Effectively sampling rectal mucus and assessing the validity of a DNA methylation assay in the detection of Colorectal Cancer.

Thesis presented for the award of **MD** by **University of Cardiff**

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Abstract

Introduction

Colorectal cancer (CRC) is one of the most common cancers in the Western world. Screening for CRC using faecal occult blood test (FOBT) is well established. There is evidence that DNA based stool tests may be more effective than FOBT.

Hypothesis

The hypothesis is that in patients with CRC, rectal mucus may contain DNA derived from colonic tumours. It is speculated that quantitative or qualitative assessment of DNA in rectal mucus may permit an improved method of CRC screening.

Aims

Using surgically resected specimens of colonic tumours:

To assess the feasibility and reliability of measuring DNA in mucus samples

To compare different devices to measure mucus DNA

To assess the amount of mucus DNA at various distances from colonic tumours

In patients with CRC and controls:

To compare the amount of DNA in rectal mucus

Using a panel of 3 DNA methylation markers, to compare the rectal mucus DNA methylation profile between patients with CRC and controls

Methods

Surgical colectomy specimens were obtained from 25 patients with CRC. The feasibility and repeatability of measuring mucus DNA amounts was established using different buffer solutions, different storage techniques and different sampling devices. Mucus DNA amounts were measured at tumour sites and various distances proximal and distal to the tumour. 58 patients referred to a colorectal outpatient clinic with suspected CRC were assessed. Rectal mucus samples were obtained using a balloon device introduced through a proctoscope. All patients were investigated by colonoscopy to clarify the presence or absence of a CRC. The amount of DNA in the mucus

samples was measured. The presence of three DNA methylation markers (NDRG4, TFP12 and GATA4) was assessed in all samples. All studies were approved by the local ethics committee.

Results

Reliable measurement of DNA from mucus samples was established using balloon, foam and brush devices and a cell lysate buffer. Higher amounts of DNA in surgical specimens were found distal to tumours compared to proximally. In patients with CRC the amount of DNA in rectal mucus was higher than in controls (no disease or benign polyps). The three DNA methylation marker panel had a sensitivity of 87% and specificity of 27.5% for the detection of CRC.

Conclusions

The results are consistent with the hypothesis that DNA detected in rectal mucus is derived from proximal tumours. Higher levels of rectal mucus DNA are obtained from patients with CRC than from controls. The selected DNA methylation panel was not sufficiently useful in our sample group to be of use as a screening technique, due to poor specificity. Further work is in progress to compare DNA abnormalities in resected tumour tissue with DNA from rectal mucus in the same patients. Future work may be required to improve the panel of DNA abnormalities assessed.

Statement by candidate:

I, Alexios Tzivanakis, confirm that:

this work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree,

this thesis is being submitted in partial fulfillment of the requirements for the degree of MD.

this thesis is the result of my own independent work/investigation, except where otherwise stated. Other sources are acknowledged by explicit references.

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……. Date: 20/07/2014

Mr Alexios Tzivanakis

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List of Abbreviations

AJCC	American Joint Committee on Cancer
APC	Adenomatous Polyposis Coli
BMPR1A	Bone morphogenetic protein receptor, type IA
BRAF	v-Raf murine sarcoma viral oncogene homolog B
BSW	Bowel Screening Wales
Cat No	Catalogue number
CCI	Commission on Chronic Illness
CE	*Conformité Européenne*, meaning European Conformity
CIN	Chromosomal Instability Pathway
CpG	CpG dinucleotide
CIMP	CpG Island Methylator Phenotype
CIMP +	CpG Island Methylator Phenotype positive
CIMP -	CpG Island Methylator Phenotype negative
CIMP-H	CpG Island Methylator Phenotype High
CIMP-L	CpG Island Methylator Phenotype Low
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
CRC	Colorectal Cancer
CTC	CT Colonography
CT	Computer Tomography
DCC	Deleted in Colorectal Carcinoma
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic Acid
EEA	European Economic Area
EPIC	European Prospective Investigation Into Caner and Nutrition
ESR1	Estrogen Receptor 1
EU	European Union
FIT	immunochemical FOBT
GATA4	GATA Binding Protein 4
GP	General Practitioner
GmbH	*Gesellschaft mit beschränkter Haftung*, meaning company with limited liability
gFOBT	GuaicFOBT
FOBT	Faecal occult blood test
FAP	Familial Adenomatous Polyposis
FDA	Food and Drug Administration (USA)
HNPCC	Hereditary Non-polyposis Colorectal Cancer
HME	High Methylation Epigenotype
HPP	Hyperplastic Polyposis
IGF-1	Insulin Growth Factor 1
IME	Intermediate Methylation Epigenotype
Inc	Incorporation
IQR	Interquartile range
ITT	Intention to Treat
JVP	Juvenile Polyposis Syndrome
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LME	Low Methylation Epigenotype

LOH	Loss of Heterozygocity
LR	Likelihood Ratio
LTD	Limited Company
MAP	MUTYH adenomatous Polyposis
MCL	Muco-cellular Layer
MINT1	Munc-18-interacting proteins 1
MINT2	Munc-18-interacting proteins 2
MINT3	Munc-18-interacting proteins 3
MMR	DNA Mismatch System
MRI	Magnetic Resonance Imaging
MSI	Micro Satellite Instability
MSS	Micro Satellite Stable
MUTYH	mutY homolog
MYOD	Myoblast determination protein
NDGRG4	N-Myc Downstream Regulated Gene 4
NHSBCSP	NHS Bowel Cancer Screening Programme
NHS	National Health Service
NORCCAP	Norwegian Colorectal Cancer Prevention
NPV	Negative Predictive Value
PBS	Phosphate Buffered Saline
PGE2	Prostagladin E2
PJS	Peutz-Jegher Syndrome
PMS2	Postmeiotic Segregation Increased 2
PPV	Positive Predictive Value
RCT	Randomised Controlled Trial
RNA	Ribonucleic acid
SDS	Sodium Dodecyl Sulfate
SMAD4	SMAD family member 4
TFPI2	Tissue Factor Pathway Inhibitor 2
TRIS	Tris[hydroxymethyl]aminomethane
UK	United Kingdom
USA	United States of America
WHO	World Health Organisation
Wnt	Wingless-related integration site

1.1 Overview of Colorectal Carcinoma

1.1.1. Epidemiology

Colorectal cancer (CRC) is the second commonest cause of cancer related mortality in men worldwide[1,2] with almost 100 people diagnosed with Colorectal Cancer (CRC) everyday in the UK[1,2]. According to the latest population statistics, in 2008 there were 39,991 new cases of CRC diagnosed in the UK, 25,551 of which were colonic and the remainder rectal. The incidence of CRC is strongly associated with patient's age. The majority of cases (60%) are found in people over the age of 60 and up to the age of 50 the incidence is equal between men and women. From then on men have increased incidence up until the age of 84 when women take over, which can be explained by the higher proportion of women in the general elderly population. The overall male to female ratio in CRC is 11:10. Incidence of CRC in the UK has been increasing across all age groups since the 1970s by about 20% and in the 60-69 age group there is a sharp increase of 11% in the period since 2006 when the national screening program was rolled out[1].

At the same period in the European Union there were 334,000 new cases of CRC diagnosed with the highest rate seen in Hungary and Denmark for men and women respectively[2]. This reflects an increasing trend in the incidence of CRC in Europe[3,4]. Interestingly the incidence of CRC has been decreasing in the US since its peak in the mid 1980s[5]. The reason behind the reduction in incidence in the US is unclear although increased use of hormone replacement[6] and aspirin[7] at the same time period have been thought to be possible reasons. The worldwide incidence of CRC is highest in industrialized economies although there is an increasing trend in less developed countries which have started adapting to a Western lifestyle and diet[8]. Overall it is estimated, based on current incidence, that up to 6% of the Western population will develop CRC in their lifetime[5].

In Great Britain the latest mortality statistics in 2008[1] showed 16,259 deaths from CRC in total, with 10,164 from colon cancer and the majority of cases (80%) in the over 65 age group. The age standardised mortality rate was 17.7/100000 population for CRC, 10.9 for Colonic and 6.9 for rectal cancers. Unlike incidence, CRC associated mortality has been decreasing in the UK since the 1970s[9] with a similar trent in the EU[10] and US[5] as well. This reflects great advances in both the diagnosis and treatment of the disease, but also in the understanding of the pathophysiology of CRC and implementation of prevention and screening programs worldwide.

1.1.2 Pathogenesis

In the late 1980s Vogelstein et al[11] described the genetic changes of the adenoma-carcinoma sequence as the basis of carcinogenesis of CRC. In summary they correlated these genetic changes to a model of tumourogenesis where there is development of adenoma from normal colonocytes (Figure 1.1) and subsequent progression to an adenocarcinoma. This process is the result of mutational activation of several oncogenetic genes and silencing of other tumour suppressor genes, that take place at different time points in the adenoma-carcinoma sequence. The initial step is inactivation of the adenomatous polyposis coli (*APC*) gene leading to the appearance of an adenoma. Following that a *KRAS* mutation allows the adenoma to grow further and when several genetic alteration take place in chromosome 18q and *p53*takes place the adenoma has become a carcinoma[11,12].

