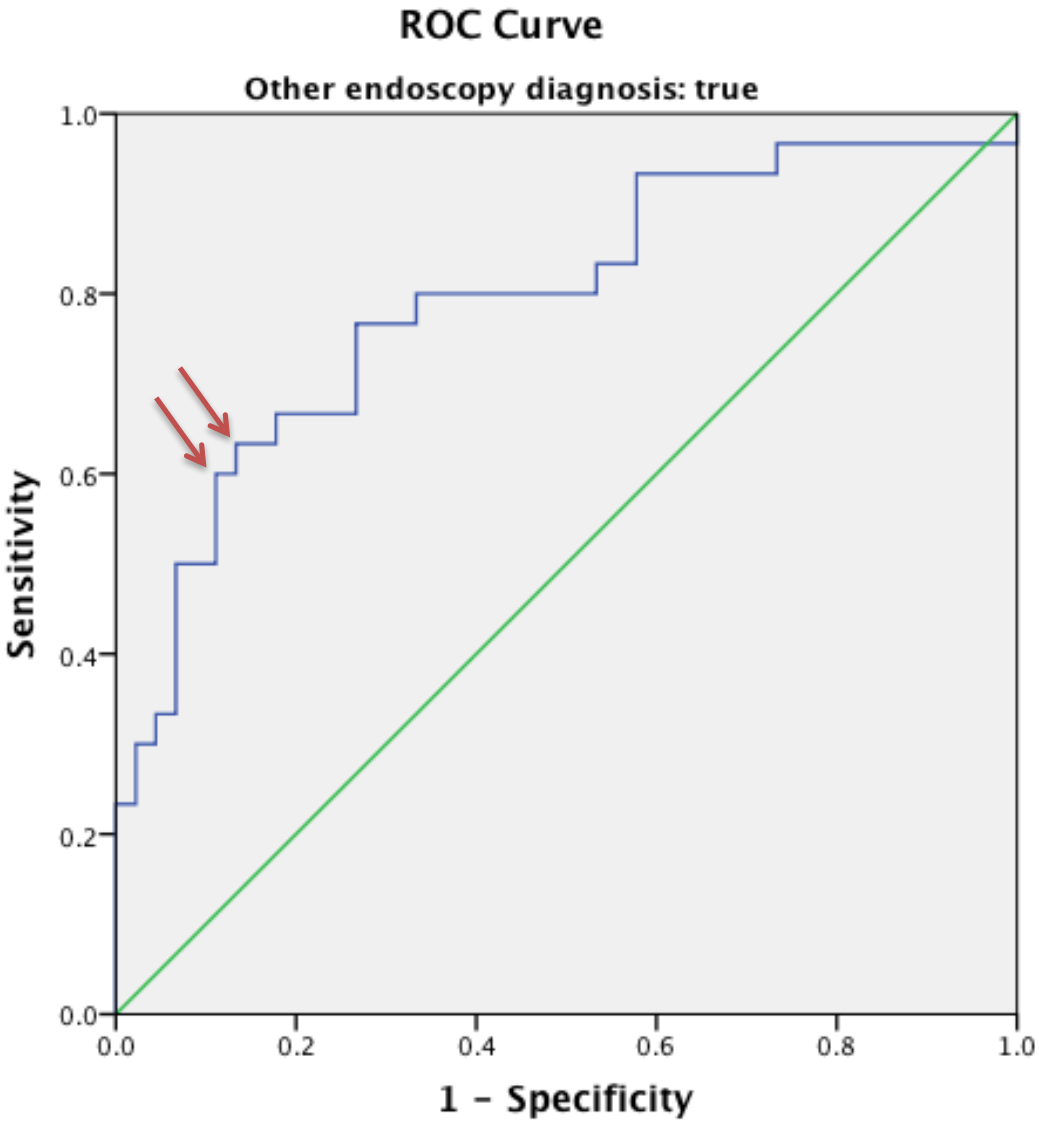
Figure 21 – MiRs 98, 186, 452, detection of adenomas in male subjects



9.2.3. Panel: MiRs 186, 452, 331-5p, detection of polyps (excluding hyperplastic polyps) in subjects with another endoscopic diagnosis.

The ROC curve shows the output of the regression analysis for the panel of miRs 186, 452 and 331-5p – indicating this model to be a good screening test in detecting polyps (excluding hyperplastic polyps) in subjects with another endoscopic diagnosis; with a sensitivity of 0.600 and a specificity of 0.889 or sensitivity 0.633 and a specificity of 0.867 – these cut points are indicated by the red arrows on figure 22.

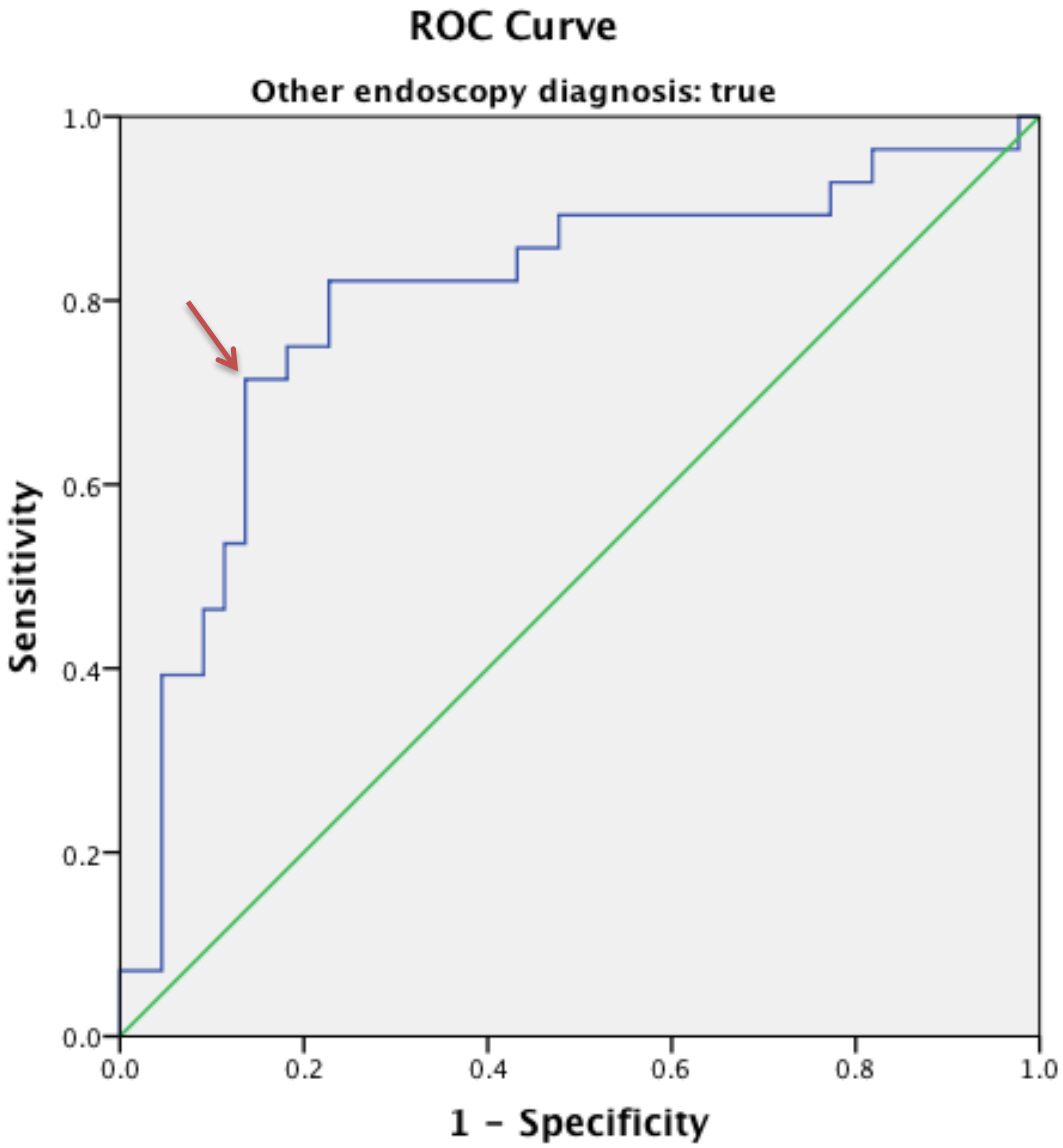
Figure 22 – MiRs 186, 452, 331-5p, polyps (excluding hyperplastic polyps) in subjects with another endoscopic diagnosis



9.2.4. Panel: MiRs 625, 452, 331-5p (normalised with miR-191), detection of adenomas in subjects with another endoscopic diagnosis.

The ROC curve shows the output of the regression analysis for the panel of miRs 625, 452 and 331-5p (normalised with miR-191) – indicating this model to be a good screening test in detecting adenomas in subjects with another endoscopic diagnosis; with a sensitivity of 0.714 and a specificity of 0.864 – this cut point is indicated by the red arrow on figure 23.

Figure 23 – MiRs 625, 452, 331-5p (normalised with miR-191), adenomas in subjects with another endoscopic diagnosis



DISCUSSION

10. Interpretation of the results

10.1. T-test results and microRNA panels as screening tests

The T-test analysis of pooled CT values are promising; identifying several candidate microRNA targets. MicroRNAs 19a, 98, 146b, 186, 625 have shown to identify adenomas with a T-test p values <0.01. MicroRNAs 331-5p and 452 have shown to be useful as part of panel when the tests are modelled by logistical regression. The statistically significant targets are pool A microRNAs, pool B targets have not been as effective in differentiation between disease groups in controls.

