Are circulating miRNAs predictive of response to therapy?

Thesis submitted for the Degree of

Doctor of Medicine

at the University of Leicester

By

Mr Lava Krishna Kannappa (MBBS, MRCS)

Leicester Cancer Research Centre

University of Leicester

2019

Abstract

Introduction: Colorectal cancer (CRC) is the third commonest cancer with nearly 1.4 million new cases identified throughout the world in 2012. There is a pressing need for new non-invasive blood based test to improve early detection and monitoring of CRC. MiRNAs are small non-coding RNAs involved in fundamental cell processes such as proliferation, survival and death. Studies have identified miRNAs in plasma of cancer patients in a stable form. This study aimed to evaluate whether circulating microRNAs are predictive of response to therapy.

Methods: 44 patients with CRC were selected from our institution's CRC surveillance programme. All selected patients at follow-up had no evidence of tumour recurrence on clinical, radiological and endoscopic assessment. Blood samples were obtained pre-treatment and at a median follow-up of 36 months. A total of 32 pairs of blood samples were matched pre- and post-treatment. Plasma RNA was extracted and target miRNAs were identified on pooled case TaqMan Low Density miRNA array (TDLA) cards and quantitative RT-PCR.

Results: Of the nine microRNAs tested, only miR-134 (P = 0.03), miR-135b (P = 0.03) and miR-431 (P = 0.031) were statistically different in post-treatment samples using a Wilcoxon signed rank test. Comparison of each miRNA with clinicopathological features using multiple linear regression tests showed miR-135b pre-treatment and miR-431 post-treatment levels to be significantly associated with both node status (positive/negative) and number of nodes involved. Pre-treatment miR-132, miR-134, miR-21, miR-27b and miR-184 were also significantly associated with node status. Further, miR-134 post-treatment was significantly associated with gender and miR-203 pre-treatment was significantly associated with all Duke's stages. However, multiple-linear regression of **all** miRNAs and clinicopathological features revealed only miR-135b levels pre-treatment to be significant in the overall model (P = 0.043).

Conclusion: MicroRNA levels of miR-134, miR-135b and miR-431 showed a potential response to therapy with higher levels pre-treatment and lower after treatment. miR-135b pre-treatment levels correlated significantly to lymph node status and number. However, larger cohorts of patients are needed to validate these findings.

Acknowledgements

First and foremost, I would like to express my sincere gratitude to Dr David Guttery for his dedication and commitment. I have learnt a lot academically and personally from Dr Guttery.

I would like to thank Dr Howard Pringle and Mr Baljit Singh for their unwavering support, mentorship, help and encouragement throughout my MD.

I would like to extend my thanks to Mr John Jamieson and Professor Donald Jones for their guidance and support over the years of my study.

I would like to thank Purnima at the Leicester General Hospital for helping me obtain the patients details, all the endoscopy departmental staff at the reception who with no expectations, helped me in arranging the patient's appointments during weekdays and even opened up the library during weekends when no spare room was available for consultation.

During my research, I had to recruit all the patients at the Leicester General Hospital and perform the lab work in Leicester Royal Infirmary. I would like to thank Angie Gillies, Linda Potter and Janine Moreton for their support in the lab and the numerous Leicester General Hospital NHS Staff, who helped me by providing vacutainers, yellow sharps bins and all other materials for blood collections for recruitment of patients, all of which were essential for me to complete blood sampling.

To my two kids Kanishk Krishna and Akshay Krishna, who are my inspiration and happiness. Finally, I would like to dedicate this to my parents, who have always been there for me and have supported me all my life.

List of Abbreviations

AKT2	V-Akt Murine Thymoma Viral Oncogene Homolog 2
ALS2CR2	STE20-Related Kinase Adaptor Beta
APC	Adenomatous Polyposis Coli
BCL2	B-Cell CLL/Lymphoma 2
BCLXL	BCL2-Like 1
CA19-9	Carbohydrate Antigen 19-9
CCND1	Cyclin D1
CDC25A	Cell Division Cycle 25A
cDNA	Complimentary DNA
CEA	Carcinoembryonic Antigen
CHFR	Checkpoint with Forkhead and Ring Finger Domains
CIMP	CpG Island Methylator Phenotype Pathway
CIN	Chromosomal Instability
CRC	Colorectal cancer
Ct	Cycle threshold
CTC	Computed Tomographic Colonography
CTNNB1	Catenin (Cadherin-Associated Protein), Beta 1, 88kDa
DNA	Deoxyribonucleic acid
EGFR	Epidermal Growth Factor Receptor
FFPE	Formalin-Fixed, Paraffin-Embedded
FIT	Faecal Immunohistochemical Test
FOB	Faecal Occult Blood Test
FOXO1	Forkhead Box O1
FOXO3A	Forkhead Box O3A
FS	Flexible Sigmoidoscopy
gFOBT	Guaiac FOB
HDI	Human Development Index
HNPCC	Hereditary Non-Polyposis CRC
IGF1R	Insulin-Like Growth Factor 1 Receptor
IRS1	Insulin Receptor Substrate 1

KRAS	Kirsten Rat Sarcoma Viral Oncogene Homologue
LOH	Loss of Heterozygosity
MAC	Modified Astler-Coller
MAPK	Mitogen Activated Protein Kinase
miRNA	MicroRNA
MLH1	MutL Homologue 1
MMR	Mismatch Repair
MTOR	Mechanistic Target of Rapamycin
MSH2	MutS Homologue 2
MSH6	MutS Homologue 6
MSI	Microsatellite Instability
NRAS	Neuroblastoma RAS Viral (V-Ras) Oncogene Homolog
PCA	Principal Component Analysis
PDCD4	Programmed Cell Death 4
PDLIM2	PDZ and LIM Domain 2
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIK3CA	PI3K, catalytic subunit alpha
PTEN	Phosphatase and Tensin Homologue
PTP4A1	Protein Tyrosine Phosphatase Type IVA, Member 1
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RAF1	Raf-1 Proto-Oncogene
RBC	Red Blood Cells
RCT	Randomised Control Trial
RNA	Ribonucleic Acid
RQ	Relative Quantification
SAM	Significance Analysis for Microarrays
SIRT1	Sirtuin 1
SMAD4	SMAD Family Member 4
SPHK1	Sphingosine Kinase 1
SPRY2	Sprouty Homologue 2
TAF12	TAF12 RNA polymerase II
Taq	*Thermus Aquaticus*

TEMS	Transanal Endoscopy Microscopic Surgery
TGFβR2	Transforming Growth Factor β Receptor 2
TIAM1	T-cell Lymphoma Invasion and Metastasis 1
TLDA	TaqMan Low Density Array
TNM	Tumour, Node, Metastasis
TP53	Tumour Protein 53
TP53INP1	TP53 Inducible Nuclear Protein 1
VEGFA	Vascular Endothelial Growth Factor A
VEGFC	Vascular Endothelial Growth Factor C
WBC	White Blood Cell
ΔC_T	Delta Cycle Threshold

Table of Contents

List of Figures

List of Tables

Chapter 1: Introduction

Chapter 1: Introduction

1 Colorectal Cancer

1.1 Demographics

Colorectal cancer is third most common cancer diagnosed worldwide accounting for about 1.36 million new cases and 694,000 deaths in 2012 (Ferlay, Soerjomataram et al. 2015). Recent updates from Globocan estimates (http://gco.iarc.fr) an incidence of 1.85 million new cases in 2018 and 880,792 deaths worldwide as shown in the figure below.

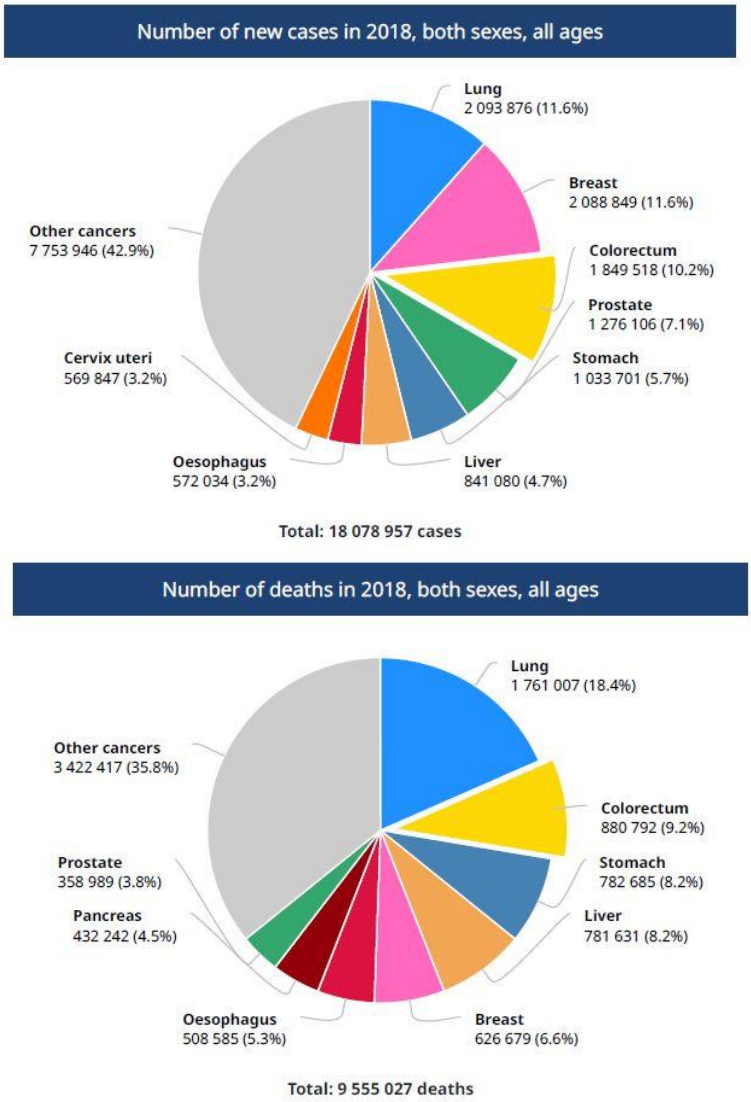


Figure 1: Number of new cases and deaths in 2018 estimated by Globocan. Adapted from Globocan (http://gco.iarc.fr).

The incidence of colorectal cancer (CRC) is expected to increase by 60% to more than 2.2 million new cases and 1.1 million cancer deaths by 2030. The occurrence of CRC varies worldwide, with two-thirds of all cases diagnosed in countries with very high human development index (HDI) (Ferlay et al. 2015), and also accounts for approximately 60% of deaths. The age standardised incidence by sex and mortality rates for colorectal cancer for 2018 is represented in the figure below.

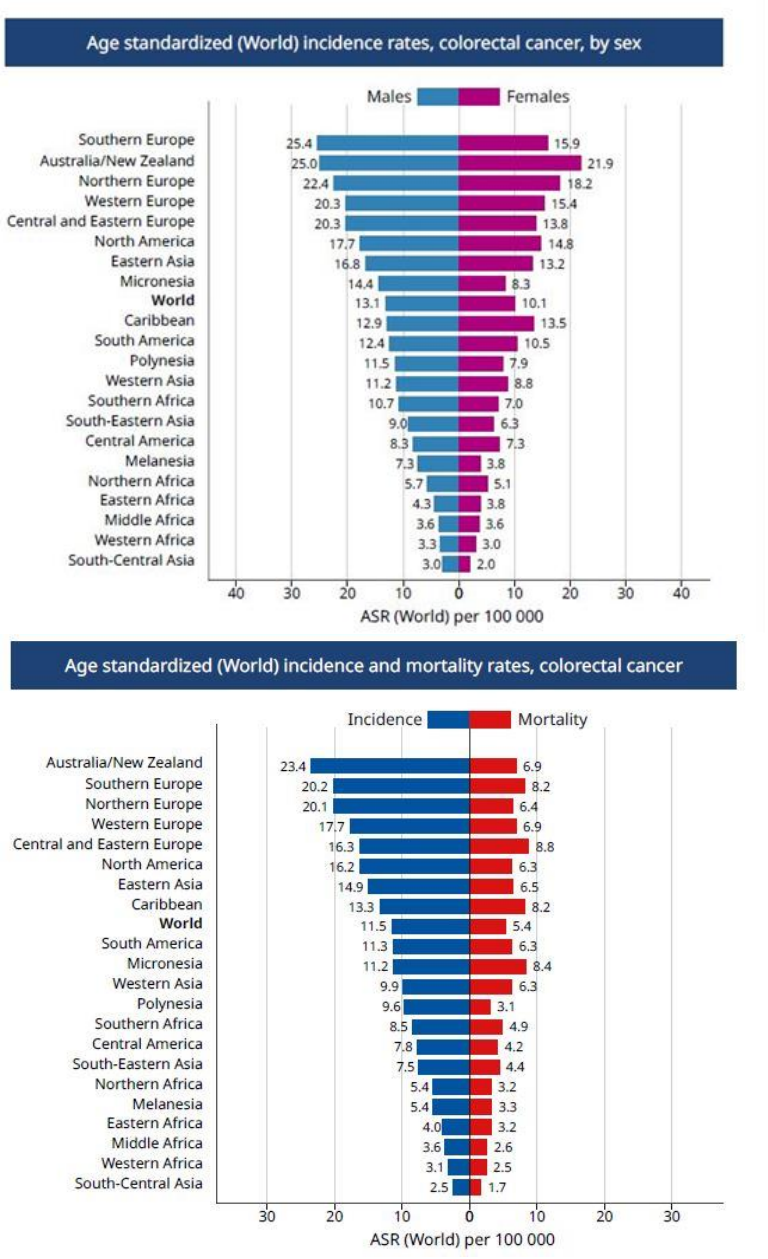


Figure 2: Age standardised incidence and mortality rates reported by Globocan 2018. Adapted from Globocan (http://gco.iarc.fr).

Arnold et al. (2017) identified three important trends worldwide and reported that CRC incidence and mortality are rising rapidly in many low income and middle income countries like Asia and South America, and have attributed this due to social and economic development (Center, Jemal et al. 2009). They also observed that there is a stabilised or decreasing trend in highly developed countries such as the USA, Australia and New Zealand (Arnold, Sierra et al. 2017). The trends observed reflect present human development levels and the adoption of Western lifestyles in the low and middle-income countries and also better screening with early detection of CRC in high income countries. CRC has always been associated with lifestyle risk factors. The continuous update project (CUP) undertaken by world cancer research, which has the largest source of scientific research on cancer prevention reported strong evidence of red meat, processed meat, alcoholic drinks and obesity (https://www.wcrf.org).

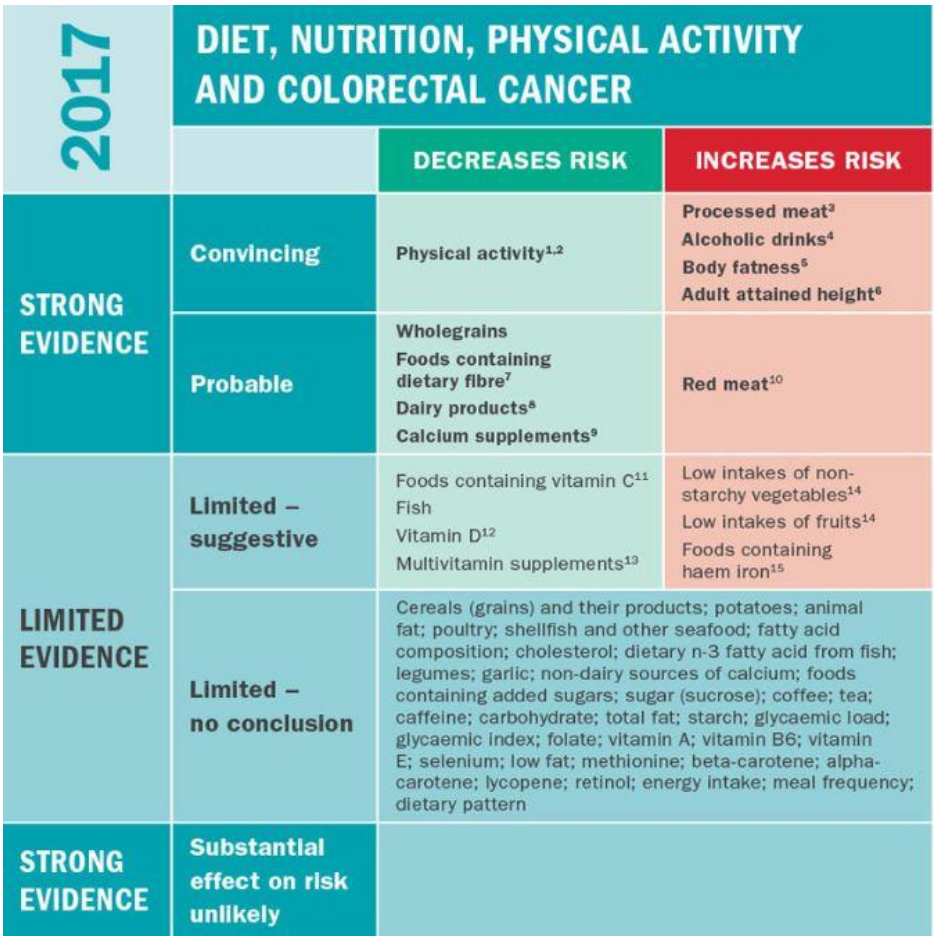


Figure 3: Evidence and risk associated with colorectal cancer. Adapted from World cancer research (https://www.wcrf.org).

1.2 Classification of colorectal cancer

Colorectal cancer has two different staging systems. The most commonly used system is the TNM (Tumour/Node/Metastasis) from the American Joint Committee of Cancer (AJCC). TNM staging (Section 1.2.1 and Figures 4 – 6) is based on three categories, `T' denotes the degree of invasion of the intestinal wall; `N' denotes degree of spread in the lymph node and `M' denotes the degree of metastasis. The TNM value is grouped on the basis of prognosis, with a higher number reflecting a poorer outcome. The other staging system is Duke's staging (Section 1.2.2 and Figure 7) first coined by British pathologist Cuthbert Dukes in 1932. However, this staging has largely been replaced by TNM staging in present practice. Dukes staging was further modified by Astler and Coller in 1954 and Turnbull in 1967 and today is sometimes referred to as Dukes or modified Astler-Coller (MAC). A comparison of features by each classification system is given in Table 1.

1.2.1 TNM classification

Tumour (T)

Tumour (T) describes the size of the tumour (area of cancer).

- **Tis:** Carcinoma in situ: intraepithelial or invasion of lamina propria
- **T1:** Tumour invades submucosa
- **T2:** Tumour invades muscularis propria
- **T3:** Tumour invades through the muscularis propria into pericolorectal tissues
- **T4:** means the tumour has grown through the outer lining of the bowel wall (into another part of the bowel, a nearby organ or structure)
    - **T4a:** Tumour penetrates to the surface of the visceral peritoneum.
    - **T4b:** Tumour directly invades or is adherent to other organs or structures

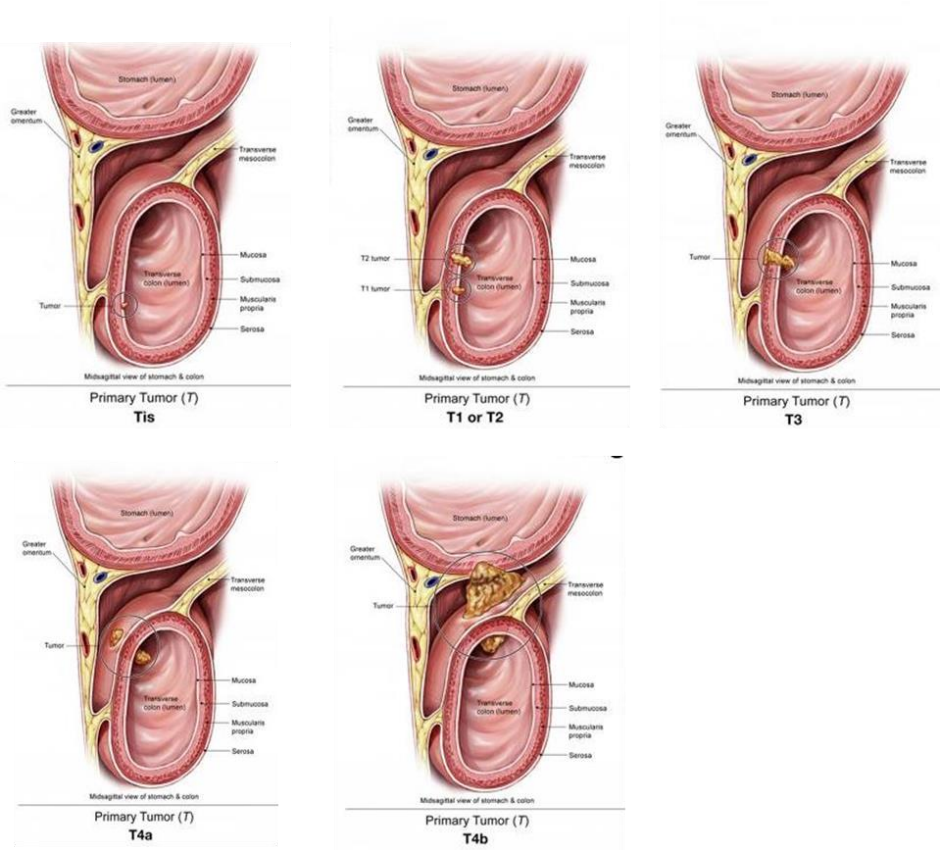


Figure 4: Tumour stages of CRC (http://www.cancer.net)

Node (N)

- N0 means there are no lymph nodes containing cancer cells
- N1 means that 1 to 3 lymph nodes close to the bowel contain cancer cells
- N2 means there are cancer cells in 4 or more nearby lymph nodes

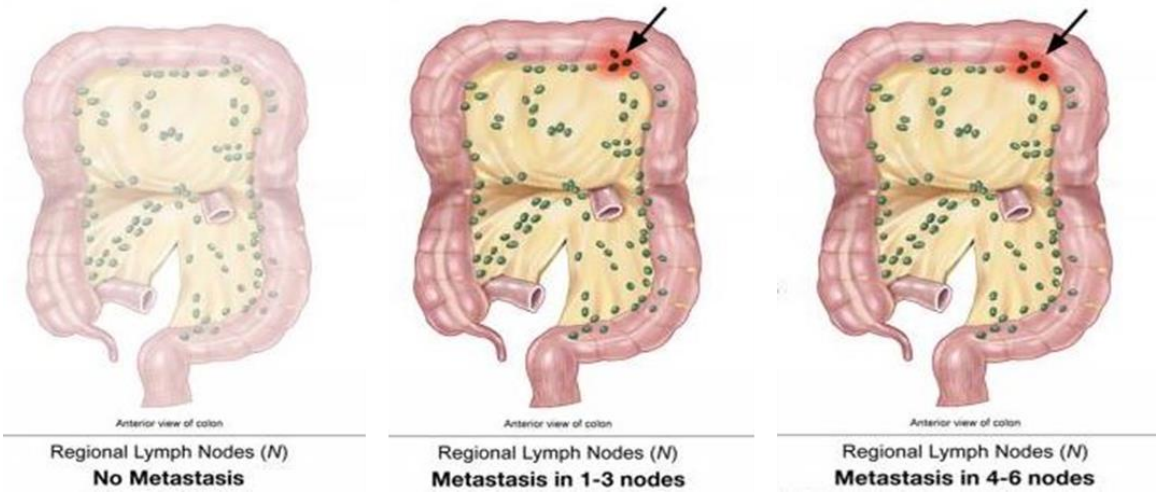


Figure 5: Lymph Node stages of CRC (http://www.cancer.net)

Metastasis (M)

- **M0:** No distant metastasis
- **M1:** Distant metastasis
- **M1a:** Metastasis confined to one organ or site (for example, liver, lung, ovary, Non-regional node
- **M1b:** Metastases in more than one organ/site or the peritoneum

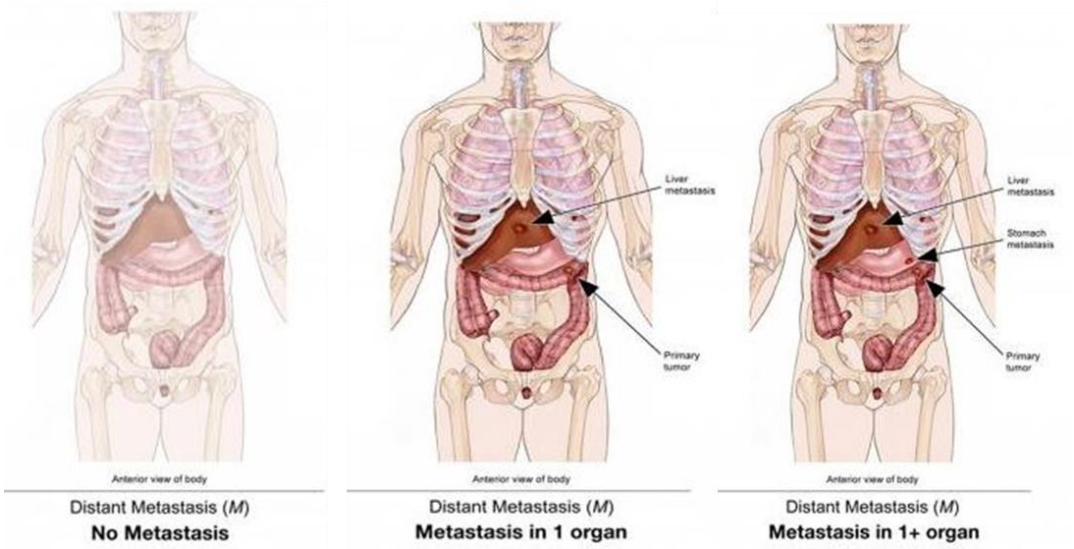


Figure 6: Metastasis stages of CRC. The above picture shows no metastasis (M0), M1a in the second picture and M1b in the third picture above. Picture taken from (http://www.cancer.net).

1.2.2 Dukes' Classification

- Stage A: limited to mucosa
- Stage B1: extending into muscularis propria but not penetrating through it; nodes not involved
- Stage B2**:** penetrating through muscularis propria; nodes not involved
- Stage C1**:** extending into muscularis propria but not penetrating through it; nodes involved
- Stage C2**:** penetrating through muscularis propria; nodes involved
- Stage D: distant metastatic spread

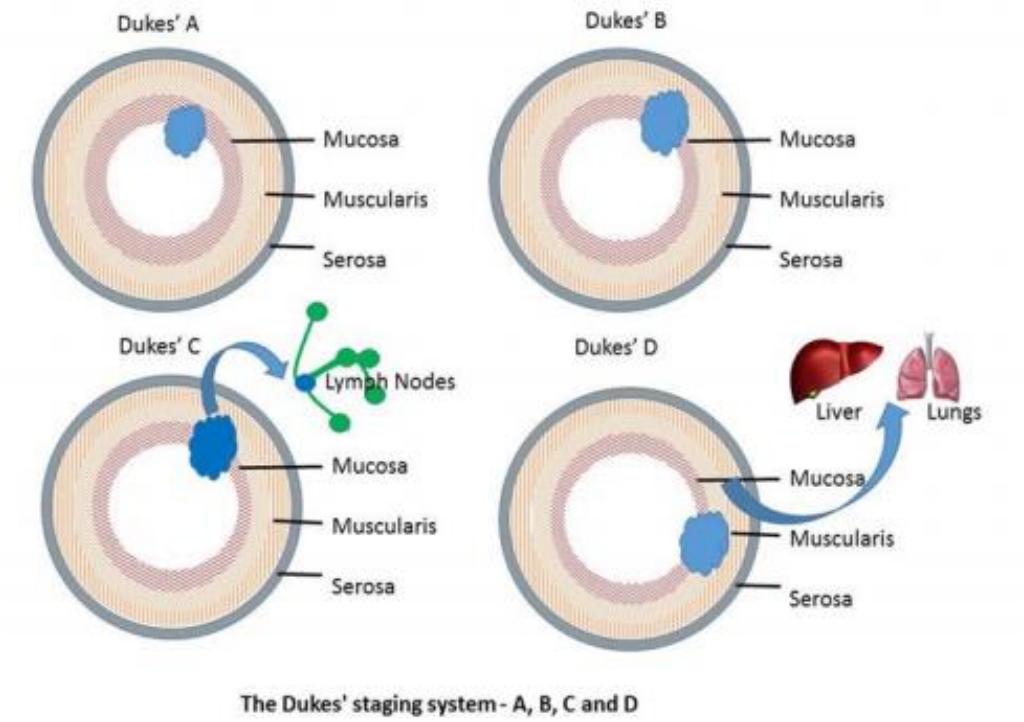


Figure 7: Dukes classification of CRC. The above figure show the simplified classification of Dukes A, B, C & D. The sub-classification of B & C is as described above.