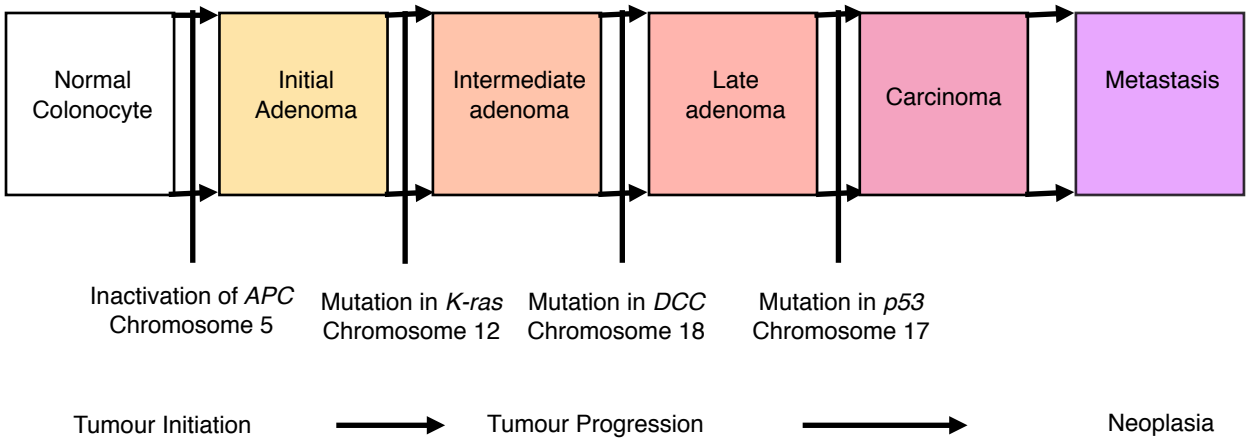

Figure 1.1: The adenoma-carcinoma sequence. Adapted from Fearon ER *et al* 1990[12]

Further understanding of the pathophysiology of CRC has led to classification of CRC carcinogenesis into three main different pathways. These are the chromosomal instability pathway (CIN), the micro satellite pathway (MSI) and the CIMP pathway which involves hypermethylation of CpG islands resulting in gene silencing. Unfortunately these pathways are not mutually exclusive of each other, resulting in tumours expressing features of more than one pathway.

Chromosomal Instability pathway (CIN)

The term CIN can be explained as an increased rate of loss and gain of whole or parts of a chromosome resulting in genetic cellular instability[13]. The result of CIN is aneuploidy (chromosomal number imbalance), high frequency of loss of heterozygosity (LOH) and sub-chromosomal genomic amplifications[14].

The CIN pathway is the combination of a characteristic set of genetic mutations in CRC in the presence of chromosomal instability. The big question of whether CIN causes these mutations or is CIN the result of such mutations is yet to be answered. The main mutations involved in the CIN pathway are *APC*, *K-RAS*, *TP53*, *18q loss* and there also frequent over expression of Cyclooxygenase-2.

Somatic *APC* mutation is found in up to 75% sporadic CRC[15,16] and activation of the *Wnt* signaling pathway is considered one of the earliest events in CRC tumourogenesis[17]. *APC* inactivation is mainly due to mutation although hypermethylation of its promoter has also been reported[18]. The result of *APC* inactivation is increased cytoplasmic levels of β-Catenin which then translocates to the nucleus triggering transcription of several genes involved in both tumourogenesis and tumour progression[14].

K-Ras mutation is found in up to 50% of CRC[19]. It regulates several cellular functions including cell growth, survival, apoptosis, cytoskeleton organisation, cell motility, inflammation and cell proliferation[14].

p53 dysfunction is considered as the hallmark of almost all human tumours and is widely considered the guardian of the genome. It is a tumour suppressor gene and it controls hundreds of genes involved in most cellular functions including apoptosis, cell cycle regulation, cell differentiation and cell migration[14]. In CRC mutated *TP53* is found in 50-75% of cases[20].

Allelic loss at chromosome 18q is found in 70% of sporadic CRC[12]. The exact gene involved is yet to be defined although several candidates have been identified[14].

Over expression of cyclooxygenase-2 is found in 86% of CRC carcinomas[21]. Its tumourogenic effect can be explained by the increased production of prostagladins (especially E2) which has been seen in both adenomas and carcinomas. PGE2 is involved in tumour proliferation migration, survival and invasion[22].

Several mechanisms have been proposed to explain the CIN phenotype. One of these involves defects in the cellular pathways responsible for ensuring that the chromosomes segregate accurately during cell division (equal number of chromosomes distributed to each daughter cell) leading to aneuploidy. Abnormal number and function of centrosomes, which coordinate the formation and function of the mitotic spindle apparatus during cell division, is also proposed as a mechanism of CIN. The end result is again aneuploidy. During chromosomal segregation the ends of the chromosomes are protected by hexameric repeats called telomeres. These sequences are yet another possible candidate to explain CIN. Telomere activation, damage or loss has been associated with dramatic genome reorganisation and is seen *in vitro* in CRC of all stages. Another possible cause of

CIN is defective cellular DNA damage-repair although the exact mechanism is not fully understood yet. Finally CIN is associated with high LOH and although several mechanisms have been proposed, the dominant theory is that LOH is due to mitotic nondisjunction, however the relationship between CIN and LOH is still not fully understood[14].

Microsatellite instability pathway

Microsatellites are repetitive DNA sequences comprising short reiterated motifs. They were first described in CRC in 1993 when it was noted that a percentage of tumours had several deleted DNA bands. When these bands were examined more closely it was clear that they were not actually deleted but had migrated further down and had shortened in length. They contained simple repetitive sequences (microsatellites) and the research groups used the term microsatellite instability to describe their observations. These tumours were more likely to be in the proximal colon and had a better prognosis[23, 24] compared to other CRC tumours.

Further research revealed that 12-18% of all CRC characteristically had large number of MSI mutations that seemed to be arising in the DNA mismatch repair system (MMR) and led to the identification of the genes (MSH2, MSH3, MLH1, MSH6, PMS1, PMS2) responsible for Lynch syndrome. As a result MSI was associated with inherited CRC despite the fact that the initial description of MSI was in sporadic CRC cases. In addition only up to 4% of all CRC is due to Lynch syndrome (see section 1.1.3.1) which can not explain why MSI is found in up to 17% of all CRC[25]. Towards the end of the 1990s it was known that most of the sporadic, MSI positive CRC had silencing of MHL1 (via hypermethylation[26]) and PMS2, and a mutation in *BRAF*[27]. These tumours were clinically different from tumours in Lynch syndrome. They occur later in life, about half of them have *BRAF* mutations and are associated with CIMP (see below).

In 1997 a classification of MSI tumours was proposed:

- MSI-High tumours where more than 30% of MS present is mutated.
- MSI-Low tumours where a tumour had MSI mutations but under the 30% threshold. These tumours have microsatellite stable tumour clinical features.
- Microsatellite Stable tumours (MSS)[28].

The mechanism of tumourogenesis in the presence of MSI involves the same pathways as in non MSI CRC tumours but different targets are involved, For example MSI CRC tumours express a normal *APC* gene but have a mutated β-*catenin* resulting in impaired *APC* function. *KRAS* (Lynch syndrome) and *BRAF* (sporadic MSI) mutations have the same result as their products both act at the mitogen activated kinase signaling pathway involved in epithelial cell proliferation[25].

CpG Island methylator phenotype (CIMP)

CpG island refers to a CpG dinucleotide which is found at specific sites in the genome. Human DNA contains 5-methylcytosine at specific sites depending on the cell type. These sites are determined during embryonic development. The pattern of methylation in human DNA is dependent on activity of methyltransferases and demethylases. Methyltransferases are able to recognise these CpG islands. They are normally found near gene promoters and transcription start sites. Several human tumours including CRC types use high levels of methylation to silence tumour suppressor genes[29].

In 1999 Toyota et al[30] divided CRC into two groups, one with CIMP (CIMP+) and one without (CIMP-). Since then several other groups also tried to define CRC based on methylation markers and several systems have been proposed, with the most recent ones in 2007[31] (CIMP1, CIMP2 and CIMP-) and in 2010[32] (high methylation epigenotype (HME), intermediate methylation epigenotype (IME) and low methylation epigenotype (LME)). At the moment there is no universally agreed classification of CRC based on methylation markers as new markers are being identified at a high rate. However, five markers are considered as the classic CIMP markers which are *CDKN2A, MINT1, MINT2, MINT31* and *MLH1*. In 2007 Jass et al[33] classified CRC into 5 subtypes based on molecular and clinical characteristics:

- 1. CIMP-H and MSI-H and *BRAF* mutations with poor differentiation, proximal tumours representing 12% of CRC,
- 2. CIMP-H, MSI-L or MSS, *BRAF* mutation, poorly differentiated proximal tumours representing 8% of CRC,
- 3. CIMP-L, MSS or MSI-L, *KRAS* mutation, well differentiated distal tumours 20% of CRC,
- 4. CIMP-MSS, *TP53* mutation, well differentiated distal tumours 57% of CRC and finally
- 5. Lynch Syndrome, CIMP-, MSI-H moderately differentiated proximal tumours 3% of CRC.

Types 1 and 2 are considered chromosomal stable while 3 and 4 have high degree of CIN. These types are not mutually exclusive of each other and there is some overlap between them. A year later in 2008 following the publication of a study[34] that demonstrated an inverse relationship between CIN and CIMP, it was suggested that the two mechanisms should be independent of each other. A further classification of CRC was proposed by Issa JR[35] into three groups/mechanism.

- Group 1 CIMP+ and MSS,
- Group 2 CIMP + and MSI
- Group 3 CIN.

The first two groups differ in their CIMP+ profile in that the MSS group has more *KRAS* mutations but can occasionally have *BRAF* mutations, a worse clinical outcome and is more closely associated with villous adenomas.

In 2006 Suzuki et al[36] noted that hypo and hypermethylation of DNA increases with age and such changes are also seen in CRC. Several genes (e.g. *ESR1* and *MYOD*) have been shown to be hypermethylated in an age related fashion in normal colonic mucosa (these were excluded from the original CIMP classification). What causes this age related hypermethylation remains unknown at the moment but it is possible to be secondary to environmental factors[29].