There are dramatic differences in T-test values when sub-analysing by gender. The microRNA expression profiles are markedly different. In men, microRNAs pick out individuals with adenomas and polyps whilst in woman they do not meaningfully pick out any disease group.

It is well known that colorectal adenomas and cancer are much more prevalent in men suggesting that gender has a crucial role in colorectal cancer biology. Whether this is a protective effect of sex hormones such as oestrogen is not known, it is debateable that sex hormones would actively be modifying the biology as the woman recruited in the study were all over 60 years old and in theory they should be post-menopausal (though some woman may be on hormone replacement therapy).

It also is questionable what the microRNA targets actually represent. They could be directly related to polyp formation or it could represent cascade reactions such as inflammation or other immune responses. The precise biology remains now and for the foreseeable future – poorly understood. The best way to conceptualise microRNA expression profiles is as a biological signature that may signpost certain disease processes.

Modelling of microRNA panels using multivariate logistical regression has shown 4 possible screening tests with sensitivities of 0.6-0.7 with specificities of

0.9 – this is comparable to FIT testing (in terms of sensitivity) with high specificity which is crucial for effective screening tests.

10.2. Detected microRNAs target in this study – role in other cancers.

To add to the plausibility of our findings. A brief literature search was performed to look at whether the target microRNAs discovered have a suggested role in other human cancers.

A Chinese study looked at **miR-19a** expression in 89 patients with oesophageal squamous cell cancer (OSCC), compared to 125 controls (80 healthy controls & 45 benign oesophageal lesions), the study also looked at **miR-19a** expression pre and post-surgical resection of OSCC in 30 patients. RT-qPCR was performed and the sensitivity of **miR-19a** for early stages of OSCC was 68.09%. Combination of **miR-19a** and cytokeratin 19 fragment 21-1 further improved the sensitivity to 78.70%. Moreover, plasma **miR-19a** level was decreased in patients after surgery. [85]

A smaller Chinese study looked at 7 patients' saliva with oesophageal cancer and compared with 3 healthy controls. **MiR-98** was associated with oesophageal cancer (with other miRs). They looked in close detail at the miRs and associated genes. **MiR-98** directly targets E2F transcription factor - associated with the cell cycle which is enriched in oesophageal cancer. [86]

Another Chinese study looked at the expression levels of 3 miRs (miR-222, miR-221 and **miR-146b**). They were analysed using RT-qPCR in 106 patients with papillary thyroid carcinoma (PTC), 35 patients with benign thyroid nodules (BTN) and 40 paired controls. Patients with either newly diagnosed PTC or BTN who were undergoing thyroidectomies were recruited for a dynamic analysis of preoperative and postoperative plasma miR levels. The results indicated that the expression levels of miR-222, miR-221 and **miR-146b** were significantly increased in patients with newly diagnosed PTC compared with controls and patients with BTN. The expression of these three miRNAs in serum was significantly associated with poorer prognostic variables, including extra-

thyroidal invasion, metastatic lymph nodes and high-risk or advanced tumour node metastasis stage. [87]

In a study, 151 Chinese patients with pancreatic ductal adenocarcinoma (PDAC) were analysed for **miR-186** and miR-326. **MiR-186** was over-expressed in PDAC patients compared with controls, especially in patients with large tumours (>2 cm), lymph node metastasis, or short-term survival (< 2 years). [88]

In a study, the diagnostic utility of microRNAs 143, 222 & **452** was assessed for discriminating patients with bladder cancer (n = 37) from controls (n = 57), including healthy individuals (n = 20) and patients with benign urological diseases (n = 37). RT-qPCR of **miR-452** and miR-222 in urine provided high accuracy in the diagnosis for bladder cancer. [89]

In another study, **miR-625** expression was determined in 96 pairs of primary CRC and adjacent non-tumour tissue by RT-qPCR. **MiR-625** expression was analysed with demographic and clinic-pathologic features and was significantly downregulated in CRC tissues and cell lines. It was also noted that the decreased expression of **miR-625** was positively associated with advanced lymph node metastasis ($p = 0.038$), liver metastasis ($p = 0.031$), and poor overall survival ($p = 0.002$). [90]

These are just a few studies published in journals. The work is mainly smaller studies undertaken in Asia. Nevertheless, it adds credence to our target microRNAs being relevant for pre-cancerous colorectal adenomas.

10.3. Limitations of the study

10.3.1. Limitations with reports

Endoscopy and histology reports are reported by operators and have a large subjective component – namely in giving a professional opinion. Reports are usually delivered in context of the indication and hence information may have been excluded from this which are actually important for research. This is difficult in reality to mitigate for.

10.3.2. Controls

The use of controls in this study does open up debate around whether they are true controls. All patients invited for BCSP screening colonoscopies have been pre-screened with faecal occult blood testing (FOBt) kits and have all been found to be positive. Individuals without polyps or cancer may have diverticulosis and/or haemorrhoids to explain the positive FOBt kit – this may not have been reported in the endoscopy reports. Pathological lesions (such as small adenomas) may have not been detected at colonoscopy. This could also represent pathology within the small bowel, upper gastrointestinal tract organs and upper respiratory tract; which may not be known to patient or clinician.

Patients may have other co-morbidities which change the microRNA expression profile. Chronic diseases, previous cancer and ongoing pre-malignant or malignant processes of which the patient and clinician may or may not be aware off is also a confounder. Again, this is difficult in reality to mitigate for. Data had been collected with regards to known co-morbidities but wasn't used for meaningful analysis as these controls are a close representation to real life controls given the prevalence of co-morbidities and gastrointestinal disorders such as haemorrhoids and diverticulosis in a middle-aged population.

For a true control group, it may be appropriate to test age and sex matched individuals who have been screened and have negative FOBt kits. This would be a better control group but they still will have pathology and co-morbidity as described in the previous paragraph as the FOBt kits (like any mass screening test) are not perfect in terms of specificity and sensitivity,

10.3.3. Statistical error

The use of statistical techniques is prone to error. False discovery is a possibility given the number of variables looked at. T-testing itself is an imperfect technique with it only being an effective method if the populations tested are normally distributed. Whilst this can be tested for (and has been), the normality testing can only prove distribution is *not* normal, it cannot prove normality (though fair presumption can be made depending on the probability

value of Anderson-Darling (and other) normality tests). Some, but not all of these potential errors can be mitigated for.

Whilst T-test p values < 0.05 are considered to be significant, the majority of the p values are < 0.01. This does make false discovery less likely. However, with the number of T-tests performed (608 in all subjects, 570 when in male subjects and 380 in female subjects), it is still possible that a small number may be due to false discovery. The logistical regression modelling of microRNA panels is much more stringent statistical technique to prove the hypothesis of our study. Also, I have clearly stated that the results of the study show promise but need to be validated in a study suitably powered to conclusively prove the effectiveness of microRNAs in detecting colorectal adenomas in patients when compared to controls.

10.3.4. Gender differences

It was only during post-hoc analysis of the data that it became evident that there are marked gender differences in microRNA expression profiles. This was not known during the microRNA array card analysis. Ideally it would be prudent to re-run the cards with gender specific pooled disease groups to see if suitable gender specific microRNA targets could be identified. The gender differences in microRNA expression profiles in patients with similar disease has not been reported previously so we could not have anticipated this.

10.3.5. Scale of study

This study is best thought of as a large pilot / discovery study. Whilst the results show promise the dataset is not large enough to power the statistics. The next step would be to analyse a large number of patients (thousands) to see if target microRNA expression can truly differentiate disease states and controls. The test should also then be repeated on the positive individuals at least once (if not several times) to prove reproducibility. This would require established automated machine testing processes which would be cheaper and less labour intensive then the discovery study we have performed. This would give improved statistical analysis to fully evaluate the merits of microRNAs as a potential diagnostic and screening test.

10.4. Strengths of the study

10.4.1. Completed data set

A comprehensive data set was collected for all patients with multiple variables recorded allowing for detailed analysis.

10.4.2. Case selection

The individuals recruited to the trail were predominately in their 60s and 70s and were typically well – in good health and mobile. The individuals with polyps were largely asymptomatic. Having a reasonably narrow control and disease group does limit error when analysing data.

10.4.3. Statistical robustness

At all stages, we were aware of potential statistical error and have mitigated for this as much as possible. We have interpreted T-test results with caution and have tested for normal distribution in the comparator populations.

10.4.4. Gender differences

The sub-analysis by gender has opened up debate regarding microRNAs. It is not generally reported in published research literature with regards to gender differences with microRNA expression profiles. Irrespective of whether this is unique to colorectal adenomas or if it pertains to gender differences in microRNA expression profiles for health and for disease, it is important to know.

10.4.5. Scale of study and quality control

Despite being a pilot / discovery study, it is the largest study published on microRNA expression in individuals with colorectal polyps and hence the results are meaningful. The quality of this study is high as there were many steps taken during each phase of the study to ensure that the results are meaningful and accurate and hence the interpretation of results produces reasonable conclusions. Our research group has extensive experience with microRNA expression and this helps with quality control also.

11. Final conclusions

11.1. Overall summary of this study

This study has proved convincingly that microRNAs have potential as plasma biomarkers for patients with adenomas when compared with controls. Several microRNA targets have shown significance when comparing disease groups with controls – with the majority of T-test p values well below 0.01.

This is a study larger than any published looking at microRNA expression in colorectal adenomas. Despite the concerns of false discovery and not powered to prove the hypothesis conclusively, the results are worthy of respect.