TNM classification (American joint commission on cancer)				**Dukes Classification**
Stages	**T**	**N**	**M**	**Stages**
Stage 0	Tis	NO	M0	
Stage 1	T1	N0	MO	A
	T2	N0	M0	B1
Stage 2	T3	N0	M0	B2
	T4	N0	M0	B2
Stage 3	T1,T2	N1 or N2	M0	C1
	T3,T4	N1 or N2	M0	C2
Stage 4	Ant T	Any N	M1	D

Table 1: TNM and Dukes Staging comparison

1.2.3 Grading

Grading the cancer helps in understanding how quickly it may grow and spread. A low grade cancer may grow more slowly and is less likely to spread than a high grade cancer. Each grade is defined as:

- Grade 1 (low grade) - the cancer cells look similar to normal cells (well differentiated)
- Grade 2 (moderate grade) – the cancer cells look more abnormal (moderately differentiated)
- Grade 3 (high grade) – the cancer cells look very abnormal (poorly differentiated).

1.3 Screening for Colorectal cancer

Bowel cancer screening is the standard screening procedure using a faecal occult blood (FOB) test, sent every 2 years to men and women aged between 60 and 74 years in England, Wales and Northern Ireland, and 50 to 74 years in Scotland. Additional one off flexible sigmoidoscopy tests are being gradually introduced into the UK at the age of 55. If the sigmoidoscopy reveals polyps, then colonoscopy is offered for the patient.

1.3.1 Faecal Occult blood Test (FOB)

FOB is a biannual screening tool used to detect CRC in the UK for all subjects aged 60 and 74 years. Faecal occult blood testing was the first CRC screening test evaluated in randomized controlled trials that began in 1975 in the United States (US) and in 1981 in Europe, with publication in the 1990's (Kronborg, Fenger et al. 1996, Hardcastle, Chamberlain et al. 1996, Mandel, Bond et al. 1993). The mortality reduction was higher for annual FOBT (33%) with rehydrated slides in the US (Mandel, Church et al. 2000) than for the programs with biannual screening (15% and 18%) (Hardcastle et al. 1996, Kronborg et al. 1996). An incidence reduction was also achieved in the US FOBT study for both annual (20%) and biannual screening (18%) after 18 years of follow up (Mandel et al. 2000). Longer-term follow-up to 30 years in this trial showed that

screening with FOBT provided a long term mortality reduction of 32% for annual screening and 18% for biannual screening. The authors attributed the long term sustained effect for CRC mortality reduction as a function of the polypectomy associated with the FOBT screening program (Shaukat, Mongin et al. 2013).

1.3.2 Flexible sigmoidoscopy (FS)

FS screens for adenomas using a flexible endoscope inserted into the distal colon, with the aim of examining at least the rectum and sigmoid and, if possible, as far as the splenic flexure. There are variations in practice with regard to the bowel cleansing preparation required (enema preparation versus full oral bowel cleansing), depth of insertion desired, medications used (none, Entonox, or intravenous sedation), and the threshold for referral for a completion colonoscopy.

There were four randomised control trials (RCTs) from Europe (Atkin, Edwards et al. 2010, Hoff, Grotmol et al. 2009, Segnan, Armaroli et al. 2011) and one from America (Schoen, Pinsky et al. 2012) conducted for groups aged between 55 to 74 years. European trials offered once only FS, unlike America which offered a repeat FS every 3-5 years. Pooled analysis of these studies estimated a risk reduction of 18% for CRC incidence and 28% for deaths from CRC (Brenner, Stock et al. 2014). FS is deemed to be a low-risk procedure (in the three RCTs of FS screening, there were two perforations in almost 54,000 procedures (Atkin, Cook et al. 2002, Gondal, Grotmol et al. 2003, Segnan, Senore et al. 2002)).

1.3.3 Colonoscopy

Examination of the entire colon using a flexible colonoscopy is the “gold standard” investigation for CRC. Screening with colonoscopy has the primary aim of detecting CRC and a secondary aim of detecting and removing adenomas. It allows direct assessment of the entire colonic mucosa, although visualization is rarely 100%.

Although strong evidence backs the use of endoscopic screening, there is currently little proof that colonoscopy is superior to FS in terms of mortality reduction. Two

large-scale RCTs are underway to explore the outcomes of screening colonoscopy (Quintero, Castells et al. 2012, Kaminski, Bretthauer et al. 2012), but no results are expected until the next decade. Cohort studies of patients undergoing colonoscopy and clearance of polyps demonstrate reduction of CRC incidence by up to 90% (Winawer, Zauber et al. 1993, Citarda, Tomaselli et al. 2001), but these are not asymptomatic populations.

In most countries, colonoscopy is used as the second step in the screening process after FS or faecal occult blood testing (with gFOBt or FIT) (Schreuders, Ruco et al. 2015). This is primarily because of cost and resource constraints and has been demonstrated to be a cost-effective measure (Schreuders et al. 2015, Tappenden, Chilcott et al. 2007).

One of the drawbacks of colonoscopy as a screening tool is the potential for complications. These include bleeding, bowel perforation, complications of sedation, and complications of bowel cleansing preparations. Overall, low rates of serious complications have been reported by the English Bowel cancer screening programme (bleeding requiring transfusion, 0.04%; perforation, 0.06%) and the French screening program (overall rate of serious complications, 0.06%)(Rees, Bevan 2013, Logan, Patnick et al. 2012, Leuraud, Jezewski-Serra et al. 2013, Rutter, Nickerson et al. 2014).

1.3.4 Radiology

Computed tomographic colonography (CTC; virtual colonoscopy) may be used to evaluate the bowel for CRC, either as an initial screening modality or after gFOBt/FIT, in the same way as colonoscopy. It requires bowel cleansing preparation with laxatives, or it can be performed with non-laxative bowel preparation with faecal tagging (wherein oral contrast is consumed and digital image manipulation is performed to provide a CT view of the bowel). Carbon dioxide is insufflated into the bowel using a small rectal catheter. In a multicenter RCT comparing CTC with colonoscopy in symptomatic patients, the detection rate of cancer or large polyps was found to be the same in both arms (11%). For lesions over 1 cm in size, CTC performed

comparably to colonoscopy, but for smaller lesions, the sensitivity of CTC dropped to 50% (Levin, Brooks et al. 2003).

CTC has been shown to be more acceptable to patients than barium enemas are, with greater satisfaction, and less pain, nausea, vomiting, and wind (von Wagner, Smith et al. 2011). When CTC was compared to colonoscopy in a randomized trial, the initial satisfaction was higher with the former, but after longer follow-up and subsequent investigations, individuals who underwent initial colonoscopy were more satisfied (von Wagner, Ghanouni et al. 2012). A meta-analysis suggested that symptomatic patients preferred colonoscopy, as opposed to screening patients, who demonstrated a preference for CTC (O. S. Lin, Kozarek et al. 2012).

1.4 Molecular Basis of Colorectal Cancer

Carcinogenesis involves multiple steps resulting from accumulation of genetic and epigenetic mutations transforming normal glandular epithelial cells into adenomas and finally into carcinoma (Vogelstein, Fearon et al. 1988). Loss of genetic stability leads to acquisition of genetic mutations leading to progression to colorectal cancer.

In colon cancer, three distinct pathways of genomic instability have been recognized: 1) Chromosomal Instability (CIN); 2) Microsatellite Instability (MSI) and 3) CpG Island Methylator Phenotype pathway (CIMP) (Figure 8).

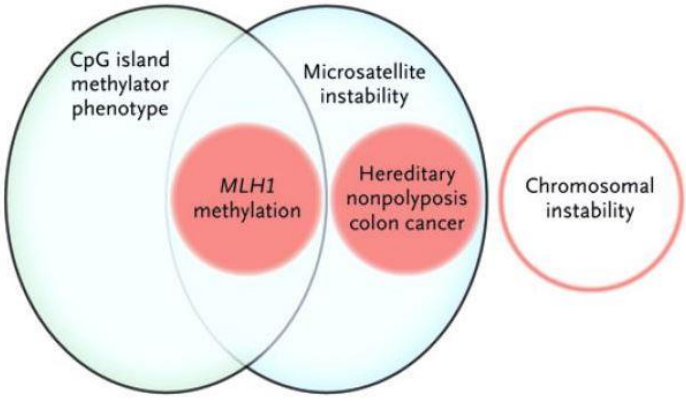


Figure 8: Genetic instability pathways of colon neoplasia: Adapted from (Markowitz, Bertagnolli 2009).

1.4.1 The Chromosomal Instability Pathway

This Chromosomal INstability (CIN) pathway also known as the adenoma-carcinoma sequence is the most common pathway and accounts for 80-85% of all the colorectal cancers arising from adenoma (Pritchard, Grady 2011). The genomic changes include activation of proto-oncogenes (*KRAS*) and inactivation of at least three tumour suppression genes, namely, loss of *APC* (chromosome region 5q21), loss of p53 (chromosome region 17p13) and loss of heterozygosity for the long arm of chromosome 18 (18q LOH). The first step in the pathway is mutation of the *APC* (Adenomatous Polyposis Coli) gene on 5q21 by activating the Wnt signalling pathway resulting in the formation of aberrant crypt focus. Further progression to early adenoma to large adenomas and early cancer requires mutations in *KRAS, TP53* and chromosome 18q. Mutation of *PIK3CA* occurs late in the pathway in a small proportion of colorectal cancers. The mechanisms leading to chromosome instability can be due to chromosome segregation, telomere regulation and DNA damage response which in turn causes germline and somatic mutations and gene amplification and over expression (Pino, Chung 2010). This is a simplified version describing the pathway as shown in the figure below (Figure 9).

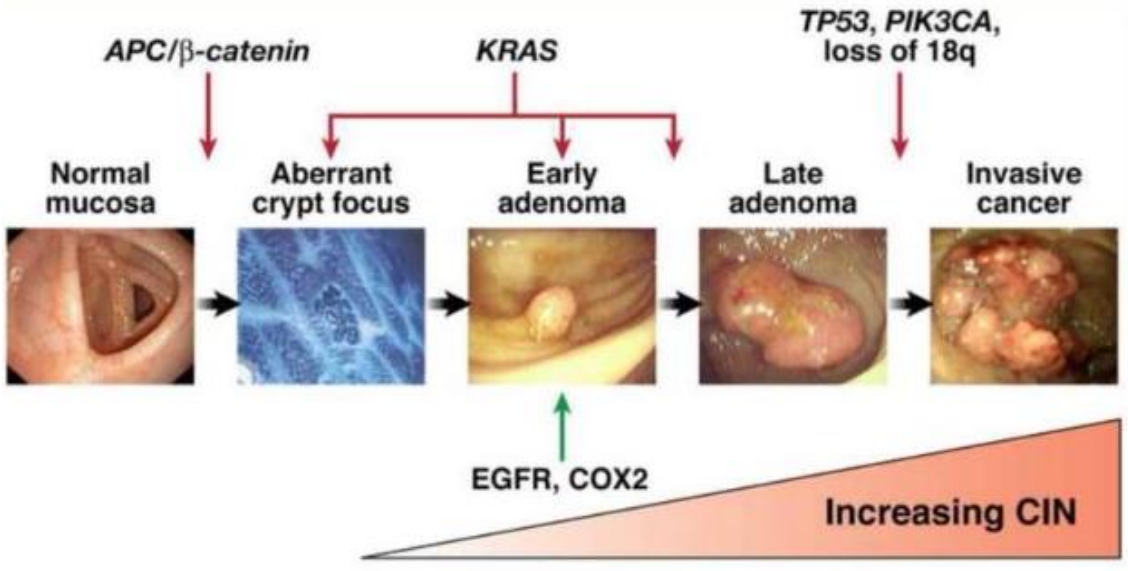


Figure 9: Multistep genetic model of Carcinogenesis of Colorectal cancer showing the formation of aberrant crypt foci (ACF) initially followed by activation of Wnt signalling pathway due to mutations in *APC* gene. Further progression to larger adenomas and early carcinoma requires activating mutations of the proto-oncogene *KRAS*, mutations in *TP53*, and loss of

heterozygosity at chromosome 18q. Activation of the mutated *PIK3CA* occurs late in the adenoma–carcinoma sequence in a small proportion of colorectal cancers. Adapted from (Pino, Chung 2010).

1.4.2 Microsatellite Instability (MSI)

MicroSatellite Instability (MSI) refers to genetic hypermutability due to impaired DNA mismatch repair (MMR). DNA mismatch repair is necessary for maintaining genome stability and integrity, and functions to correct biosynthetic errors, DNA damage surveillance and prevention of recombination of non-identical sequences. Failure of the function leads to cancer (Peltomaki 2003). Mismatch repair (MMR) deficiency is seen in approximately 15% of colorectal cancers and up to 90% of hereditary nonpolyposis colorectal cancer (HNPCC) patients (Findeisen, Kloor et al. 2005). The genes responsible for mismatch repair are called MMR genes (*MLH1*, *MSH2*, *MSH6* or *PMS2)* and inactivation by mutation will result in accumulation of errors in microsatellites causing MSI. Microsatellites are short sequence nucleotides sequences scattered out over the whole genome and are prone to DNA replication errors (Al-Sohaily, Biankin et al. 2012).

The National Cancer Institute guidelines for MSI testing recommend a panel of five microsatellite loci, including three dinucleotide repeat markers (D2S123, D5S346, D17S250) and two mononucleotide repeat markers (BAT 25 and BAT 26). This panel is called the *Bethesda* panel (Buhard, Cattaneo et al. 2006, Kawakami, Zaanan et al. 2015). High-frequency MSI (MSI-H) is defined as instability in two or more of the five markers and low-frequency MSI (MSI-L) is defined as instability in one unstable marker. Microsatellite stable (MSS) status is established when none of the markers shows instability.

When a mismatch is detected, MSH2 binds with MSH6 or MSH3 to form MutSα or MutSβ complexes and MLH1 interacts with PMS2, PMS1 or MLH3 to form MutLα, MutLβ or MutLγ complexes, respectively (Jiricny 2006). Excision of the mismatch is performed by proteins such as exonuclease 1 and proliferating-cell-nuclear antigen, with the complex of MutS and a MutL recognising mismatches and insertion-deletion

loops. Final involvement of the MLH1/PMS2 complex will degrade the mutated stretch and initiates re-synthesis (Boland, Goel 2010, Baretti, Le 2018). The steps of the MSI pathway are as shown in figure 10.

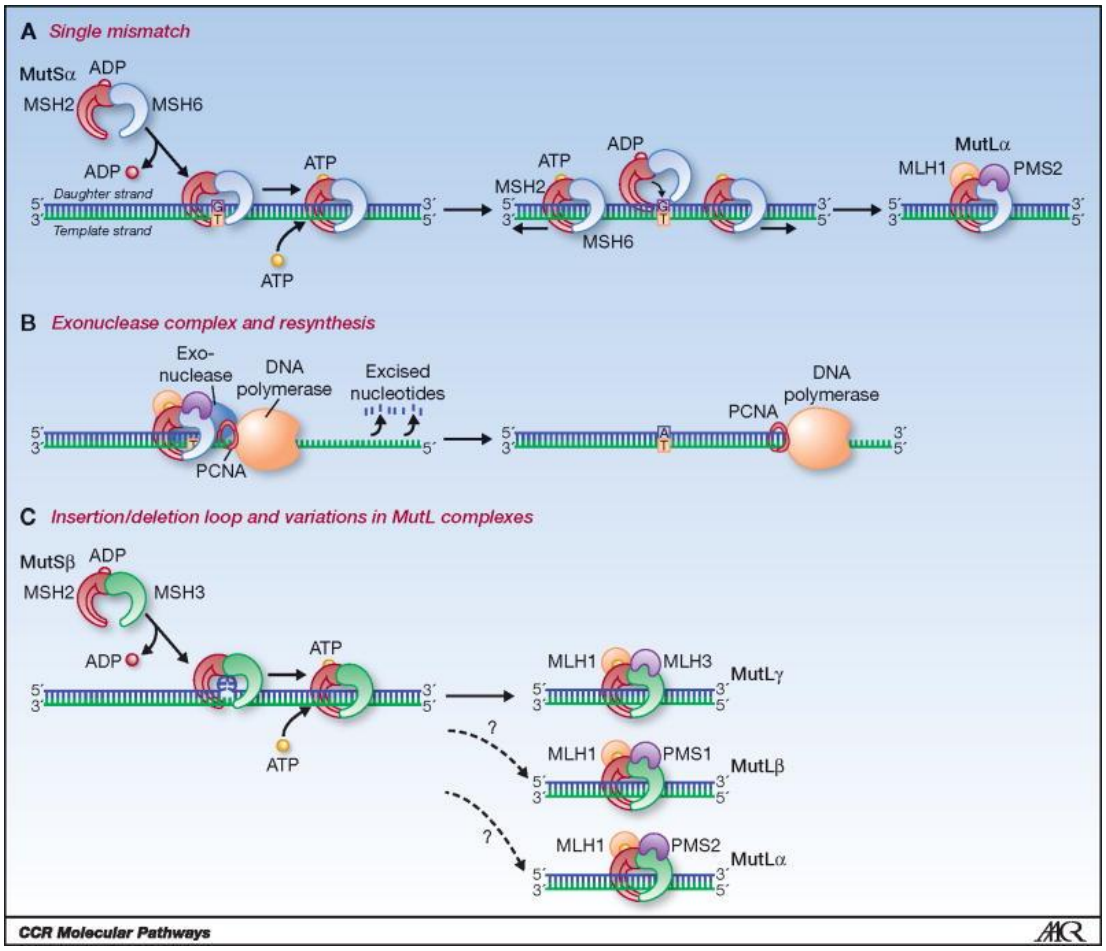


Figure 10: The MSI Pathway: **A)** ***Single mismatch*****:** MSH2–MSH6 (MutS*α*) recognizes single base-pair mismatches, where DNA polymerase has matched the wrong base (G) with the T on the template and creates a sliding clamp around the DNA resulting in the exchange of adenosine triphosphate (ATP) for adenosine diphosphate (ADP) (by MSH2, but not MSH6 or MSH3). The complex is bound by the MLH1-PMS2 (MutL*α*) complex. This "matchmaker" complex moves along the new DNA chain until it encounters the DNA polymerase complex. **B)** ***Exonuclease Complex and resynthesis*****: The** DNA MMR protein interacts with exonuclease-1, proliferating cell nuclear antigen (PCNA), and DNA polymerase forming a complex. This complex excises the daughter strand back to the site of the mismatch and detaches from the DNA leading to resynthesis and correction of error. **C*) Insertion/deletion Loop and variations in MutL Complexes*****:** MSH2–MSH3 (MutS*β*) recognises larger IDLs (Insertion-deletion loops) interacting with different MutL dimers, as MLH1 can dimerize with PMS2, PMS1, or MLH3. The

preferred interaction with MSH2–MSH3 is MLH1–MLH3 (MutL*γ*), but the precise roles of the other MutL heterodimers in this reaction are not entirely understood. Taken from (Boland, Goel 2010, Sinicrope, Sargent 2012).

1.4.3 CpG Island Methylator Phenotype pathway (CIMP)

The CpG Island Methylator Phenotype pathway, also known as epigenetic changes/ epigenetic silencing is another carcinogenic pathway in colorectal cancer and was first reported in 1999 (Toyota, Ahuja et al. 1999). Epigenetics refers to heritable alterations that are not due to changes in the DNA sequence. The epigenetic mechanisms in cancer mainly involve DNA methylation of cytosine bases at CpG islands, histone modification post-transcription, regulation of expression by miRNAs and nucleosome positioning/occupancy. CpG island methylator phenotype (CIMP) refers to hyper-methylation of promoter CpG islands resulting in inactivation of tumour suppressor genes (Lao, Grady 2011). DNA hyper-methylation remains the most studied and dysregulated epigenetic mechanism in colorectal cancer. DNA methylation occurs due to attachment of a methyl group of a cytosine residue to CpG dinucleotides (C-phosphodiester-G bond) sequences (Bird 1986, Cooper, Youssoufian 1988). Most of the CpG dinucleotides are methylated; however, there are also unmethylated CpG rich sequences of the DNA called CpG islands in normal healthy cells (Okugawa, Grady et al. 2015) (Figure 11). CpG islands are found in approximately 70% of the promoter region of the tumour suppressor genes and are involved in regulating gene expression (Deaton, Bird 2011, Saxonov, Berg et al. 2006). Normally, CpG islands are protected from methylation, but can become aberrantly methylated in cancer (Lao, Grady 2011).

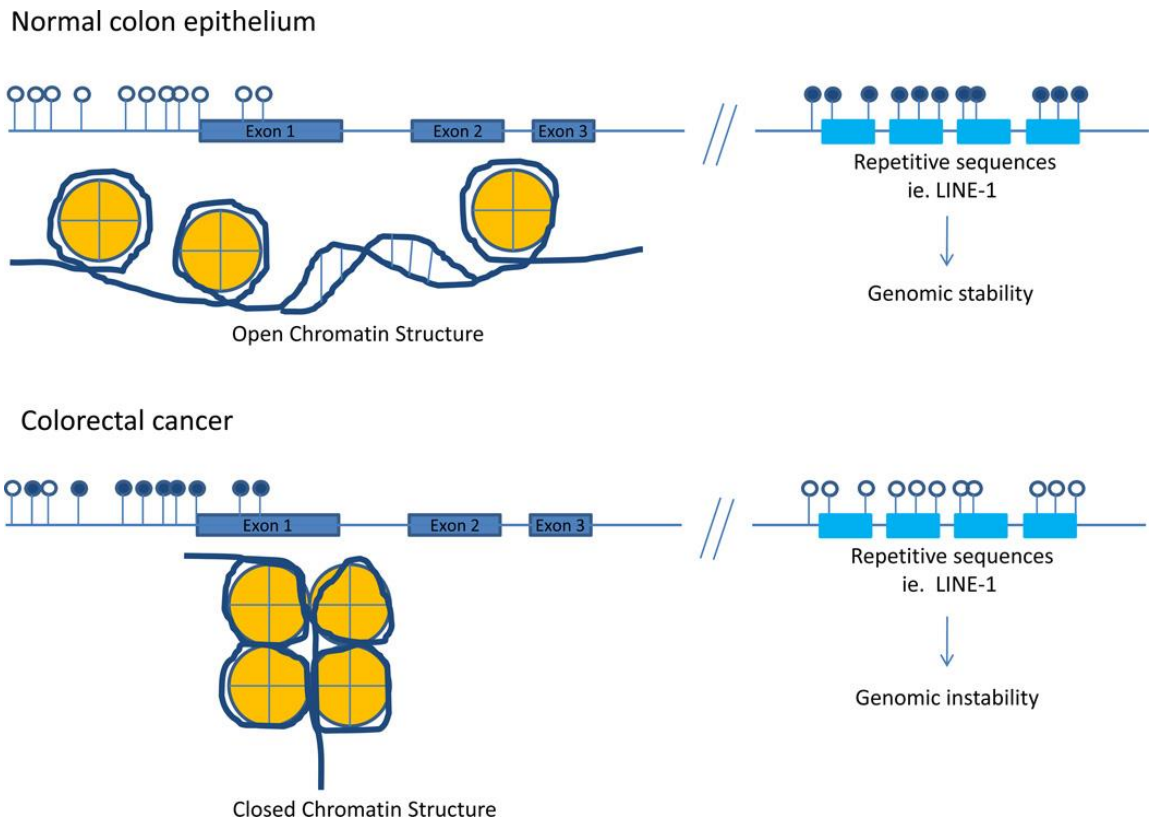


Figure 11: CpG island DNA methylation. The above figure shows the CpG island DNA hypermethylation (promoter is methylated) and hypomethylation (no methylation) process in a tumour suppressor gene. The closed and open lollipops represent the methylated and unmethylated CpG sites in the genome, respectively. Unmethylated CpG islands are seen in the promoter region of the genes with an open chromatin structure in normal colonic epithelium. A closed chromatic structure is seen in colorectal cancer with CpG islands that are condensed associated with transcriptional silencing. In addition, hypomethylation in *LINE-1* sequence is associated with local CpG island hypermethylation as colonic neoplasm progress. Taken from (Lao, Grady 2011).

1.5 Biomarkers of colorectal cancer

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (De Gruttola, Clax et al. 2001). The National Institute of Health (NIH) defines a biological marker (biomarker) as a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease (De Gruttola et al. 2001). In addition to

screening for disease, a biomarker may be used to evaluate how well the body responds to a treatment and can also be called a molecular marker or signature molecule (De Gruttola et al. 2001, Langan, Mullinax et al. 2013).

The definition of a biomarker mostly refers to DNA, RNA, miRNA (miRNA), protein expression, epigenetic changes or antibodies. A term tumour marker, by some researchers considered as a synonym of biomarker, refers to substances (most typically proteins, glycolipids) representing biological structures, which can be attributed to the development of normal cells or carcinogenesis at different cell development stages *e.g.*, tumour-associated antigens (TAAs), which are the largest group of clinically significant markers (Lech, Slotwinski et al. 2016). These markers can be divided into three different groups: diagnostic, predictive, and prognostic. Diagnostic markers permit an early diagnosis and risk stratification. Predictive biomarkers are useful for predicting the patient's response to a given therapy and so patients can be selected to undergo a particular treatment on the basis of a likely positive response. They can even be used to identify the right drug dose and to prevent its toxicity (Kalia 2013, Duffy, O'Donovan et al. 2011, Iaffaioli, Facchini et al. 2006, Strocchi, Iaffaioli et al. 2004). Prognostic biomarkers allow estimating the natural course of the disease and dividing tumours in two groups: the ones with a good outcome and the ones with a bad outcome (Sawyers 2008). They can be molecules involved in different process, such as cellular proliferation, differentiation, angiogenesis, invasion, and metastasis (Kalia 2013). Examples of biomarkers used in colorectal cancer are given in Table 2.

Type of Biomarker	Analysis
Genetic	Gene Mutations
	Tumour suppressor genes
DNA	Gene Amplification
	Microsatellite Instability
	Mitochondrial DNA
Epigenetics	DNA methylation
RNA	miRNAs
Protein	Faecal Haemoglobin, CEA
Carbohydrate	CA19-9

Table 2: Biomarkers of colorectal cancer

1.5.1 Carcinoembryonic antigen (CEA)

CarcinoEmbryonic Antigen (CEA) is an oncofetal antigen first described by Gold and freedman in 1965 and is found in foetal colon and colon adenocarcinoma (GOLD, FREEDMAN 1965, Gold, Freedman 1965). CEA was initially considered to be a raised only in CRC, but has since been found in cancers of pancreas, liver, breast and lung. High levels are also found in benign conditions in liver cirrhosis, inflammatory bowel disease, pancreatitis, diverticulitis and smoking (Goldstein, Mitchell 2005). Even though, CEA has been used as a biomarker for many years for CRC, Recent Cochrane review of 52 studies estimated sensitivity range from 41% to 97% and specificity from 52% to 100% (Nicholson, Shinkins et al. 2015). CEA sensitivity also depends on the threshold used in the test, with the sensitivity of CEA varying with disease progression being reported at 3%, 25% 45% and 65% for Dukes A, B C and D, respectively at CEA > 5µg/L (Sturgeon, Lai et al. 2009).