1.1.3 Risk Factors

1.1.3.1 Familial and Hereditary Colorectal Cancer

Up to 20% of CRC is associated with an inherited predisposition as shown by twin studies[37] and genetic testing studies[38]. For the majority of cases of CRC with inherited predisposition, the underlying genetic mechanism is well understood, but there is a significant minority for which the exact process is not fully understood[39].

Lynch Syndrome (hereditary non-polyposis colorectal cancer)

In 1966 Henry Lynch[40] reported an increased incidence of colorectal cancer in two kindreds and called it cancer family syndrome. Later, the terms Hereditary NonPolyposis Colorectal Cancer (HNPCC) and Lynch syndromes were used but lately the latter has been used more frequently. About 2-4% of all CRC patients will have Lynch syndrome[41].

Patients with Lynch syndrome tend to develop CRC earlier in life than sporadic CRC patients and they rarely have polyposis. In addition there is a higher incidence of proximal tumours compared to sporadic CRC cases[39]. There is an increased incidence of extra-colonic cancers, namely endometrial cancer with a lifetime risk of up to 60%[42] while the CRC lifetime risk ranges from 50-80%[43]. Table 1.1 summarises tumours associated with Lynch syndrome[44].

Genetically Lynch syndrome is characterised by the presence of a germline mutation affecting the DNA Mismatch-repair system (*hMSH2, hMLH1, hMSH6, hPMS2*) which is responsible for correcting single base mismatches and insertion deletion loops during DNA replication. These mutations are inherited in a autosomal dominant fashion and cause high micro satellite instability (MSI-H)[39].

Site	Frequency**
Colon	30-75%
Endometrium	30-70*%
Stomach	5-10%
Ovary	5-10*%
Urinary Tract	5%
Other (Small bowel, pancreas, brain)	<5%

Table 1.1: Tumours associated with Lynch Syndrome[44]

*refers to women cancer cases

**Frequency: cumulative cancer incidences

Diagnosis of such patients is difficult as it is not possible to do genetic testing of all family members of all CRC patients as these tests are costly and labour intensive .The Amsterdam criteria I and II (table 1.2)[45] have been developed to assist clinicians in deciding which patients should be sent for genetic testing to identify Lynch Syndrome. Surprisingly up to 50% of families meeting the Amsterdam criteria did not have MMR germline mutations and the tumours were MSI negative[46,47] implying that a large proportion of families with Lynch syndrome do not meet the Amsterdam criteria. In such families testing tissue from CRC in family members could assist clinicians to diagnose Lynch syndrome. As mentioned above such tests can be expensive, take time and are labour intensive and therefore the Bethesda guidelines[48] have been used to identify the patients where tumour tissue should be examined for MSI.

In the cases where the patient and his/her family meet the criteria but the genetic profile does not fit that of Lynch Syndrome the term Familial colorectal cancer type X is used[46].

Amsterdam I	Amsterdam II
At least 3 relatives with CRC, 1 has to be 1st degree relative of the other two	At least 3 relatives with Lynch Tumour (Table 1) 1 has to be 1st degree relative of the other two
At least two successive generations involved	At least two successive generations involved
At least one CRC <50yrs old	At least one CRC <50yrs old
FAP excluded	FAP excluded
Histological verification of tumours	Histological verification of tumours

Table 1.2: Amsterdam criteria I and II to assist clinicians to identify families with possible Lynch syndrome[45].

Familial Adenomatous Polyposis (Classic and Attenuated) and MAP

Following Lynch syndrome, FAP is the second most common CRC syndrome in the familial CRC group and unlike Lynch syndrome it is characterised by multiple colonic polyps in early adolescence. The average age of diagnosis of CRC is 39 years with the majority (95%) of FAP patients developing CRC by the age of 50 years[39] if they remain untreated. In addition to classical FAP, there is attenuated FAP where patients develop significantly less polyps and CRC at an older age with an increased prevalence of proximal tumours[49]. Both FAP and its attenuated form are due to a germline mutation in the *APC* gene on chromosome 5q (tumour suppressor gene, part of the *wnt*-signaling pathway) that is inherited in a autosomal dominant fashion[39]. The location of the mutation in *APC* has been correlated with the severity of polyposis, as well as the presence of extra-colonic features such as desmoids[50]. About 25% of FAP cases are the result of new mutations or *de novo* mutations[39].

Classical FAP is characterised by hundreds of adenomatous colonic polyps at a young age (15-30yrs old)[51]. Those patients who have between 10-100 adenomatous polyps and usually present at a later age, are more likely to have either attenuated FAP or MYH-polyposis (MAP see below) Extra colonic polyposis especially of the upper GI tract is common in FAP with >50% of patients having duodenal and about 50% having gastric polyps[52]. The life-time risk of developing duodenal cancer is about 12%[53] compared to gastric cancer which is much lower (1%)[51] making duodenal malignancy the second commonest cause of cancer in FAP and attenuated FAP[53]. Non malignant features of FAP include desmoids (about 10% of FAP patients), osteomas (skull and mandible), fibromas, dental abnormalities, epidermoid cysts and others[54]. These features are less common in attenuated FAP.

MAP is clinically very similar to attenuated FAP, both in the number of polyps present but also in the age of onset of CRC and higher proximal lesion incidence[55] but unlike FAP or its attenuated form, is due to a biallelic *MUTYH* mutation (mutY human homologue gene on chromosome 1p). FAP-associated non malignant features such desmoids etc have not been seen in MAP, although a higher incidence of ovarian and badder cancer has been observed[39]. In addition there is some evidence of an association between MAP and both hyperplastic and serrated polyps but this is based on a small study[56].

Hamartomatous Polyposis (Peutz-Jeghers (PJS) and Juvenile Polyposis (JPS) Syndromes)

Hamartoma is defined as a tumour-like, non-neoplastic disordered proliferation of mature tissues that are native to the site of origin[57]. PJS is due to a germline mutation in *STK11* while JPS is due to mutations in two genes (SMAD4 or BMPR1A) but these mutations are only seen in up to 70% in PJS and only 40% in JPS[39].

Both PJS and JPS are characterised by hamartomatous polyps in the gastrointestinal tract and are associated with an increased risk of CRC[58]. In PJS the dominant site of polyposis is the small bowel[59] (95% of cases) followed by the colon[39] (30% of cases). Small bowel obstruction and PR bleeding are the predominant symptoms that usual start in the teenage years with the almost pathognomonic feature of buccal and lip pigmentation. A combination of small bowel polyps and/or pigmentation and/or family history of PJS is used to diagnose patients. The life time risk of developing CRC in PJS patients is estimated[60] at 39%. Unlike PJS, JPS does not have any pathognomonic features such as pigmentation to facilitate diagnosis and as a result the diagnosis is based on finding more than 3 juvenile polyps in the colon and /or multiple GI polyps or a family history of JPS. A minority of JPS patient have GI arteriovenous malformations as well as pulmonary arteriovenous malformations[39]. The lifetime risk of CRC in JPS[61] is 39%.

Hyperplastic Polyposis (HPP) or Serrated Polyposis

Little is known regarding this rare syndrome where there are multiple and/or large colonic polyps. The current WHO criteria for diagnosing HPP are 30 cumulative hyperplastic polyps of any size in the colon, or >5 hyperplastic polyps proximal to sigmoid, or at least one hyperplastic polyp in an individual with a family history (first degree relative) of HPP.

HPP is associated with an increased risk of developing CRC and there is an association between the number of hyperplastic polyps and the presence of serrated adenomas. The exact genetic mechanism of HPP is not yet understood[39].

Common Familial Colorectal Cancer

This subgroup of inherited CRC includes patients who have an increased incidence of CRC in their family but do not have the high penetrance genetic mutations of the above syndromes. Individuals with a first degree relative that had CRC diagnosed <50yrs or two first degree relatives with CRC have a 6-fold increase in their lifetime risk of developing CRC[62]. Several (up to 170) chromosomal regions have been identified[63] as possible candidates to explain this increased risk but they all have low penetrance unlike the genetically well defined syndromes. As there are no specific genetic

markers for this group of patients, specific guidelines based on number of first degree relatives diagnosed with CRC and the age that they developed CRC, are used to identify high risk cases[39].

1.1.3.2 Diet

Fibre

It is has been known for over 40 years that high fibre based diets seem to offer protection against CRC[64]. However the scientific evidence to support that observation appears to be controversial. A meta-analysis[65] of 37 observational epidemiological studies and 16 case-controlled studies and a pooled analysis of 13 case-controlled[66] studies concluded that there was a 40-50% reduction in CRC risk in individuals with a high fibre diet.

In contrast, a pooled analysis of 13 prospective cohort studies in 2005 by Park et al[67] showed a weak association between a high fibre diet and a reduction in risk of CRC, which was lost when they repeated the analysis correcting for other possible dietary factors such folate or Vitamin B. In 2009 the EPIC study[68], a large prospective study showed a 40% risk reduction with a high fibre diet. Finally several randomised controlled studies[69-71] where the intervention was a high fibre diet (in the form of increased vegetables, supplements and fruit) have showed no difference between the intervention and control groups.

Red Meat

The evidence that red meat is associated with a higher risk of developing CRC is less controversial and mostly universally accepted. The EPIC study in 2005[72] gave a risk of 1.8% of developing CRC in 10 years for a 50 year old eating large amounts of red meat and only 1.3% if less red meat was consumed. The mechanism by which red meat exerts its effect remains unclear at the moment but there is some evidence that red meat which is grilled, fried or cooked at high temperatures is rich in heterocyclic amines which are mutagenic and can cause adenoma[73].

Carbohydrates

Diets rich in refined carbohydrates can stimulate short lived surges of insulin which in turn can be carcinogenic (see below section on obesity) in CRC[73]. In addition several studies[74-76] have demonstrated a link between carbohydrates and CRC.