The observation that microRNA expression profiles differ in males & females is a new finding with no published studies on microRNA expression in cancer or pre-cancerous conditions showing this phenomenon with convincing results.

The results themselves do allow a panel of microRNAs to act as a potential screening test when modelled with a ROC curve profile which is comparable to many tests used in clinical practice currently.

A larger study with many more adenomas and controls focusing in on gender differences and adenomas as a whole would undoubtedly reveal the full potential of microRNAs as a screening biomarker and also address concerns around false discovery. It would validate the findings of this study.

11.2. Current state of colorectal cancer screening

11.1.1. Bowel Scope Screening

In 2017, bowel scope flexible sigmoidoscopy screening continues to be rolled out across England. Despite the high number of patient needed to screen to prevent colorectal cancer (191) and colorectal cancer death (489). [29]

The landmark UK Flexible Sigmoidoscopy Screening Trial revealed an adenoma detection rate of 12.1%. The adenoma detection rate in Bowel Scope examinations is overall approximately 7%. Wendy Atkin – the lead investigator

in the trial addressed the difference in adenoma detection rate at the British Society of Gastroenterology Annual Meeting 2016 (Liverpool). She stated the differences are due to a younger age group of patients being screened in Bowel Scope (55 year) compared with the trial (60 years) and the fact that the endoscopists within the trial ended up doing hundreds to thousands of procedures and became more adept at detecting subtle adenomas. She stated that adenoma detection rates of 9-10% should be achievable.

Despite Wendy Atkins commentary on mitigating factors, the Bowel Scope adenoma detection rate is disappointingly low and efforts are being made to improve adenoma detection. The B-ADENOMA trial has started in the 2nd quarter of 2017 which involves the addition of an endocuff vision device to the endoscope to improve visualisation on endoscope withdrawal to improve adenoma detection. The trial aims to recruit 3222 patients across a randomised treatment and non-treatment arm to see if this device improves adenoma detection. It has already been shown to be extremely effective in screening colonoscopies after a positive FOBt within the BCSP with an improvement in ADR of approximately 7% (data awaiting publication).

11.1.2. Bowel Cancer Screening Programme

As opposed to Bowel Scope, BCSP has been deemed as successful with an ADR of 45% and a cancer detection rate of 8% with a reduction in cancer mortality of approximately 15%. [27]

The effect of BCSP screening is evident in the presentation of colorectal cancer. In 389 analysed CRC cases presenting to Kettering General Hospital in 2013-16, 127 patients (32.6%) had engaged with BCSP of which 112/127 (88.2%) were screen detected cancers, 262 had not (67.4%). CRC is more common in men and in the screened cohort the male to female ratio is 1.9:1. In the non-screened cohort, the ratio is only 1.2:1.

In the smaller cohort, 78/127 (61.4%) are staged Dukes' A or B which are associated with a favourable outcome from surgery. In the larger non-screened

CRC cohort 143/262 (54.6%) are staged Dukes' C or D which are likely to be non-curable (palliative) ($p = <0.05$).

Despite woman engaging in screening more than men, screening seems to benefit men more than woman with nearly double the cancers detected in patients who had undergone bowel cancer screening.

11.2. Role of plasma based screening

Despite the existence of two bowel cancer screening programmes, uptake remains below 60% and this means large cohorts of the general public are not gaining the established advantages of screening for colorectal cancer.

There is no data to confirm this as yet but you would imagine the individuals who do not engage in Bowel Scope screening at 55 years old will also not engage in FOBt/FIT Bowel Cancer Screening Programme at ages 60-75.

Blood tests are commonplace in middle age as part of health screening by employers annual medical testing and those done by General Practice. If a microRNA based blood test could be verified as an effective diagnostic and screening tool, the strength of a blood based (plasma) screening test based on microRNA targets is to identify individuals who are likely to have colorectal adenomas so they can be encourage to have colonoscopy and polypectomy. This could be done for individuals aged 60 years old which is a typical age for blood test screening in middle age. This would be as a form of "salvage' for a vulnerable unscreened cohort.

The other use may be to stratify risk for patient who are FOBt positive. A suitably powered blood based test could be used in addition or conjunction with FOBt or FIT testing to give an individualised risk profile and to deliver appropriate (in terms of modality and urgency) diagnostic tests and therapies.

APPENDICES

Appendix 1 – Publications resulting from project.

Verma AM, Patel M, Aslam MI, Jameson J, Pringle JH, Wurm P, et al. **P0381 Plasma microRNAs as screening biomarkers for colorectal adenomas.** *United European Gastroenterology Journal*. 2014;**2**(1_suppl):A132-A605

Poster presentation at an international Gastroenterology conference.
United European Gastroenterology (UEG) Week 2014
ACV, Vienna; 22-24th October 2014

Verma AM, Patel M, Aslam MI, Jameson J, Pringle JH, Wurm P, et al. **Circulating plasma microRNAs as a screening method for detection of colorectal adenomas**. *Lancet*. 2015;**385 Suppl 1**:S100.

Poster presentation at a national Academy of Medical Sciences Meeting:
2015 Spring Meeting for Clinician Scientists in Training
Royal College of Physicians, London; 26th February 2015.

Verma AM, Patel M, Aslam MI, Wurm P, Jameson J, Pringle JH, et al. **OC-029 Non-invasive screening for colorectal adenomas using plasma microRNAs**. *Gut.* 2015;**64**(Suppl 1):A15.1-A.

Oral presentation at a national combined societies' meeting:
Digestive Disorders Federation Conference
ExCeL, London; 22-25th June 2015.

Appendix 2 – Study documentation.

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

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: ……

University Hospitals of Leicester NHS
NHS Trust

Participant Information Sheet

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.

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

University Hospitals of Leicester NHS
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: .....

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.