These findings of variation in sensitivities render the positive predictive value of CEA low with little benefit for screening healthy subjects (Midgley, Kerr 1999, Duffy 2001). As a result, The European Group on Tumour Markers, European Society of Medical Oncology and American Society of Clinical Oncology guidelines does not recommend

CEA for use in screening tests (Locker, Hamilton et al. 2006, Labianca, Nordlinger et al. 2010, Duffy, Lamerz et al. 2014).

The recent Cochrane review conducted in 2016 of 15 RCT studies, which included 5403 patients, concluded no overall survival benefit for intensifying the follow-up of patients after curative surgery for colorectal cancer (Jeffery, Hickey et al. 2016). The CEA second-look (CEASL) trial also debated about increases in overall mortality due to higher chances of identifying asymptomatic recurrences and treating them (Treasure, Monson et al. 2014). On the other hand, the Follow-up after Colorectal Surgery (FACS) randomized clinical trial, one of the largest trials conducted across 39 National Health Service hospitals in the United Kingdom, with 1202 eligible participants concluded CEA screening or CT imaging noted an increased rate of curative surgery for recurrence(Primrose, Perera et al. 2014). Identification of CEA is a relatively simple, low-cost biomarker that can be detected by a blood test, which is still being used in the clinical setting all over the world for follow up in CRC. The National Institute for Health and Clinical Excellence (NICE) recommended follow-up from four to six weeks following curative treatment with CEA measurement at least every six months in the first three years (NICE 2012). Further, The European Society of Medical Oncology (ESMO) recommends CEA determination every three to six months for the first three years, and every six to 12 months in years four and five (http://www.esmo.org).

1.5.2 Genetic and epigenetic markers

A genetic biomarker refers to analysing DNA mutations in genes such as *KRAS*, *TP53*, *APC* and *BRAF*. Epigenetic markers broadly refer to detecting microsatellite instability (MSI) and hyper-methylation processes.

KRAS

KRAS protein operates physiologically along two cellular pathways: mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K) (Figure 12). In the RAS/MAPK pathway, KRAS transmits the signal from outside the cell into the cell's nucleus, and these signals make the cell grow and divide (proliferate) or to mature and

perform specific functions (differentiate). The *KRAS* gene is also an oncogene and when mutated can cause disruption or deregulation of the MAPK or PI3K pathway causing a transformation of a normal cell to a cancerous one (https://ghr.nlm.nih.gov/gene). *KRAS* mutation is found in approximately 30-40% of all colorectal tumours (Adjei 2001, Vaughn, Zobell et al. 2011). *KRAS* mutations have been identified as a potent predictor of resistance to EGFR directed antibodies such as cetuximab or panitumumab (van Krieken, Jung et al. 2008). In 2015 the American Society for Clinical Oncology (ASCO) strongly suggested testing for mutations in *KRAS* exon 2 (codons 12 and 13) before treatment with anti-EGFR antibody therapy. Furthermore, patients with metastatic CRC should have their tumour tested for mutations in *KRAS* exons 3 (codons 59 and 61) and 4 (codons 117 and 146) and *NRAS* exons 2 (codons 12 and 13), 3 (codons 59 and 61), and 4 (codons 117 and 146) (Allegra, Rumble et al. 2016).

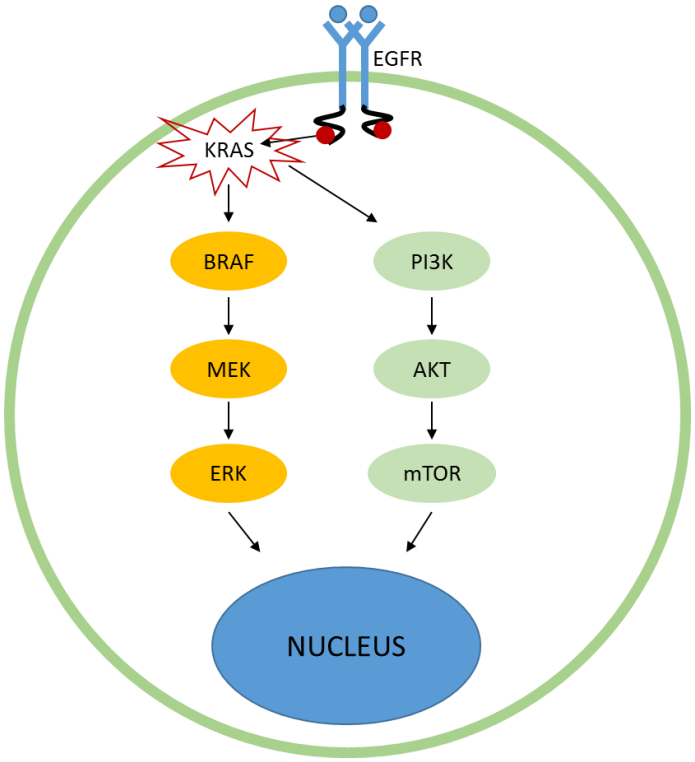


Figure 12: Mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K) pathways for KRAS.

The first step towards activating the RAS/MAPK pathway occurs by a ligand (such as EGF) binding to a receptor (e.g. EGFR). Following the binding of the ligand, the EGFR receptor becomes dimerised, phosphorylated and activated. KRAS protein is activated

and further activates both phosphatidylinositol 3-kinase (PI3K) and BRAF, resulting in cellular proliferation, survival and migration (Brand, Wheeler 2012, McCain 2013).

BRAF

The *BRAF* gene can be mutated in approximately 5% to 10% of colon cancers (Fearon 2011), the predominant mutation being V600E (Tran, Kopetz et al. 2011a). *BRAF* mutations in colorectal cancer are most commonly associated with female sex, right sided location in the colon and poor differentiation histologically (C. C. Lin, Lin et al. 2014). They are rarely found in the left side of the colon and rectal cancers (Tie, Desai 2015a). *BRAF* mutations also have a prognostic role. Tran et al. showed a median overall survival of 10.4 months for *BRAF* mutant tumours and 34.7 months for *BRAF* wild type tumours (Tran, Kopetz et al. 2011b). Results from a phase one extension study showed *BRAF* mutant colorectal cancer patients treated with PLX4032 has a partial response, confirming that *BRAF* as a potential therapeutic target in colorectal cancer (Kopetz, Desai et al. 2015, Tie, Desai 2015b).

1.5.3 Carbohydrate Antigen 19-9 (CA19-9)

Carbohydrate Antigen 19-9 (CA19-9), also called cancer antigen 19-9 or sialylated Lewis antigen is the carbohydrate determinant that functions as an adhesion molecule and plays a role in the process of tumour progression (Del Villano, Brennan et al. 1983).

CA19-9 is present in human serum and found elevated in various diseases (Mann, Edwards et al. 2000) including cancers of the digestive tract such as the pancreas (Ballehaninna, Chamberlain 2012), bile ducts (Kikkawa, Sogawa et al. 2012), stomach (Kim, Oh et al. 2011), and colon (Yamashita, Watanabe 2009). CA19-9 is recommended by medical societies and study groups for managing cancers of the pancreas but not of the colon (Duffy, Sturgeon et al. 2010, Duffy, van Dalen et al. 2003, Duffy, van Dalen et al. 2007a). Studies have reported that CA19-9 has no prognostic impact of postoperative chemotherapy but, in patients with stage IV CRC who underwent curative resection, the combination of post-CEA and post-CA19-9 three months after surgery was a predictive indicator for recurrence (Abe, Kawai et al. 2016). Another

study of 73 patients with stage IV CRC, who underwent curative resection were categorized into normal and high CA19-9 groups. The 3-year relapse-free survival and overall of the high CA19-9 group was significantly worse than that of the normal CA19-9 group. Also, the preoperative serum CA19-9 level is a good predictive marker of tumour recurrence and prognosis in patients with stage IV CRC who have undergone curative resection (Ozawa, Ishihara et al. 2016).

1.5.4 Faecal Haemoglobin

Stool-based detection of CRC is probably the simplest, least costly and the least invasive method of screening available (Duffy, van Dalen et al. 2007b) (Figure 13). The Guaiac based faecal occult blood test detects haemoglobin enzymatically and relies on pseudo-peroxidase-like activity of haem, which oxidises guaiac when hydrogen peroxide is added and can originate from any source. The source could be bleeding from both upper and lower gastrointestinal tracts, ingestion of certain foods like red meat, fruits and vegetables and medications like non-steroidal anti-inflammatory drugs can lead to false-positive results (Tanaka, Tanaka et al. 2010, Tinmouth, Lansdorp-Vogelaar et al. 2015). Despite the faecal occult blood test being one of the easiest non-invasive cancer screening test used, its sensitivity is poor: 13-25% (Health Quality Ontario 2009). Furthermore, the proportion of people undertaking the FOBT remains disappointingly low, at 55-60% in England (http://www.cancerresearchuk.org).

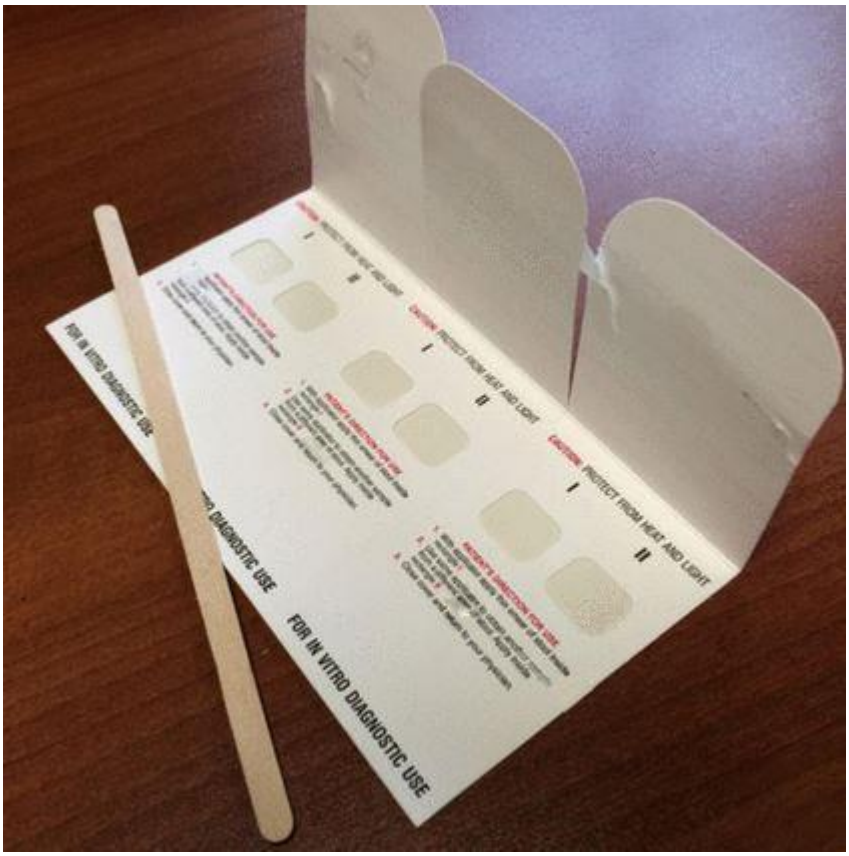


Figure 13: Guaiac-based faecal occult blood test card: There are six windows in total for 3 consecutive days with two faecal samples each day separately using a wooden applicator. Image adapted from (Tinmouth et al. 2015).

1.5.5 Monoclonal and polyclonal antibodies

The use of monoclonal or polyclonal antibodies specific to human haemoglobin detects globin through immunochemical reactions (van Dam, Kuipers et al. 2010). The Faecal Immunohistochemical Test (FIT), being specific to human haemoglobin does not require dietary restrictions and is also specific to lower gastrointestinal bleeding (Binefa, Rodriguez-Moranta et al. 2014, Allison, Fraser et al. 2014). There are two types of FIT: “Qualitative” based on immunochromatography and “Quantitative” based on latex agglutination immunoturbidimetry. Quantitative FIT is preferred over Qualitative for diagnostic accuracy for removing reader and inter-batch variability (Mowat, Digby et al. 2016). FIT requires fewer samples than gFOBT, but requires samples to be stored in the refrigerator since false negatives occur with storage at high temperature (Grazzini, Ventura et al. 2010). A meta-analysis by Lee et al. (2014) on the accuracy of

FIT for CRC showed the pooled sensitivity and specificity to be approximately 79% and 94%, respectively (J. K. Lee, Liles et al. 2014).

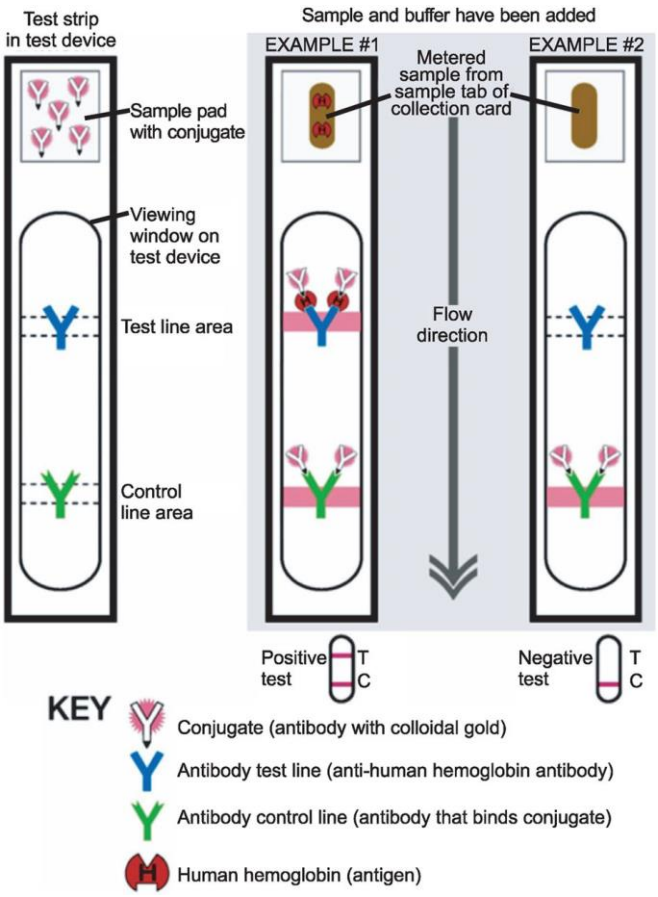


Figure 14: Lateral flow immunochromatographic analysis principle of a faecal immunochemical test for haemoglobin. Image Adapted from (Allison et al. 2014).

1.6 MiRNA

MiRNAs (miRNAs) are a class of endogenous, small (17-25 nucleotides), noncoding, single-stranded, evolutionarily conserved RNA molecules that play an important role in gene regulation and post-transcriptional repression (Bartel 2009).

Lin-4 was the first miRNA to be discovered in 1993 in the worm *Caenorabditis elegans* during their development, where lin-4 was involved in the post-transcriptional regulation of *the LIN14* gene (Wightman, Ha et al. 1993, E. J. Lee, Baek et al. 2008). However the role of miRNAs was established when let-7 was found across all species in

2000 (Reinhart, Slack et al. 2000). Let-7 itself was discovered in the genomes of humans, fly and 11 other animals (Pasquinelli, Reinhart et al. 2000). Since its discovery, there has been vast progress in determining the role of miRNAs in cancer. MiRNAs have been found to play a key role in the regulation of various biological functions including growth, proliferation, survival, differentiation and cell death (Stahlhut, Slack 2013, Kasinski, Slack 2011). In cancer, the loss of tumour-suppressive miRNAs and increased expression of the oncogenic miRNAs (oncomiRs) can enhance the expression of target oncogenes or repress target tumor suppressor genes respectively (Kasinski, Slack 2011). According to the latest miRBase release 22 (http://www.mirbase.org), 1917 precursors and 2654 mature miRNAs have been identified.

1.6.1 Biogenesis of miRNAs

MiRNA biogenesis starts in the nucleus where the miRNA gene is transcribed by RNA polymerase II into a long primary miRNA (pri-miRNA) in a hairpin loop structure (Y. Lee, Kim et al. 2004), which will become the final miRNA after several steps. This long primary miRNA becomes a substrate for initial processing by Drosha (a RNA polymerase III enzyme) along with a RNA binding protein called DGCR8 (also known as Pasha). DROSHA and DGCR8 forms the microprocessor complex and cleaves the pri-miRNA into a small precursor miRNA (pre-miRNA) approximately 60-70 nucleotides in length. This pre-miRNA is recognised by a protein called EXPORTIN 5 in the nucleus and is exported to the cytoplasm (Brownawell, Macara 2002). Further processing happens in the cytoplasm, where DICER (another RNase III enzyme) cleaves the precursor into the 21–24 nucleotide duplex miRNA. The duplex RNA contains the guide and the passenger strands. After the strands bind with ARGONATE, the guide strand is retained forming the miRNA induced silencing complex (miRISC) along with other proteins. The miRISC complex binds its target RNA resulting in gene silencing (if the miRNA is perfectly complementary) or translational expression (if the miRNA has a mismatch in its sequence). Biogenesis and the function of miRNAs are as shown in the figure below (Figure 15).

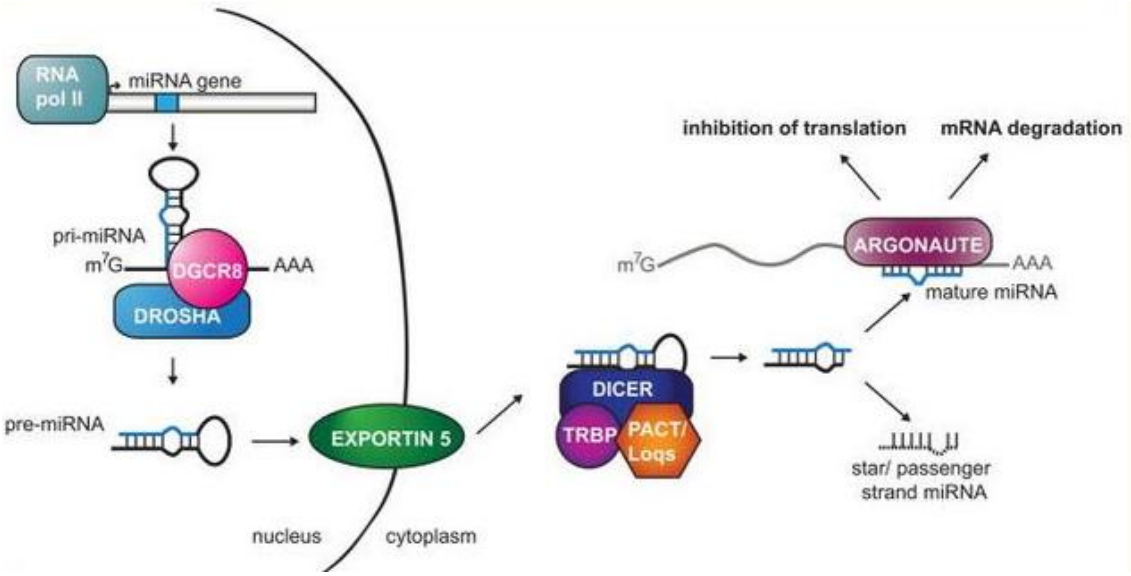


Figure 15: Biogenesis and role of miRNAs (taken from (Finnegan, Pasquinelli 2013))

1.7 MiRNA profiling in plasma and colorectal cancer

MiRNAs have been identified from various sources in the body such as plasma, saliva, urine and faeces and are found to be inherently stable (Weber, Baxter et al. 2010, Kanaan, Rai et al. 2012, Chevillet, Lee et al. 2014). Although not very clear, several possibilities have been hypothesised.

Cell free circulating miRNAs are packaged in lipid vesicles or as a protein complex preventing them from RNase degradation. In the lipid vesicles, they are likely to be bound to larger 50–100 nm diameter membrane vesicles called exosomes (Valadi, Ekstrom et al. 2007, Chevillet et al. 2014). They have also been shown to be derived from platelets (Pritchard, Kroh et al. 2012a), which are abundant in typical plasma preparations. Cell free circulating miRNA have been found to be mostly associated with Argonaute-2, but a few studies have independently associated miRNAs with high density lipoprotein (Vickers, Palmisano et al. 2011) and nucleophosmin (K. Wang, Zhang et al. 2010).

miRNAs can be extracted from wide range of cell and tissue sources like cell lines, formalin-fixed paraffin-embedded (FFPE) tissues, fresh tissues, plasma, serum, urine

and other bodily fluids (Table 3) (Weber et al. 2010). Of all the sources for miRNA extraction, cell lines and tissues have been found to have highest yield of miRNA compared to plasma, serum or urine (Table 3). MiRNA extraction from blood and plasma is difficult and more challenging compared to other sources due to high levels of endogenous RNAse activity, centrifugation conditions, white cell counts and red blood cell haemolysis, which can have a huge impact on miRNA quantitation (Mitchell, Parkin et al. 2008, McDonald, Milosevic et al. 2011, Duttagupta, Jiang et al. 2011, Leidinger, Backes et al. 2015, Pritchard, Kroh et al. 2012b). Furthermore, specimen processing conditions can also play a major factor in miRNA profiles (Arroyo, Chevillet et al. 2011, Page, Guttery et al. 2013).

Sample type	**Typical miRNA yield**	**Considerations**
Cell Lines	> 1000 ng	High quality miRNA
Fluorescence activated cell sorting	1-100 ng	Lower yield but less cell type heterogeneity
Fresh tissue	> 1000 ng	cell type heterogeneity
Formalin fixed paraffin embedded tissue	1-100 ng	More reliable than miRNA as an analyte in FFPE
Laser capture micro dissected tissue	<1-10 ng	Less heterogeneity, But lower yield, Never completely pure.
Plasma or serum	<1-10 ng	RNases ,low yield; typically cannot measure quantity of extracted RNA
Urine	<1-10 ng	Can evaluate cell platelet versus supernatant

Table 3: Sample type and miRNA yields. Adapted from (Pritchard, Cheng et al. 2012).

1.7.1 Approaches for miRNA profiling

Three main approaches are currently well established for miRNA profiling: 1) Quantitative reverse transcription PCR (qRT-PCR); 2) Hybridization-based methods (for example, DNA microarrays) and 3) High-throughput sequencing (that is, RNA sequencing) (Figure 16 and Table 4).

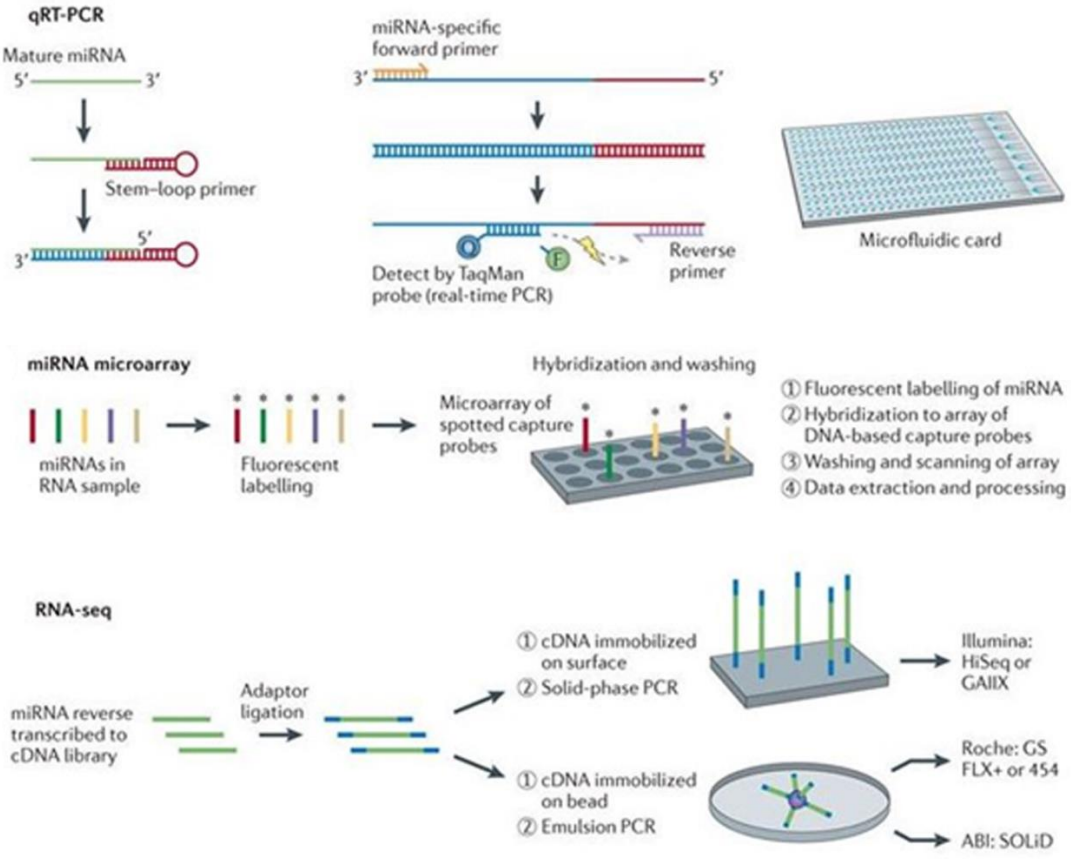


Figure 16: Methods of miRNA analysis. ***qRT-PCR****:* TaqMan qRT-PCR involves reverse transcription (RT) reactions and stem loop primers specific to the 3′ end of the miRNA for specificity. Amplicons are generated using a miRNA-specific forward primer, where DNA polymerase proceeds along template, the TaqMan probe is hydrolysed so the quencher is freed from fluorescent dye, resulting in light emission. ***miRNA Microarray:*** DNA-based capture probes (which may or may not incorporate LNA-modified bases) are used to capture fluorescently-tagged miRNAs, followed by scanning of slides and quantification of fluorescence. Several variations on this approach exist. ***RNA Sequencing:*** Current established RNA sequencing platforms begin with reverse transcription of miRNA to a cDNA library. Adaptor ligation then allows the library to either be affixed to a solid phase as in the Illumina

platform or to beads for emulsion PCR as in the Roche and ABI platforms (Pritchard et al. 2012a).

Advantages	**Disadvantages**	**Assay or platform**	**Vendor**	**RNA required**	**Material cost per sample**
Quantitative reverse transcription PCR (qRT-PCR)					
Established method, sensitive and specific. Can be used for absolute quantification	Cannot identify novel miRNAs (miRNAs) (which is problematic for less well-studied organisms in which the miRNA repertoire is not well-defined). Only medium-throughput with respect to the number of samples processed per day	TaqMan individual assays	ABI	<ng or ng–µg	$$
		miRCURY LNA qPCR	Exiqon		
		TaqMan Open Array	ABI		
		TaqMan TLDA microfluidics card	ABI		
		Biomark HD system	Fluidigm		
		SmartChip human miRNA	Wafergen		
		miScript miRNA PCR array	SABiosciences/Qiagen		
MiRNA microarray					
Established method. Easily adapted to existing microarray workflow	Lower specificity than RNAseq. Cannot be used to determine absolute quantification.	Geniom Biochip miRNA	CBC (febit)	ng–µg	$
		miRCURY LNA™ miRNA Array	Exiqon		
		µParaFlo™ Biochip Array	LC Biosciences		
		MiRNA Microarray	Agilent		
		GeneChip® miRNA Array	Affymetrix		
		OneArray®	Phalanx Biotech		
		Sentrix® Array Matrix and BeadChips	Illumina		
		GenoExplorer™	Genosensor		
RNA Sequencing* (*High Throughput Next Generation Sequencing Platforms)					
High accuracy and sensitivity. Can detect novel miRNAs	Significant computational support needed for data analysis. Cannot be used to determine absolute quantification.	HiSeq™ 2000 (Genome Analyzer IIX)	Illumina	ng–µg or > µg	$$$
		SOLiD™	ABI		
		GS FLX+ (454)	Roche		

Table 4: Each method for analysis has its own merits and disadvantages (for a comprehensive comparison of each, please see (Pritchard et al. 2012).