1.1.3.3 Lifestyle

Alcohol

A pooled analysis of 8 prospective and case control studies[77] and the EPIC study[78] both demonstrated an increased risk of CRC if >30g/day of alcohol is consumed. It is unclear how alcohol leads to carcinogenesis although it has been suggested that it might contribute to abnormal DNA methylation[79] or that it has systemic effects affecting the immunological surveillance of tumours or DNA repair mechanisms[80].

Tobacco

Epidemiological studies in the USA have concluded that up to 20% of CRC can be attributed to smoking[81-83] with the effect seen in ex smokers despite stopping smoking for up to 31 years[84]. Two recent meta-analyses in 2009 demonstrated not only an association between tobacco smoking and CRC[85], but also a dose response effect with 38% risk with 40 cigarettes/day, and also duration (20% increased risk with every 40 years of smoking) and age of initiation of smoking[86]. The carcinogenic effect of tobacco smoking is due to the release of several carcinogens such as nitrosamines, aromatic amines, heterocyclic amines and polynuclear aromatic hydrocarbons[87].

Obesity

In 2007 a meta analysis of prospective studies demonstrated an increased risk for both men (33%) and women (16%) with increasing waist circumference and waist to hip ratio independent of other lifestyle factors[88]. The mechanism behind this effect is unclear but evidence of increased CRC risk in patients with diabetes mellitus indicates that mitogenic properties of insulin along with obesity associated insulin resistance trigger carcinogenesis possibly via the release of IGF-1[89]. High levels of circulating insulin affects susceptible cells via either direct binding onto insulin receptors or IGF receptors by increasing free IGF-1 levels by reducing serum IGF binding proteins. Activation of such receptors could lead to increased cell proliferation and at the same time reducing apoptosis, hence increasing the risk of tumourogenesis[73, 90]. In 2007 a large prospective study[91] showed a 37% increase in CRC in patients with high levels of C-peptide (associated with insulin secretion). In addition patients with diabetes mellitus have an increased risk of CRC[92-94] which further supports the above observations.

Physical activity

The inverse relationship between physical activity and CRC is well described in many studies of varied design and power[95, 96] in a dose response fashion. Recently a large meta analysis[97] showed that higher physical reduced the risk of CRC by 30%. Once again the exact mechanism why physical activity has such an effect is unclear but it could be secondary to the reducing effect of physical activity on insulin secretion[98,99].

1.1.3.4 Medications

Aspirin

There have been several meta-analyses of randomised control trials (RCT) to assess whether a daily dose of aspirin is beneficial in reducing CRC risk. In 2007[100] a meta analysis looked at two RCTs with follow up data of up to 20 years concluding that daily high dose (300mg) of aspirin for 5 years reduced the incidence of CRC (RR at 10–19 years [0.60; 95% CI, 0.42-0.87]). In 2010 a further meta-analysis[101] of four RCTs with similar follow up periods (20 years) confirmed the finding of the 2007 analysis with a reduction in incidence and mortality for proximal colon (adjusted incidence HR = 0.35; 95% CI, 0.20–0.63; adjusted mortality HR = 0.24; 95% CI, 0.11–0.52) and rectal (RR = 0.58; 95% CI, 0.36–0.92). It also showed no increased benefit with doses exceeding 75mg per day for 5 years. Finally, in 2011, an analysis[102] of eight RCTs showed similar results as the previous ones and the 20 year HR mortality for CRC was 0.60 (95% CI, 0.45–0.81). This effect is most likely secondary to inhibition of COX-2 and its involvement in CRC carcinogenesis[103,104]. COX-2's role in CRC carcinogenesis has been shown in animal models where inhibition of COX-2 has stopped adenoma development in *APC* mutant mice, as well as COX-2 and not COX-1 over-expression in human adenomas and CRC tumours[73]. Although daily aspirin is beneficial in CRC it has a significant side effect profile which almost outweighs this beneficial effect. In 2009 an international consensus was published suggesting that further research should be done in high risk patients for CRC where maybe its benefit might outweigh its harms[105].

1.1.4 Diagnosis, staging and prognosis

Colon and rectal carcinoma is usually diagnosed following a full clinical examination followed by investigations that can provide a tissue diagnosis via biopsy of bowel lesions (colonoscopy or flexible endoscopy). The patient will then have a CT examination of the chest, abdomen and pelvis to assess for distant metastasis and/or synchronous tumours and to contribute to the overall staging of the disease. In the case of rectal cancer pre treatment high resolution MRI can provide useful information regarding local disease involvement (mesorectal fascia and assessment of the circumferential resection margin) and also in treatment decision making. Over the last few years the use of endo-rectal ultrasound scan[106] has been used more frequently to assess the depth of bowel muscle involvement, allowing more accurate pre treatment staging. The purpose of the pre-treatment investigations is to allow accurate staging of the disease to guide multidisciplinary teams to plan treatment strategy.

Staging of Colorectal cancer

Staging of cancer describes how advanced or severe a person's cancer is. It allows doctors involved with diagnosis and management of cancer patients to plan treatment and to estimate prognosis. In colorectal cancer there are two commonly used staging systems. The original Dukes[107] system:

- Dukes A: tumour confined to the bowel wall
- Dukes B: tumour beyond the bowel wall
- Dukes C: lymph nodes involved, Later modified to C1(apical node not involved) and C2 (apical node involved)
- Dukes D: distant metastasis present .

Dukes Stage	Relative 5 year survival rate
A	93.2%
B	77.0%
C	47.7%
D	6.6%

Table 1.3 5-year survival rates based on Dukes staging of CRC[108].

The second commonly used staging system for CRC is the American Joint Committee on Cancer (AJCC) TNM[109] system:

- T refers to bowel wall involvement:

  TX=primary tumour cannot be assessed
  T0=no evidence of primary tumour
  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 subserosa or into non- peritonealised pericolic or perirectal tissues
  T4a=tumour penetrates the surface of the visceral peritoneum
  T4b=tumour directly invades or is histologically adherent to other organs or structures

- N refers to lymph node involvement:

  NX=regional lymph nodes cannot be assessed N0=no regional lymph node metastasis
  N1a=metastasis in one regional lymph nodes
  N1b=metastasis in two to three regional lymph nodes
  N1c=Tumour deposits in subserosa, mesentery or non-peritonealised pericolic or perirectal tissues without regional nodal metastasis
  N2a=metastasis in four to six regional lymph nodes
  N2b=metastasis in seven or more regional lymph nodes

- M stands for metastatic disease:

  MX=distant metastasis cannot be assessed
  M0=no distant metastasis
  M1a=distant metastasis to one site
  M1b=distant metastasis to more than one site or peritoneum

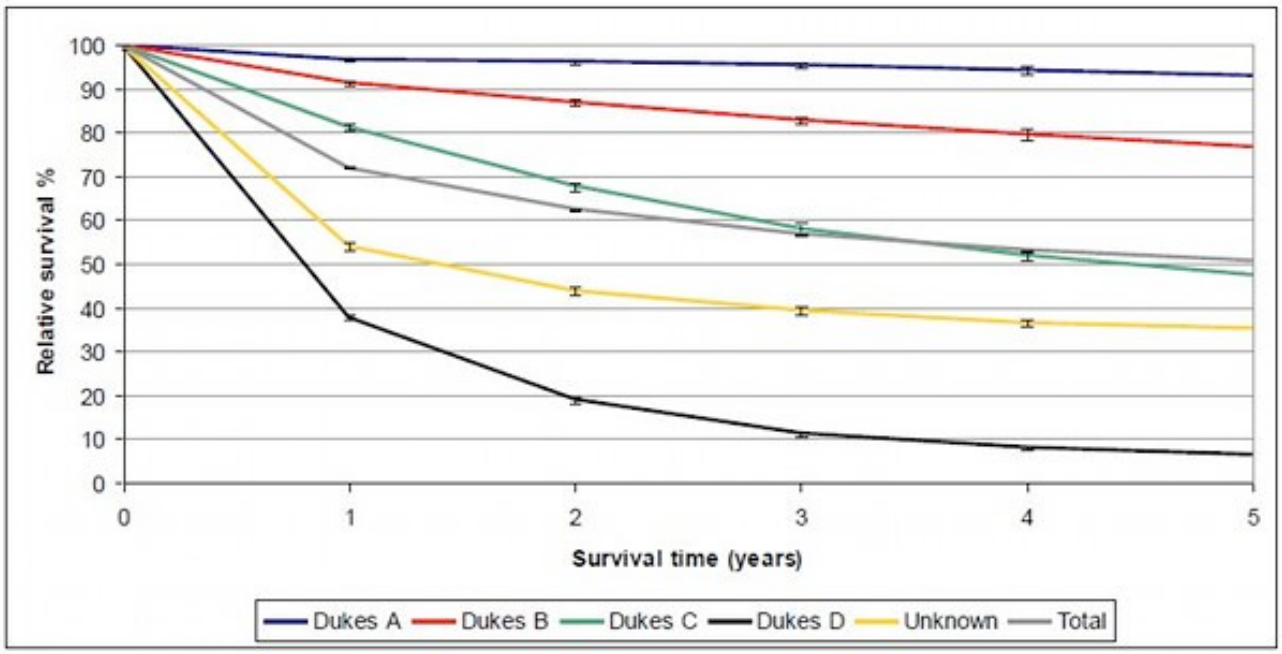

Figure 1.2 Survival rate based on staging of Colorectal cancer (Dukes Staging)[108]

It is not possible to stage patients fully before treatment starts and clinicians can modify the stage of a patient as more information becomes available. In the majority of patients the final staging is usually done following histological examination of the surgical specimen.