Appendix 3 – Spreadsheet; analysis of microassay array data

Pool A microRNA targets

Detector	FC 2-8	FC >8	T-test	Male controls	Female controls	Controls divert	Controls haem	High grade	Polyp cancer	CRC	Sessile serrated	Villous	Lt large sessile	Rt large sessile	Mult sub 10mm
hsa-miR-518d-001159	0	0	0.002	26.339132	26.30071	27.720118	25.884981	27.003773	31.546465	27.6046	27.816717	27.699665	29.201746	31.349274	28.852438
hsa-let-7e-002406	4	0	0.004	19.439337	19.649738	19.631338	18.849813	19.424374	18.425566	18.2425	17.902498	18.411673	18.810373	17.980902	17.635317
hsa-miR-30c-000419	3	0	0.005	18.429523	18.152063	18.953419	18.052202	18.311493	16.211351	17.7543	17.250326	16.356173	18.252241	17.321804	16.632185
hsa-miR-671-3p-002322	0	0	0.006	25.891273	25.819307	26.3976	25.64962	26.178297	25.242807	24.9479	24.675282	25.096786	25.512245	24.932365	25.013865
hsa-miR-186-002285	5	3	0.006	18.907875	20.70043	19.798439	18.67882	18.906298	15.653839	17.9588	17.646223	15.070822	17.546219	17.61868	15.26613
hsa-miR-146b-001097	2	2	0.007	21.126896	20.847254	21.27557	20.52906	21.166636	17.307064	20.0307	19.51251	17.434715	20.261168	19.757874	17.389698
hsa-miR-98-000577	8	0	0.008	25.439528	25.871132	25.883993	24.127275	24.471352	22.962706	23.3242	22.143764	23.127296	23.304466	23.170761	22.334003
hsa-miR-19a-000395	4	2	0.008	20.471369	20.945578	21.588987	21.170176	20.917316	18.300945	20.6126	19.982527	16.45644	19.596851	19.406284	16.980688
hsa-miR-548b-5p-002408	4	0	0.009	32.018925	31.744217	32.457497	32.0569	31.951664	31.974047	30.387	30.982729	31.539589	30.712238	30.753725	30.213371
hsa-miR-200c-002300	1	0	0.009	25.080128	24.582138	25.046186	24.801382	24.960238	24.9145	23.8696	23.420446	23.939224	23.306368	23.5814	23.652397
hsa-let-7d-002283	1	0	0.009	21.11663	21.183872	21.983093	20.859137	19.60539	20.720474	20.1115	19.790428	20.606096	20.370014	20.140656	20.001566
hsa-miR-214-002306	0	0	0.01	28.135136	27.602869	28.3301	28.372871	29.174528	31.679302	28.5486	28.387537	30.773272	27.695019	29.128475	31.112087
hsa-miR-452-002329	5	0	0.011	26.192268	26.846548	26.793211	26.604702	25.920889	26.622528	25.9453	26.31541	24.177	25.1399	23.89602	24.392647
hsa-miR-25-000403	0	0	0.012	21.37726	21.357105	22.606987	21.688164	23.483408	22.612808	21.8978	21.11473	22.837097	22.735106	22.423197	22.516481
hsa-miR-625-002431	7	1	0.013	28.27423	28.449041	29.72669	26.883806	27.601854	25.507488	26.564	25.578192	25.624302	26.416039	25.393005	23.89835
hsa-miR-24-000402	0	0	0.018	16.896175	16.873022	17.063524	16.379017	16.816368	16.464512	15.9344	15.467276	16.496525	16.263178	15.940756	15.619397
hsa-miR-629-002436	0	0	0.019	28.663364	28.025167	29.158285	28.43349	31.285212	29.226387	28.8175	28.627155	28.675266	29.055157	28.685167	28.507908
hsa-miR-15b-000390	0	0	0.025	20.317724	20.1716	20.97632	20.307007	20.654867	20.12311	19.6777	19.224464	19.62511	19.943306	19.255318	19.23497
hsa-miR-331-5p-002233	2	4	0.027	29.780571	29.748655	29.246683	27.280363	29.112387	24.656397	24.4264	24.088057	28.579287	29.447361	27.005611	21.968077
hsa-miR-548d-001605	0	0	0.028	30.994806	28.437378	30.11307	29.245472	31.596333	31.65185	28.9388	28.878038	40	40	32.3051	32.709473
hsa-miR-146b-3p-002361	4	0	0.028	30.41184	30.516317	31.734858	30.398066	30.227255	29.4408	29.0976	29.216183	29.863852	29.543983	29.460484	29.41039
hsa-miR-486-001278	0	0	0.03	20.831497	21.120632	22.07307	19.90905	23.776781	24.315582	20.1559	19.793644	22.964643	22.278397	21.763786	23.831888
hsa-miR-502-001109	0	0	0.032	29.370268	30.189177	30.79487	29.390465	30.380249	31.825737	30.6385	30.882856	30.153744	30.882013	29.466042	30.262331
hsa-miR-339-3p-002184	5	0	0.033	23.4345	23.392082	23.170998	22.26338	22.967636	23.121185	21.8523	21.42263	22.073809	22.16571	21.543192	20.891249
hsa-miR-139-5p-002289	1	0	0.033	21.219788	21.486437	21.553053	20.839912	21.462187	21.247932	20.4924	19.563389	20.571718	20.687185	19.894426	20.256086
hsa-miR-376b-001102	1	1	0.035	31.531258	21.28368	22.87411	22.509874	32.301304	34.97859	21.7531	20.829134	32.11763	32.11434	40	40
hsa-miR-521-001122	1	5	0.036	38.803467	40	40	37.41558	34.045624	32.177147	40	35.893738	31.977055	40	32.49156	40
hsa-miR-513-5p-002090	3	2	0.037	40	39.56018	40	40	34.971985	39.78949	39.2767	40	37.318634	40	37.37533	36.073215
hsa-miR-519a-002415	3	6	0.038	32.532158	33.69622	37.65758	40	32.959766	26.190592	32.9533	33.30038	22.913631	32.27119	31.92779	24.542097
hsa-miR-520f-001120	1	4	0.039	40	40	40	40	37.874744	20.54852	40	32.942837	20.985256	40	38.882412	22.470972
hsa-miR-574-3p-002349	3	0	0.039	22.092508	21.636448	22.168842	21.162336	21.744598	21.559835	20.8645	20.184725	21.51644	20.501318	20.863392	20.336712
hsa-let-7g-002282	0	0	0.04	21.645487	21.276085	22.21471	21.094755	21.659138	20.788237	20.6387	20.26578	20.609215	20.878725	20.468372	20.25446
hsa-miR-204-000508	4	0	0.04	27.16827	27.057898	27.121939	25.982702	26.692263	27.4328	25.8573	24.791943	25.210098	25.766953	24.792559	25.577585
mmu-miR-495-001663	0	3	0.041	23.74331	23.497446	24.271893	24.091518	23.527298	18.634947	24.4254	22.68791	18.364492	23.054634	22.57567	18.764793
hsa-miR-147b-002262	0	4	0.046	40	39.233852	40	40	40	21.904837	40	27.746944	20.856436	40	40	22.252274
hsa-miR-744-002324	0	0	0.048	22.190186	22.358774	23.111752	21.893597	22.124428	21.240807	21.5285	21.197376	22.069162	21.950182	21.270754	20.794415
hsa-miR-210-000512	2	1	0.049	23.901873	24.430439	25.368582	23.795483	24.2871	21.21608	24.2086	22.979303	19.34735	23.780445	23.835157	21.270327
mmu-miR-140-001187	1	0	0.05	21.407589	21.36891	21.582026	21.273548	21.88209	21.29641	20.4487	20.040703	20.950876	21.140121	20.42187	19.615263
hsa-miR-377-000566	1	1	0.051	30.410696	24.349373	24.526197	22.279076	34.40225	32.04511	23.6042	21.550898	40	32.267563	32.408867	26.251535

Pool B microRNA targets

Detector	FC 2-8	FC >8	T-test	Male controls	Female controls	Controls divert	Controls haem	High grade	Polyp cancer	CRC	Sessile serrated	Villous	Lt large sessile	Rt large sessile	Mult sub 10mm	Left pedunc
hsa-miR-1300-002902	0	0	0.006	32.658066	32.320026	31.986734	33.973717	32.594048	40	40	32.453117	34.589863	37.746178	40	37.330776	32.95619
hsa-miR-623-001555	0	6	0.012	40	40	40	40	34.488075	40	31.9325	40	39.251644	26.028376	36.597782	29.92733	28.391685
hsa-miR-633-001574	2	4	0.014	40	40	40	40	40	40	33.9229	35.839226	35.11495	40	37.692116	33.705315	37.244526
hsa-miR-617-001591	4	3	0.014	40	40	40	40	35.77062	33.10704	40	36.907894	40	29.59716	37.723446	37.3451	37.659435
hsa-miR-516-3p-0011	2	6	0.015	39.48293	34.62948	40	39.420055	37.29471	33.820133	34.2543	36.844788	30.231623	28.366709	36.931126	26.512045	27.808222
hsa-miR-605-001568	4	4	0.015	35.66674	38.60553	35.98282	36.792484	33.983932	32.110344	40	34.386406	31.62309	29.520092	33.778572	31.897003	33.67745
hsa-miR-629-001562	4	0	0.018	29.016588	29.26154	29.082289	28.660467	28.19327	29.479744	28.537	27.053484	28.259056	28.547613	27.61342	27.055223	27.690384
hsa-miR-149#-002164	3	3	0.02	40	39.74561	39.371704	39.388073	35.28611	36.592243	37.5452	35.64354	36.086998	40	40	38.10918	39.611008
hsa-miR-92b#-002343	0	4	0.027	40	40	40	40	40	39.55921	40	34.77386	33.435284	33.559635	39.833523	31.434902	38.61283
hsa-miR-222#-002097	5	2	0.029	31.307335	31.049566	32.48399	32.815407	30.488306	32.144924	31.6746	29.826199	28.51259	28.767176	30.429329	28.892122	28.720972
hsa-miR-29b-2#-0021	2	3	0.034	33.962437	31.804974	32.394657	33.759148	32.26957	30.561916	32.8221	32.576057	30.63164	29.449905	32.481846	29.696535	29.388
U6 snRNA-001973	6	0	0.034	26.595041	25.5993	26.46161	25.902472	25.535627	26.260895	26.7125	24.486935	24.293829	24.540417	24.890507	24.359196	24.21027
U6 snRNA-001973	6	0	0.034	26.595041	25.5993	26.46161	25.902472	25.535627	26.260895	26.7125	24.486935	24.293829	24.540417	24.890507	24.359196	24.21027
U6 snRNA-001973	6	0	0.034	26.595041	25.5993	26.46161	25.902472	25.535627	26.260895	26.7125	24.486935	24.293829	24.540417	24.890507	24.359196	24.21027
U6 snRNA-001973	6	0	0.034	26.595041	25.5993	26.46161	25.902472	25.535627	26.260895	26.7125	24.486935	24.293829	24.540417	24.890507	24.359196	24.21027
hsa-miR-564-001531	3	2	0.034	34.37055	34.297966	33.65892	33.99906	30.22561	33.36709	34.2207	33.3656	32.103954	31.352623	35.54696	31.481508	29.46325
hsa-miR-363#-001283	3	6	0.037	36.08504	33.901894	40	40	34.13229	32.740486	32.339	35.79267	32.16365	25.794382	34.98898	26.454496	34.616962
hsa-miR-192#-002272	0	0	0.045	31.284567	32.241154	32.178326	31.452108	35.14398	34.660034	33.3298	32.334927	32.615807	31.857687	30.984985	33.89564	40
hsa-miR-99a#-002141	1	0	0.046	30.13582	31.656227	30.985775	31.317139	31.277603	31.247791	33.7112	35.4385	33.14648	32.30328	32.673862	37.852615	28.991491
hsa-miR-1180-002847	0	0	0.048	30.96837	31.484142	30.608519	29.897552	35.325943	38.04798	32.9726	31.471846	31.406294	30.884188	30.493023	32.477306	30.323597
hsa-miR-1270-002807	1	6	0.049	35.52831	38.181267	34.609665	40	32.556477	37.346695	37.1016	34.140358	31.460281	30.662985	33.67937	32.622997	30.615036
hsa-miR-125b-1#-002	1	3	0.06	40	40	40	40	38.705963	40	40	38.98039	40	34.139755	40	33.359886	31.560068
hsa-miR-191#-002678	2	0	0.061	27.851437	27.741667	27.552206	27.587336	27.145082	28.648405	27.4333	26.44454	26.175766	27.618097	26.732914	26.43638	26.287651
hsa-miR-106a#-00217	1	3	0.062	40	40	40	40	40	40	40	40	33.410877	34.7067	40	38.199226	34.372902
hsa-miR-1247-002893	1	7	0.062	31.375221	27.366478	26.700953	31.625029	24.915972	24.884295	30.9186	24.697906	24.364683	25.493366	26.764597	25.076387	24.166948
hsa-miR-488-001106	1	3	0.066	40	40	40	40	33.112743	40	40	35.3544	37.687088	40	40	32.514545	40
hsa-miR-10a#-002288	2	5	0.067	40	36.40889	40	40	36.50902	36.699768	34.624	34.91153	34.120136	40	40	35.369072	34.693768
hsa-miR-937-002180	2	2	0.067	40	40	40	40	32.967827	40	40	40	36.67821	40	40	32.790462	36.96703
hsa-miR-626-001559	0	3	0.076	40	40	40	40	38.934464	40	40	40	33.34844	40	40	32.312046	34.09993
hsa-miR-1244-002791	4	0	0.079	32.264965	31.340961	32.167965	31.236517	31.392017	31.852917	32.1592	29.369402	30.228357	31.067492	30.700787	30.20847	30.231386
hsa-miR-609-001573	1	4	0.08	40	38.10474	40	40	40	40	40	35.83683	37.938366	40	35.423294	30.306284	31.59463
hsa-miR-664-002897	3	0	0.083	24.233994	23.08292	23.283754	23.14061	23.163116	24.025398	23.8319	21.537302	22.413174	22.06165	22.472586	21.97006	22.176401
hsa-miR-587-001540	1	6	0.088	33.589134	40	36.411526	37.68384	31.413027	32.32344	40	33.883198	38.676247	28.237581	30.188078	31.337887	32.819496
hsa-miR-9#-002231	0	0	0.096	28.097874	27.382359	27.800138	27.526857	28.961378	29.183306	28.1486	27.838661	27.490429	27.589226	27.935923	27.265316	28.719805