1.7.2 Oncogenic miRNAs in CRC

Depending on the target genes, miRNAs can be referred to as tumour suppressive or oncogenic. Deviation in the expression of miRNAs has been shown in various cancers including colorectal cancer (for comprehensive review of miRNAs in cancer, see (Thomas, Ohtsuka et al. 2015). Oncogenic miRNAs or (OncomiRs) target and downregulate endogenous tumour suppressor genes. Tumour suppressive miRNAs play a major role in downregulating genes associated with growth and metastasis (see Tables 5, 6 and 7 for examples in CRC, as well as diagnostic miRNAs).

miRNA	Target	Role in cancer	References
miR-21	PDCD4,TIAM1 SPRY2,PTEN, TGFBR2, CDC25A	Proliferation, Apoptosis, Invasion, Migration, CSC maintenance	(Yu, Kanwar et al. 2012, Asangani, Rasheed et al. 2008, Cottonham, Kaneko et al. 2010, Sayed, Rane et al. 2008)
miR-92a	PTEN	Proliferation, Invasion, EMT	(Zhang, Zhou et al. 2014)
miR-96	TP53INP1, FOXO1,FOXO 3A	Proliferation	(Gao, Wang 2015)
miR-135a	APC	Proliferation	(Nagel, le Sage et al. 2008)
miR-135b	APC	Proliferation	(Nagel et al. 2008)
miR-155	MLH1, MSH2, MSH6	DNA damage response	(Nagel et al. 2008)
miR-214	PTEN, PDLIM2	Inflammation	(Polytarchou, Hommes et al. 2015)
miR-224	SMAD4	Metastasis	(Ling, Pickard et al. 2016)

Table 5: OncomiRs associated with CRC

miRNA	Target	Role in cancer	References
let-7	KRAS	Proliferation	(Johnson, Grosshans et al. 2005)
miR-7	EGFR, RAF1	Proliferation	(Suto, Yokobori et al. 2015)
miR-18a*	KRAS	Proliferation	(Tsang, Kwok 2009)
miR-26b	TAF12, PTP4A1, CHFR, ALS2CR2	Proliferation, Apoptosis, Invasion, Migration	(Ma, Zhang et al. 2011)
miR-27b	VEGFC	Proliferation, Angiogenesis	(Ye, Wu et al. 2013a)
miR-34a	SIRT1	Apoptosis	(Yamakuchi, Ferlito et al. 2008)
miR-101	SPHK1	Angiogenesis	(M. B. Chen, Yang et al. 2015)
miR-126	VEGFA	Angiogenesis	(Stiegelbauer, Perakis et al. 2014)
miR-143	KRAS, IGF1R	Proliferation	(X. Chen, Guo et al. 2009)
miR-144	MTOR	Proliferation	(Iwaya, Yokobori et al. 2012)
miR-145	IRS1, NRAS, IGF1R	Proliferation, Invasion, Migration, Angiogenesis	(J. Su, Liang et al. 2014)
miR-194	AKT2	Proliferation, Apoptosis, Invasion, Migration	(Zhao, Ren et al. 2014)
miR-195	BCL2	Apoptosis	(L. Liu, Chen et al. 2010)
miR-320a	CTNNB1	Proliferation	(J. Y. Sun, Huang et al. 2012)
miR-365	BCL2, CCND1	Apoptosis	(Nie, Liu et al. 2012)
miR-491	BCLXL	Apoptosis	(Nakano, Miyazawa et al. 2010)

Table 6: Tumour suppressor miRNAs associated with CRC

1.7.3 Circulating miRNAs in CRC

To decrease morbidity and mortality due to CRC, early detection of colorectal cancer by a non-invasive test having high sensitivity, specificity and at the same time being cost effective is the ultimate aim. Since the discovery by Mitchell et al. (2008) of the remarkably stable miRNAs present in human plasma, their utilisation as blood based markers set researchers to further investigate the feasibility of utilising circulating miRNA in cancer (Mitchell et al. 2008). The ease of detection using Quantitative polymerase chain reaction (qPCR) and its relatively low expensive made miRNAs an even more attractive option in the field of CRC detection and treatment. Several

studies have since been performed suggesting miRNAs as a potential biomarker which could be used for diagnostic, prognostic and predictive purposes.

Kanaan et al. demonstrated a panel of 8 plasma miRNAs (miR-532-3p, miR-331, miR-195, miR-17, miR-142-3p, miR-15b, miR-532, and miR-652, which identified adenoma patients with a sensitivity of 88% and a specificity of 64% (Kanaan, Roberts et al. 2013). Xu et al. (2014) demonstrated in plasma samples, miR-375 and miR-206 were dysregulated and could discriminate CRC patients from healthy controls (L. Xu, Li et al. 2014). Ristau et al (2014) for the first time compared pre- and post-surgical plasma samples and showed a decreasing trend in expression levels of miR-92a, miR-18a, miR-320a, miR-106a, miR-16-2, miR-20a, miR-223, miR-17, miR-143 pre-surgery, post-surgery (2–7 days after surgery) and at six months follow-up highlighting the utility of plasma miRNAs as predictive biomarkers (Ristau, Staffa et al. 2014a).

However, despite their initial discovery, there are multiple questions which have been raised about extracting miRNAs from plasma/serum. Lack of standardised sample processing procedures leading to contamination of samples with white blood cells and platelets, handling steps that cause haemolysis leading to alteration of miRNA in the sample, the use of anticoagulants such as heparin (a potent inhibitor of reverse transcriptase during cDNA synthesis) and low yield of RNA isolated from plasma have all been shown to hinder downstream miRNA analysis and reproducibility. Further, different studies have used different methods of normalisation, and there are issues of specificity of the circulating miRNA for particular cancers (Fesler, Jiang et al. 2014). Nevertheless, there remains promise in utilisation of miRNA as a diagnostic and prognostic biomarker. Various studies from the literature showing plasma miRNAs in CRC are shown in the Table 7.

miRNA	Origin	Expression	References
miR-29a	Plasma	Upregulated in CRC plasma, associated with advanced TNM stages	(Xing, Wan et al. 2012)
miR-92a	Plasma	Upregulated in CRC plasma; could distinguish CRC from other GI cancers and IBD; not associated with TNM stages	(Dews, Homayouni et al. 2006)
miR-17-3p	Plasma	Upregulated in CRC plasma	(Sureban, May et al. 2009)
miR-25	Plasma	Distinguished CRC with controls-Diagnostic, Correlates with stage I and II CRC.	(Wikberg, Myte et al. 2018)
miR-22	Plasma	Distinguished CRC with controls –Diagnostic, Correlates with stage I and II CRC.	(Wikberg et al. 2018)
miR-18	Plasma	Distinguished CRC with controls –Diagnostic, Correlates with stage I and II CRC.	(Wikberg et al. 2018)
miR-92a	Plasma	Upregulated in CRC plasma; not associated with TNM stages	(Xing et al. 2012)
miR 21	Plasma	Distinguish both CRC and advanced adenomas from controls, Correlates with decreased overall survival.	(Toiyama, Takahashi et al. 2013, G. H. Liu, Zhou et al. 2013)
miR-141	Plasma	Distinguishes Stage IV CRC patients from other stages, Correlates with poorer survival.	(Cheng, Zhang et al. 2011)
miR-601	Plasma	Distinguishes CRC patients from controls ,Lowest expression in Stage IV	(Q. Wang, Huang et al. 2012)
miR-760	Plasma	Distinguishes CRC patients from controls, Lowest expression in Stage IV.	(Q. Wang et al. 2012)
miR-200c	Plasma	Increased expression in CRC vs control, Highest in Stage IV compared to Stage I-III, Associated with distant metastasis and poorer survival.	(Toiyama, Hur et al. 2014)
miR-29	Plasma	Distinguish both CRC and advanced adenomas from controls. Distinguish patients with liver metastasis from those without liver metastasis	(Huang, Huang et al. 2010, L. G. Wang, Gu 2012)
miR-20b-5p	Plasma	Predictive in Metastatic Colorectal Cancer. Higher levels better outcome	(Ulivi, Canale et al. 2018)
miR-155-5p	Plasma	Predictive in Metastatic Colorectal Cancer treated with bevacizumab. High basal levels indicates better outcome.	(Ulivi et al. 2018)

Table 7: Various studies showing diagnostic and predictive plasma miRNAs

1.8 Background leading to the study

The work outlined in this study was a continuation of previous work by PhD student Mr Imran Aslam in the Leicester Cancer Research Centre. This study used TaqMan Low Density Array (TLDA) cards to identify a panel of 18 plasma miRNAs (miR-135b, miR-34a, miR-431, miR-16, miR-369-5p, miR-23b, miR-191, miR-21, miR-589, miR-487b, miR-95, miR-484, miR-195, miR-181C*, miR-410, miR-532-5p, miR-192*, miR-203), which accurately differentiated pre-treatment CRC adenomas and carcinomas from healthy controls with 92% sensitivity and 88% specificity. A major conclusion of this study was that expression levels of these miRNAs could be correlated with a prospectively maintained database with details of CRC treatments, follow-ups and outcomes.

In Mr Aslam's study, a total of 265 participants were recruited through the University Hospitals of Leicester NHS trust as part of the National Bowel Cancer Screening Programme (NBCSP) and agreed towards blood sample collection. Ethical permission was obtained from the National Patient Safety Agency and National Research Ethics Committee for Leicestershire, Northamptonshire and Rutland (REC 05/Q2502/27 and 05/Q2502/28) to collect both blood, and matched tissue samples should there be a confirmed diagnosis of cancer by colonoscopy. Of these, 100 participants had confirmed cancer diagnosed by colonoscopy and had tissue collected for the study during surgery. Participants with normal colonoscopy or findings of diverticular disease, haemorrhoids or mild colitis were used as controls. Participants with diagnosis of cancer, polyps of any type (except with hyperplastic polyps) were used as the diseased group.

The inclusion and exclusion criteria were as follows:

Inclusion criteria:

I. Patients aged 25-90 years
II. Patients undergoing surgical resection of colorectal neoplasia
III. Patients undergoing surgical resection of bowel diverticular disease
IV. Asymptomatic healthy controls without any bowel symptoms

V. Patients undergoing colonoscopic examination of large bowel for:
- Family history of CRC or IBD
- Surveillance after CRC and polyp resection
- Surveillance for dysplasia in the background of IBD
- Surveillance colonoscopy for FAP/HNPCC
- Symptoms of bowel disease
- Positive FOBT

Exclusion Criteria

I. Synchronous carcinoma of other body organ

II. Age >90 and <25 years

III. Pregnancy

IV. HIV

V. Hepatitis C with or without anti-viral treatment

VI. Patients with needle phobia.

To develop a panel of miRNAs that can distinguish pre-treatment CRC adenomas and carcinomas from healthy controls, an initial discovery phase was performed on plasma miRNAs from 32 of the participants recruited to the study (11 normal, 9 adenomas and 12 carcinomas) using Taqman® MicroRNA Array, Megaplex™ RT and pre-amplification primers Human Pool A v.2.1 and Pool B v.2.0. Plasma miRNA expression normalised to MammU6 was analysed using Z-scores, principal component analysis, hierarchical cluster analysis, bioinformatics and student's t-test. This highlighted 27 miRNAs that can significantly discriminate CRC adenomas and carcinomas from healthy controls (miR-16, miR-23b, miR-34a, miR-92a, miR-95, miR-135b, miR-181c, miR-181c*, miR-182, miR-182*, miR-192*, miR-200a*, miR-203, miR-205, miR-369-5p, miR-410, miR-431, miR-486-3P, miR-486-5p miR-487b, miR-502- 5p, miR-532-5p, miR-564, miR-566, miR-589, miR-592 & miR-624*).

Next, the panel of 27 potentially discriminatory miRNAs, plus 2 additional miRNAs known to be significantly associated with CRC (miR-191 and miR-195) were taken forward for validation in an independent cohort of 94 symptomatic participants (32

controls, 28 adenomas and 34 carcinomas). Receiver operating characteristics (ROC) and logistic regression analysis to assess the diagnostic accuracy of individual miRNAs and different groups/panels of miRNAs revealed a panel of 18 miRNAs (miR-135b, miR-34a, miR-431, miR-16, miR-369-5p, miR-23b, miR-191, miR-21, miR-589, miR-487b, miR-95, miR-484, miR-195, miR-181C*, miR-410, miR-532-5p, miR-192*, miR-203) that could accurately distinguish adenomas and carcinoma with 92% sensitivity and 88% specificity.

Finally, due to it having the highest diagnostic accuracy in the validation cohort, miR-135b was selected and further validated in an additional independent cohort of 96 patients (25 asymptomatic controls, 30 adenomas and 41 carcinomas), differentiating asymptomatic controls from adenomas and carcinomas with 80% sensitivity and 84% specificity. Figure 17 gives a diagrammatic view of this study.

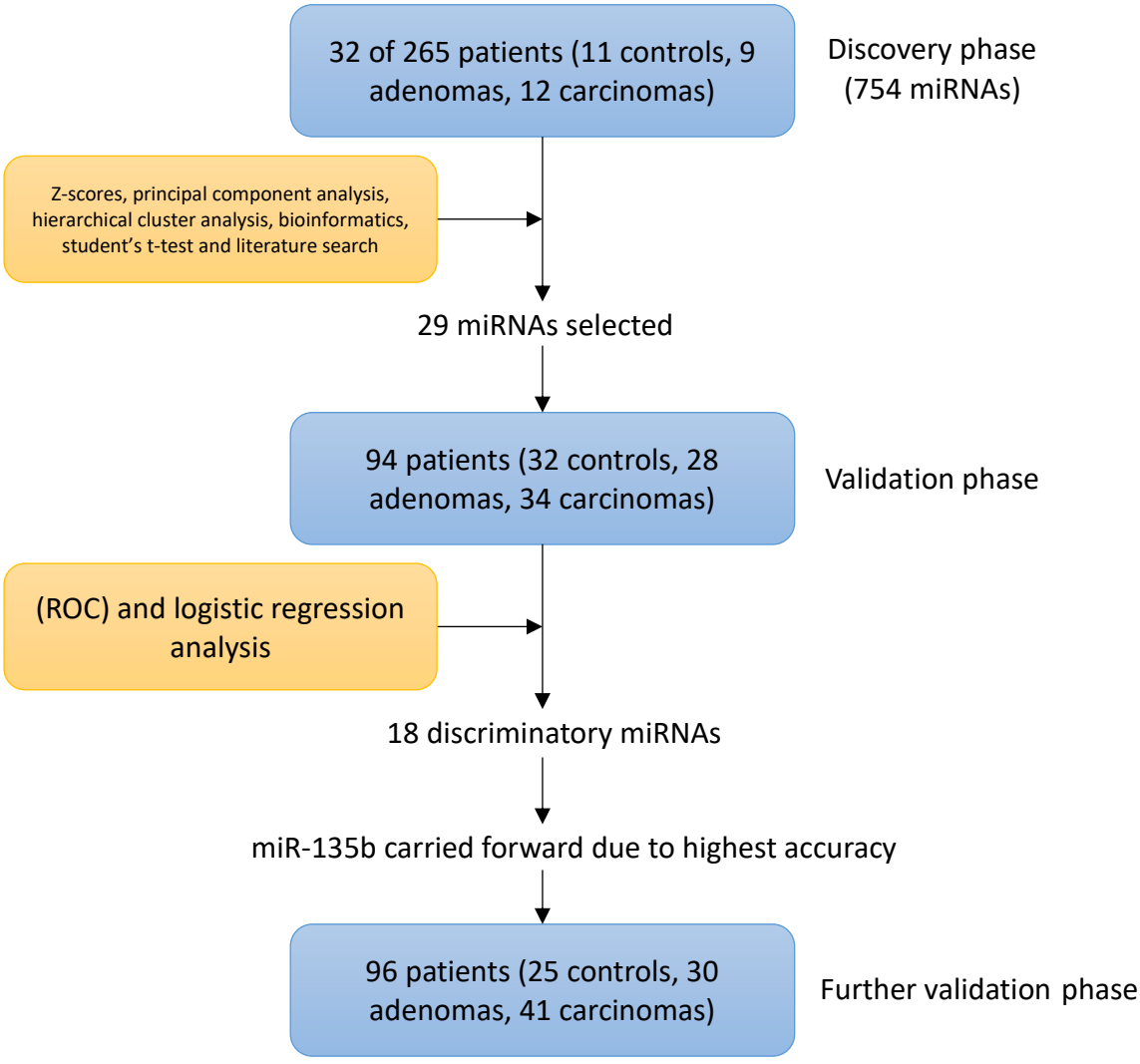


Figure 17: Diagrammatic representation of recruitment of Mr Aslam's study

A major conclusion of this study was that expression levels of these miRNAs should be correlated with a prospectively maintained database with details of CRC treatments, follow ups and outcomes.

Hypothesis: Based on Mr Aslam's previous study and in follow up, the hypothesis tested here was that *circulating miRNAs are accurate biomarkers for monitoring the response to therapy*.

The **aim** of this project was to follow up all 100 patients diagnosed with colorectal cancer by radiology and histology based on endoscopy, verify the dataset and collect matched paired blood samples post-treatment (surgery/chemotherapy/radiotherapy) to determine which miRNAs may be useful markers of response to therapy.

The specific objectives were:

1) To verify the dataset and recruit each patient towards obtaining follow-up samples for analysis of the 18 discriminatory miRNAs highlighted as part of Mr Imran Aslam's study;

2) To select and validate 4 of the 18 discriminatory miRNAs, as well as 5 miRNAs known to be indictors of therapy response in CRC using qRT-PCR in both pre- and post-treatment samples to determine whether they are also indicative of treatment response;

3) To correlate each miRNA from matched pre- and post-treatment samples with clinicopathological features using multilinear regression and non-parametric tests.

Chapter 2: Materials and Methods

2.0 Materials & Methods

2.1 Ethical approval

Ethical approval was obtained from the Nottingham National Research and Ethics Committee (NREC) (Reference: Biomarkers of Bowel Disease - 10/H0408/11, Markers of tumour progression in colorectal cancer - 05/Q2502/28) and locally from the University Hospitals of Leicester (UHL) research and development (R&D) department (REGPR11005). Each has been included in Appendices 1-4.

2.2 Sample collection and processing

Blood samples were collected into Monovette 10 ml K2 EDTA tubes (Sarstedt, Germany) after venepuncture and were centrifuged within 2 hours to avoid white blood cell (WBC) lysis. Blood samples collected were mixed and inverted 5 times immediately and stored on ice before being transported from Leicester General Hospital to the Robert Kilpatrick Clinical Sciences Building for centrifugation.

Fresh blood samples were spun in a Jouan centrifuge at 1000 x g at 4 °C for 10 minutes. This separated the blood into 3 layers: the upper plasma, middle buffy coat and lower packed red blood cells (RBCs) as shown in Figure 17. The plasma was removed and transferred to a fresh 15 ml tube, taking care not to disturb the buffy coat and the red cell layer. Extreme care was taken to leave 2-3 mls of plasma above the buffy coat. Four hundred microliters of buffy coat and 1 ml of packed RBCs were transferred to seperate 1.5 ml eppendorfs for storage at -80 °C. Plasma was centrifuged for a second time at 2000 rpm at 4 °C for 10 minutes. Post-centrifugation, plasma was transferred as 1 ml aliquots in 1.5 ml Eppendorf tubes for storage at -80 °C.

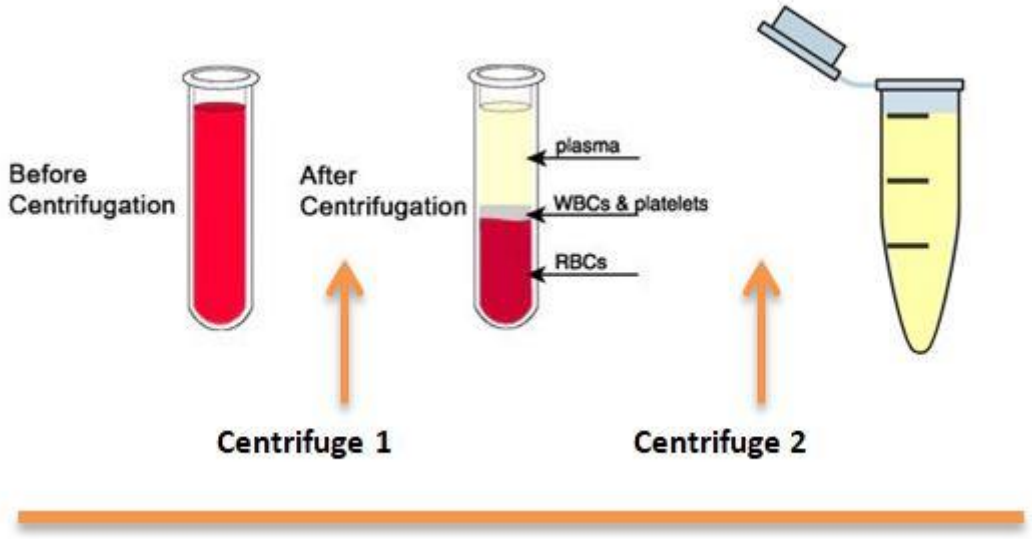


Figure 18: Plasma extraction after centrifugation cycles from whole blood sample.

2.3 miRNA analysis

2.3.1 Extraction of miRNA from plasma

Prior to extraction of miRNA, a 1 ml aliquot of plasma for each patient was thawed and 250 µl taken for miRNA extraction. The remaining plasma was returned to -80 °C. Samples were centrifuged at 3500 rpm for 5 minutes at room temperature (15-25 °C) and 200 µl transferred into a fresh Eppendorf tube. The rest of the procedure was performed in a fume hood.

One ml of QIAzol reagent was added to the plasma and vortexed for 15 seconds with incubation for 5 minutes at room temperature and intermittent mixing. Two hundred µl of chloroform was added and mixed by inverting the tube 20 times, with further incubation at room temperature for 3 minutes. Samples were centrifuged for 15 min at 13,000 rpm at 4 °C. After centrifuging, 600 µl of the upper aqueous phase was aspirated into a fresh 1.5 ml Eppendorf tube and the remaining fluid containing the red lower layer and white interface discarded. Nine hundred µl of absolute ethanol was added into the 600 µl of upper aqueous phase and the tube inverted 20 times to mix. Seven hundred µl of the sample was added to an RNeasy mini spin column and centrifuged at 13,000 rpm at room temperature for 15 seconds. The flow-through was

discarded. The sample was then added to a new column and centrifuged at 13,000 rpm at room temperature for 15 seconds. Seven hundred µl of buffer RWT was added to the column and centrifuged at 13,000 rpm for 15 seconds, and the flow-through discarded. Five hundred µl of buffer RPE was added to the column and the centrifuged at 13,000 rpm for 15 seconds. This was then repeated using 500 µl of 80% ethanol. The RNeasy mini spin column was transferred into a fresh 2 ml collection tube and centrifuged at 13,000 rpm for 5 minutes to dry the column. The column was then transferred into a clean 1.5 ml Eppendorf 14 µl of RNase free water added directly on the membrane. The column was incubated at room temperature for one minute and centrifuged at 13,000 rpm for 60 seconds to elute RNA. The eluted RNA was stored at -80 °C until required.

2.3.2 Reverse transcription for cDNA production

RNA samples were reverse transcribed using the TaqMan® MiRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Megaplex reactions were conducted using Taqman Human Pool A Megaplex™ RT ™ v2.1 primers and Human Pool B Megaplex™ RT v3.0 to convert RNA to cDNA. For each sample a mastermix of the following was prepared:

Mastermix per sample	
Reagent	**Volume**
10 x RT Primers	0.8 µl
100 mM dNTPs	0.2 µl
MultiScribe™ Reverse Transcriptase (50 units/µl)	1.5 µl
10 x RT Buffer	0.8 µl
$MgCl_2$	0.9 µl
RNase inhibitor (20U/µl)	0.1 µl
Nuclease free water	0.2 µl
Total Volume	**4.5 µl**

Table 8: Mastermix Preparation for RT

Three µl of RNA was added to the mastermix to make a total volume of 7.5 µl. A negative control (-RT) master mix solution was also prepared for three reactions by replacing the MultiScribe™ Reverse Transcriptase (50 U/µl) with RNAase-free water Reverse transcription (RT) was performed using a Veriti thermal cycler (Applied Biosystems, Foster City, CA) to the following conditions:

Stage	Temperature	Time
Initial incubation	16 °C	2 minutes
40 cycles	16 °C	1 minutes
	50 °C	1 sec
Hold	85 °C	5 minutes
Hold	4 °C	∞

Table 9: Thermal cycling profiles for RT

2.3.3 Pre-amplification (Pre-amp) of cDNA

cDNA sequences were pre-amplified using Megaplex™ PreAmp Primers, Human Pool A & B v2.1 (Applied Biosystems), thereby enhancing the sensitivity of real time RT-PCR to detect miRNAs at low levels in the sample. Firstly, a mastermix was produced as follows:

Mastermix per sample	
Reagent	**Volume**
TaqMan Pre-amp Master Mix (2x)	6.25 µl
Megaplex Pre-amp Primer Pool (10x)	1.25 µl
Nuclease free water	3.75 µl
Total Volume	**11.25 µl**

Table 10: Mastermix preparation for Pre-amp.

cDNA (1.25 µl) was added to 11.25 µl of mastermix to give a final reaction volume of 12.5 µl. The sample was mixed well and incubated in ice for 5 minutes. Pre-

amplification was performed using a Veriti thermal cycler (Applied Biosystems, Foster City, CA) under the following conditions:

Stage	Temperature	Time
Initial incubation	95 °C	10 minutes
Hold	55 °C	2 minutes
Hold	72 °C	2 minutes
12 cycles	95 °C	4 minutes
	60 °C	4 minutes
Final incubation	99.9 °C	10 minutes
Hold	4 ° C	∞

Table 11: Thermal cycling profiles for Pre-amp

After pre-amplification, 37.5 µl of 0.1 X TE buffer, pH 8.0 was added to each sample to dilute, and the diluted pre-amplified product stored at -20 °C until required.

2.3.4 Quantitative Real-Time PCR (qRT-PCR)

Prior to qRT-PCR analysis, pre-amplified products were diluted 1:20 in 0.1 X TE buffer, pH 8.0 and incubated on ice. A mastermix for each reaction was produced as follows:

Mastermix per sample	
Reagent	**Volume**
TaqMan® Universal PCR Master Mix No AmpErase® UNG (2x)	5 µl
RNAase/ DNAase free water	1.5 µl
Taqman MiRNA PCR assay (20x)	0.5 µl
Total Volume	**7 µl**

Table 12: Mastermix preparation for qRT-PCR

Three µl of diluted cDNA (1:20 dilution) was added to each mastermix and mixed well. PCR was performed using the Step-One-Plus™ real-time PCR system (Applied Biosystems, Foster City, California, USA) using the following conditions:

Stage	Temperature	Time
Initial denaturation	95 °C	10 minutes
45 cycles	95 °C	15 seconds
	60 °C	1 second
Hold	4 °C	∞

Table 13: Thermal cycling profiles for qRT-PCR

Data was collected during the log phase of amplification and the C_T threshold automatically set by the machine. For each miRNA, data was exported into Microsoft Office Excel.

2.4 Statistical analysis

All statistical analyses were performed using SPSS v22 (IBM, New York, USA). For comparisons of miRNA levels in matched pre- and post-treatment samples, a non-parametric Wilcoxon signed-rank test was performed due to the non-normal distribution of the data using ΔC_T values for each miRNA. For correlation of each miRNA with various clinicopathological characteristics in pre- and post-treatment samples, multiple linear regression was performed using ΔC_T values for each miRNA.