TNM	Colon 5 year survival rate	Rectal 5 year survival Rate
T1, T2 N0	97.1%	94.4%
T3 N0	87.5%	78.7%
T4 N0	71.5%	61.4%
T1, T2 N1	87.7%	85.1%
T1, T2 N2	75.0%	63.9%
T3 N1	68.7%	63.3%
T3 N2	47.3%	43.7%
T4 N1	50.5%	47.1%
T4 N2	27.1%	29.5%

Table 1.4: 5-year survival rates based on TNM staging of Colon and rectal caners[108-111]

The survival rates of CRC depend highly on the stage of disease (Figure 1.2, tables 1.3 and 1.4 summarise the latest survival rates using both the Dukes[107] and the AJCC TNM systems for colon[110] and rectal[111] cancers).

It is clear from these data that there is a significant survival benefit with early stage Colon and Rectal cancer and therefore early diagnosis and treatment is essential.

It is this survival benefit of early disease detection and treatment along with the presence of a premalignant stage in the natural history of colorectal cancer that make CRC a candidate for screening. CRC screening principles, outcomes and challenges will be discussed in detail in the next section.

1.2. Colorectal Cancer Screening

1.2.1. Principles of screening for disease

Screening in medicine is unique in that it is a process which aims to identify those individuals within a population with an asymptomatic or pre-symptomatic disease. In other words it is aimed at otherwise healthy individuals. It was defined by the Commission on Chronic Illness (CCI) in 1951 Conference on Preventive Aspects of Chronic Disease as "the presumptive identification of unrecognized disease or defect by the application of tests, examinations, or other procedures which can be applied rapidly. Screening tests sort out apparently well persons who probably have a disease from those who probably do not"[112]. The basic principle behind screening for disease is the identification of a disease at an early stage (secondary prevention) to allow early intervention and therefore significantly reduce morbidity and mortality associated with that particular disease. At the same time screening aims to avoid exposing patients to unnecessary investigation or treatment.

In 1968 the WHO identified 10 principles of screening[113]:

- The condition screened should be an important health problem. As described in the previous section (1.1) CRC is a major health problem both based on its prevalence but also as a cause of significant mortality and morbidity.
- There should be an accepted treatment for the condition. The management of CRC has not been discussed in this thesis as it is a large topic and not relevant to the content of the thesis. However CRC can be successfully treated and as discussed in section 1.1.4 there is a significant advantage in early treatment of CRC as stage at presentation correlates with outcome (Figure 1.2, tables 1.3 and 1.4).
- Facilities and resources for both diagnosis and treatment of the disease must be available.
- The disease needs to have a recognisable latent/early symptomatic stage. CRC as demonstrated by the adenoma-carcinoma sequence (section 1.1.3.) has a premalignant stage which is recognisable and detectable, making CRC a good candidate for screening.
- There should be a suitable test or examination. As described later in this section, at the moment there is no perfect screening test for CRC and various screening programs around the world use different tests in their screening programs.
- This test needs to be acceptable to the population screened.

- The natural history of the disease, especially the transition from latent to active (declared) disease should be well understood. CRC carcinogenesis is a widely researched subject with constant flow of new information almost on a weekly basis.
- There should be an agreed treatment policy for patients with the disease.
- The total cost of identifying patients should be balanced against medical expenditure.
- Screening should be continuous and not a once for all process.

There are three types of screening:

- Mass screening: large scale screening of entire population groups.
- Selective screening: screening of a selected high-risk groups. It can be as large scale as mass screening depending on the population group selected.
- Multiple screening: screening program where at every screening episode more than one disease is screened for.

Sensitivity and specificity of screening test

The WHO in 1968 also defined how to measure the efficiency of a screening test[113]. In a screened population there are four categories of results:

- those with the disease and with a positive test (true positive)
- those without the disease and a positive test (false positive)
- those without the disease and a negative test (true negative)
- those with the disease and a negative test (false negatives).

Based on the above four categories of results we can define sensitivity and specificity:

$$Sensitivity = \frac{True\ Positives}{True\ Positives + False\ Negatives}$$

$$Specificity = \frac{True\ Negatives}{True\ Negatives + False\ Positives}$$

Essentially sensitivity refers to the ability of the test to correctly identify those individuals within the screened population with the disease (false negative rate) while specificity refers to correctly identifying those without the disease (false positive rate).

Bias in screening

In CRC, as in most diseases, tumours detected via screening tend to be diagnosed at an earlier stage and therefore should have a better prognosis compared to symptomatic tumours. That does not automatically means that this benefit is purely due to screening because screening has three major inherent biases:

- Lead time bias: One of the major aims of screening is to detect asymptomatic disease. It is inherent that tumours will be diagnosed earlier than symptomatic tumours. Therefore the survival time since diagnosis is extended compared to symptomatic patients. If in both cases patients die at the same time and we compared the two survival times since diagnosis, the screened detected patient will have an apparently improved survival compared to the symptomatic patient.
- Length time bias: This refers to the different biological characteristics that screen detected tumours may have compared to symptomatic tumours. In other words, screen detected tumours may have a slower, less aggressive biological profile to symptomatic tumours.
- Selection bias: Here the bias is due to the different attitude that different groups of the population have towards participation in screening program. Persons with, for example, a strong family history of CRC (higher risk population by definition) are more likely to participate in a screening exercise.

The best way to eliminate such biases, is to use large RCTs when evaluating a screening test/ programme. In the next section we will discuss the available and potential screening methods for CRC as well as a brief discussion of the current NHS Bowel Cancer Screening Programme (NHSBCSP).

1.2.2. Methods of Colorectal Cancer Screening

There are several proposed methods of CRC screening, some well established and in current use throughout the world, others that are gaining acceptance by both patients and healthcare professionals and others still experimental. They can been grossly classified into those based on stool sampling and those based on structural colonic examinations such as endoscopy or radiology.

1.2.2.1 Stool based tests

GuaiacFOBT(gFOBT)

This is the most widely used screening method around the world. It works by detecting blood in the stool sample tested, by testing the peroxidase activity of the haem group of the haemoglobin molecule. Therefore it is not specific to only human blood. As described below the immunochemical FOBT (FIT) works around that problem by being specific to human blood. A positive gFOBT will trigger a referral for consideration of colonoscopy.

The main reason gFOBT is so popular with screening programs around the world is that it is simple to use by the patient and is relatively low cost compared to other CRC screening tests. The detection rates of gFOBT improve significantly if the patient provides more than one stool sample taken on separate days with better results if at least three samples are taken[114]. Due to its inability to distinguish human haem group from dietary haem (red meat, poultry etc) and the inhibitory effect of Vitamin C on peroxidase, gFOBT has high false positive and negative rates. In 2011 a Cochrane review[115] summarised the performance of gFOBT in four RCTs that involved 372,043 patients in four countries (USA, UK, Sweden and Denmark). The screening rounds were mainly biannual (Minnesota trial was randomised to annual and biannual) with age groups varying from 45 to 80years within the four trials. The follow up ranged from 11.7(UK) to 18 (USA) years. Interestingly in three trials (USA, Sweden and Denmark) participants were asked to modify their diets prior to sampling stool for the gFOBT. In addition, in two out of the four trials (Sweden, USA) the samples were rehydrated prior to analysing the stool samples in the lab as this has been shown to increase the sensitivity of the gFOBT[116]. This was reflected by the low test positivity reported in the non-rehydrated trials 0.8-3.8% with a positive predictive value for cancer of 5-18.7% compared to the other two trials which rehydrated the samples with a test positivity of 1.7-15.4% and PPV of 0.9% to 6.1%. Similarly the test sensitivity was lower in the non hydrated trials (55-57% v 82-92%) as

summarised in Table 1.5. The low PPV for cancer implies that almost 80% of the positive gFOBT were false positive with the majority of the patients being exposed to unnecessary colonoscopy (with its associated complications) and psychological stress. However the combined CRC mortality reduction from gFOBT screening was 16% with ITT analysis and 25% for those who actually attended the screening rounds.

RCTs	*Rehyd.*	*Positive Rate*	*Sensitivity*	*PPV (CRC)*	*PPV (Aden)*
Nottingham	No	1.2-2.7%	57.2%	9.9-17.1%	42.8-54.5%
Funen	No	0.8-3.8%	55%	5.2-18.7%	14.6-38.3%
Goteborg	Yes	1.7-14.3%	82%	4.8%	14.0%
Minnesota	No	1.9%	NR	NR	NR
	Yes	3.9-15.4%	92.2%	0.9-6.1%	6.0-11.0%
	No	1.4-5.3%	80.8%	5.6%	NR

Table 1.5: Sensitivity, PPV for both cancer and adenoma, Rehydration of samples and positivity rates for the four RCTs. Adapted from Hewitson P et al[116] 2008.

The participation rates in the four trials varied with 60-78% of patients attending at least one round of screening. The attendance was reduced to 60% in the Swedish trial with subsequent rounds while the Danish trial only re-invited those patients that attended the initial screening round yielding very high subsequent rounds compliance 94%). In addition, in all four RCTs, staging of the cancers detected was significantly more favourable compared to the control arm with more Dukes As and Bs detected (table 1.6) which satisfies the screening test criterion of detecting cancer at earlier stage. Interestingly no trial showed any reduction in CRC incidence by using gFOBT screening.

	Screening Group				Control Group			
RCTs	A	B	C	D	A	B	C	D
Notting-ham	20%	32%	24%	22%	11%	33%	31%	21%
Funen	22%	34%	19%	20%	11%	37%	23%	24%
Goteborg	26%	28%	32%	14%	9%	34%	21%	17%
Minnesota A	30%	29%	23%	9%	22%	31%	21%	17%
Minnesota B	27%	26%	26%	11%	-	-	-	-

Table 1.6: Percentages of CRC stage detected in the two arms of the four RCTs Adapted from Hewitson P et al[116] 2008.