Appendix 4 - REMARK Guidelines

(REporting recommendations for tumour MARKer prognostic studies)

Introduction

1. State the marker examined, the study objectives, and any prespecified hypotheses.

Materials and Methods

Patients

2. Describe the characteristics (e.g. disease stage or comorbidities) of the study patients, including their source and inclusion and exclusion criteria.

3. Describe treatments received and how chosen (e.g. randomised or rule-based).

Specimen characteristics

4. Describe type of biological material used (including control samples), and methods of preservation and storage.

Assay methods

5. Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments, quantitation methods, and scoring and reporting protocols. Specify whether and how assays were performed blinded to the study end point.

Study design

6. State the method of case selection, including whether prospective or retrospective and whether stratification or matching (e.g. by stage of disease or age) was employed. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time.

7. Precisely define all clinical end points examined.

8. List all candidate variables initially examined or considered for inclusion in models.

9. Give rationale for sample size; if the study was designed to detect a specified effect size, give the target power and effect size.

Statistical analysis methods

10. Specify all statistical methods, including details of any variable selection procedures and other model-building issues, how model assumptions were verified, and how missing data were handled.

11. Clarify how marker values were handled in the analyses; if relevant, describe methods used for cutpoint determination.

Results

Data

12. Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, both overall and for each subgroup extensively examined report the numbers of patients and the number of events.

13. Report distributions of basic demographic characteristics (at least age and sex), standard (disease-specific) prognostic variables, and tumour marker, including numbers of missing values.

Analysis and presentation

14. Show the relation of the marker to standard prognostic variables.

15. Present univariate analyses showing the relation between the marker and outcome, with the estimated effect (e.g. hazard ratio and survival probability). Preferably provide similar analyses for all other variables being analysed. For the effect of a tumour marker on a time-to-event outcome, a Kaplan–Meier plot is recommended.

16. For key multivariable analyses, report estimated effects (e.g. hazard ratio) with confidence intervals for the marker and, at least for the final model, all other variables in the model.

17. Among reported results, provide estimated effects with confidence intervals from an analysis in which the marker and standard prognostic variables are included, regardless of their significance.

18. If done, report results of further investigations, such as checking assumptions, sensitivity analyses, internal validation.

Discussion

19. Interpret the results in the context of the prespecified hypotheses and other relevant studies; include a discussion of limitations of the study.

20. Discuss implications for future research and clinical value.

Appendix 5 – Tables showing MicroRNA T-test data

Pool A – microRNA 98

Disease group	MiR-98	Δ MiR-98 – miR-484	Δ MiR-98 – miR-U6
All polyps	**0.0224**	0.0880	0.0694
Adenomas (excl. sessile serrated adenomas)	**0.0266**	0.0747	0.0574
Left colon adenomas	0.1989	0.2804	0.1453
Left colon large (≥10 mm) pedunc. adenomas	0.2147	0.4149	0.3661
Left colon large sessile adenomas	0.3926	0.4318	0.3272
Left colon small (<10 mm) adenomas	0.5860	0.5640	0.3191
Multiple adenomas on both sides of colon	0.0616	0.0980	**0.0084**
Multiple large adenomas both sides of colon	0.7115	0.7422	0.1732
Multiple small adenomas both sides of colon	**0.0029**	**0.0213**	**0.0135**
Right colon adenomas	**0.0310**	0.1300	0.9874
Right colon large adenomas	0.0704	0.2224	0.3012
Right colon small adenomas	0.1512	0.3119	0.5976
Sessile serrated adenomas	0.1860	0.1791	0.0726
Colorectal cancer	0.3022	0.7237	0.8982
All cancer (CRC & malignant polyps)	0.4336	0.9206	0.8288
Disease (all polyps excl. hyperplastic, & CRC)	**0.0233**	0.0878	0.0796

Disease group	MiR-98	Δ MiR-98 – miR-484	Δ MiR-98 – miR-U6
All polyps	0.0008	0.0012	0.0274
Adenomas (excl. sessile serrated adenomas)	0.0005	0.0006	0.0153
Left colon adenomas	0.0191	0.0251	0.0712
Left colon large (≥10 mm) pedunc. adenomas	0.1309	0.2647	0.2063
Left colon large sessile adenomas	0.0370	0.0954	0.3178
Left colon small (<10 mm) adenomas	0.2834	0.1663	0.2577
Multiple adenomas both sides of colon	0.0015	0.0016	0.0022
Multiple large adenomas both sides of colon	0.1717	0.1582	0.1913
Multiple small adenomas both sides of colon	0.0000	0.0002	0.0002
Right colon adenomas	0.0520	0.0696	0.8862
Right colon large adenomas	0.1616	0.3053	0.5524
Right colon small adenomas	0.1768	0.1528	0.8041
Colorectal cancer	0.3662	0.6178	0.7973
All cancer (CRC & malignant polyps)	0.1496	0.3159	0.6240
Disease (all polyps excl. hyperplastic, & CRC)	0.0008	0.0009	0.0220
All polyps	0.7680	0.7439	0.6805
Adenomas (excl. sessile serrated adenomas)	0.9655	0.5580	0.8207
Left colon adenomas	0.8727	0.6066	0.8165
Left colon large (≥10 mm) pedunc. adenomas	0.3405	0.4057	0.9421
Left colon large sessile adenomas	0.2137	0.2213	0.6837
Left colon small (<10 mm) adenomas	0.5768	0.2490	0.9998
Multiple adenomas both sides of colon	0.2245	0.1544	0.8167
Right colon adenomas	0.1055	0.6318	0.9834
Right colon small adenomas	0.3742	0.9495	0.7132
Disease (all polyps excl. hyperplastic, & CRC)	0.7601	0.6811	0.8812

Pool A – microRNA 19a

Disease group	MiR-19a	Δ MiR-19a – miR-484	Δ MiR-19a – miR-U6
All polyps	**0.0233**	0.0781	**0.0485**
Adenomas (excl. sessile serrated adenomas)	**0.0339**	0.0800	**0.0430**
Left colon adenomas	0.2761	0.3420	0.1522
Left colon large (≥10 mm) pedunc. adenomas	0.4293	0.6117	0.4216
Left colon large sessile adenomas	0.3362	0.3666	0.3128
Left colon small (<10 mm) adenomas	0.6205	0.6190	0.3383
Multiple adenomas on both sides of colon	0.0590	0.0776	**0.0048**
Multiple large adenomas both sides of colon	0.6597	0.6940	0.1385
Multiple small adenomas both sides of colon	**0.0150**	**0.0199**	**0.0078**
Right colon adenomas	0.0716	0.1643	0.8308
Right colon large adenomas	0.2275	0.3708	0.4345
Right colon small adenomas	0.1811	0.2900	0.7192
Sessile serrated adenomas	0.4784	0.2997	0.2598
Colorectal cancer	0.1659	0.5755	0.7991
All cancer (CRC & malignant polyps)	0.4342	0.9875	0.7758
Disease (all polyps excl. hyperplastic, & CRC)	**0.0324**	0.0992	0.0687

Disease group	MiR-19a	Δ MiR-19a – miR-484	Δ MiR-19a – miR-U6
All polyps	0.0009	0.0020	0.0182
Adenomas (excl. sessile serrated adenomas)	0.0010	0.0019	0.0130
Left colon adenomas	0.0544	0.0570	0.0845
Left colon large (≥10 mm) pedunc. adenomas	0.3690	0.4730	0.2430
Left colon large sessile adenomas	0.0886	0.1307	0.3854
Left colon small (<10 mm) adenomas	0.2328	0.1721	0.2322
Multiple adenomas both sides of colon	0.0005	0.0008	0.0013
Multiple large adenomas both sides of colon	0.0748	0.0786	0.1517
Multiple small adenomas both sides of colon	0.0003	0.0004	0.0002
Right colon adenomas	0.0746	0.0958	0.6767
Right colon large adenomas	0.5697	0.6435	0.8995
Right colon small adenomas	0.0898	0.0951	0.6566
Colorectal cancer	0.1289	0.3315	0.5505
All cancer (CRC & malignant polyps)	0.0371	0.1504	0.4250
Disease (all polyps excl. hyperplastic, & CRC)	0.0011	0.0021	0.0163
All polyps	0.8250	0.7038	0.6848
Adenomas (excl. sessile serrated adenomas)	0.9709	0.6124	0.7705
Left colon adenomas	0.9384	0.6574	0.7805
Left colon large (≥10 mm) pedunc. adenomas	0.3569	0.3773	0.8974
Left colon large sessile adenomas	0.5208	0.4213	0.5737
Left colon small (<10 mm) adenomas	0.5682	0.3618	0.9057
Multiple adenomas both sides of colon	0.3863	0.1546	0.7317
Right colon adenomas	0.3835	0.7616	0.9910
Right colon small adenomas	0.9319	0.6064	0.4380
Disease (all polyps excl. hyperplastic, & CRC)	0.9300	0.5477	0.9719