Chapter 3: Results

Chapter 3: Results

3.1 Dataset verification

3.1.1 Patient recruitment

The 100 CRC patients included for follow-up blood sample collection in this study were previously recruited by Mr Imran Aslam between 2008 and 2011. All had a histological diagnosis of CRC by endoscopy and were undergoing curative resection of cancer with laparoscopic/open or Transanal Microscopic Surgery (TEMS) at Leicester General Hospital, University Hospital of Leicester (UHL) NHS Trust. Up-to-date Information for each patient was verified through the UHL's NHS database. Patient details verified were: demographics, cancer status, disease stage, serum marker levels (CEA), type of surgery, chemotherapy/radiotherapy details, blood samples available, radiological and histopathological findings.

From this, the following findings were identified:

- 16 patients had died prior to recruitment to this study.
- 6 patients were excluded from the study because of the histology results, which were as follows:
    - 3 patients had diverticular disease;
    - 2 patients had a diagnosis of gastric cancer as the primary cancer;
    - 1 patient had a diagnosis of ovarian cancer as the primary cancer.

Seventy-eight (78) patients were included for recruitment to this study. All 78 patients were sent a personalised letter with a phone number for correspondence in order to participate in the study. Patients who did not respond to the letter by text message or by phone were called to confirm their participation in the study. The results were as follows:

- 25 patients did not want to participate in the study;
- 9 patients were undergoing palliative treatment and were not able to participate.

The 44 remaining participants recruited to this study all consented to have blood samples taken post-treatment.

3.1.2 Matching post-treatment samples with stored pre-treatment samples

For each of the 44 patients recruited to the study, matched pre-treatment samples were sourced. Unfortunately, matched pre-treatment samples could only be found for 32 patients. Figure 19 below illustrates the above information.

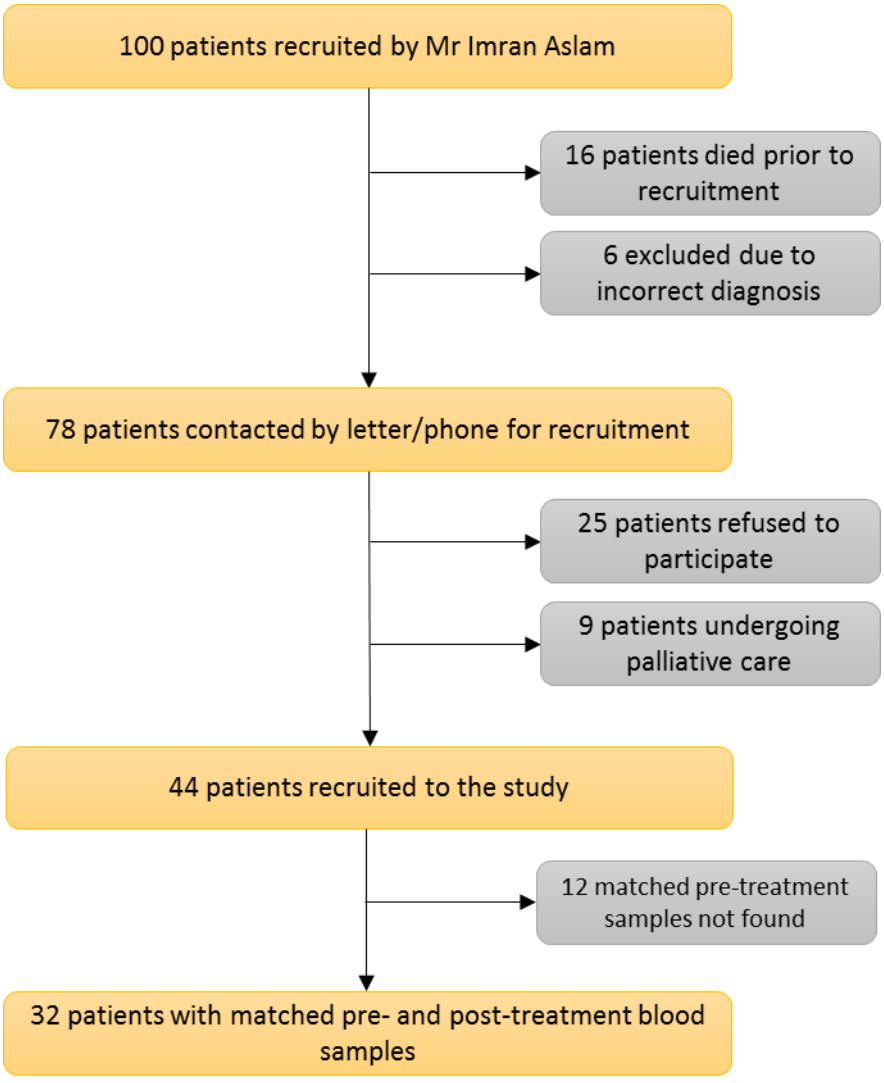


Figure 19: Dataset verification and recruitment of patients to the study for post-treatment sample collection.

3.1.3 Patient characteristics

The clinicopathological characteristics for the 32 patients recruited to the study with matching pre- and post-treatment samples are given in Table 14.

Table 14: Clinicopathological features of the patient cohort

Clinico-pathological characteristics			**Matched Pre- and Post-treatment samples (n = 32)**
Age (Median)	≤ 72.5		19
	>72.5		9
Gender	Male		19
	Female		13
Dukes Staging	Dukes A		3
	Dukes B		14
	Dukes C		14
	Dukes D		1
TNM Staging T (Tumour size)	T1		1
	T2		4
	T3		26
	T4		1
TNM N (Lymph Node)	N0 (No node involvement)		18
	N1 (1-3 nodes involved)		8
	N2 (4 or more nodes involved)		6
Lymph Node	Node Positive		18
	Node Negative		14
Metastasis (M)	Metastasis		31
	No metastasis		1
Differentiation	Well differentiated		2
	Moderately differentiated		29
	Poorly differentiated		1
Site of tumour	Colon	Right	10
		Left	10
	Rectum		12
Treatment	Surgery		19
	Surgery & Chemotherapy		3
	Surgery and radiotherapy		5
	Surgery and Chemo/radiotherapy		5
Margin Resection	Complete		31
	Incomplete		1
CEA Levels (Post Treatment)	Normal levels		29
	Elevated		3

3.2 MiRNA data analysis

3.2.1 Selection of candidate miRNAs

TaqMan Low Density Array (TLDA) card analyses performed in a discovery study of 32 patients (11 normal, 9 adenomas and 12 carcinomas) by Mr Imran Aslam highlighted 29 miRNAs that could significantly discriminate CRC adenomas and carcinomas from healthy controls using Z-scores, principal component analysis, hierarchical cluster analysis, bioinformatics and student's t-test (see section 1.8 for details and Table 15).

Table 15 : Twenty-nine discriminatory miRNAs highlighted by Mr Aslam's study

	miRNA	PCA	Z-Score	t-test	Bioinfor-matics	Star vs. nonstar	Specific patterns	Literature
1	miR-16	✓	✓	✓			✓	✓
2	miR-21	✓		✓				✓
3	miR-23b	✓	✓					
4	miR-34a	✓	✓				✓	✓
5	miR-92a	✓	✓	✓	✓			✓
6	miR-95		✓	✓				
7	miR-135b	✓	✓	✓				✓
8	miR-181c*					✓		
9	miR-182	✓	✓			✓		✓
10	miR-182*			✓	✓	✓		
11	miR-191							✓
12	miR-192*	✓	✓					
13	miR-195							✓
14	miR-200a*	✓	✓					
15	miR-203		✓				✓	
16	miR-205		✓				✓	
17	miR-369-5p	✓	✓	✓	✓			
18	miR-410	✓	✓	✓				
19	miR-431	✓	✓	✓				
20	miR-486-3p						✓	
21	miR-486-5p						✓	
22	miR-487b	✓	✓	✓				
23	miR-502-5p	✓	✓	✓	✓			
24	miR-532-5p						✓	
25	miR-564	✓	✓	✓				
26	miR-566						✓	
27	miR-589	✓		✓	✓			
28	miR-592	✓	✓					
29	miR-624*					✓		

A further validation study of these 29 discriminatory miRNAs in 94 symptomatic patients (32 controls, 28 adenomas and 34 carcinomas) using ROC curve analysis further elucidated a panel of 18 miRNAs (miR-135b, miR-34a, miR-431, miR-16, miR-369-5p, miR-23b, miR-191, miR-21, miR-589, miR-487b, miR-95, miR-484, miR-195, miR-181C*, miR-410, miR-532-5p, miR-192*, miR-203) that could distinguish normal controls from adenomas and carcinomas with high specificity.

In this study, four miRNAs (miR-21, miR-92a, miR-135b and miR-431) were selected from this panel for further validation in post-treatment samples to determine whether they were also predictive of treatment response. MiR-21, miR-135b and miR-431 were selected for this study due their ability to accurately distinguish adenomas and carcinomas from controls in Mr Imran Aslam's study using ROC analysis (all $P > 0.01$). Although miR-92a did not significantly distinguish adenomas from carcinomas in Mr Imran Aslam's validation study of 94 symptomatic patients, it was used in this study due to its known association with treatment response as part of the miR-17/92 cluster (Tsuchida, Ohno et al. 2011). Further to this, an additional 5 miRNAs were selected for validation in this cohort due to their known roles in CRC (see Table 16). MammU6 was selected as an endogenous normaliser since this is used on TDLA cards.

Table 16: miRNAs selected for further validation based on literature searches

MiRNA	Function	Reference
miR-27b	Inhibits tumour progression and angiogenesis in Colorectal Cancer	(Ye, Wu et al. 2013b)
miR-132	Inhibits colorectal cancer invasion and metastasis	(Zheng, Luo et al. 2014, Ng, Chong et al. 2009)
miR-134	Downregulated in CRC	(Bandres, Cubedo et al. 2006)
miR-184	Polymorphism within *CD86* in CRC	(Landi, Gemignani et al. 2008)
miR-203	Low expression in colorectal cancer tissues & associated with proliferation and invasion	(Chiang, Song et al. 2011)

3.2.2 Comparison of miRNA levels pre- and post-treatment

To determine which miRNAs are significantly associated with treatment response, C_T values were normalised to MammU6 levels to obtain a ΔC_T value for statistical analyses. A D'Agostino-Pearson omnibus test was first applied to test the normality of the data, which showed that the data for each miRNA was not normally distributed. Therefore, a non-parametric Wilcoxon signed-rank test was used to compare pre- and post-treatment samples.

Of the nine miRNAs tested, only miR-134, miR-135b and miR-431 were statistically different in post-treatment samples (Figure 20).

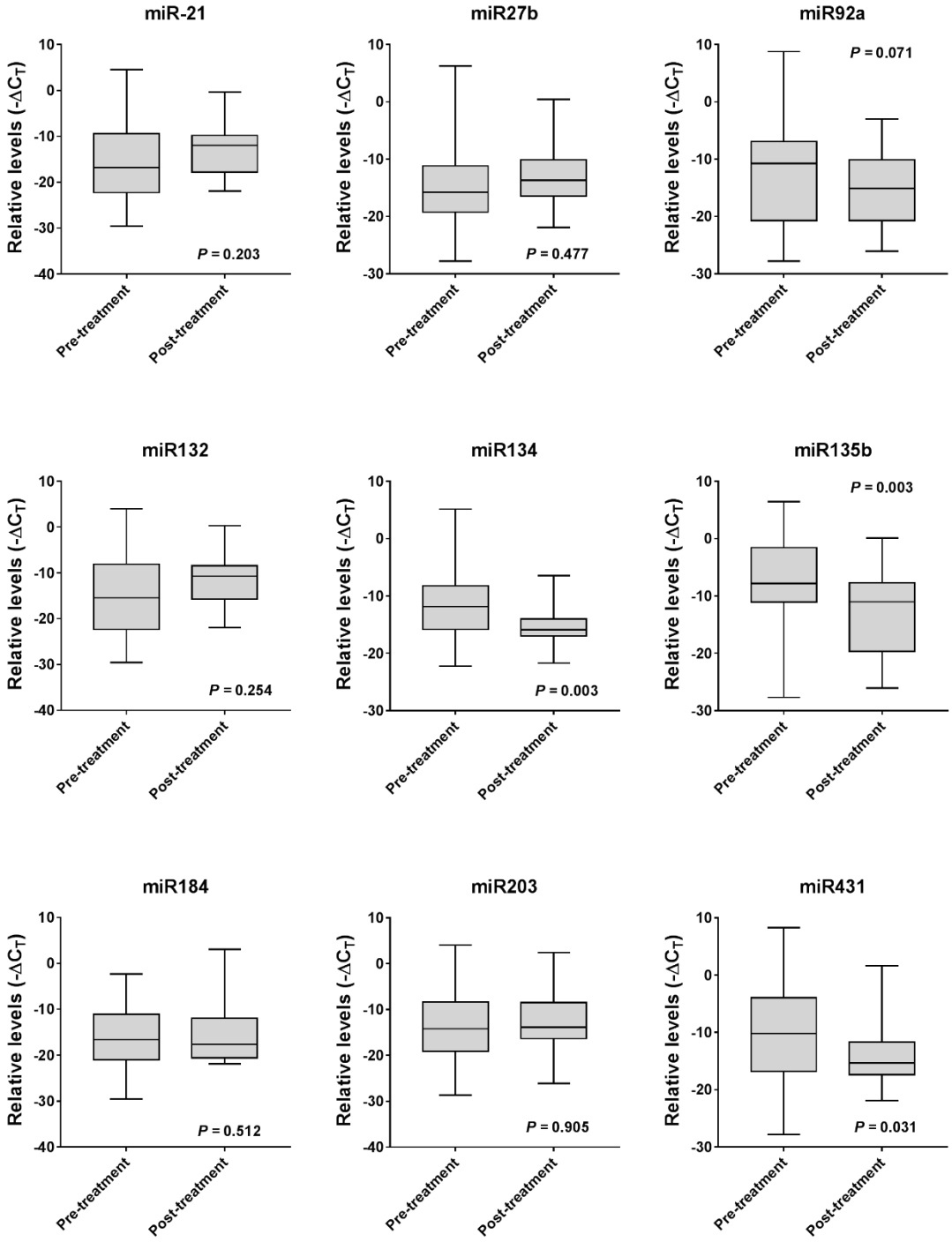


Figure 20: Wilcoxon signed-rank test showing statistical significance for MiRNAs-134, 135b and miR-431. P values for each miRNA is given on each graph. Relative levels are expressed as $-\Delta C_T$ to illustrate comparative levels correctly.

3.2.3 Correlation of miRNA levels with clinicopathological features

To determine whether any relationship exists between miRNA levels in both pre- and post-treatment samples, and clinicopathological features for each patient including age, gender, Dukes stage etc., a multiple-linear regression analysis was performed using delta CT values for each miRNA.

When analysed **individually** using multiple linear regression, miR-135b pre-treatment and miR-431 post-treatment were significantly associated with both node status (positive/negative) and number of nodes involved (Table 16). Pre-treatment miR-132, miR-134, miR-21, miR-27b, miR-184 and miR-203 were also significantly associated with node status (Table 16). Further, miR-134 post-treatment was significantly associated with gender, as was miR-92a, and miR-203 pre-treatment was significantly associated with all Duke's stages (Table 17). However, multiple-linear regression of **all** miRNAs and clinicopathological features revealed only miR-135b levels pre-treatment to be significant in the overall model by ANOVA ($R = 0.754$, $R^2 = 0.569$, $P = 0.043$ – for summary of all miRNAs, see Table 18).

Table 17: Summary of significant miRNAs using multiple-linear regression analysis

Dependent variable	Clinicopathological feature	Unstandardized Coefficients	*P*
miR-135b pre-treatment	**Number of nodes involved**	**-11.422**	**0.009**
miR-135b pre-treatment	**Node status (positive/negative)**	**33.578**	**<0.001**
miR-431 post-treatment	Number of nodes involved	-11.422	0.045
miR-431 post-treatment	Node status (positive/negative)	-12.577	0.033
miR-132 pre-treatment	Node status (positive/negative)	16.433	0.003
miR-134 pre-treatment	Node status (positive/negative)	16.965	0.032
miR-134 post-treatment	Gender	-2.739	0.027
miR-92a post-treatment	Age	-5.882	0.033
miR-92a post-treatment	Gender	-5.902	0.013
miR-21 pre-treatment	Node status (positive/negative)	29.839	0.004
miR-27b pre-treatment	Node status (positive/negative)	30.259	0.003
miR-184 pre-treatment	Node status (positive/negative)	17.295	0.030
miR-203 pre-treatment	Duke's Stage (all stages)	-13.670	0.027
miR-203 pre-treatment	Node Status (positive/negative)	28.401	0.002

Note that miR-135b is highlighted in red in Table 17 as it is the only miRNA that is significant in the **overall** model (see Table 18 for full details).

Table 18: Summary of significant miRNAs using multiple-linear regression analysis ANOVA and overall model summary

		ANOVA				Model summary	
miRNA	**Time point**	**Regression df**	**Residual df**	**F-Ratio**	**Significance**	**R**	**R Square**
miR-135b	Pre-Treatment	11	20	2.400	***0.043***	0.754	0.569
	Post-Treatment	11	20	0.505	0.909	0.448	0.201
miR-132	Pre-Treatment	11	20	2.005	0.085	0.724	0.524
	Post-Treatment	11	20	1.211	0.341	0.632	0.400
miR-134	Pre-Treatment	11	20	1.125	0.393	0.618	0.382
	Post-Treatment	11	20	1.219	0.402	0.616	0.379
miR-21	Pre-Treatment	11	20	1.269	0.309	0.641	0.411
	Post-Treatment	11	20	0.937	0.527	0.583	0.340
miR-27b	Pre-Treatment	11	20	1.623	0.167	0.687	0.472
	Post-Treatment	11	20	0.332	0.968	0.393	0.154
miR-92a	Pre-Treatment	11	20	2.090	0.073	0.731	0.535
	Post-Treatment	11	20	1.716	0.142	0.697	0.485
miR-203	Pre-Treatment	11	20	2.106	0.071	0.733	0.537
	Post-Treatment	11	20	0.279	0.983	0.365	0.133
miR-431	Pre-Treatment	11	20	0.976	0.497	0.591	0.349
	Post-Treatment	11	20	1.092	0.415	0.613	0.375
miR-184	Pre-Treatment	11	20	1.119	0.348	0.630	0.397
	Post-Treatment	11	20	1.237	0.326	0.636	0.405

The table shows the coefficient of determination showing R square (Proportion of variance) and R values (Strength of the relationship between the dependent variable and all of the predictor variables).

Chapter 4: Discussion

Chapter 4: Discussion

4.1: Discussion

This study has shown that plasma levels of miR-135b, miR-134 and miR-431 from patients with CRC are significantly lower post-treatment (Figure 20), regardless of therapeutic intervention (i.e. surgery, chemotherapy or radiotherapy). A previous study by Mr Imran Aslam showed a panel of 18 miRNAs (miR-135b, miR-34a, miR-431, miR-16, miR-369-5p, miR-23b, miR-191, miR-21, miR-589, miR-487b, miR-95, miR-484, miR-195, miR-181C*, miR-410, miR-532-5p, miR-192*, miR-203) were significantly altered in plasma from CRC patients and could accurately distinguish CRC adenomas and carcinomas from healthy controls with 91% sensitivity and 88% specificity (https://lra.le.ac.uk/handle/2381/37943). Another miRNA (miR-92a) was not found to be significant, but nonetheless was investigated in this study. This study investigated 3 of the 18 miRNAs with significantly altered levels in CRCs identified by Mr Imran Aslam (miR-135b, miR-431 and miR-21) and showed that only miR-135b and miR-431 were significantly reduced post-treatment by Wilcoxon signed rank analysis, suggesting a potential role as markers for therapeutic response. Further, miR-134 (selected for analysis here due to being identified as a prominent miRNA important in CRC in a literature search) was significantly reduced post-treatment. Previous studies have shown miR-135b to be significantly upregulated in CRC (both in tissue and plasma) (X. M. Xu, Qian et al. 2012). To date, there are no studies in the literature which have also shown miR-134 and miR-431 expression levels pre and post treatment in plasma. However, a Chinese study has recently shown lower miR-431 expression levels in CRC tissue compared to normal tissue, which is a contradiction to our findings (W. B. Su, Liu 2018). Another Chinese study by Xie et al. (2015) showed miR-134 expression was significantly downregulated in CRC cancer tissues and cell lines, which is again in contradiction to our findings (Xie, Song et al. 2015). However, to our knowledge, this is the first study to show a significant decrease in plasma levels of these miRNAs in a longitudinal study and therefore, suggests that these miRNAs are potential markers of therapeutic response.

Valeri et al. (2014) performed studies in mouse models of CRC and showed miR-135b expression levels to be upregulated by an average of 4-fold in cancer compared to paired normal tissue and correlated this expression with tumour progression. Tumour stage and nodal status were confirmed as prognostic markers in our cohort of sporadic CRC, suggesting that miR-135b is a CRC specific deregulated miRNA; thus, a valuable biomarker and potential target for therapy. Loss of *APC* is the initiating mutation in CRC. MicroRNA-135b expression follows the accumulation of mutations in *APC*, *PI3KCA* and *SRC.* The figure below shows several genes potentially controlled by miR-135b leading to their targets transforming growth factor β receptor 2 (*TGFβR2*), death-associated protein kinase 1 (*DAPK1*), and *FIH.* The downstream effect of the mutated genes eventually leads to activation of interleukin-8, promoting invasion, apoptosis and proliferation (Valeri, Braconi et al. 2014).

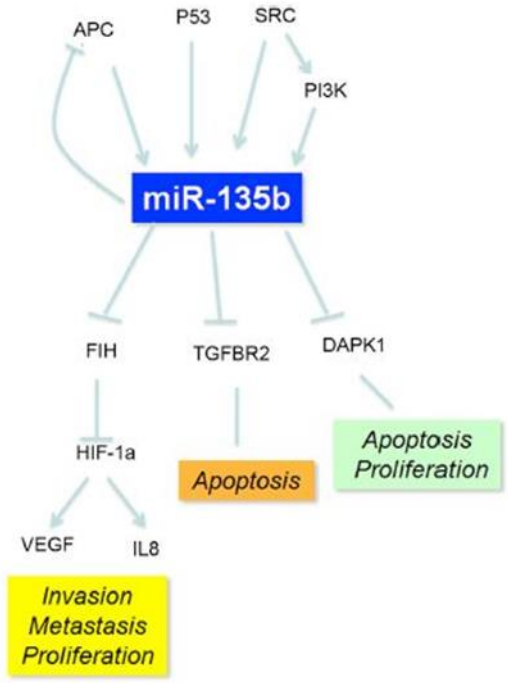


Figure 21: MiR-135b functions like a Key Oncogenic Hub Mediating the Cancer Phenotype Downstream of Genes Frequently Mutated in CRC and their representation of genetic aberrations promoting miR-135b overexpression and miR-135b downstream targets. Figure taken from (Valeri et al. 2014).

Although levels of miR-92a were not shown to be significantly altered in this study, in 2014 a study of 27 patients by Ristau et al. (2014) involved collecting blood samples

the day before surgery, 2-7 days post-surgery and 6 months after surgery. Analyses of plasma miRNA levels over all three time points demonstrated a statistically significant decrease 2-7 days post-surgery of four circulating miRNAs (**miR-92a**, miR-320a, miR-106a, and miR-18a) (Ristau, Staffa et al. 2014b), but returned to pre-surgical levels at 6 months. In this study, follow-up samples was obtained 3 – 5 years post-surgery and therefore may explain the lack of significance.

Similarly, miR-21 was not significantly altered in this study; however, Toiyama et al. (2014) analysed paired pre- and post-operative serum samples in 45 CRC patients who underwent curative surgical resection and 15 patients with non-curative resection (multiple hepatic metastases and underwent primary resection to prevent bleeding and bowel obstruction). The study showed that postoperative reductions in serum miR-21 levels occurred exclusively among patients with potentially curative surgery, but no statistically significant differences were observed before or after surgery in patients with non-curative resections (Toiyama et al. 2013).

The largest longitudinal study to date by Yuan et al. (2017) on 122 patients analysed plasma miRNA levels prior to surgery, at the time of resection, follow up at 6, 12 and 24 months. This study showed that although the majority of miRNA did not show any significant changes over time, miR-203 (identified as a candidate miRNA in this study) was not detectable at all in the plasma. Of the miRNAs that were significantly altered post-operatively in the Yuan study (2017), none of them correlated with this study. Plasma miR-141 levels decreased 24 months after surgery, compared to baseline and 12 months after surgery in 9 pairs of patients. Plasma miR-16 levels increased 12 months after surgery compared to baseline in the recurrence group in 10 pairs of Patient samples. The study also showed higher levels of miR-31 were associated with a three-fold increased risk of recurrence across all time points (Yuan, Baker et al. 2017).

A multiple linear regression analysis highlighted only pre-treatment miR-135b levels to be significantly associated with lymph node involvement. Few studies have shown miRNA-135b levels to be significantly elevated in CRC plasma samples and increasing trend with stage tumour stages II, III, IV and is involved in CRC development and

progression (Eslamizadeh, Heidari et al. 2018, X. M. Xu et al. 2012). Our study is the first in the literature to associate miRNA-135b with lymph node involvement in plasma samples, complementing studies in mouse models performed by Valeri et al. (2014) and discussed earlier. Although a number of miRNA were significantly associated with other clinicopathological features when analysed individually, only miR-135b was significantly associated with any features in the **overall model.** This is important as it highlights that when all of the miRNAs are analysed *together*, although other miRNAs may contribute the model, only miR-135b contributed significantly to the overall model and therefore can be the only miRNA that can truly be considered as being associated with any clinicopathological feature (that being nodal involvement).

4.2 Limitations of the study

4.2.1 Large number of patients with no follow-up samples

The sample size was 32 patients with matched pre- and post-treatment samples, which was far less than the predicted number of patients initially thought to have been recruited to Mr Aslams's study. Of the initial 100 recruited to Mr Alsam's study, the 44 patients who ultimately agreed to participate in this study could not all be matched with the pre–treatment group due to the non-availability of the samples. This could possibly explain lack of statistically significant associations with clinic pathological parameters.

Further, the data consisted of healthy patients who were free of cancer proven by radiology and endoscopy in the follow up since their treatment. The dataset also contained variables including metastasis (one metastasis out of 32 patients), differentiation (29 moderately differentiated, 2 well-differentiated and 1 poorly differentiated), CEA levels (one abnormal level out of 32) and margin resection (one out of 32), which were not included in the regression model due to the small numbers potentially leading to statistical errors.

4.2.2 Normalisation

Despite their importance, at present there is no consensus regarding housekeeping miRNAs for normalising circulating miRNA data. Therefore, measurement of relative expression levels of circulating miRNAs has been a common approach in published studies. Numerous studies have used *MammU6* as an endogenous control miRNA, which was also used in this study. However, this miRNA was analysed on separate qPCR plates and not run alongside other candidate miRNAs, which could affect the normalisation of the data. However, to attempt to minimize the potential variation that may occur as a result of this strategy, exact the same sample of diluted cDNA was used to analyse each miRNA. Should more cDNA needed to be diluted, this was used to analyse all miRNAs and not just *MammU6,* ensuring the same sample was used to analyse each miRNA.

4.2.3 Lack of consistency with other studies

The limited number of paired sample studies discussed above raise the question of the inconsistencies in miRNA expression levels determined across various studies in the plasma in pre and post-operative samples at different time points. Presently, no miRNA biomarkers have been proven to be good enough for use in the clinical setting. This is attributed to the inconsistency of reproducibility of the results of the published studies (Schubert, Junker et al. 2016). The poor reproducibility of biomarker miRNA studies is likely due to small sample sizes, lack of validation cohorts, different assay technologies, differences in normalisation methods across studies and differences in pre-analytical sample handling protocols (Y. Sun, Liu et al. 2016, Yamada, Horimatsu et al. 2015).

4.3 Strengths of the study

4.3.1 The dataset

Despite the large dropout in this study regarding recruitment of patients for follow-up samples, the data included thirty two matched pre- and post-operative samples, which

represents one of the largest longitudinal studies to date and as a result allowed us to performed more rigorous data analysis statistically.