FIT

This test uses antibodies specific to human proteins (haem, albumin or blood components) and has therefore lower false positive tests and does not get affected by diet. In addition only one sample is sufficient. It has a sensitivity of 66-82% in detecting CRC and advanced adenoma (high grade dysplasia or polyps more than 1cm) of up to 30% with a high specificity[117, 118] of up to 97%. Two studies compared FIT and gFOBT in patients participating in screening for CRC, with the immunochemical test showing higher detection rates for advanced colonic neoplasia (2.4% v 1.2%) and much higher compliance rates of 61%[119]. More and more countries in the EU have started using FIT instead of gFOBT with the Welsh and Scottish screening programs using it as a second line test for unclear gFOBT results and with the English program due to switch to FIT over the next few years.

Stool DNA tests

Stool DNA testing aims to detect DNA exfoliated by CRC tumours and adenomas into the bowel lumen and incorporated within the stool. That DNA then needs to be isolated from the stool and tested for markers to detect CRC or advanced adenoma. As described previously in section 1.1.2 the genetics of CRC are highly complex and not yet fully understood which does not allow for a universal marker to be used to detect CRC or advanced adenomas. As a result a panel of markers needs to be used to allow for the molecular heterogenicity of CRC. Only a few markers have been

identified as present in more than 40% of CRC (mutated *p53, APC, KRAS*)[120, 121]. Cell exfoliation in CRC and normal colonocytes is discussed in detail in section 1.3 but in summary, exfoliation from colorectal neoplasms is a continuous process and more frequent compared to normal colonocytes. In addition neoplastic cells seem to have a higher survival potential once exfoliated into the lumen (loss of the anoikis effect) a process discussed below in section 1.3.

Mutant *KRAS* was the the first DNA marker to be tested as a stool marker in 1992[122] to detect CRC in stools from nine patients with CRC with known *KRAS* mutations. Following that, several studies have used panels of DNA markers in a screening setting to detect advanced colorectal neoplasms. Imperiale *et al*[123] in 2004 used a pre-commercial panel panel of 21 mutations (PreGenPlus by Exact Sciences (Maynard, USA)) (3 in the K-ras gene, 10 in the APC gene, and 8 in the p53 gene) and the microsatellite-instability marker BAT-26 along with a marker of long DNA thought to reflect disordered apoptosis of cancer cells sloughed into the colonic lumen, versus gFOBT in a screening population. The patients were invited to provide a stool sample of up to 30g into a special container which was returned to the laboratory, perform a non rehydrated gFOBT(see above) and were invited to undergo a colonoscopy irrespective of the stool based test result. The DNA panel detected 52% v 13% (for gFOBT) of cancers and 18% v 11% of advanced adenomas with similar specificity between the two tests. In 2008 Ahlquist *et al*[124] used the same DNA panel as well as the new 3 marker panel SDT2 (methylated vimentin, mutant KRAS, and mutant APC) in a similarly designed study. The authors reported similar results with 46% v 10% for gFOBT for advanced neoplasms. In this study however the false positives were higher in the DNA test (48%) compared to 21% in gFOBT.

At the moment stool DNA tests are not used in any screening program in the world, however the American Colleges of Radiology[125] and Gastroenterology[126] have included stool DNA testing in the recommended screening modalities if used every 3 years. As new markers, sampling, isolation and analysis technology improves DNA tests will most likely replace FOBT in the future.

1.2.2.2 Structural Colonic Examinations

Endoscopy

There are two endoscopic methods to examine the colon and rectum, flexible sigmoidoscopy and colonoscopy. The main difference between the two is that flexible sigmoidoscopy examines only part of the colon while colonoscopy examines the entire length of the large bowel. In addition flexible sigmoidoscopy does not require any sedation, is quicker and can be performed outside a designated endoscopy unit. Both procedures require the patient to receive some form of bowel

preparation with most units using enemas for sigmoidoscopy and full bowel preparation (patient receives strong laxative the day before the procedure and follows a special dietary intake) for colonoscopy.

Flexible sigmoidoscopy in CRC screening

In 2010 Atkin *et al*[127] published the results of a RCT looking at the effectiveness of once only flexible sigmoidoscopy as a screening tool for CRC. All patients between 55 and 64 years that were registered with a GP were invited to participate. The patients were excluded if they were unable to provide informed consent, had a history of bowel cancer or adenoma, inflammatory bowel disease, life expectancy of less than 5 years or had lower gastro-intestinal endoscopy within the last 3 years. In addition patients were asked whether if invited they would take up screening test for CRC screening. Those who answered yes were randomised to the intervention arm (flexible sigmoidoscopy) or control group. Patients in the intervention group received one flexible sigmoidoscopy in their local endoscopy unit. If adenomas were identified they were removed and if patients deemed high risk were subsequently referred for full colonoscopy. The primary outcomes of the study were incidence of CRC and mortality of CRC with a median follow up of 11.2 years. In an intention to treat analysis they showed a 31% (hazard ration 0.69, CI 0.45-0.72) reduction in mortality from CRC in the intervention group and a 23% (0.77, 070-0.84) reduction in incidence of CRC. When correcting for self selection bias, mortality was reduced by 43% (0·57, 0·45–0·72) and the incidence of CRC by 33% (0·67, 0·60–0·76) in those participating in screening. 191 persons needed to be screened to prevent one cancer. The main limitation of this trial was that due to the way patients were recruited (only those that answered yes to the question 'would you participate in a screening program?') meant that the uptake was significantly higher than that expected if this was done in a population based study. Interestingly the interim report of the population based NORCCAP trial[128], which also looked at once only flexible sigmoidoscopy in a similar age group to the UK trial, showed no difference in CRC incidence. It also showed a significant reduction in mortality from CRC (59%, hazard ratio 0.4. 95% CI 0.21-0.82). One explanation the authors gave was that the study follow up period (7 years) was shorter than the time that an adenoma will take to turn malignant especially in view of the results of the UK trial. Two further ongoing trials are due to report results in the next few years[129, 130]. With flexible sigmoidoscopy the procedure related complications (bleeding, perforation) are relatively low (0.03%)[127, 128]. However there are significant differences in adenoma detection rate between endoscopists of varying experience. This,

along with the inability to accurately measure the length of scope insertion makes quality assurance of a screening program challenging[131].

Based on the above evidence several countries are considering introducing flexible sigmoidoscopy into their bowel cancer screening programs including England as discussed below.

Colonoscopy

Unlike flexible sigmoidoscopy, colonoscopy requires formal bowel preparation, allows inspection of the entire colon and can be a diagnostic and a therapeutic modality (polypectomy, biopsy etc). However it requires sedation, takes longer to perform and hence is more resource intense than flexible sigmoidoscopy. The higher complication rate (0.1-0.3%)[132, 133] of colonoscopy namely perforation 0.001% (0.002% after polypectomy) and bleeding 0.1%[134] reflects its interventional potential. Colonoscopy remains the end point of all screening programs throughout the world. Surprisingly only a few countries have colonoscopy as the first line screening method with the American College of Gastroenterology recommending it as its preferred method of screening at a 10 year interval[126] beginning at the age of 50. It remains the gold standard for detection of significant colonic neoplasia but it still caries a significant adenoma miss rate of 20-26% for all adenomas with 2.6% for adenoma >1cm[135, 136] due to both patient factors and quality standards of the examination. On the other hand a negative colonoscopy can reassure both patient and healthcare professionals as the risk of developing CRC following a negative test is very low even 10 years later[137-139]. Several studies[138, 140] have shown a significant reduction (53-72%) in CRC incidence as well as a 31% mortality reduction from CRC. Interestingly Baxter et al[140] showed that the reduction in mortality was mainly from lesions detected in the distal colon (sigmoid and rectum) and not from proximal colonic sites, raising the point that these lesions could have been detected by a flexible sigmoidoscopy based screening program.

CT Colonography (CTC)

CTC is a radiological investigation which can image the entire colon like a colonoscopy (hence the term virtual colonoscopy has also been used to describe it). It involves CO_2 insufflation via a small rectal catheter to allow better visualization of the gut. Spiral X-ray images are obtained and the computer software reconstructs the images into a two or three dimensional image. It does not require full bowel preparation but the patient has to go to a modified diet for a few days prior to the test. It also offers imaging of extra colonic structures at the same sitting. There are no significant complication rates observed in large series of CTC in a screening cohort[141] with the main issue

being the exposure of a healthy patient to ionising radiation in the context of a screening program. This is addressed by using low radiation dose protocols of up to 12.5mGy with most scans delivering about 10mGy (1 Gray= Absorption of 1 Joule of ionising energy by 1 kilogram of biological tissue)[142]. For comparison a standard CT of the abdomen and pelvis exposes the patient to 10mGy, equivalent to three years of natural background radiation or 100 chest X-rays.

Several large studies examined the performance of CTC as a screening method for CRC. In 2003 1233 asymptomatic patients were recruited to a trial[143] where they received a CTC followed by colonoscopy on the same day. The performance of both tests was compared. The results were comparable between the two tests with CTC having high sensitivity for large adenomas (94% v 81% in colonoscopy) but lower for smaller polyps (94% v 92% for polyps up to 8mm, 88% v 92% for up to 6mm) and a specificity of about 96% overall for CTC. Kim *et al*[141] compared primary screening using CTC in one arm and colonoscopy in the other (about 3000 patients in each arm) in consecutive asymptomatic patients with no randomisation. The end point of the study was detection of advanced colorectal neoplasia. Patients within the CTC arm that had polyps >6mm were offered colonoscopy and polypectomy while patients with more than 2 smaller polyps were offered CTC surveillance. This study demonstrated comparable yields of diagnosis of advanced colorectal neoplasia between the two arms. A further study[144] in 2008 demonstrated a detection rate of 90% for polyps more than 10mm with a false positive rate of 14%. The main limitation of CTC lies in its inability to accurately detect smaller polyps as they can be difficult to distinguish from feacal residue[145]. However studies[143, 146] have shown that such polyps detected at colonoscopy have low risk malignant potential (<50%) with the chance of advanced histology being 1.7%.