Pool A – microRNA 146b

Disease group	MiR-146b	Δ MiR-146b – miR-484	Δ MiR-146b – miR-U6
All polyps	**0.0030**	**0.0146**	**0.0136**
Adenomas (excl. sessile serrated adenomas)	**0.0045**	**0.0146**	**0.0118**
Left colon adenomas	0.1083	0.1508	0.0726
Left colon large (≥10 mm) pedunc. adenomas	0.1208	0.2161	0.1843
Left colon large sessile adenomas	0.2211	0.2486	0.2198
Left colon small (<10 mm) adenomas	0.7010	0.6687	0.3535
Multiple adenomas on both sides of colon	**0.0194**	**0.0321**	**0.0021**
Multiple large adenomas both sides of colon	0.3943	0.4229	0.0877
Multiple small adenomas both sides of colon	**0.0023**	**0.0067**	**0.0051**
Right colon adenomas	**0.0076**	**0.0320**	0.5846
Right colon large adenomas	0.0568	0.1602	0.2519
Right colon small adenomas	0.0502	0.0990	0.8841
Sessile serrated adenomas	0.4101	0.2054	0.1234
Colorectal cancer	0.1936	0.6228	0.8361
All cancer (CRC & malignant polyps)	0.3011	0.8384	0.8730
Disease (all polyps excl. hyperplastic, & CRC)	**0.0050**	**0.0215**	**0.0221**

Disease group	MiR-146b	Δ MiR-146b – miR-484	Δ MiR-146b – miR-U6
All polyps	0.0002	0.0003	0.0089
Adenomas (excl. sessile serrated adenomas)	0.0002	0.0002	0.0055
Left colon adenomas	0.0242	0.0234	0.0535
Left colon large (≥10 mm) pedunc. adenomas	0.1347	0.2056	0.1315
Left colon large sessile adenomas	0.0443	0.0735	0.2765
Left colon small (<10 mm) adenomas	0.3922	0.2588	0.2967
Multiple adenomas both sides of colon	0.0007	0.0014	0.0011
Multiple large adenomas both sides of colon	0.1109	0.1311	0.1303
Multiple small adenomas both sides of colon	0.0000	0.0003	0.0002
Right colon adenomas	0.0146	0.0205	0.4820
Right colon large adenomas	0.1646	0.2787	0.5277
Right colon small adenomas	0.0540	0.0442	0.7130
Colorectal cancer	0.0896	0.2740	0.5375
All cancer (CRC & malignant polyps)	0.0589	0.2011	0.5082
Disease (all polyps excl. hyperplastic, & CRC)	0.0003	0.0004	0.0089
All polyps	0.3963	0.8082	0.4052
Adenomas (excl. sessile serrated adenomas)	0.5739	0.9872	0.5154
Left colon adenomas	0.6931	0.9610	0.5565
Left colon large (≥10 mm) pedunc. adenomas	0.2218	0.2319	0.7278
Left colon large sessile adenomas	0.4104	0.1998	0.5456
Left colon small (<10 mm) adenomas	0.8455	0.5659	0.9351
Multiple adenomas both sides of colon	0.5868	0.3292	0.5378
Right colon adenomas	0.1515	0.4986	0.8791
Right colon small adenomas	0.4990	0.9643	0.6528
Disease (all polyps excl. hyperplastic, & CRC)	0.4649	0.9632	0.6364

Pool A – microRNA 186

Disease group	MiR-186	Δ MiR-186 – miR-484	Δ MiR-186 – miR-U6
All polyps	**0.0004**	**0.0023**	**0.0039**
Adenomas (excl. sessile serrated adenomas)	**0.0008**	**0.0029**	**0.0039**
Left colon adenomas	**0.0381**	0.0616	**0.0412**
Left colon large (≥10 mm) pedunc. adenomas	0.1051	0.1974	0.2017
Left colon large sessile adenomas	0.2572	0.2431	0.2255
Left colon small (<10 mm) adenomas	0.2477	0.2733	0.1765
Multiple adenomas on both sides of colon	**0.0226**	**0.0261**	**0.0036**
Multiple large adenomas both sides of colon	0.3540	0.3482	0.1555
Multiple small adenomas both sides of colon	**0.0047**	**0.0070**	**0.0009**
Right colon adenomas	**0.0041**	**0.0105**	0.3247
Right colon large adenomas	**0.0140**	**0.0278**	0.0890
Right colon small adenomas	0.0684	0.1027	0.9980
Sessile serrated adenomas	0.2330	0.0952	0.0997
Colorectal cancer	0.5714	0.9690	0.9577
All cancer (CRC & malignant polyps)	0.4450	0.8926	0.8723
Disease (all polyps excl. hyperplastic, & CRC)	**0.0008**	**0.0044**	**0.0075**

Disease group	MiR-186	Δ MiR-186 – miR-484	Δ MiR-186 – miR-U6
All polyps	0.0005	0.0009	0.0113
Adenomas (excl. sessile serrated adenomas)	0.0002	0.0004	0.0051
Left colon adenomas	0.0150	0.0189	0.0411
Left colon large (≥10 mm) pedunc. adenomas	0.0487	0.1007	0.0878
Left colon large sessile adenomas	0.1748	0.1779	0.4337
Left colon small (<10 mm) adenomas	0.2684	0.2152	0.2260
Multiple adenomas both sides of colon	0.0078	0.0096	0.0085
Multiple large adenomas both sides of colon	0.1979	0.1922	0.2836
Multiple small adenomas both sides of colon	0.0033	0.0080	0.0002
Right colon adenomas	0.0053	0.0049	0.2312
Right colon large adenomas	0.0769	0.1165	0.3333
Right colon small adenomas	0.0383	0.0263	0.4592
Colorectal cancer	0.3601	0.6070	0.7798
All cancer (CRC & malignant polyps)	0.3656	0.5931	0.8041
Disease (all polyps excl. hyperplastic, & CRC)	0.0006	0.0009	0.0102
All polyps	0.1178	0.3004	0.1721
Adenomas (excl. sessile serrated adenomas)	0.3528	0.6453	0.3561
Left colon adenomas	0.6906	0.9251	0.5611
Left colon large (≥10 mm) pedunc. adenomas	0.9185	0.9744	0.5444
Left colon large sessile adenomas	0.8652	0.9439	0.3859
Left colon small (<10 mm) adenomas	0.6751	0.9413	0.6064
Multiple adenomas both sides of colon	0.7058	0.7448	0.2500
Right colon adenomas	0.2525	0.5163	0.8286
Right colon small adenomas	0.7990	0.8322	0.5712
Disease (all polyps excl. hyperplastic, & CRC)	0.2223	0.5358	0.3868

Pool A – microRNA 331-5p

Disease group	MiR-331-5p	Δ MiR-331-5p – miR-484	Δ MiR-331-5p – miR-U6
All polyps	0.0630	0.1591	0.0957
Adenomas (excl. sessile serrated adenomas)	0.1222	0.2107	0.1104
Left colon adenomas	0.3074	0.3587	0.1814
Left colon large (≥10 mm) pedunc. adenomas	0.3062	0.4589	0.4072
Left colon large sessile adenomas	0.2850	0.3263	0.2471
Left colon small (<10 mm) adenomas	0.9330	0.9771	0.5274
Multiple adenomas on both sides of colon	0.7211	0.8276	0.0817
Multiple large adenomas both sides of colon	0.5726	0.5392	0.6358
Multiple small adenomas both sides of colon	0.0774	0.1829	**0.0242**
Right colon adenomas	**0.0142**	0.0731	0.7319
Right colon large adenomas	0.0692	0.1979	0.2161
Right colon small adenomas	0.0722	0.1825	0.8624
Sessile serrated adenomas	0.0625	**0.0077**	**0.0181**
Colorectal cancer	0.2257	0.5747	0.7828
All cancer (CRC & malignant polyps)	0.2793	0.5967	0.8825
Disease (all polyps excl. hyperplastic, & CRC)	0.0612	0.1536	0.1032