4.3.2 Statistical analysis

The choice of statistical test depends on the type of the data being tested. The ΔC_T expression levels of the candidate miRNA tested were found to be being continuous, paired and dependent, so are likely to be not normalised in distribution as confirmed using a D'Agostino-Pearson omnibus test. Therefore, it was necessary to perform a non-parametric Wilcoxon rank test to determine the relationship between ΔC_T values of both pre- and post-treatment groups.

The study also wanted to determine the relationship between the pre- and post-treatment miRNA expression levels and various clinical and pathological characteristics of the patients. Multiple linear regressions therefore the most appropriate statistical test and subsequently was chosen to predict miRNA levels based on multiple independent variables of the Clinico-pathological characteristics of the patients. Multiple linear regression allowed us to determine the overall fit of the model with variations and the relative contribution from each of the predictors. Statistical advice was taken from a statistician (Dr Mark Rutherford) during the analysis.

4.4 Future work

This pilot study investigated the pre- and post-treatment levels of miRNAs in response to therapy. The follow up required for these patients was difficult and time consuming due to voluntary participation and terminally ill patients not able to attend during the recruitment process. Going forward, a longitudinal study with follow up blood samples collected at multiple end points including pre-surgery, one week after surgery, and 3, 6, 9 and 12 months would be able to accurately track plasma miRNA levels throughout the disease process. Further, our study could also involve lifestyle risk factors including meat consumption, alcohol intake, smoking habits, body fat (BMI) etc.

4.5 Conclusion

This study identified miR-134; miR-135b and miR-431 expression levels may highlight miRNAs that are associated with response to therapy. Further, while the recruitment of the patients was lower than expected, this was still a larger study in comparison to the current literature, highlighting a panel of miRNAs which could be potentially serve as biomarkers of response in CRC. Overall, although this is a pilot study in a small cohort of patients, this study has highlighted the highly heterogeneous results obtained from numerous miRNA studies, further suggesting that more appropriate standardisation of circulating miRNA studies is required before they can be considered as a validated clinical biomarker. This is a pilot project with preliminary data and should become a precedent for future studies on a larger scale.

Appendix 1: Presentations arising from this thesis

1) Plasma microRNA 135b: Diagnostic Biomarker and predicts lymph node stage in colorectal cancer patients. *American Society of colon and rectal surgeons*, June 2017, Seattle, USA.

2) Plasma microRNA 21: Diagnostic Biomarker and predicts positive and negative lymph nodes in colorectal cancer, *American Society of colon and rectal surgeons* June 2017, Seattle, USA.

3) Do Plasma miRNAs have a role before and after treatment in colorectal cancer.*14th international congress on Targeted Anticancer Therapies*, March 2017, Washington DC, USA.

4) Plasma microRNA 132: Diagnostic Biomarker and correlates with Node, Tumour size an Dukes Staging, *7th SICCR meeting Rome*, 2017.

5) Plasma microRNA 27b: Diagnostic Biomarker and correlates with Lymph node and surgery, 7th SICCR meeting Rome, 2017.

6) Plasma microRNA 21: Diagnostic Biomarker and predicts positive and negative lymph nodes in colorectal cancer*, 7th SICCR meeting Rome*, 2017.

7) Diagnostic Biomarker and predicts Lymph nodes in Colorectal cancer patients, *7th SICCR meeting Rome, 2017.*

8) Plasma microRNA 184: Diagnostic Biomarker and Correlates with Node stage site of Cancer, *7th SICCR meeting Rome, 2017.*

Appendix 2: Study Documentation

1: Ethics 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 Hotmanski**
R&D Manager: **Carolyn Maloney**

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

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

03/03/2011

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

Dear Dr James Howard Pringle

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

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

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

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

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

Version 5, 20.04.10

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

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

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

We wish you every success with your research.

Yours sincerely

Carolyn Maloney
R&D Manager

2: Consent forms

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: .....

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: .....

3 Information leaflet for Patients

Participant Information Sheet

University Hospitals of Leicester NHS
NHS Trust

STUDY TITLE: BIOMARKERS FOR BOWEL DISEASE PROGRESSION

University of Leicester - Department of Molecular Medicine

Robert Kilpatrick Building Level 3 Leicester Royal Infirmary

Researchers: Miss Patel, Mr. Aslam, Mr. Singh, Mr. Jameson.

Principle Investigator: Dr Pringle

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

1. Why have I been chosen?

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

We will also study a sample of your blood, which will be taken around the time of your procedure.

2. What is the purpose of the study?

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

3. Do I have to take part?

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

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

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

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

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

6. What are the possible benefits of taking part?

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

7. What if new information becomes available?

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

8. What if something goes wrong?

The chance of any problems arising because of your inclusion in the study is extremely small. If you do feel that taking part in this research project has harmed you, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may

have grounds for a legal action. If you wish to complain, or have any concerns regarding the way you have been approached or treated throughout the study, you may contact National Health Service complaints department in the normal way.

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

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

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

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

11. Who is organising and funding the research?

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

12. Who has reviewed the study?

All research that involves NHS patients, staff, and 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.

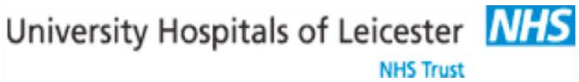
13. Contacts for Further Information

Dr. Howard Pringle	Miss Maleene Patel
Department of Molecular Medicine	Department of Molecular Medicine.
Robert Kilpatrick Building	Level 3, Robert Kilpatrick Building
Leicester Royal Infirmary	Leicester Royal Infirmary
Leicester LE2 7LX	Leicester LE2 7LX
E-mail: JHP@le.ac.uk	Email: mp364@le.ac.uk
Phone: 0116 252 3227	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.

Participant Information Sheet



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.

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.

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.

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.

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.

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.

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.

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

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).

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

OR Research Office

Directorate of Research & Development

University Hospitals of Leicester NHS Trust

Leicester General Hospital

Gwendolen Road

Leicester LE5 4PW

Tel: +44 116 2523227 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: Summary of qRT-PCR data

	miR-135b				miR-132				miR-134			
	Pre-treatment		Post-treatment		Pre-treatment		Post-treatment		Pre-treatment		Post-treatment	
Patient	Ct	delta Ct	Ct	delta Ct	Ct	delta Ct	Ct	delta Ct	Ct	delta Ct	Ct	delta Ct
H91	25.32	1.45	25.48	6.52	40.00	16.13	40.00	21.04	32.55	8.68	34.58	15.62
H92	26.44	3.09	40.00	22.06	38.92	15.57	28.94	11.00	33.04	9.68	34.94	17.00
H93	28.52	-2.36	25.42	8.69	27.28	-3.60	25.29	8.55	33.12	2.23	33.62	16.88
H95	26.65	6.94	40.00	22.71	40.00	20.29	27.44	10.16	32.97	13.26	34.81	17.52
H96	33.58	0.81	25.62	7.50	36.43	3.65	29.05	10.92	32.66	-0.12	28.84	10.71
H97	26.12	15.66	40.00	18.98	40.00	29.54	28.18	7.16	32.68	22.22	36.46	15.44
H99	25.39	-3.20	25.89	7.58	40.00	11.41	40.00	21.70	32.72	4.12	33.79	15.49
H100	25.60	-1.06	40.00	19.23	40.00	13.34	33.94	18.67	31.94	5.28	34.52	13.76
H101	40.00	27.66	25.32	6.12	40.00	27.66	25.30	6.10	33.29	19.95	34.82	15.62
H102	31.24	-6.46	25.00	5.84	33.69	-4.01	28.31	9.15	32.59	-5.11	33.88	14.71
H103	40.00	25.56	25.23	5.04	38.17	23.72	25.03	4.84	33.25	18.81	33.68	13.50
H105	28.25	11.57	25.82	-0.12	40.00	23.31	36.43	10.49	32.55	15.86	34.57	8.63
H106	26.10	9.47	40.00	19.79	40.00	23.37	25.98	5.77	32.63	16.00	34.33	14.12
H107	34.63	13.94	37.73	23.79	40.00	19.30	25.25	11.31	33.05	12.35	27.88	13.94
H108	40.00	7.95	40.00	26.04	40.00	7.95	27.91	3.96	33.091	1.04	30.76	16.80
H110	25.39	5.53	31.48	11.75	25.94	6.08	40.00	20.27	33.26	13.40	38.44	18.71
H111	25.46	2.00	38.84	20.58	25.66	2.20	33.16	14.90	32.85	9.38	34.59	16.34
H112	29.14	-5.66	29.21	10.82	40.00	5.30	26.74	8.35	32.69	-2.01	34.97	16.58
H113	36.56	11.84	40.00	21.72	40.00	15.28	33.45	15.17	33.20	8.48	40.00	21.72
H115	25.14	0.70	26.71	8.61	40.00	15.56	40.00	21.91	32.57	8.12	37.52	19.43
H116	25.27	7.53	27.12	7.10	40.00	22.26	40.00	19.98	28.41	10.67	38.26	18.24
H119	25.36	7.68	40.00	16.94	40.00	22.32	36.22	3.16	32.64	14.96	35.90	12.83
H120	25.28	9.14	40.00	11.14	40.00	23.86	28.54	-0.32	26.79	10.65	35.31	6.45
H121	40.00	22.18	40.00	19.82	40.00	22.18	30.25	10.08	33.59	15.77	34.82	14.64
H125	25.48	2.38	33.48	13.64	36.48	13.38	26.62	6.79	33.40	10.29	35.78	15.95
H126	25.33	7.90	31.84	12.55	28.94	11.51	30.51	11.22	32.47	15.04	35.12	15.83
H127	25.01	12.80	28.43	7.95	40.00	27.79	39.71	9.23	27.97	5.76	36.77	16.29
H128	26.75	9.20	27.53	8.23	25.99	8.44	29.28	9.99	33.56	16.01	35.40	16.11
H130	27.55	8.77	27.45	8.02	25.78	7.00	30.01	10.58	33.21	14.43	36.45	17.01
H131	25.31	4.07	28.18	9.85	30.32	9.07	34.22	15.89	32.55	11.30	37.15	18.82
H132	25.10	9.18	36.12	16.51	30.24	14.32	29.60	9.98	32.74	16.83	35.59	15.98
H133	25.57	8.61	27.91	10.22	37.83	20.87	25.31	7.62	33.28	16.31	31.25	13.57

	miR-21				miR-27b				miR-92a			
	Pre-treatment		Post-treatment		Pre-treatment		Post-treatment		Pre-treatment		Post-treatment	
Patient	Ct	delta Ct	Ct	delta Ct	Ct	delta Ct	Ct	delta Ct	Ct	delta Ct	Ct	delta Ct
H91	40.00	16.13	35.13	16.17	35.00	11.13	34.79	15.83	31.15	7.27	29.93	10.97
H92	40.00	16.65	29.12	11.18	39.05	15.70	34.56	16.62	31.10	7.74	26.12	8.18
H93	34.12	3.23	32.91	16.18	33.50	2.61	35.04	18.31	29.22	-1.66	28.73	12.00
H95	40.00	20.29	35.38	18.09	36.19	16.48	35.38	18.09	30.28	10.57	29.12	11.84
H96	40.00	7.22	34.68	16.55	40.00	7.22	32.17	14.04	32.24	-0.54	27.20	9.07
H97	40.00	29.54	37.87	16.85	33.65	23.19	34.20	13.18	27.61	17.16	28.90	7.87
H99	40.00	11.41	40.00	21.70	22.37	-6.22	34.86	16.55	40.00	11.41	28.84	10.53
H100	40.00	13.34	40.00	19.23	38.20	11.54	34.23	13.46	27.39	0.73	40.00	19.23
H101	40.00	27.66	39.73	20.54	36.06	23.72	36.84	17.65	29.68	17.33	29.14	9.94
H102	40.00	2.30	37.41	18.25	35.09	-2.61	35.32	16.16	28.94	-8.76	28.69	9.53
H103	40.00	25.56	28.79	8.60	33.91	19.47	34.96	14.78	36.26	21.82	28.04	7.85
H105	29.50	12.81	38.58	12.65	35.26	18.57	36.06	10.13	27.35	10.66	28.94	3.00
H106	30.21	13.58	31.69	11.49	28.67	12.04	34.15	13.94	40.00	23.37	28.89	8.69
H107	40.00	19.30	25.30	11.36	36.51	15.82	25.29	11.35	31.29	10.60	40.00	26.06
H108	27.48	-4.57	25.68	11.72	26.54	-5.51	25.10	11.14	40.00	7.95	40.00	26.04
H110	40.00	20.14	40.00	20.27	38.52	18.66	40.00	20.27	29.37	9.51	31.26	11.53
H111	28.62	5.15	25.47	7.21	27.29	3.82	25.82	7.56	40.00	16.53	40.00	21.74
H112	40.00	5.30	27.80	9.41	35.12	0.42	26.15	7.76	29.67	-5.03	40.00	21.61
H113	34.00	9.28	30.47	12.19	35.98	11.26	29.48	11.20	29.64	4.92	40.00	21.72
H115	26.44	2.00	40.00	21.91	40.00	15.56	40.00	21.91	40.00	15.56	31.19	13.09
H116	40.00	22.26	40.00	19.98	40.00	22.26	40.00	19.98	40.00	22.26	33.32	13.30
H119	40.00	22.32	32.44	9.37	33.79	16.11	37.23	14.16	40.00	22.32	40.00	16.94
H120	40.00	23.86	29.20	0.34	40.00	23.86	28.45	-0.41	40.00	23.86	40.00	11.14
H121	40.00	22.18	30.38	10.20	40.00	22.18	29.04	8.86	40.00	22.18	40.00	19.82
H125	40.00	16.90	26.59	6.75	36.24	13.14	27.54	7.71	28.01	4.91	40.00	20.17
H126	34.96	17.53	30.41	11.12	35.18	17.74	30.47	11.18	28.24	10.81	40.00	20.71
H127	40.00	27.79	32.72	12.24	40.00	27.79	34.42	13.94	40.00	27.79	40.00	19.52
H128	27.33	9.78	28.35	9.06	31.85	14.29	29.08	9.78	40.00	22.45	40.00	20.71
H130	31.83	13.05	29.10	9.66	34.63	15.85	29.82	10.38	28.21	9.44	40.00	20.56
H131	40.00	18.76	31.47	13.14	36.17	14.93	32.24	13.91	28.07	6.83	40.00	21.67
H132	35.76	19.84	30.26	10.65	35.54	19.62	29.71	10.10	29.26	13.34	40.00	20.39
H133	40.00	23.04	28.27	10.58	35.31	18.35	27.66	9.97	28.55	11.59	40.00	22.31

	miR-203				miR-431				miR-184			
	Pre-treatment		Post-treatment		Pre-treatment		Post-treatment		Pre-treatment		Post-treatment	
Patient	Ct	delta Ct	Ct	delta Ct	Ct	delta Ct	Ct	delta Ct	Ct	delta Ct	Ct	delta Ct
H91	34.13	10.26	34.18	15.22	29.18	5.31	33.93	14.97	40.00	16.13	40.00	21.04
H92	34.95	11.59	35.89	17.95	29.76	6.40	36.45	18.51	40.00	16.65	34.63	16.69
H93	31.48	0.60	32.23	15.49	29.45	-1.44	35.02	18.29	40.00	9.11	32.60	15.86
H95	35.57	15.86	34.92	17.64	25.63	5.93	32.22	14.93	40.00	20.29	38.48	21.19
H96	32.91	0.14	34.13	16.00	30.69	-2.09	35.44	17.31	40.00	7.22	33.14	15.01
H97	39.10	28.64	34.58	13.56	25.28	14.83	32.97	11.95	40.00	29.54	40.00	18.98
H99	29.01	0.41	35.94	17.64	27.93	-0.66	34.22	15.92	34.24	5.64	40.00	21.70
H100	36.89	10.23	34.55	13.78	30.56	3.91	34.78	14.01	40.00	13.34	40.00	19.23
H101	35.58	23.24	33.58	14.39	28.84	16.50	34.61	15.41	40.00	27.66	40.00	20.80
H102	33.66	-4.04	35.63	16.47	32.02	-5.68	40.00	20.84	40.00	2.30	40.00	20.84
H103	34.97	20.52	33.17	12.98	27.14	12.69	30.10	9.91	40.00	25.56	37.88	17.70
H105	35.22	18.53	34.41	8.47	29.18	12.49	36.27	10.33	40.00	23.31	40.00	14.06
H106	40.00	23.37	35.00	14.79	25.14	8.51	35.49	15.29	33.85	17.22	40.00	19.79
H107	40.00	19.30	40.00	26.06	27.55	6.86	25.07	11.13	40.00	19.30	25.49	11.55
H108	31.30	-0.75	40.00	26.04	28.30	-3.75	26.12	12.16	40.00	7.95	29.91	15.95
H110	35.36	15.50	31.76	12.03	28.12	8.26	40.00	20.27	40.00	20.14	38.64	18.91
H111	31.09	7.62	26.73	8.47	27.45	3.99	40.00	21.74	40.00	16.53	40.00	21.74
H112	39.70	5.00	25.01	6.62	26.39	-8.31	25.43	7.04	40.00	5.30	40.00	21.61
H113	33.01	8.30	29.52	11.24	29.32	4.60	34.44	16.16	40.00	15.28	35.77	17.49
H115	33.10	8.66	34.93	16.83	26.88	2.44	40.00	21.91	40.00	15.56	40.00	21.91
H116	26.27	8.53	34.55	14.54	40.00	22.26	36.48	16.46	33.99	16.25	40.00	19.98
H119	33.20	15.53	25.28	2.22	35.05	17.37	38.97	15.91	35.51	17.84	28.67	5.60
H120	40.00	23.86	26.42	-2.44	40.00	23.86	27.21	-1.65	26.46	10.31	25.76	-3.10
H121	39.57	21.74	25.05	4.87	40.00	22.18	27.98	7.80	33.75	15.92	40.00	19.82
H125	34.49	11.38	31.68	11.85	37.55	14.45	40.00	20.17	40.00	16.90	30.99	11.15
H126	34.81	17.38	29.04	9.74	34.78	17.35	33.76	14.46	40.00	22.57	25.29	6.00
H127	25.66	13.45	34.34	13.86	40.00	27.79	36.99	16.52	27.07	14.87	33.71	13.23
H128	31.47	13.92	27.74	8.45	36.28	18.73	30.88	11.59	27.51	9.95	26.74	7.45
H130	37.25	18.48	40.00	20.56	34.53	15.75	31.35	11.91	40.00	21.22	29.20	9.76
H131	35.64	14.40	32.24	13.91	33.08	11.84	34.01	15.68	40.00	18.76	26.48	8.15
H132	35.25	19.33	28.09	8.48	32.83	16.91	36.99	17.38	40.00	24.08	37.38	17.77
H133	35.41	18.44	25.89	8.20	32.78	15.82	27.08	9.39	40.00	23.04	32.58	14.90

Appendix 4: Summary of multiple linear regression analyses

	miR-135b		miR-132		miR-134	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Age	0.775	0.211	0.875	0.892	0.936	0.862
Gender	0.898	0.378	0.946	0.158	0.723	0.027
Dukes Stage	0.279	0.702	0.254	0.472	0.322	0.463
Dukes Stage (A/B vs. C/D)	0.304	0.841	0.519	0.709	0.416	0.633
TNM	0.538	0.354	0.861	0.161	0.845	0.974
TNM Stage (T1/T2 vs. T3/T4	0.409	0.725	0.745	0.424	0.842	0.496
Number of Nodes	0.009	0.891	0.134	0.153	0.661	0.345
Node status	<0.001	0.663	0.003	0.088	0.032	0.11
Site (Colon/Rectum)	0.511	0.706	0.463	0.208	0.884	0.461
Surgery (SC vs. SR. vs. SCR)	0.171	0.405	0.324	0.276	0.748	0.889
Surgery vs. SCR	0.093	0.529	0.217	0.697	0.407	0.556

	miR-21		miR-27b		miR-92a	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Age	0.343	0.063	0.679	0.176	0.125	0.033
Gender	0.522	0.556	0.842	0.595	0.937	0.013
Dukes Stage	0.312	0.416	0.069	0.76	0.673	0.061
Dukes Stage (A/B vs. C/D)	0.646	0.303	0.864	0.769	0.422	0.295
TNM	0.684	0.69	0.483	0.83	0.226	0.413
TNM Stage (T1/T2 vs. T3/T4	0.887	0.429	0.808	0.693	0.869	0.839
Number of Nodes	0.093	0.275	0.053	0.487	0.278	0.316
Node status	0.004	0.289	0.003	0.57	0.059	0.464
Site (Colon/Rectum)	0.994	0.577	0.608	0.914	0.172	0.484
Surgery (SC vs. SR. vs. SCR)	0.842	0.632	0.699	0.935	0.797	0.519
Surgery vs. SCR	0.877	0.544	0.2	0.884	0.769	0.888

All tables show P values for linear regression analyses

	miR-203		miR-431		miR-184	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Age	0.594	0.769	0.49	0.48	0.348	0.21
Gender	0.313	0.516	0.605	0.128	0.953	0.32
Dukes Stage	0.027	0.872	0.975	0.506	0.078	0.52
Dukes Stage (A/B vs. C/D)	0.93	0.867	0.525	0.767	0.39	0.91
TNM	0.457	0.818	0.604	0.89	0.79	0.386
TNM Stage (T1/T2 vs. T3/T4	0.31	0.468	0.721	0.921	0.685	0.34
Number of Nodes	0.322	0.419	0.089	0.045	0.738	0.14
Node status	0.002	0.591	0.057	0.033	0.03	0.471
Site (Colon/Rectum)	0.469	0.406	0.525	0.664	0.284	0.851
Surgery (SC vs. SR. vs. SCR)	0.387	0.849	0.827	0.556	0.612	0.085
Surgery vs. SCR	0.267	0.893	0.617	0.985	0.272	0.133

All tables show P values for linear regression analyses

References

ABE, S., KAWAI, K., ISHIHARA, S., NOZAWA, H., HATA, K., KIYOMATSU, T., TANAKA, T. and WATANABE, T., 2016. Prognostic impact of carcinoembryonic antigen and carbohydrate antigen 19-9 in stage IV colorectal cancer patients after R0 resection. *The Journal of surgical research,* **205**(2), pp. 384-392.

ADJEI, A.A., 2001. Blocking oncogenic Ras signaling for cancer therapy. *Journal of the National Cancer Institute,* **93**(14), pp. 1062-1074.

ALLEGRA, C.J., RUMBLE, R.B., HAMILTON, S.R., MANGU, P.B., ROACH, N., HANTEL, A. and SCHILSKY, R.L., 2016. Extended RAS Gene Mutation Testing in Metastatic Colorectal Carcinoma to Predict Response to Anti-Epidermal Growth Factor Receptor Monoclonal Antibody Therapy: American Society of Clinical Oncology Provisional Clinical Opinion Update 2015. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* **34**(2), pp. 179-185.

ALLISON, J.E., FRASER, C.G., HALLORAN, S.P. and YOUNG, G.P., 2014. Population screening for colorectal cancer means getting FIT: the past, present, and future of colorectal cancer screening using the fecal immunochemical test for hemoglobin (FIT). *Gut and liver,* **8**(2), pp. 117-130.

AL-SOHAILY, S., BIANKIN, A., LEONG, R., KOHONEN-CORISH, M. and WARUSAVITARNE, J., 2012. Molecular pathways in colorectal cancer. *Journal of gastroenterology and hepatology,* **27**(9), pp. 1423-1431.

ARNOLD, M., SIERRA, M.S., LAVERSANNE, M., SOERJOMATARAM, I., JEMAL, A. and BRAY, F., 2017. Global patterns and trends in colorectal cancer incidence and mortality. *Gut,* **66**(4), pp. 683-691.

ARROYO, J.D., CHEVILLET, J.R., KROH, E.M., RUF, I.K., PRITCHARD, C.C., GIBSON, D.F., MITCHELL, P.S., BENNETT, C.F., POGOSOVA-AGADJANYAN, E.L., STIREWALT, D.L., TAIT, J.F. and TEWARI, M., 2011. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proceedings of the National Academy of Sciences of the United States of America,* **108**(12), pp. 5003-5008.

ASANGANI, I.A., RASHEED, S.A., NIKOLOVA, D.A., LEUPOLD, J.H., COLBURN, N.H., POST, S. and ALLGAYER, H., 2008. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene,* **27**(15), pp. 2128-2136.

ATKIN, W.S., COOK, C.F., CUZICK, J., EDWARDS, R., NORTHOVER, J.M., WARDLE, J. and UK FLEXIBLE SIGMOIDOSCOPY SCREENING TRIAL INVESTIGATORS, 2002. Single flexible sigmoidoscopy screening to prevent colorectal cancer: baseline findings of a UK multicentre randomised trial. *Lancet (London, England),* **359**(9314), pp. 1291-1300.

ATKIN, W.S., EDWARDS, R., KRALJ-HANS, I., WOOLDRAGE, K., HART, A.R., NORTHOVER, J.M., PARKIN, D.M., WARDLE, J., DUFFY, S.W., CUZICK, J. and UK FLEXIBLE SIGMOIDOSCOPY TRIAL INVESTIGATORS, 2010. Once-only flexible sigmoidoscopy screening in prevention of colorectal cancer: a multicentre randomised controlled trial. *Lancet (London, England),* **375**(9726), pp. 1624-1633.

BALLEHANINNA, U.K. and CHAMBERLAIN, R.S., 2012. The clinical utility of serum CA 19-9 in the diagnosis, prognosis and management of pancreatic adenocarcinoma: An evidence based appraisal. *Journal of gastrointestinal oncology,* **3**(2), pp. 105-119.

BANDRES, E., CUBEDO, E., AGIRRE, X., MALUMBRES, R., ZARATE, R., RAMIREZ, N., ABAJO, A., NAVARRO, A., MORENO, I., MONZO, M. and GARCIA-FONCILLAS, J., 2006. Identification by Real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues. *Molecular cancer,* **5**, pp. 29-4598-5-29.

BARETTI, M. and LE, D.T., 2018. DNA mismatch repair in cancer. *Pharmacology & therapeutics,* **189**, pp. 45-62.

BARTEL, D.P., 2009. MicroRNAs: target recognition and regulatory functions. *Cell,* **136**(2), pp. 215-233.

BINEFA, G., RODRIGUEZ-MORANTA, F., TEULE, A. and MEDINA-HAYAS, M., 2014. Colorectal cancer: from prevention to personalized medicine. *World journal of gastroenterology,* **20**(22), pp. 6786-6808.

BIRD, A.P., 1986. CpG-rich islands and the function of DNA methylation. *Nature,* **321**(6067), pp. 209-213.

BOLAND, C.R. and GOEL, A., 2010. Microsatellite instability in colorectal cancer. *Gastroenterology,* **138**(6), pp. 2073-2087.e3.

BRAND, T.M. and WHEELER, D.L., 2012. KRAS mutant colorectal tumors: past and present. *Small GTPases,* **3**(1), pp. 34-39.

BRENNER, H., STOCK, C. and HOFFMEISTER, M., 2014. Effect of screening sigmoidoscopy and screening colonoscopy on colorectal cancer incidence and mortality: systematic review and meta-analysis of randomised controlled trials and observational studies. *BMJ (Clinical research ed.),* **348**, pp. g2467.

BROWNAWELL, A.M. and MACARA, I.G., 2002. Exportin-5, a novel karyopherin, mediates nuclear export of double-stranded RNA binding proteins. *The Journal of cell biology,* **156**(1), pp. 53-64.

BUHARD, O., CATTANEO, F., WONG, Y.F., YIM, S.F., FRIEDMAN, E., FLEJOU, J.F., DUVAL, A. and HAMELIN, R., 2006. Multipopulation analysis of polymorphisms in five mononucleotide repeats used to determine the microsatellite instability status of human tumors. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* **24**(2), pp. 241-251.

CENTER, M.M., JEMAL, A., SMITH, R.A. and WARD, E., 2009. Worldwide variations in colorectal cancer. *CA: a cancer journal for clinicians,* **59**(6), pp. 366-378.