Conclusions

There is good evidence for gFOBT based screening programs which could improve further compliance with the introduction of FIT[119], as only one sample will be required per patient. In addition flexible sigmoidoscopy once at the age of 55 has shown significantly higher mortality reduction compared to gFOBT and a combination of the two might be an appealing screening method. There are some good candidates for alternative screening methods namely CTC with comparable detection rates to colonoscopy for large polyps. Colonoscopy is the gold standard examination of the large bowel and is the end point of most screening programs around the world. However it has its limitations in detecting polyps and has a significant complication rate and impact on healthcare resources with lower patient uptake. Stool DNA tests show promising potential and

will be strong candidates as sampling, isolation and analysis technology improves over the next years as well as the discovery of new potential markers.

The ideal screening test will be patient friendly, with minimal complications, low cost with high sensitivity and specificity.

1.2.3. Screening for colorectal cancer in the United Kingdom

England

In the late 1990s the National Screening Comittee comissioned the first colorectal cancer screening pilot to assess the effectiveness of screening for CRC using FOBT. This was based on evidence from four RCTs that demonstrated significant reduction in mortality from CRC(discussed in section 1.2.2.). The pilot began in 2000 in the West Midlands in England and three sites in Scotland and 486,355 patients were offered screening. The pilot demonstrated that the key beneficial results observed in the RCTs were repeated in a population based programme[147]. In 2003 the Department of Health commissioned a second round pilot into bowel cancer screening. The aim of this round was to evaluate key outcomes and analyse the impact on hospital services. It was designed to build on the evaluation of the first round pilot. As the sensitivity of screening had already been evaluated in the first round, the emphasis of this pilot was shifted toward the impact of screening on resources and uptake by patients. 127,746 patients aged 50-69 were invited to participate in the second pilot. Only 52.1% of the invitees returned a screening kit (58.4% in first round), with higher uptake rates in those that had previously participated in the first pilot. As before the uptake was lower in areas with a high proportion of people form the Indian Sub-Continent. However colonoscopy uptake had improved compared to the first round (82.8% v 80.5%) with no differences in uptake due to ethnicity or sex. Overall the sensitivity of screening was comparable to the Nottingham RCT results. The effect of screening on endoscopy unit increased workload ranged from 14 to 28%, similar to the first round. Healthcare personnel had a positive attitude towards bowel screening despite the increased pressure on resources. The second pilot recommendations were in favour of bowel cancer screening overall with specific recommendations on maximising patient uptake, patient and healthcare education and ongoing evaluation of the screening programme[148].

In 2006 the National Health Service Bowel Cancer Screening Program (NHSBCSP) was rolled out across England. Local screening centers were established within the 5 bowel screening program hub areas covering England. The initial target age group was 60-69 years old with a biannual cycle of screening. In 2008 the screening age was extended across screening centers in England to include

women and men up to 75years old. This process is expected to be completed in all 58 local screening centers during 2013.

Patients initially received an invitation letter followed by the FOBT kit. The patients are asked to collect two samples from each of three separate bowel motions. They then post the kit back to their local screening centre for analysis. Patients receive their results within 14 days of sending in their kit. There are five possible results:

- Normal. No blood detected in any of the six samples. The patient will be sent another kit in two years time.
- Unclear. 1-4 positive samples. A letter is sent to patient with an explanation of the result along with a second kit to repeat the test. If the second FOBT is normal the patient is sent a third kit and if that is again normal the patient goes back into biannual testing. If the second or third FOBT is unclear or abnormal, the patient is given an appointment to see a screening nurse to discuss colonoscopy at the local screening centre.
- Abnormal. 5-6 positive samples. The patient receives a letter and an appointment to see the screening nurse in the local centre within a week of the receipt of the letter. Patients GP is notified.
- Technical failure. There has been a problem in the laboratory processing the samples. A repeat kit is sent to the patient.
- Spoilt Kit. The kit can not be processed as it was not used correctly. The patient is sent a instructions and a new kit.

Patients with an abnormal test are seen by a specialist screening nurse who assesses them for fitness for bowel preparation and colonoscopy. If the patients is deemed unfit for colonoscopy alternative colonic imaging might be offered. Colonoscopy for bowel screening is performed by designated screening endoscopists in each centre. These endoscopists have been trained and passed the colonoscopy driving test, to perform screening colonoscopy that meets specific procedural criteria to be deemed complete. About 5 in 10 screening colonoscopies are normal, with 4 in 10 demonstrating polyps and 1 in 10 cancer. Depending on the result of the colonoscopy the patient is either discharged back to the screening program (normal endoscopy) or enters endoscopic surveillance depending on the findings (number and size of polyps found). If CRC is detected the patient is referred to the local CRC MDT for further investigation and management.

In April 2011 NHSBCSP announced that it will introduce a flexible sigmoidoscopy screening pilot in addition to the current program. This is expected to start in 2013 in six bowel screening centers and will invite men and women around their 55th birthday. Later in 2011 NHSCBCSP announced

that the programme will be looking to replace the guaiac based FOBT to the more accurate and specific immunochemical FOBT over the next few years (discussed in detail in section 1.2.2.2).

Wales

Bowel screening in Wales was introduced in 2008 by Bowel Screening Wales[149] (BSW). Initially men and women between the age of 60-69 were invited to complete FOBT as in England. The used kits are sent by the patient to the central screening laboratory and results sent back to patients as described above for England. Patients with positive results are invited for a telephone assessment with a specialist screening practitioner (specially trained nurse) based at local assessment centers throughout Wales and are usually offered colonoscopy. One major difference between the Wales and England screening programs is that those patients with an unclear FOBT, they get an iFOBT instead of receiving a second FOBT as they do in England. The pathway for the patient in Wales following colonoscopy is exactly the same as described above for England program. Once again the quality of colonoscopy is very intensely monitored and audited. BSW have also extended their target age group to include those in the 70-74 years from 2010 and 50-59 years by 2012.

Scotland

As with the England and Wales programs, bowel cancer screening was introduced to Scotland following the two successful pilots (first pilot included three sites in Scotland) in 2007. Unlike England and Wales the program started with a different age group to include every patient registered with a GP between the age of 50-74 years. As with England and Wales the patients are sent a FOBT kit which they complete and send back. If the result is unclear an iFOBT is sent to the patient. If the result of the FOBT is positive then the patients are sent an information leaflet with details of their local NHS Board where they are pre-assessed for fitness to undergo colonoscopy. This pre-assessment can be done over the telephone or with an interview and depends on local NHS Board policy and patient preferences. Once patients have their colonoscopy their pathways are similar to those in England and Wales. The Scottish Bowel Screening Programme[150] is monitored and coordinated by the National Screening Co-ordinator.

Northern Ireland

As with the rest of the UK, NI introduced bowel cancer screening in 2010 initially to cover population between 60 and 69 years old and is scheduled to cover patients up to 74 years old over the next few years. The used kits are sent by the patient to the central screening laboratory and

results sent back to patients as described above for England. Patients with positive results are invited for an assessment with a specialist screening practitioner (specially trained nurse) and are usually offered colonoscopy. As with the Wales program patients with unclear first FOBT they get an iFOBT instead of receiving a second FOBT as they do in England. The pathway for the patient in Wales following colonoscopy is exactly the same as described above for England program[151].

1.3 Cell Exfoliation in human colon

1.3.1 Normal colonocyte mucosa

The large bowel wall consists of 4 layers (Figure 1.3): the mucosa, submucosa, muscularis propria and serosa. The serosa (formed by visceral peritoneum) and the muscularis propria (contains inner circular and longitudinal smooth muscles as well as myenteric Auerbach nerve plexi) mainly form the structural support for the bowel wall while the submucosa contains the Meisner nerve plexi as well as blood vessels. In this section we will concentrate on the mucosa which contains the differentiated epithelial cells.

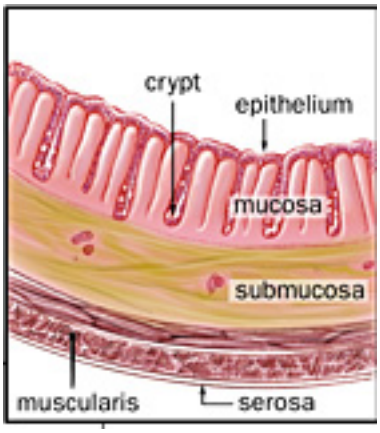

Figure 1.3: The layers of the human colon.

In human colon there are four types of differentiated epithelial cells. The colonocyte (columnar epithelial cell) is the principle cell of the mucosa, followed by the mucus secreting Goblet cells. Enteroendocrine cells and Paneth cells (mainly found in small intestine and ascending colon) are the other much less numerous cell types found in colonic mucosa. Unlike the small intestine the colon does not have villi (protrusions of the epithelium) but like the small bowel it has crypts (shallow pockets of epithelium)[152]. In normal physiological circumstances the gut mucosa undergoes complete self renewal every 2-7 days[153]. This impressive co-existence of both proliferating and differentiated cell in the gut mucosa is driven by gastrointestinal stem cells found at the base of the crypts. Although the exact position of these stem cells is subject to debate it is generally accepted that they are found at the bottom of the crypts in a so called stem cell compartment or niche[153]. The remaining lower two thirds of the crypt are occupied by proliferating colonocytes while the surface epithelium (luminal) and top one third of the crypt are occupied by differentiated cells. These stems cells do not migrate out of the crypt[152]. The differentiated

colonocytes spend a few days on the luminal surface to the mucosa where they are replaced by newer cells migrating from the bottom of the crypt. This constant flow of new colonocytes from the crypt to the mucosal surface requires an efficient mechanism of disposing the old colonocytes. This can be achieved in two ways and initially it was widely believed that colonocytes were simply removed from the luminal mucosa by exfoliation into the lumen[154-157]. The second way is via apoptosis either *in situ* or via the process of anoikis (apoptosis induced by loss of cell matrix interactions)[158]. In the normal colon there is dual anchorage of epithelial cells to both the basement membrane and to their neighbors via cell-cell adhesion mechanisms of their lateral membranes. At the same time the basement membrane has different distribution of various adhesion molecules at the base of the crypt compare to luminal surface giving it a more sticky profile the deeper into the crypt. Interestingly a similar pattern is seen in the expression of the Bcl-2 and caspase family of proteins in the colonocyte depending to its geographical position in the mucosa, with pro apoptotic proteins expressed at the surface[159]. It is now generally accepted that in the normal colonic epithelium apoptosis takes place *in situ* and cells are mainly removed by mucosal macrophages via phagocytosis and some colonocytes are exfoliated and eliminated by anoikis and disposed into the lumen[160-163]. This mechanism is almost reversed in the neoplastic colonic epithelium which will be discussed in detail below.