Disease group	MiR-331-5p	Δ MiR-331-5p – miR-484	Δ MiR-331-5p – miR-U6
All polyps	0.0194	0.0180	0.0477
Adenomas (excl. sessile serrated adenomas)	0.0193	0.0138	0.0318
Left colon adenomas	0.0664	0.0515	0.0806
Left colon large (≥10 mm) pedunc. adenomas	0.1251	0.1775	0.1553
Left colon large sessile adenomas	0.1470	0.1840	0.3858
Left colon small (<10 mm) adenomas	0.6052	0.3675	0.3776
Multiple adenomas both sides of colon	0.2972	0.2918	0.0494
Multiple large adenomas both sides of colon	0.8343	0.8285	0.6017
Multiple small adenomas both sides of colon	0.0883	0.0843	0.0031
Right colon adenomas	0.0032	0.0154	0.4565
Right colon large adenomas	0.0971	0.2866	0.4801
Right colon small adenomas	0.0099	0.0251	0.6653
Colorectal cancer	0.2876	0.4563	0.5921
All cancer (CRC & malignant polyps)	0.0464	0.0950	0.2900
Disease (all polyps excl. hyperplastic, & CRC)	0.0151	0.0118	0.0313
All polyps	0.5187	0.9615	0.5448
Adenomas (excl. sessile serrated adenomas)	0.9737	0.6596	0.8053
Left colon adenomas	0.7319	0.5207	0.9145
Left colon large (≥10 mm) pedunc. adenomas	0.9096	0.9887	0.5348
Left colon large sessile adenomas	0.9880	0.8931	0.3932
Left colon small (<10 mm) adenomas	0.4676	0.2417	0.7383
Multiple adenomas both sides of colon	0.6585	0.5660	0.6216
Right colon adenomas	0.3612	0.7455	0.9744
Right colon small adenomas	0.8044	0.8808	0.7206
Disease (all polyps excl. hyperplastic, & CRC)	0.5719	0.9233	0.7540

Pool A – microRNA 452

Disease group	MiR-452	Δ MiR-452 – miR-484	Δ MiR-452 – miR-U6
All polyps	0.1008	0.1982	0.1123
Adenomas (excl. sessile serrated adenomas)	0.2029	0.2851	0.1420
Left colon adenomas	0.5219	0.5412	0.2429
Left colon large (≥10 mm) pedunc. adenomas	0.6150	0.7582	0.5371
Left colon large sessile adenomas	0.7891	0.6620	0.4379
Left colon small (<10 mm) adenomas	0.6095	0.6067	0.3478
Multiple adenomas on both sides of colon	0.2517	0.3084	**0.0300**
Multiple large adenomas both sides of colon	0.8176	0.7712	0.4850
Multiple small adenomas both sides of colon	**0.0251**	**0.0358**	**0.0038**
Right colon adenomas	0.2577	0.3918	0.8812
Right colon large adenomas	0.2271	0.3347	0.3627
Right colon small adenomas	0.7397	0.8570	0.3521
Sessile serrated adenomas	0.5432	0.4510	0.3188
Colorectal cancer	0.6901	0.8430	0.8255
All cancer (CRC & malignant polyps)	0.6371	0.8915	0.7151
Disease (all polyps excl. hyperplastic, & CRC)	0.1893	0.3180	0.1909

Disease group	MiR-452	Δ MiR-452 – miR-484	Δ MiR-452 – miR-U6
All polyps	0.1050	0.0935	0.1462
Adenomas (excl. sessile serrated adenomas)	0.1192	0.0866	0.1206
Left colon adenomas	0.4093	0.2898	0.2470
Left colon large (≥10 mm) pedunc. adenomas	0.7762	0.8091	0.4360
Left colon large sessile adenomas	0.4799	0.3533	0.7055
Left colon small (<10 mm) adenomas	0.5044	0.3935	0.3435
Multiple adenomas both sides of colon	0.0688	0.0767	0.0249
Multiple large adenomas both sides of colon	0.6613	0.6613	0.5615
Multiple small adenomas both sides of colon	0.0160	0.0209	0.0004
Right colon adenomas	0.4608	0.3838	0.7621
Right colon large adenomas	0.6015	0.6401	0.8322
Right colon small adenomas	0.6235	0.4299	0.5142
Colorectal cancer	0.7280	0.9857	0.9220
All cancer (CRC & malignant polyps)	0.4611	0.7026	0.9144
Disease (all polyps excl. hyperplastic, & CRC)	0.1566	0.1208	0.1629
All polyps	0.3199	0.6297	0.3634
Adenomas (excl. sessile serrated adenomas)	0.6588	0.9987	0.5892
Left colon adenomas	0.7869	0.9761	0.6146
Left colon large (≥10 mm) pedunc. adenomas	0.4825	0.4894	0.9881
Left colon large sessile adenomas	0.7082	0.5333	0.4508
Left colon small (<10 mm) adenomas	0.9929	0.7100	0.8463
Multiple adenomas both sides of colon	0.7579	0.6146	0.5968
Right colon adenomas	0.3171	0.6397	0.8965
Right colon small adenomas	0.9857	0.6489	0.5562
Disease (all polyps excl. hyperplastic, & CRC)	0.4711	0.8790	0.6265

Pool A – microRNA 625

Disease group	MiR-625	Δ MiR-625 – miR-484	Δ MiR-625 – miR-U6
All polyps	**0.0010**	**0.0030**	**0.0026**
Adenomas (excl. sessile serrated adenomas)	**0.0049**	**0.0092**	**0.0060**
Left colon adenomas	0.1435	0.1691	0.0883
Left colon large (≥10 mm) pedunc. adenomas	0.1468	0.2225	0.1913
Left colon large sessile adenomas	0.4067	0.3806	0.2861
Left colon small (<10 mm) adenomas	0.5488	0.5451	0.3653
Multiple adenomas on both sides of colon	**0.0130**	**0.0142**	**0.0015**
Multiple large adenomas both sides of colon	0.1616	0.1799	0.0907
Multiple small adenomas both sides of colon	**0.0205**	**0.0105**	**0.0002**
Right colon adenomas	**0.0049**	**0.0082**	0.2639
Right colon large adenomas	**0.0117**	**0.0218**	0.0800
Right colon small adenomas	0.0712	0.0812	0.9348
Sessile serrated adenomas	0.1835	0.1180	0.1160
Colorectal cancer	0.1494	0.3224	0.4831
All cancer (CRC & malignant polyps)	0.0649	0.1922	0.4215
Disease (all polyps excl. hyperplastic, & CRC)	**0.0018**	**0.0050**	**0.0046**

Disease group	MiR-625	Δ MiR-625 – miR-484	Δ MiR-625 – miR-U6
All polyps	0.0060	0.0056	0.0151
Adenomas (excl. sessile serrated adenomas)	0.0083	0.0066	0.0145
Left colon adenomas	0.1239	0.1059	0.1107
Left colon large (≥10 mm) pedunc. adenomas	0.1213	0.1766	0.1092
Left colon large sessile adenomas	0.5144	0.4444	0.6881
Left colon small (<10 mm) adenomas	0.4587	0.3756	0.3555
Multiple adenomas both sides of colon	0.0111	0.0123	0.0060
Multiple large adenomas both sides of colon	0.1987	0.2127	0.2407
Multiple small adenomas both sides of colon	0.0041	0.0032	0.0000
Right colon adenomas	0.0369	0.0272	0.4310
Right colon large adenomas	0.0680	0.1102	0.3059
Right colon small adenomas	0.1714	0.1144	0.8658
Colorectal cancer	0.1546	0.2726	0.4203
All cancer (CRC & malignant polyps)	0.1526	0.2618	0.4330
Disease (all polyps excl. hyperplastic, & CRC)	0.0065	0.0055	0.0138
All polyps	0.0464	0.1079	0.0656
Adenomas (excl. sessile serrated adenomas)	0.1880	0.3231	0.1884
Left colon adenomas	0.6115	0.7657	0.4948
Left colon large (≥10 mm) pedunc. adenomas	0.6535	0.6792	0.9432
Left colon large sessile adenomas	0.6067	0.6053	0.1721
Left colon small (<10 mm) adenomas	0.9967	0.7966	0.8800
Multiple adenomas both sides of colon	0.4688	0.4462	0.1418
Right colon adenomas	0.0546	0.1095	0.4454
Right colon small adenomas	0.2479	0.4154	0.9682
Disease (all polyps excl. hyperplastic, & CRC)	0.0808	0.1897	0.1475

Pool B – microRNA 222#

Disease group	MiR-222#	∆ MiR-222# – miR-U6
All polyps	0.7257	0.8784
Adenomas (excl. sessile serrated adenomas)	0.4548	0.7760
Left colon adenomas	0.3815	0.4745
Left colon large (≥10 mm) pedunc. adenomas	0.4598	0.6202
Left colon large sessile adenomas	0.5464	0.4518
Left colon small (<10 mm) adenomas	**0.0496**	**0.0365**
Multiple adenomas on both sides of colon	0.5931	0.5820
Multiple large adenomas both sides of colon	0.8445	0.7611
Multiple small adenomas both sides of colon	0.2724	0.5802
Right colon adenomas	0.9967	0.3419
Right colon large adenomas	0.3178	0.4929
Right colon small adenomas	0.4594	0.5066
Sessile serrated adenomas	0.3429	0.4250
Colorectal cancer	0.7014	0.8352
All cancer (CRC & malignant polyps)	0.4258	0.5768
Disease (all polyps excl. hyperplastic, & CRC)	0.7975	0.9348