CHEN, M.B., YANG, L., LU, P.H., FU, X.L., ZHANG, Y., ZHU, Y.Q. and TIAN, Y., 2015. MicroRNA-101 down-regulates sphingosine kinase 1 in colorectal cancer cells. *Biochemical and biophysical research communications,* **463**(4), pp. 954-960.

CHEN, X., GUO, X., ZHANG, H., XIANG, Y., CHEN, J., YIN, Y., CAI, X., WANG, K., WANG, G., BA, Y., ZHU, L., WANG, J., YANG, R., ZHANG, Y., REN, Z., ZEN, K., ZHANG, J. and ZHANG, C.Y., 2009. Role of miR-143 targeting KRAS in colorectal tumorigenesis. *Oncogene,* **28**(10), pp. 1385-1392.

CHENG, H., ZHANG, L., COGDELL, D.E., ZHENG, H., SCHETTER, A.J., NYKTER, M., HARRIS, C.C., CHEN, K., HAMILTON, S.R. and ZHANG, W., 2011. Circulating plasma MiR-141 is a

novel biomarker for metastatic colon cancer and predicts poor prognosis. *PloS one,* **6**(3), pp. e17745.

CHEVILLET, J.R., LEE, I., BRIGGS, H.A., HE, Y. and WANG, K., 2014. Issues and prospects of microRNA-based biomarkers in blood and other body fluids. *Molecules (Basel, Switzerland),* **19**(5), pp. 6080-6105.

CHIANG, Y., SONG, Y., WANG, Z., CHEN, Y., YUE, Z., XU, H., XING, C. and LIU, Z., 2011. Aberrant expression of miR-203 and its clinical significance in gastric and colorectal cancers. *Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract,* **15**(1), pp. 63-70.

CITARDA, F., TOMASELLI, G., CAPOCACCIA, R., BARCHERINI, S., CRESPI, M. and ITALIAN MULTICENTRE STUDY GROUP, 2001. Efficacy in standard clinical practice of colonoscopic polypectomy in reducing colorectal cancer incidence. *Gut,* **48**(6), pp. 812-815.

COOPER, D.N. and YOUSSOUFIAN, H., 1988. The CpG dinucleotide and human genetic disease. *Human genetics,* **78**(2), pp. 151-155.

COTTONHAM, C.L., KANEKO, S. and XU, L., 2010. miR-21 and miR-31 converge on TIAM1 to regulate migration and invasion of colon carcinoma cells. *The Journal of biological chemistry,* **285**(46), pp. 35293-35302.

DE GRUTTOLA, V.G., CLAX, P., DEMETS, D.L., DOWNING, G.J., ELLENBERG, S.S., FRIEDMAN, L., GAIL, M.H., PRENTICE, R., WITTES, J. and ZEGER, S.L., 2001. Considerations in the evaluation of surrogate endpoints in clinical trials. summary of a National Institutes of Health workshop. *Controlled clinical trials,* **22**(5), pp. 485-502.

DEATON, A.M. and BIRD, A., 2011. CpG islands and the regulation of transcription. *Genes & development,* **25**(10), pp. 1010-1022.

DEL VILLANO, B.C., BRENNAN, S., BROCK, P., BUCHER, C., LIU, V., MCCLURE, M., RAKE, B., SPACE, S., WESTRICK, B., SCHOEMAKER, H. and ZURAWSKI, V.R., 1983. Radioimmunometric assay for a monoclonal antibody-defined tumor marker, CA 19-9. *Clinical chemistry,* **29**(3), pp. 549-552.

DEWS, M., HOMAYOUNI, A., YU, D., MURPHY, D., SEVIGNANI, C., WENTZEL, E., FURTH, E.E., LEE, W.M., ENDERS, G.H., MENDELL, J.T. and THOMAS-TIKHONENKO, A., 2006. Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster. *Nature genetics,* **38**(9), pp. 1060-1065.

DUFFY, M.J., 2001. Carcinoembryonic antigen as a marker for colorectal cancer: is it clinically useful? *Clinical chemistry,* **47**(4), pp. 624-630.

DUFFY, M.J., LAMERZ, R., HAGLUND, C., NICOLINI, A., KALOUSOVA, M., HOLUBEC, L. and STURGEON, C., 2014. Tumor markers in colorectal cancer, gastric cancer and gastrointestinal stromal cancers: European group on tumor markers 2014 guidelines update. *International journal of cancer,* **134**(11), pp. 2513-2522.

DUFFY, M.J., O'DONOVAN, N. and CROWN, J., 2011. Use of molecular markers for predicting therapy response in cancer patients. *Cancer treatment reviews,* **37**(2), pp. 151-159.

DUFFY, M.J., STURGEON, C., LAMERZ, R., HAGLUND, C., HOLUBEC, V.L., KLAPDOR, R., NICOLINI, A., TOPOLCAN, O. and HEINEMANN, V., 2010. Tumor markers in pancreatic

cancer: a European Group on Tumor Markers (EGTM) status report. *Annals of oncology : official journal of the European Society for Medical Oncology,* **21**(3), pp. 441-447.

DUFFY, M.J., VAN DALEN, A., HAGLUND, C., HANSSON, L., HOLINSKI-FEDER, E., KLAPDOR, R., LAMERZ, R., PELTOMAKI, P., STURGEON, C. and TOPOLCAN, O., 2007a. Tumour markers in colorectal cancer: European Group on Tumour Markers (EGTM) guidelines for clinical use. *European journal of cancer (Oxford, England : 1990),* **43**(9), pp. 1348-1360.

DUFFY, M.J., VAN DALEN, A., HAGLUND, C., HANSSON, L., HOLINSKI-FEDER, E., KLAPDOR, R., LAMERZ, R., PELTOMAKI, P., STURGEON, C. and TOPOLCAN, O., 2007b. Tumour markers in colorectal cancer: European Group on Tumour Markers (EGTM) guidelines for clinical use. *European journal of cancer (Oxford, England : 1990),* **43**(9), pp. 1348-1360.

DUFFY, M.J., VAN DALEN, A., HAGLUND, C., HANSSON, L., KLAPDOR, R., LAMERZ, R., NILSSON, O., STURGEON, C. and TOPOLCAN, O., 2003. Clinical utility of biochemical markers in colorectal cancer: European Group on Tumour Markers (EGTM) guidelines. *European journal of cancer (Oxford, England : 1990),* **39**(6), pp. 718-727.

DUTTAGUPTA, R., JIANG, R., GOLLUB, J., GETTS, R.C. and JONES, K.W., 2011. Impact of cellular miRNAs on circulating miRNA biomarker signatures. *PloS one,* **6**(6), pp. e20769.

ESLAMIZADEH, S., HEIDARI, M., AGAH, S., FAGHIHLOO, E., GHAZI, H., MIRZAEI, A. and AKBARI, A., 2018. The Role of MicroRNA Signature as Diagnostic Biomarkers in Different Clinical Stages of Colorectal Cancer. *Cell journal,* **20**(2), pp. 220-230.

FEARON, E.R., 2011. Molecular genetics of colorectal cancer. *Annual review of pathology,* **6**, pp. 479-507.

FERLAY, J., SOERJOMATARAM, I., DIKSHIT, R., ESER, S., MATHERS, C., REBELO, M., PARKIN, D.M., FORMAN, D. and BRAY, F., 2015. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *International journal of cancer,* **136**(5), pp. E359-86.

FESLER, A., JIANG, J., ZHAI, H. and JU, J., 2014. Circulating microRNA testing for the early diagnosis and follow-up of colorectal cancer patients. *Molecular diagnosis & therapy,* **18**(3), pp. 303-308.

FINDEISEN, P., KLOOR, M., MERX, S., SUTTER, C., WOERNER, S.M., DOSTMANN, N., BENNER, A., DONDOG, B., PAWLITA, M., DIPPOLD, W., WAGNER, R., GEBERT, J. and VON KNEBEL DOEBERITZ, M., 2005. T25 repeat in the 3' untranslated region of the CASP2 gene: a sensitive and specific marker for microsatellite instability in colorectal cancer. *Cancer research,* **65**(18), pp. 8072-8078.

FINNEGAN, E.F. and PASQUINELLI, A.E., 2013. MicroRNA biogenesis: regulating the regulators. *Critical reviews in biochemistry and molecular biology,* **48**(1), pp. 51-68.

GAO, F. and WANG, W., 2015. MicroRNA-96 promotes the proliferation of colorectal cancer cells and targets tumor protein p53 inducible nuclear protein 1, forkhead box protein O1 (FOXO1) and FOXO3a. *Molecular medicine reports,* **11**(2), pp. 1200-1206.

GOLD, P. and FREEDMAN, S.O., 1965. Demonstration of Tumor-Specific Antigens in Human Colonic Carcinomata by Immunological Tolerance and Absorption Techniques. *The Journal of experimental medicine,* **121**, pp. 439-462.

GOLD, P. and FREEDMAN, S.O., 1965. Specific carcinoembryonic antigens of the human digestive system. *The Journal of experimental medicine,* **122**(3), pp. 467-481.

GOLDSTEIN, M.J. and MITCHELL, E.P., 2005. Carcinoembryonic antigen in the staging and follow-up of patients with colorectal cancer. *Cancer investigation,* **23**(4), pp. 338-351.

GONDAL, G., GROTMOL, T., HOFSTAD, B., BRETTHAUER, M., EIDE, T.J. and HOFF, G., 2003. The Norwegian Colorectal Cancer Prevention (NORCCAP) screening study: baseline findings and implementations for clinical work-up in age groups 50-64 years. *Scandinavian Journal of Gastroenterology,* **38**(6), pp. 635-642.

GRAZZINI, G., VENTURA, L., ZAPPA, M., CIATTO, S., CONFORTINI, M., RAPI, S., RUBECA, T., VISIOLI, C.B. and HALLORAN, S.P., 2010. Influence of seasonal variations in ambient temperatures on performance of immunochemical faecal occult blood test for colorectal cancer screening: observational study from the Florence district. *Gut,* **59**(11), pp. 1511-1515.

HARDCASTLE, J.D., CHAMBERLAIN, J.O., ROBINSON, M.H., MOSS, S.M., AMAR, S.S., BALFOUR, T.W., JAMES, P.D. and MANGHAM, C.M., 1996. Randomised controlled trial of faecal-occult-blood screening for colorectal cancer. *Lancet (London, England),* **348**(9040), pp. 1472-1477.

HEALTH QUALITY ONTARIO, 2009. Fecal occult blood test for colorectal cancer screening: an evidence-based analysis. *Ontario health technology assessment series,* **9**(10), pp. 1-40.

HOFF, G., GROTMOL, T., SKOVLUND, E., BRETTHAUER, M. and NORWEGIAN COLORECTAL CANCER PREVENTION STUDY GROUP, 2009. Risk of colorectal cancer seven years after flexible sigmoidoscopy screening: randomised controlled trial. *BMJ (Clinical research ed.),* **338**, pp. b1846.

HTTP://GCO.IARC.FR, .

HTTPS://GHR.NLM.NIH.GOV/GENE, .

HTTPS://LRA.LE.AC.UK/HANDLE/2381/37943, .

HTTPS://WWW.WCRF.ORG, .

HUANG, Z., HUANG, D., NI, S., PENG, Z., SHENG, W. and DU, X., 2010. Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. *International journal of cancer,* **127**(1), pp. 118-126.

IAFFAIOLI, R.V., FACCHINI, G., TORTORIELLO, A., CROVELLA, F., ROMANO, G., FORMATO, R., SANTANGELO, M., BARLETTA, E., FIORE, F., IACCARINO, V., MEMOLI, B., D'ANGELO, R., PILATI, P.L., ROSSI, R., GUADAGNI, S., DERACO, M., PIGNATA, S., DANIELE, B., ROSATI, G., MASSIDDA, B., MANTOVANI, G., DI SALVO, E., MARZANO, N., MEMEO, V., PARISI, V., MALLARINI, G. and COLUCCI, G., 2006. Stop Flow in abdominal and pelvic cancer relapses. *Frontiers in bioscience : a journal and virtual library,* **11**, pp. 1284-1288.

IWAYA, T., YOKOBORI, T., NISHIDA, N., KOGO, R., SUDO, T., TANAKA, F., SHIBATA, K., SAWADA, G., TAKAHASHI, Y., ISHIBASHI, M., WAKABAYASHI, G., MORI, M. and MIMORI, K., 2012. Downregulation of miR-144 is associated with colorectal cancer

progression via activation of mTOR signaling pathway. *Carcinogenesis,* **33**(12), pp. 2391-2397.

JEFFERY, M., HICKEY, B.E., HIDER, P.N. and SEE, A.M., 2016. Follow-up strategies for patients treated for non-metastatic colorectal cancer. *The Cochrane database of systematic reviews,* **11**, pp. CD002200.

JIRICNY, J., 2006. The multifaceted mismatch-repair system. *Nature reviews.Molecular cell biology,* **7**(5), pp. 335-346.

JOHNSON, S.M., GROSSHANS, H., SHINGARA, J., BYROM, M., JARVIS, R., CHENG, A., LABOURIER, E., REINERT, K.L., BROWN, D. and SLACK, F.J., 2005. RAS is regulated by the let-7 microRNA family. *Cell,* **120**(5), pp. 635-647.

KALIA, M., 2013. *Personalized oncology: Recent advances and future challenges.*

KAMINSKI, M.F., BRETTHAUER, M., ZAUBER, A.G., KUIPERS, E.J., ADAMI, H.O., VAN BALLEGOOIJEN, M., REGULA, J., VAN LEERDAM, M., STEFANSSON, T., PAHLMAN, L., DEKKER, E., HERNAN, M.A., GARBORG, K. and HOFF, G., 2012. The NordICC Study: rationale and design of a randomized trial on colonoscopy screening for colorectal cancer. *Endoscopy,* **44**(7), pp. 695-702.

KANAAN, Z., RAI, S.N., EICHENBERGER, M.R., ROBERTS, H., KESKEY, B., PAN, J. and GALANDIUK, S., 2012. Plasma miR-21: a potential diagnostic marker of colorectal cancer. *Annals of Surgery,* **256**(3), pp. 544-551.

KANAAN, Z., ROBERTS, H., EICHENBERGER, M.R., BILLETER, A., OCHERETNER, G., PAN, J., RAI, S.N., JORDEN, J., WILLIFORD, A. and GALANDIUK, S., 2013. A plasma microRNA panel for detection of colorectal adenomas: a step toward more precise screening for colorectal cancer. *Annals of Surgery,* **258**(3), pp. 400-408.

KASINSKI, A.L. and SLACK, F.J., 2011. Epigenetics and genetics. MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy. *Nature reviews.Cancer,* **11**(12), pp. 849-864.

KAWAKAMI, H., ZAANAN, A. and SINICROPE, F.A., 2015. Microsatellite instability testing and its role in the management of colorectal cancer. *Current treatment options in oncology,* **16**(7), pp. 30-015-0348-2.

KIKKAWA, S., SOGAWA, K., SATOH, M., UMEMURA, H., KODERA, Y., MATSUSHITA, K., TOMONAGA, T., MIYAZAKI, M., YOKOSUKA, O. and NOMURA, F., 2012. Identification of a Novel Biomarker for Biliary Tract Cancer Using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. *International journal of proteomics,* **2012**, pp. 108609.

KIM, D.H., OH, S.J., OH, C.A., CHOI, M.G., NOH, J.H., SOHN, T.S., BAE, J.M. and KIM, S., 2011. The relationships between perioperative CEA, CA 19-9, and CA 72-4 and recurrence in gastric cancer patients after curative radical gastrectomy. *Journal of surgical oncology,* **104**(6), pp. 585-591.

KOPETZ, S., DESAI, J., CHAN, E., HECHT, J.R., O'DWYER, P.J., MARU, D., MORRIS, V., JANKU, F., DASARI, A., CHUNG, W., ISSA, J.P., GIBBS, P., JAMES, B., POWIS, G., NOLOP, K.B., BHATTACHARYA, S. and SALTZ, L., 2015. Phase II Pilot Study of Vemurafenib in Patients With Metastatic BRAF-Mutated Colorectal Cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* **33**(34), pp. 4032-4038.

KRONBORG, O., FENGER, C., OLSEN, J., JORGENSEN, O.D. and SONDERGAARD, O., 1996. Randomised study of screening for colorectal cancer with faecal-occult-blood test. *Lancet (London, England),* **348**(9040), pp. 1467-1471.

LABIANCA, R., NORDLINGER, B., BERETTA, G.D., BROUQUET, A., CERVANTES, A. and ESMO GUIDELINES WORKING GROUP, 2010. Primary colon cancer: ESMO Clinical Practice Guidelines for diagnosis, adjuvant treatment and follow-up. *Annals of oncology : official journal of the European Society for Medical Oncology,* **21 Suppl 5**, pp. v70-7.

LANDI, D., GEMIGNANI, F., NACCARATI, A., PARDINI, B., VODICKA, P., VODICKOVA, L., NOVOTNY, J., FORSTI, A., HEMMINKI, K., CANZIAN, F. and LANDI, S., 2008. Polymorphisms within micro-RNA-binding sites and risk of sporadic colorectal cancer. *Carcinogenesis,* **29**(3), pp. 579-584.

LANGAN, R.C., MULLINAX, J.E., RAIJI, M.T., UPHAM, T., SUMMERS, T., STOJADINOVIC, A. and AVITAL, I., 2013. Colorectal cancer biomarkers and the potential role of cancer stem cells. *Journal of Cancer,* **4**(3), pp. 241-250.

LAO, V.V. and GRADY, W.M., 2011. Epigenetics and colorectal cancer. *Nature reviews.Gastroenterology & hepatology,* **8**(12), pp. 686-700.

LECH, G., SLOTWINSKI, R., SLODKOWSKI, M. and KRASNODEBSKI, I.W., 2016. Colorectal cancer tumour markers and biomarkers: Recent therapeutic advances. *World journal of gastroenterology,* **22**(5), pp. 1745-1755.

LEE, E.J., BAEK, M., GUSEV, Y., BRACKETT, D.J., NUOVO, G.J. and SCHMITTGEN, T.D., 2008. Systematic evaluation of microRNA processing patterns in tissues, cell lines, and tumors. *RNA (New York, N.Y.),* **14**(1), pp. 35-42.

LEE, J.K., LILES, E.G., BENT, S., LEVIN, T.R. and CORLEY, D.A., 2014. Accuracy of fecal immunochemical tests for colorectal cancer: systematic review and meta-analysis. *Annals of Internal Medicine,* **160**(3), pp. 171-1484.

LEE, Y., KIM, M., HAN, J., YEOM, K.H., LEE, S., BAEK, S.H. and KIM, V.N., 2004. MicroRNA genes are transcribed by RNA polymerase II. *The EMBO journal,* **23**(20), pp. 4051-4060.

LEIDINGER, P., BACKES, C., RHEINHEIMER, S., KELLER, A. and MEESE, E., 2015. Towards Clinical Applications of Blood-Borne miRNA Signatures: The Influence of the Anticoagulant EDTA on miRNA Abundance. *PloS one,* **10**(11), pp. e0143321.

LEURAUD, K., JEZEWSKI-SERRA, D., VIGUIER, J. and SALINES, E., 2013. Colorectal cancer screening by guaiac faecal occult blood test in France: Evaluation of the programme two years after launching. *Cancer epidemiology,* **37**(6), pp. 959-967.

LEVIN, B., BROOKS, D., SMITH, R.A. and STONE, A., 2003. Emerging technologies in screening for colorectal cancer: CT colonography, immunochemical fecal occult blood tests, and stool screening using molecular markers. *CA: a cancer journal for clinicians,* **53**(1), pp. 44-55.

LIN, C.C., LIN, J.K., LIN, T.C., CHEN, W.S., YANG, S.H., WANG, H.S., LAN, Y.T., JIANG, J.K., YANG, M.H. and CHANG, S.C., 2014. The prognostic role of microsatellite instability, codon-specific KRAS, and BRAF mutations in colon cancer. *Journal of surgical oncology,* **110**(4), pp. 451-457.

LIN, O.S., KOZAREK, R.A., GLUCK, M., JIRANEK, G.C., KOCH, J., KOWDLEY, K.V., IRANI, S., NGUYEN, M. and DOMINITZ, J.A., 2012. Preference for colonoscopy versus computerized tomographic colonography: a systematic review and meta-analysis of observational studies. *Journal of general internal medicine,* **27**(10), pp. 1349-1360.

LING, H., PICKARD, K., IVAN, C., ISELLA, C., IKUO, M., MITTER, R., SPIZZO, R., BULLOCK, M., BRAICU, C., PILECZKI, V., VINCENT, K., PICHLER, M., STIEGELBAUER, V., HOEFLER, G., ALMEIDA, M.I., HSIAO, A., ZHANG, X., PRIMROSE, J., PACKHAM, G., LIU, K., BOJJA, K., GAFA, R., XIAO, L., ROSSI, S., SONG, J.H., VANNINI, I., FANINI, F., KOPETZ, S., ZWEIDLER-MCKAY, P., WANG, X., IONESCU, C., IRIMIE, A., FABBRI, M., LANZA, G., HAMILTON, S.R., BERINDAN-NEAGOE, I., MEDICO, E., MIRNEZAMI, A., CALIN, G.A. and NICOLOSO, M.S., 2016. The clinical and biological significance of MIR-224 expression in colorectal cancer metastasis. *Gut,* **65**(6), pp. 977-989.

LIU, G.H., ZHOU, Z.G., CHEN, R., WANG, M.J., ZHOU, B., LI, Y. and SUN, X.F., 2013. Serum miR-21 and miR-92a as biomarkers in the diagnosis and prognosis of colorectal cancer. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine,* **34**(4), pp. 2175-2181.

LIU, L., CHEN, L., XU, Y., LI, R. and DU, X., 2010. microRNA-195 promotes apoptosis and suppresses tumorigenicity of human colorectal cancer cells. *Biochemical and biophysical research communications,* **400**(2), pp. 236-240.

LOCKER, G.Y., HAMILTON, S., HARRIS, J., JESSUP, J.M., KEMENY, N., MACDONALD, J.S., SOMERFIELD, M.R., HAYES, D.F., BAST, R.C.,Jr and ASCO, 2006. ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* **24**(33), pp. 5313-5327.

LOGAN, R.F., PATNICK, J., NICKERSON, C., COLEMAN, L., RUTTER, M.D., VON WAGNER, C. and ENGLISH BOWEL CANCER SCREENING EVALUATION COMMITTEE, 2012. Outcomes of the Bowel Cancer Screening Programme (BCSP) in England after the first 1 million tests. *Gut,* **61**(10), pp. 1439-1446.

MA, Y.L., ZHANG, P., WANG, F., MOYER, M.P., YANG, J.J., LIU, Z.H., PENG, J.Y., CHEN, H.Q., ZHOU, Y.K., LIU, W.J. and QIN, H.L., 2011. Human embryonic stem cells and metastatic colorectal cancer cells shared the common endogenous human microRNA-26b. *Journal of Cellular and Molecular Medicine,* **15**(9), pp. 1941-1954.

MANDEL, J.S., BOND, J.H., CHURCH, T.R., SNOVER, D.C., BRADLEY, G.M., SCHUMAN, L.M. and EDERER, F., 1993. Reducing mortality from colorectal cancer by screening for fecal occult blood. Minnesota Colon Cancer Control Study. *The New England journal of medicine,* **328**(19), pp. 1365-1371.

MANDEL, J.S., CHURCH, T.R., BOND, J.H., EDERER, F., GEISSER, M.S., MONGIN, S.J., SNOVER, D.C. and SCHUMAN, L.M., 2000. The effect of fecal occult-blood screening on the incidence of colorectal cancer. *The New England journal of medicine,* **343**(22), pp. 1603-1607.

MANN, D.V., EDWARDS, R., HO, S., LAU, W.Y. and GLAZER, G., 2000. Elevated tumour marker CA19-9: clinical interpretation and influence of obstructive jaundice. *European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology,* **26**(5), pp. 474-479.

MARKOWITZ, S.D. and BERTAGNOLLI, M.M., 2009. Molecular origins of cancer: Molecular basis of colorectal cancer. *The New England journal of medicine,* **361**(25), pp. 2449-2460.

MCCAIN, J., 2013. The MAPK (ERK) Pathway: Investigational Combinations for the Treatment Of BRAF-Mutated Metastatic Melanoma. *P & T : a peer-reviewed journal for formulary management,* **38**(2), pp. 96-108.

MCDONALD, J.S., MILOSEVIC, D., REDDI, H.V., GREBE, S.K. and ALGECIRAS-SCHIMNICH, A., 2011. Analysis of circulating microRNA: preanalytical and analytical challenges. *Clinical chemistry,* **57**(6), pp. 833-840.

MIDGLEY, R. and KERR, D., 1999. Colorectal cancer. *Lancet (London, England),* **353**(9150), pp. 391-399.

MITCHELL, P.S., PARKIN, R.K., KROH, E.M., FRITZ, B.R., WYMAN, S.K., POGOSOVA-AGADJANYAN, E.L., PETERSON, A., NOTEBOOM, J., O'BRIANT, K.C., ALLEN, A., LIN, D.W., URBAN, N., DRESCHER, C.W., KNUDSEN, B.S., STIREWALT, D.L., GENTLEMAN, R., VESSELLA, R.L., NELSON, P.S., MARTIN, D.B. and TEWARI, M., 2008. Circulating microRNAs as stable blood-based markers for cancer detection. *Proceedings of the National Academy of Sciences of the United States of America,* **105**(30), pp. 10513-10518.

MOWAT, C., DIGBY, J., STRACHAN, J.A., WILSON, R., CAREY, F.A., FRASER, C.G. and STEELE, R.J., 2016. Faecal haemoglobin and faecal calprotectin as indicators of bowel disease in patients presenting to primary care with bowel symptoms. *Gut,* **65**(9), pp. 1463-1469.

NAGEL, R., LE SAGE, C., DIOSDADO, B., VAN DER WAAL, M., OUDE VRIELINK, J.A., BOLIJN, A., MEIJER, G.A. and AGAMI, R., 2008. Regulation of the adenomatous polyposis coli gene by the miR-135 family in colorectal cancer. *Cancer research,* **68**(14), pp. 5795-5802.

NAKANO, H., MIYAZAWA, T., KINOSHITA, K., YAMADA, Y. and YOSHIDA, T., 2010. Functional screening identifies a microRNA, miR-491 that induces apoptosis by targeting Bcl-X(L) in colorectal cancer cells. *International journal of cancer,* **127**(5), pp. 1072-1080.

NG, E.K., CHONG, W.W., JIN, H., LAM, E.K., SHIN, V.Y., YU, J., POON, T.C., NG, S.S. and SUNG, J.J., 2009. Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. *Gut,* **58**(10), pp. 1375-1381.

NICE 2012, .

NICHOLSON, B.D., SHINKINS, B., PATHIRAJA, I., ROBERTS, N.W., JAMES, T.J., MALLETT, S., PERERA, R., PRIMROSE, J.N. and MANT, D., 2015. Blood CEA levels for detecting recurrent colorectal cancer. *The Cochrane database of systematic reviews,* **(12):CD011134. doi**(12), pp. CD011134.

NIE, J., LIU, L., ZHENG, W., CHEN, L., WU, X., XU, Y., DU, X. and HAN, W., 2012. microRNA-365, down-regulated in colon cancer, inhibits cell cycle progression and promotes apoptosis of colon cancer cells by probably targeting Cyclin D1 and Bcl-2. *Carcinogenesis,* **33**(1), pp. 220-225.

OKUGAWA, Y., GRADY, W.M. and GOEL, A., 2015. Epigenetic Alterations in Colorectal Cancer: Emerging Biomarkers. *Gastroenterology,* **149**(5), pp. 1204-1225.e12.

OZAWA, T., ISHIHARA, S., KAWAI, K., NOZAWA, H., YAMAGUCHI, H., KITAYAMA, J. and WATANABE, T., 2016. Prognostic Significance of Preoperative Serum Carbohydrate Antigen 19-9 in Patients With Stage IV Colorectal Cancer. *Clinical colorectal cancer,* **15**(4), pp. e157-e163.

PAGE, K., GUTTERY, D.S., ZAHRA, N., PRIMROSE, L., ELSHAW, S.R., PRINGLE, J.H., BLIGHE, K., MARCHESE, S.D., HILLS, A., WOODLEY, L., STEBBING, J., COOMBES, R.C. and SHAW, J.A., 2013. Influence of plasma processing on recovery and analysis of circulating nucleic acids. *PloS one,* **8**(10), pp. e77963.

PASQUINELLI, A.E., REINHART, B.J., SLACK, F., MARTINDALE, M.Q., KURODA, M.I., MALLER, B., HAYWARD, D.C., BALL, E.E., DEGNAN, B., MULLER, P., SPRING, J., SRINIVASAN, A., FISHMAN, M., FINNERTY, J., CORBO, J., LEVINE, M., LEAHY, P., DAVIDSON, E. and RUVKUN, G., 2000. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature,* **408**(6808), pp. 86-89.

PELTOMAKI, P., 2003. Role of DNA mismatch repair defects in the pathogenesis of human cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* **21**(6), pp. 1174-1179.

PINO, M.S. and CHUNG, D.C., 2010. The chromosomal instability pathway in colon cancer. *Gastroenterology,* **138**(6), pp. 2059-2072.

POLYTARCHOU, C., HOMMES, D.W., PALUMBO, T., HATZIAPOSTOLOU, M., KOUTSIOUMPA, M., KOUKOS, G., VAN DER MEULEN-DE JONG, A.E., OIKONOMOPOULOS, A., VAN DEEN, W.K., VORVIS, C., SEREBRENNIKOVA, O.B., BIRLI, E., CHOI, J., CHANG, L., ANTON, P.A., TSICHLIS, P.N., POTHOULAKIS, C., VERSPAGET, H.W. and ILIOPOULOS, D., 2015. MicroRNA214 Is Associated With Progression of Ulcerative Colitis, and Inhibition Reduces Development of Colitis and Colitis-Associated Cancer in Mice. *Gastroenterology,* **149**(4), pp. 981-92.e11.

PRIMROSE, J.N., PERERA, R., GRAY, A., ROSE, P., FULLER, A., CORKHILL, A., GEORGE, S., MANT, D. and FACS TRIAL INVESTIGATORS, 2014. Effect of 3 to 5 years of scheduled CEA and CT follow-up to detect recurrence of colorectal cancer: the FACS randomized clinical trial. *Jama,* **311**(3), pp. 263-270.

PRITCHARD, C.C., CHENG, H.H. and TEWARI, M., 2012. MicroRNA profiling: approaches and considerations. *Nature reviews.Genetics,* **13**(5), pp. 358-369.

PRITCHARD, C.C. and GRADY, W.M., 2011. Colorectal cancer molecular biology moves into clinical practice. *Gut,* **60**(1), pp. 116-129.

PRITCHARD, C.C., KROH, E., WOOD, B., ARROYO, J.D., DOUGHERTY, K.J., MIYAJI, M.M., TAIT, J.F. and TEWARI, M., 2012a. Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. *Cancer prevention research (Philadelphia, Pa.),* **5**(3), pp. 492-497.

PRITCHARD, C.C., KROH, E., WOOD, B., ARROYO, J.D., DOUGHERTY, K.J., MIYAJI, M.M., TAIT, J.F. and TEWARI, M., 2012b. Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. *Cancer prevention research (Philadelphia, Pa.),* **5**(3), pp. 492-497.

QUINTERO, E., CASTELLS, A., BUJANDA, L., CUBIELLA, J., SALAS, D., LANAS, A., ANDREU, M., CARBALLO, F., MORILLAS, J.D., HERNANDEZ, C., JOVER, R., MONTALVO, I., ARENAS, J., LAREDO, E., HERNANDEZ, V., IGLESIAS, F., CID, E., ZUBIZARRETA, R., SALA, T., PONCE, M., ANDRES, M., TERUEL, G., PERIS, A., RONCALES, M.P., POLO-TOMAS, M., BESSA, X., FERRER-ARMENGOU, O., GRAU, J., SERRADESANFERM, A., ONO, A., CRUZADO, J., PEREZ-RIQUELME, F., ALONSO-ABREU, I., DE LA VEGA-PRIETO, M., REYES-MELIAN, J.M., CACHO, G., DIAZ-TASENDE, J., HERREROS-DE-TEJADA, A., POVES, C., SANTANDER, C., GONZALEZ-NAVARRO, A. and COLONPREV STUDY INVESTIGATORS, 2012. Colonoscopy versus fecal immunochemical testing in colorectal-cancer screening. *The New England journal of medicine,* **366**(8), pp. 697-706.

REES, C.J. and BEVAN, R., 2013. The National Health Service Bowel Cancer Screening Program: the early years. *Expert review of gastroenterology & hepatology,* **7**(5), pp. 421-437.

REINHART, B.J., SLACK, F.J., BASSON, M., PASQUINELLI, A.E., BETTINGER, J.C., ROUGVIE, A.E., HORVITZ, H.R. and RUVKUN, G., 2000. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature,* **403**(6772), pp. 901-906.

RISTAU, J., STAFFA, J., SCHROTZ-KING, P., GIGIC, B., MAKAR, K.W., HOFFMEISTER, M., BRENNER, H., ULRICH, A., SCHNEIDER, M., ULRICH, C.M. and HABERMANN, N., 2014a. Suitability of circulating miRNAs as potential prognostic markers in colorectal cancer. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* **23**(12), pp. 2632-2637.

RISTAU, J., STAFFA, J., SCHROTZ-KING, P., GIGIC, B., MAKAR, K.W., HOFFMEISTER, M., BRENNER, H., ULRICH, A., SCHNEIDER, M., ULRICH, C.M. and HABERMANN, N., 2014b. Suitability of circulating miRNAs as potential prognostic markers in colorectal cancer. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* **23**(12), pp. 2632-2637.

RUTTER, M.D., NICKERSON, C., REES, C.J., PATNICK, J. and BLANKS, R.G., 2014. Risk factors for adverse events related to polypectomy in the English Bowel Cancer Screening Programme. *Endoscopy,* **46**(2), pp. 90-97.

SAWYERS, C.L., 2008. The cancer biomarker problem. *Nature,* **452**(7187), pp. 548-552.

SAXONOV, S., BERG, P. and BRUTLAG, D.L., 2006. A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *Proceedings of the National Academy of Sciences of the United States of America,* **103**(5), pp. 1412-1417.

SAYED, D., RANE, S., LYPOWY, J., HE, M., CHEN, I.Y., VASHISTHA, H., YAN, L., MALHOTRA, A., VATNER, D. and ABDELLATIF, M., 2008. MicroRNA-21 targets Sprouty2 and promotes cellular outgrowths. *Molecular biology of the cell,* **19**(8), pp. 3272-3282.

SCHOEN, R.E., PINSKY, P.F., WEISSFELD, J.L., YOKOCHI, L.A., CHURCH, T., LAIYEMO, A.O., BRESALIER, R., ANDRIOLE, G.L., BUYS, S.S., CRAWFORD, E.D., FOUAD, M.N., ISAACS, C., JOHNSON, C.C., REDING, D.J., O'BRIEN, B., CARRICK, D.M., WRIGHT, P., RILEY, T.L., PURDUE, M.P., IZMIRLIAN, G., KRAMER, B.S., MILLER, A.B., GOHAGAN, J.K., PROROK, P.C., BERG, C.D. and PLCO PROJECT TEAM, 2012. Colorectal-cancer incidence

and mortality with screening flexible sigmoidoscopy. *The New England journal of medicine,* **366**(25), pp. 2345-2357.

SCHREUDERS, E.H., RUCO, A., RABENECK, L., SCHOEN, R.E., SUNG, J.J., YOUNG, G.P. and KUIPERS, E.J., 2015. Colorectal cancer screening: a global overview of existing programmes. *Gut,* **64**(10), pp. 1637-1649.

SCHUBERT, M., JUNKER, K. and HEINZELMANN, J., 2016. Prognostic and predictive miRNA biomarkers in bladder, kidney and prostate cancer: Where do we stand in biomarker development? *Journal of cancer research and clinical oncology,* **142**(8), pp. 1673-1695.

SEGNAN, N., ARMAROLI, P., BONELLI, L., RISIO, M., SCIALLERO, S., ZAPPA, M., ANDREONI, B., ARRIGONI, A., BISANTI, L., CASELLA, C., CROSTA, C., FALCINI, F., FERRERO, F., GIACOMIN, A., GIULIANI, O., SANTARELLI, A., VISIOLI, C.B., ZANETTI, R., ATKIN, W.S., SENORE, C. and SCORE WORKING GROUP, 2011. Once-only sigmoidoscopy in colorectal cancer screening: follow-up findings of the Italian Randomized Controlled Trial--SCORE. *Journal of the National Cancer Institute,* **103**(17), pp. 1310-1322.

SEGNAN, N., SENORE, C., ANDREONI, B., ASTE, H., BONELLI, L., CROSTA, C., FERRARIS, R., GASPERONI, S., PENNA, A., RISIO, M., ROSSINI, F.P., SCIALLERO, S., ZAPPA, M., ATKIN, W.S. and SCORE WORKING GROUP--ITALY, 2002. Baseline findings of the Italian multicenter randomized controlled trial of "once-only sigmoidoscopy"--SCORE. *Journal of the National Cancer Institute,* **94**(23), pp. 1763-1772.

SHAUKAT, A., MONGIN, S.J., GEISSER, M.S., LEDERLE, F.A., BOND, J.H., MANDEL, J.S. and CHURCH, T.R., 2013. Long-term mortality after screening for colorectal cancer. *The New England journal of medicine,* **369**(12), pp. 1106-1114.

SINICROPE, F.A. and SARGENT, D.J., 2012. Molecular pathways: microsatellite instability in colorectal cancer: prognostic, predictive, and therapeutic implications. *Clinical cancer research : an official journal of the American Association for Cancer Research,* **18**(6), pp. 1506-1512.

STAHLHUT, C. and SLACK, F.J., 2013. MicroRNAs and the cancer phenotype: profiling, signatures and clinical implications. *Genome medicine,* **5**(12), pp. 111.

STIEGELBAUER, V., PERAKIS, S., DEUTSCH, A., LING, H., GERGER, A. and PICHLER, M., 2014. MicroRNAs as novel predictive biomarkers and therapeutic targets in colorectal cancer. *World journal of gastroenterology,* **20**(33), pp. 11727-11735.

STROCCHI, E., IAFFAIOLI, R.V., FACCHINI, G., MANTOVANI, G., RICCI, S., CAVALLO, G., TORTORIELLO, A., D'ANGELO, R., FORMATO, R., ROSATO, G., FIORE, F., IACCARINO, V., PETRELLA, G., MEMOLI, B., SANTANGELO, M. and CAMAGGI, C.M., 2004. Stop-flow technique for loco-regional delivery of high dose chemotherapy in the treatment of advanced pelvic cancers. *European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology,* **30**(6), pp. 663-670.

STURGEON, C.M., LAI, L.C. and DUFFY, M.J., 2009. Serum tumour markers: how to order and interpret them. *BMJ (Clinical research ed.),* **339**, pp. b3527.

SU, J., LIANG, H., YAO, W., WANG, N., ZHANG, S., YAN, X., FENG, H., PANG, W., WANG, Y., WANG, X., FU, Z., LIU, Y., ZHAO, C., ZHANG, J., ZHANG, C.Y., ZEN, K., CHEN, X. and

WANG, Y., 2014. MiR-143 and MiR-145 regulate IGF1R to suppress cell proliferation in colorectal cancer. *PloS one,* **9**(12), pp. e114420.

SU, W.B. and LIU, Z.Y., 2018. MiR-431 inhibits colorectal cancer cell invasion via repressing CUL4B. *European review for medical and pharmacological sciences,* **22**(10), pp. 3047-3052.

SUN, J.Y., HUANG, Y., LI, J.P., ZHANG, X., WANG, L., MENG, Y.L., YAN, B., BIAN, Y.Q., ZHAO, J., WANG, W.Z., YANG, A.G. and ZHANG, R., 2012. MicroRNA-320a suppresses human colon cancer cell proliferation by directly targeting beta-catenin. *Biochemical and biophysical research communications,* **420**(4), pp. 787-792.

SUN, Y., LIU, Y., COGDELL, D., CALIN, G.A., SUN, B., KOPETZ, S., HAMILTON, S.R. and ZHANG, W., 2016. Examining plasma microRNA markers for colorectal cancer at different stages. *Oncotarget,* **7**(10), pp. 11434-11449.

SUREBAN, S.M., MAY, R., RAMALINGAM, S., SUBRAMANIAM, D., NATARAJAN, G., ANANT, S. and HOUCHEN, C.W., 2009. Selective blockade of DCAMKL-1 results in tumor growth arrest by a Let-7a MicroRNA-dependent mechanism. *Gastroenterology,* **137**(2), pp. 649-59, 659.e1-2.

SUTO, T., YOKOBORI, T., YAJIMA, R., MORITA, H., FUJII, T., YAMAGUCHI, S., ALTAN, B., TSUTSUMI, S., ASAO, T. and KUWANO, H., 2015. MicroRNA-7 expression in colorectal cancer is associated with poor prognosis and regulates cetuximab sensitivity via EGFR regulation. *Carcinogenesis,* **36**(3), pp. 338-345.

TANAKA, T., TANAKA, M., TANAKA, T. and ISHIGAMORI, R., 2010. Biomarkers for colorectal cancer. *International journal of molecular sciences,* **11**(9), pp. 3209-3225.

TAPPENDEN, P., CHILCOTT, J., EGGINGTON, S., PATNICK, J., SAKAI, H. and KARNON, J., 2007. Option appraisal of population-based colorectal cancer screening programmes in England. *Gut,* **56**(5), pp. 677-684.

THOMAS, J., OHTSUKA, M., PICHLER, M. and LING, H., 2015. MicroRNAs: Clinical Relevance in Colorectal Cancer. *International journal of molecular sciences,* **16**(12), pp. 28063-28076.

TIE, J. and DESAI, J., 2015a. Targeting BRAF mutant metastatic colorectal cancer: clinical implications and emerging therapeutic strategies. *Targeted oncology,* **10**(2), pp. 179-188.

TIE, J. and DESAI, J., 2015b. Targeting BRAF mutant metastatic colorectal cancer: clinical implications and emerging therapeutic strategies. *Targeted oncology,* **10**(2), pp. 179-188.

TINMOUTH, J., LANSDORP-VOGELAAR, I. and ALLISON, J.E., 2015. Faecal immunochemical tests versus guaiac faecal occult blood tests: what clinicians and colorectal cancer screening programme organisers need to know. *Gut,* **64**(8), pp. 1327-1337.

TOIYAMA, Y., HUR, K., TANAKA, K., INOUE, Y., KUSUNOKI, M., BOLAND, C.R. and GOEL, A., 2014. Serum miR-200c is a novel prognostic and metastasis-predictive biomarker in patients with colorectal cancer. *Annals of Surgery,* **259**(4), pp. 735-743.

TOIYAMA, Y., TAKAHASHI, M., HUR, K., NAGASAKA, T., TANAKA, K., INOUE, Y., KUSUNOKI, M., BOLAND, C.R. and GOEL, A., 2013. Serum miR-21 as a diagnostic and

prognostic biomarker in colorectal cancer. *Journal of the National Cancer Institute,* **105**(12), pp. 849-859.

TOYOTA, M., AHUJA, N., SUZUKI, H., ITOH, F., OHE-TOYOTA, M., IMAI, K., BAYLIN, S.B. and ISSA, J.P., 1999. Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. *Cancer research,* **59**(21), pp. 5438-5442.

TRAN, B., KOPETZ, S., TIE, J., GIBBS, P., JIANG, Z.Q., LIEU, C.H., AGARWAL, A., MARU, D.M., SIEBER, O. and DESAI, J., 2011a. Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. *Cancer,* **117**(20), pp. 4623-4632.

TRAN, B., KOPETZ, S., TIE, J., GIBBS, P., JIANG, Z.Q., LIEU, C.H., AGARWAL, A., MARU, D.M., SIEBER, O. and DESAI, J., 2011b. Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. *Cancer,* **117**(20), pp. 4623-4632.

TREASURE, T., MONSON, K., FIORENTINO, F. and RUSSELL, C., 2014. The CEA Second-Look Trial: a randomised controlled trial of carcinoembryonic antigen prompted reoperation for recurrent colorectal cancer. *BMJ open,* **4**(5), pp. e004385-2013-004385.

TSANG, W.P. and KWOK, T.T., 2009. The miR-18a* microRNA functions as a potential tumor suppressor by targeting on K-Ras. *Carcinogenesis,* **30**(6), pp. 953-959.

TSUCHIDA, A., OHNO, S., WU, W., BORJIGIN, N., FUJITA, K., AOKI, T., UEDA, S., TAKANASHI, M. and KURODA, M., 2011. miR-92 is a key oncogenic component of the miR-17-92 cluster in colon cancer. *Cancer science,* **102**(12), pp. 2264-2271.

ULIVI, P., CANALE, M., PASSARDI, A., MARISI, G., VALGIUSTI, M., FRASSINETI, G.L., CALISTRI, D., AMADORI, D. and SCARPI, E., 2018. Circulating Plasma Levels of miR-20b, miR-29b and miR-155 as Predictors of Bevacizumab Efficacy in Patients with Metastatic Colorectal Cancer. *International journal of molecular sciences,* **19**(1), pp. 10.3390/ijms19010307.

VALADI, H., EKSTROM, K., BOSSIOS, A., SJOSTRAND, M., LEE, J.J. and LOTVALL, J.O., 2007. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature cell biology,* **9**(6), pp. 654-659.

VALERI, N., BRACONI, C., GASPARINI, P., MURGIA, C., LAMPIS, A., PAULUS-HOCK, V., HART, J.R., UENO, L., GRIVENNIKOV, S.I., LOVAT, F., PAONE, A., CASCIONE, L., SUMANI, K.M., VERONESE, A., FABBRI, M., CARASI, S., ALDER, H., LANZA, G., GAFA', R., MOYER, M.P., RIDGWAY, R.A., CORDERO, J., NUOVO, G.J., FRANKEL, W.L., RUGGE, M., FASSAN, M., GRODEN, J., VOGT, P.K., KARIN, M., SANSOM, O.J. and CROCE, C.M., 2014. MicroRNA-135b promotes cancer progression by acting as a downstream effector of oncogenic pathways in colon cancer. *Cancer cell,* **25**(4), pp. 469-483.

VAN DAM, L., KUIPERS, E.J. and VAN LEERDAM, M.E., 2010. Performance improvements of stool-based screening tests. *Best practice & research.Clinical gastroenterology,* **24**(4), pp. 479-492.

VAN KRIEKEN, J.H., JUNG, A., KIRCHNER, T., CARNEIRO, F., SERUCA, R., BOSMAN, F.T., QUIRKE, P., FLEJOU, J.F., PLATO HANSEN, T., DE HERTOGH, G., JARES, P., LANGNER, C., HOEFLER, G., LIGTENBERG, M., TINIAKOS, D., TEJPAR, S., BEVILACQUA, G. and ENSARI, A., 2008. KRAS mutation testing for predicting response to anti-EGFR therapy for

colorectal carcinoma: proposal for an European quality assurance program. *Virchows Archiv : an international journal of pathology,* **453**(5), pp. 417-431.

VAUGHN, C.P., ZOBELL, S.D., FURTADO, L.V., BAKER, C.L. and SAMOWITZ, W.S., 2011. Frequency of KRAS, BRAF, and NRAS mutations in colorectal cancer. *Genes, chromosomes & cancer,* **50**(5), pp. 307-312.

VICKERS, K.C., PALMISANO, B.T., SHOUCRI, B.M., SHAMBUREK, R.D. and REMALEY, A.T., 2011. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nature cell biology,* **13**(4), pp. 423-433.

VOGELSTEIN, B., FEARON, E.R., HAMILTON, S.R., KERN, S.E., PREISINGER, A.C., LEPPERT, M., NAKAMURA, Y., WHITE, R., SMITS, A.M. and BOS, J.L., 1988. Genetic alterations during colorectal-tumor development. *The New England journal of medicine,* **319**(9), pp. 525-532.

VON WAGNER, C., GHANOUNI, A., HALLIGAN, S., SMITH, S., DADSWELL, E., LILFORD, R.J., MORTON, D., ATKIN, W., WARDLE, J. and SIGGAR INVESTIGATORS, 2012. Patient acceptability and psychologic consequences of CT colonography compared with those of colonoscopy: results from a multicenter randomized controlled trial of symptomatic patients. *Radiology,* **263**(3), pp. 723-731.

VON WAGNER, C., SMITH, S., HALLIGAN, S., GHANOUNI, A., POWER, E., LILFORD, R.J., MORTON, D., DADSWELL, E., ATKIN, W., WARDLE, J. and SIGGAR INVESTIGATORS, 2011. Patient acceptability of CT colonography compared with double contrast barium enema: results from a multicentre randomised controlled trial of symptomatic patients. *European radiology,* **21**(10), pp. 2046-2055.

WANG, K., ZHANG, S., WEBER, J., BAXTER, D. and GALAS, D.J., 2010. Export of microRNAs and microRNA-protective protein by mammalian cells. *Nucleic acids research,* **38**(20), pp. 7248-7259.

WANG, L.G. and GU, J., 2012. Serum microRNA-29a is a promising novel marker for early detection of colorectal liver metastasis. *Cancer epidemiology,* **36**(1), pp. e61-7.

WANG, Q., HUANG, Z., NI, S., XIAO, X., XU, Q., WANG, L., HUANG, D., TAN, C., SHENG, W. and DU, X., 2012. Plasma miR-601 and miR-760 are novel biomarkers for the early detection of colorectal cancer. *PloS one,* **7**(9), pp. e44398.

WEBER, J.A., BAXTER, D.H., ZHANG, S., HUANG, D.Y., HUANG, K.H., LEE, M.J., GALAS, D.J. and WANG, K., 2010. The microRNA spectrum in 12 body fluids. *Clinical chemistry,* **56**(11), pp. 1733-1741.

WIGHTMAN, B., HA, I. and RUVKUN, G., 1993. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell,* **75**(5), pp. 855-862.

WIKBERG, M.L., MYTE, R., PALMQVIST, R., VAN GUELPEN, B. and LJUSLINDER, I., 2018. Plasma miRNA can detect colorectal cancer, but how early? *Cancer medicine,* **7**(5), pp. 1697-1705.

WINAWER, S.J., ZAUBER, A.G., HO, M.N., O'BRIEN, M.J., GOTTLIEB, L.S., STERNBERG, S.S., WAYE, J.D., SCHAPIRO, M., BOND, J.H. and PANISH, J.F., 1993. Prevention of colorectal cancer by colonoscopic polypectomy. The National Polyp Study Workgroup. *The New England journal of medicine,* **329**(27), pp. 1977-1981.

WWW.CANCER.NET, .

WWW.CANCERRESEARCHUK.ORG, .

WWW.ESMO.ORG, .

XIE, Y., SONG, J., ZONG, Q., WANG, A., YANG, Y., LIU, F. and MENG, X., 2015. Decreased Expression of MIR-134 and its Clinical Significance in Human Colorectal Cancer. *Hepato-gastroenterology,* **62**(139), pp. 615-619.

XING, J., WAN, S., ZHOU, F., QU, F., LI, B., MYERS, R.E., FU, X., PALAZZO, J.P., HE, X., CHEN, Z. and YANG, H., 2012. Genetic polymorphisms in pre-microRNA genes as prognostic markers of colorectal cancer. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* **21**(1), pp. 217-227.

XU, L., LI, M., WANG, M., YAN, D., FENG, G. and AN, G., 2014. The expression of microRNA-375 in plasma and tissue is matched in human colorectal cancer. *BMC cancer,* **14**, pp. 714-2407-14-714.

XU, X.M., QIAN, J.C., DENG, Z.L., CAI, Z., TANG, T., WANG, P., ZHANG, K.H. and CAI, J.P., 2012. Expression of miR-21, miR-31, miR-96 and miR-135b is correlated with the clinical parameters of colorectal cancer. *Oncology letters,* **4**(2), pp. 339-345.

YAMADA, A., HORIMATSU, T., OKUGAWA, Y., NISHIDA, N., HONJO, H., IDA, H., KOU, T., KUSAKA, T., SASAKI, Y., YAGI, M., HIGURASHI, T., YUKAWA, N., AMANUMA, Y., KIKUCHI, O., MUTO, M., UENO, Y., NAKAJIMA, A., CHIBA, T., BOLAND, C.R. and GOEL, A., 2015. Serum miR-21, miR-29a, and miR-125b Are Promising Biomarkers for the Early Detection of Colorectal Neoplasia. *Clinical cancer research : an official journal of the American Association for Cancer Research,* **21**(18), pp. 4234-4242.

YAMAKUCHI, M., FERLITO, M. and LOWENSTEIN, C.J., 2008. miR-34a repression of SIRT1 regulates apoptosis. *Proceedings of the National Academy of Sciences of the United States of America,* **105**(36), pp. 13421-13426.

YAMASHITA, K. and WATANABE, M., 2009. Clinical significance of tumor markers and an emerging perspective on colorectal cancer. *Cancer science,* **100**(2), pp. 195-199.

YE, J., WU, X., WU, D., WU, P., NI, C., ZHANG, Z., CHEN, Z., QIU, F., XU, J. and HUANG, J., 2013a. miRNA-27b targets vascular endothelial growth factor C to inhibit tumor progression and angiogenesis in colorectal cancer. *PloS one,* **8**(4), pp. e60687.

YE, J., WU, X., WU, D., WU, P., NI, C., ZHANG, Z., CHEN, Z., QIU, F., XU, J. and HUANG, J., 2013b. miRNA-27b targets vascular endothelial growth factor C to inhibit tumor progression and angiogenesis in colorectal cancer. *PloS one,* **8**(4), pp. e60687.

YU, Y., KANWAR, S.S., PATEL, B.B., OH, P.S., NAUTIYAL, J., SARKAR, F.H. and MAJUMDAR, A.P., 2012. MicroRNA-21 induces stemness by downregulating transforming growth factor beta receptor 2 (TGFbetaR2) in colon cancer cells. *Carcinogenesis,* **33**(1), pp. 68-76.

YUAN, Z., BAKER, K., REDMAN, M.W., WANG, L., ADAMS, S.V., YU, M., DICKINSON, B., MAKAR, K., ULRICH, N., BOHM, J., WURSCHER, M., WESTERHOFF, M., MEDWELL, S., MOONKA, R., SINANAN, M., FICHERA, A., VICKERS, K. and GRADY, W.M., 2017. Dynamic plasma microRNAs are biomarkers for prognosis and early detection of recurrence in colorectal cancer. *British journal of cancer,* **117**(8), pp. 1202-1210.

ZHANG, G., ZHOU, H., XIAO, H., LIU, Z., TIAN, H. and ZHOU, T., 2014. MicroRNA-92a functions as an oncogene in colorectal cancer by targeting PTEN. *Digestive diseases and sciences,* **59**(1), pp. 98-107.

ZHAO, H.J., REN, L.L., WANG, Z.H., SUN, T.T., YU, Y.N., WANG, Y.C., YAN, T.T., ZOU, W., HE, J., ZHANG, Y., HONG, J. and FANG, J.Y., 2014. MiR-194 deregulation contributes to colorectal carcinogenesis via targeting AKT2 pathway. *Theranostics,* **4**(12), pp. 1193-1208.

ZHENG, Y.B., LUO, H.P., SHI, Q., HAO, Z.N., DING, Y., WANG, Q.S., LI, S.B., XIAO, G.C. and TONG, S.L., 2014. miR-132 inhibits colorectal cancer invasion and metastasis via directly targeting ZEB2. *World journal of gastroenterology,* **20**(21), pp. 6515-6522.