Disease group	MiR-222#	∆ MiR-222# – miR-U6
All polyps	0.2317	0.2484
Adenomas (excl. sessile serrated adenomas)	0.1877	0.1808
Left colon adenomas	0.1354	0.1608
Left colon large (≥10 mm) pedunc. adenomas	0.2761	0.4119
Left colon large sessile adenomas	0.9748	0.9490
Left colon small (<10 mm) adenomas	0.0021	0.0001
Multiple adenomas both sides of colon	0.5249	0.3975
Multiple large adenomas both sides of colon	0.7126	0.4092
Multiple small adenomas both sides of colon	0.5428	0.7030
Right colon adenomas	0.5778	0.6291
Right colon large adenomas	0.6169	0.8600
Right colon small adenomas	0.3446	0.6217
Colorectal cancer	0.5814	0.9949
All cancer (CRC & malignant polyps)	0.9391	0.5120
Disease (all polyps excl. hyperplastic, & CRC)	0.3397	0.2583
All polyps	0.3966	0.1662
Adenomas (excl. sessile serrated adenomas)	0.7221	0.3428
Left colon adenomas	0.6470	0.6847
Left colon large (≥10 mm) pedunc. adenomas	0.7795	0.9698
Left colon large sessile adenomas	0.5758	0.4874
Left colon small (<10 mm) adenomas	0.8850	0.9102
Multiple adenomas both sides of colon	0.7393	0.8021
Right colon adenomas	0.7282	0.1668
Right colon small adenomas	0.8858	0.2787
Disease (all polyps excl. hyperplastic, & CRC)	0.5085	0.1953

Pool B – microRNA 664

Disease group	MiR-664	∆ MiR-664 – miR-U6
All polyps	0.8382	0.3126
Adenomas (excl. sessile serrated adenomas)	0.9466	0.4416
Left colon adenomas	0.5105	0.8089
Left colon large (≥10 mm) pedunc. adenomas	0.0634	0.1629
Left colon large sessile adenomas	0.3891	0.4501
Left colon small (<10 mm) adenomas	0.9884	0.9277
Multiple adenomas on both sides of colon	0.7836	0.9214
Multiple large adenomas both sides of colon	0.4705	**0.0225**
Multiple small adenomas both sides of colon	0.8015	0.1246
Right colon adenomas	0.2753	**0.0442**
Right colon large adenomas	0.3292	0.7583
Right colon small adenomas	0.4977	**0.0326**
Sessile serrated adenomas	0.2437	0.4003
Colorectal cancer	0.8197	0.1742
All cancer (CRC & malignant polyps)	0.9198	0.6634
Disease (all polyps excl. hyperplastic, & CRC)	0.8749	0.4747

Disease group	MiR-664	Δ MiR-664 – miR-U6
All polyps	0.6262	0.9675
Adenomas (excl. sessile serrated adenomas)	0.7679	0.9231
Left colon adenomas	0.3229	0.6195
Left colon large (≥10 mm) pedunc. adenomas	0.0571	0.2384
Left colon large sessile adenomas	0.7104	0.6627
Left colon small (<10 mm) adenomas	0.9382	0.9611
Multiple adenomas both sides of colon	0.8248	0.7370
Multiple large adenomas both sides of colon	0.5236	0.0214
Multiple small adenomas both sides of colon	0.8129	0.3590
Right colon adenomas	0.3522	0.2962
Right colon large adenomas	0.1816	0.6357
Right colon small adenomas	0.8347	0.3650
Colorectal cancer	0.9292	0.2206
All cancer (CRC & malignant polyps)	0.8280	0.1763
Disease (all polyps excl. hyperplastic, & CRC)	0.8646	0.8834
All polyps	0.3697	0.1645
Adenomas (excl. sessile serrated adenomas)	0.8070	0.3764
Left colon adenomas	0.8707	0.9913
Left colon large (≥10 mm) pedunc. adenomas	0.6074	0.3483
Left colon large sessile adenomas	0.4615	0.6265
Left colon small (<10 mm) adenomas	0.9259	0.8809
Multiple adenomas both sides of colon	0.8077	0.9723
Right colon adenomas	0.6239	0.0856
Right colon small adenomas	0.4672	0.0378
Disease (all polyps excl. hyperplastic, & CRC)	0.6394	0.2581

Pool B – microRNA 1247

Disease group	MiR-1247	∆ MiR-1247 – miR-U6
All polyps	0.3848	**0.0455**
Adenomas (excl. sessile serrated adenomas)	0.3734	**0.0470**
Left colon adenomas	0.8667	0.4164
Left colon large (≥10 mm) pedunc. adenomas	0.3712	0.9067
Left colon large sessile adenomas	0.2163	0.3216
Left colon small (<10 mm) adenomas	0.7016	0.6110
Multiple adenomas on both sides of colon	0.1369	**0.0430**
Multiple large adenomas both sides of colon	0.1491	0.3780
Multiple small adenomas both sides of colon	0.6195	**0.0101**
Right colon adenomas	0.8245	0.1661
Right colon large adenomas	**0.0389**	0.2421
Right colon small adenomas	0.3800	0.2302
Sessile serrated adenomas	0.6279	0.9472
Colorectal cancer	0.8853	0.1398
All cancer (CRC & malignant polyps)	0.6894	0.6859
Disease (all polyps excl. hyperplastic, & CRC)	0.3446	0.0949

Disease group	MiR-1247	∆ MiR-1247 – miR-U6
All polyps	0.3641	0.0978
Adenomas (excl. sessile serrated adenomas)	0.2355	0.0708
Left colon adenomas	0.6296	0.1586
Left colon large (≥10 mm) pedunc. adenomas	0.6453	0.3690
Left colon large sessile adenomas	0.5011	0.3913
Left colon small (<10 mm) adenomas	0.0769	0.1226
Multiple adenomas both sides of colon	0.1662	0.0854
Multiple large adenomas both sides of colon	0.2595	0.3777
Multiple small adenomas both sides of colon	0.3736	0.0251
Right colon adenomas	0.6277	0.4816
Right colon large adenomas	0.0875	0.4631
Right colon small adenomas	0.6107	0.5844
Colorectal cancer	0.4417	0.2733
All cancer (CRC & malignant polyps)	0.7504	0.1922
Disease (all polyps excl. hyperplastic, & CRC)	0.2193	0.1967
All polyps	0.5538	0.2251
Adenomas (excl. sessile serrated adenomas)	0.8536	0.3143
Left colon adenomas	0.9646	0.8067
Left colon large (≥10 mm) pedunc. adenomas	0.5736	0.1911
Left colon large sessile adenomas	0.1984	0.5420
Left colon small (<10 mm) adenomas	0.4429	0.5129
Multiple adenomas both sides of colon	0.3902	0.3043
Right colon adenomas	0.8316	0.2391
Right colon small adenomas	0.5039	0.3028
Disease (all polyps excl. hyperplastic, & CRC)	0.7865	0.2554

Other microRNA targets

Disease group	Pool A miR-484	Pool A miR-U6	Pool B miR-U6
All polyps	0.6331	0.5299	0.3050
Adenomas (excl. sessile serrated adenomas)	0.8596	0.4155	0.3036
Left colon adenomas	0.8994	0.3288	0.5396
Left colon large (≥10 mm) pedunc. adenomas	0.7851	0.7597	0.3967
Left colon large sessile adenomas	0.6739	0.4614	0.9008
Left colon small (<10 mm) adenomas	0.8184	0.3044	0.8796
Multiple adenomas on both sides of colon	0.7432	0.0626	0.7748
Multiple large adenomas both sides of colon	0.8771	0.1999	0.2187
Multiple small adenomas both sides of colon	0.7506	0.1505	0.0790
Right colon adenomas	0.7029	0.2631	0.3143
Right colon large adenomas	0.7327	0.9914	0.2289
Right colon small adenomas	0.8186	0.2233	0.1793
Sessile serrated adenomas	0.9394	0.3782	0.0856
Colorectal cancer	0.2427	0.4714	0.1159
All cancer (CRC & malignant polyps)	0.2215	0.3307	0.4330
Disease (all polyps excl. hyperplastic, & CRC)	0.6470	0.5791	0.4876

Disease group	Pool A miR-484	Pool A miR-U6	Pool B miR-U6
All polyps	0.5933	0.6457	0.4989
Adenomas (excl. sessile serrated adenomas)	0.4080	0.4872	0.5733
Left colon adenomas	0.4097	0.3857	0.3784
Left colon large (≥10 mm) pedunc. adenomas	0.9913	0.4880	0.2015
Left colon large sessile adenomas	0.4201	0.9621	0.9387
Left colon small (<10 mm) adenomas	0.3444	0.3037	0.9587
Multiple adenomas both sides of colon	0.8889	0.1438	0.9631
Multiple large adenomas both sides of colon	0.9577	0.6485	0.4234
Multiple small adenomas both sides of colon	0.8794	0.0633	0.3898
Right colon adenomas	0.5410	0.2184	0.8089
Right colon large adenomas	0.8163	0.7304	0.1803
Right colon small adenomas	0.5556	0.2073	0.4940
Colorectal cancer	0.4346	0.6113	0.0527
All cancer (CRC & malignant polyps)	0.4764	0.5750	0.1022
Disease (all polyps excl. hyperplastic, & CRC)	0.5013	0.5665	0.9557
All polyps	0.2481	0.7763	0.5132
Adenomas (excl. sessile serrated adenomas)	0.2686	0.7798	0.4366
Left colon adenomas	0.4650	0.7270	0.8327
Left colon large (≥10 mm) pedunc. adenomas	0.8711	0.4083	0.6105
Left colon large sessile adenomas	0.7642	0.4170	0.9845
Left colon small (<10 mm) adenomas	0.3374	0.7813	0.9219
Multiple adenomas both sides of colon	0.7642	0.3578	0.7103
Right colon adenomas	0.1496	0.6314	0.2908
Right colon small adenomas	0.3217	0.5631	0.2870
Disease (all polyps excl. hyperplastic, & CRC)	0.1772	0.9825	0.4064

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