1.3.2 Colonic exfoliation in neoplasia

As described above in the normal colon differentiated colonocytes are believed to arise from stem cells found at the bottom of the crypts and migrate towards the surface where they end their short lives. This has led to the "bottom-up" theory of morphogenesis of colorectal neoplasia summarised below (Figure 1.4A).

Studies of mice and human mucosa that harbor the *APC* mutation demonstrated that adenomas expanded through the process of crypt fission, where the crypt is initially bifurcated at its base followed by longitudinal division of the entire crypt[164,165]. These events are under the control of intestinal stem cells and adenomatous crypts do indeed arise from normal crypts[165]. In 2002 Wong et al[166] examined biopsies taken at colonoscopy of normal colonic mucosa, hyperplastic polyps and adenomas (hereditary colorectal cancer patients were excluded) which were then micro-dissected to individual crypts. The morphology of the cells, their proliferating characteristics and fission indices of crypts were analysed. They showed that crypts in colorectal adenomas and hyperplastic polyps were significantly larger than normal mucosa and at the same time crypt fission was very common

in polyps but very rare in normal mucosa. There was also an upwards expansion of the proliferation compartment in adenomas i.e. mitoses were equally distributed along the crypt continuum. It is widely believed that stem cells at the base of the crypts which are normally slow to divide and tethered to a niche at the base of the crypt accumulate oncogenetic mutations that result in dysplastic crypts which will result in neoplastic lesions at the surface of the epithelium[153, 155, 166]. However histological examinations of early adenomas have shown the presence of dysplastic cells at the orifice and luminal surface rather the bottom of the crypts which contradicts the "bottom up" theory[167,168]. In 2001 a different theory of cacrinogenesis was put forward by Shih et al[169] describing a "top-down" morphogenesis in colorectal tumours (Figure 1.4B). The molecular characteristics of cells isolated from both the base and the orifices of the same crypts in adenomas were examined.

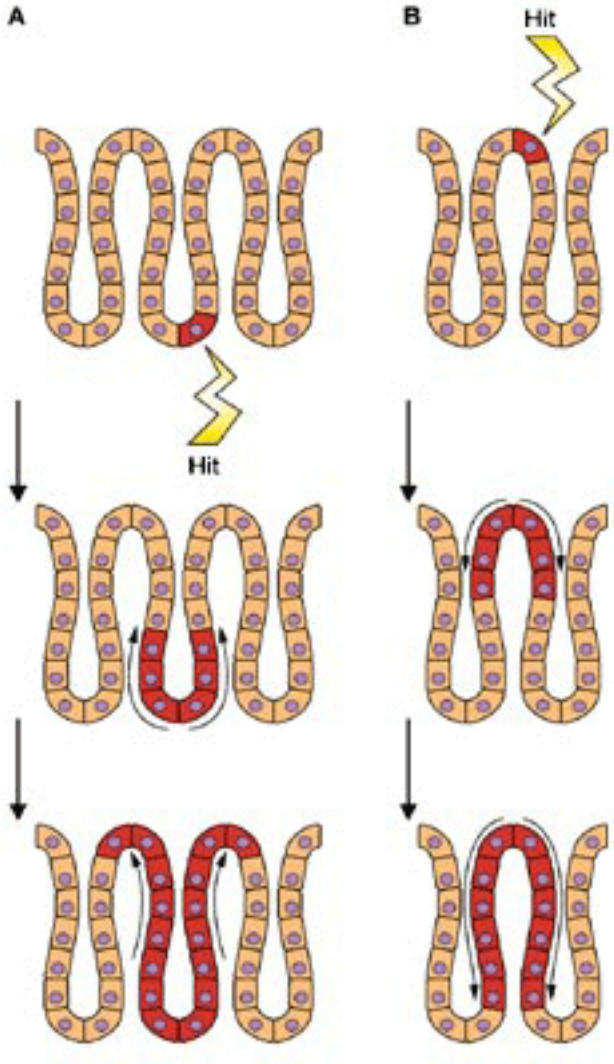

Figure 1.4: The bottom up (A) and top-down theories of morphogenesis in colorectal tumours. (www.medascape.com, accessed February 2013)

They showed that dysplastic cell at the tops of the crypts had genetic alterations of APC and neoplastic associated patterns of gene expression, but at the same time cells at the base of the same crypt did not have these molecular characteristics. They suggested two mechanisms through which

top-bottom morphogenesis takes place. Firstly that it could be that the stem cells do not reside at the bottom of the crypt but at its inter-cryptal zones and grow laterally pushing normal cells towards the bottom of the crypt. A second explanation was suggested which accepts that the stem cell does indeed reside at the bottom of the crypt then it moves to the top where it continues proliferating starting to replace and push normal colonocytes towards the bottom of the crypt. At the moment both theories are accepted with no one more dominant than the the other. It is widely agreed that the luminal epithelium is involved very early on in the neoplastic process.

Colonocyte exfoliation during early stages of neoplasia is not well understood unlike that in established cancer where both deregulation of apoptosis[170,171] and loss of cell to cell adhesion are associated with malignancy[172-174] and suggest that tumour colonocytes end up in the colonic lumen. Indeed several studies have reported increased colonocyte exfoliation in colorectal cancer patients[160, 175, 176] both in stool samples but also in the mucocellular layer. More specifically Ahlquist et al [160] examined tissue samples from 20 resected colorectal cancers specimens. They looked at the mucocellular layer (MCL) both directly over tumour sites as well as adjacent normal mucosa using immunocytochemistry measuring mean cell density. This was found to be significantly higher 2,639/mm^2 compared to adjacent mucosa 184/mm^2. Interestingly they observed that the cells found in the MCL above tumours were not apoptotic, and were mostly colonocytes along with chronic inflammatory cells while those cells over normal mucosa were mostly apoptotic. Numerous studies have reported secondary distal tumours[177-179] in colorectal cancer patients, via re-implantation of viable tumour cell at sites at distal distances to the tumour. Two recent studies[180,181] used a ballon device to collect rectal mucus from patients referred to outpatients for colonic symptoms (change in bowel habit, rectal bleeding) and measured the DNA concentration in their samples. They both found significant DNA counts in patients diagnosed with malignancy compared with patients with normal colonic investigations (colonoscopy). Although DNA extracted from stool would be a useful target for identification of biomarkers, extracting DNA of sufficient amount and quality from stool remains challenging.

The above observations, namely higher exfoliation of colonocyte in tumour, detection of viable colonocytes in the MCL over tumours, distal metastasis of tumours within the colon and DNA in the stool and rectal mucus, suggest accumulation of DNA from the tumours towards the rectum along the MCL which forms the basis of our hypothesis.

2. Hypothesis

The hypothesis is that in patients with colorectal cancer, rectal mucus may contain DNA derived from colonic tumours. These tumours may exfoliate cells which pass distally to the rectum. Sampling of mucus in the rectum may permit assessment of DNA derived from proximal tumour cells. It is speculated that quantitative or qualitative assessment of DNA in rectal mucus may permit an improved method of CRC screening.

3. Aims

1. Detection of DNA in mucus from colon

- To establish whether it is possible to perform quantitative measurements of DNA in mucus samples taken from human surgical colonic specimens in a repeatable and reproducible method
- To establish an appropriate buffer solution to store the mucus collection device following sampling of human colonic mucus and should it be stored at room temperature or refrigerated

2. To establish which collection material will yield the highest DNA content from colonic mucus.

3. Is there a gradient of DNA concentration in colonic mucus from the tumour site proximally or distally?

- To establish the amount of DNA at tumour sites and set distances proximally and distally from the tumour site and to identify if there is a pattern or not.

4. Can we use DNA isolated from rectal mucus to detect colorectal cancer?

- To identify if there is a significant difference in the amount of DNA extracted from sampled rectal mucus between patients with neoplasia and those without.
- To identify if a patient has CRC by using a three marker methylation panel assay on DNA isolated from rectal mucus in symptomatic patients referred to a colorectal clinic.

4. Detection of DNA in mucus from colon surgical specimens

In order to test our hypothesis we first needed to prove that it was possible to collect, safely store and accurately measure the DNA content of colonic mucus. This chapter describes the experiments that address these issues.

4.1 Objectives

i. To establish whether it was possible to perform quantitative measurements of DNA in a mucus sample taken from human surgical colonic specimens in a repeatable and reproducible method.

ii.To establish an appropriate buffer solution in which to store the mucus collection device following sampling of human colonic mucus.

iii. To determine the optimum temperature at which the samples should be stored.

4.2 Methodology

4.2.1 Is it possible to perform quantitative measurements of DNA in mucus samples taken from human surgical colonic specimens in a repeatable and reproducible method ?

Preparation of collecting device

Glove

A piece of Encore® under-glove (Ansell Healthcare Ltd) was fashioned by cutting the glove into a 1x4cm rectangle. The pieces were then placed in a sterile pot for storage. This type of glove is made of natural rubber latex.

Collection of surgical specimens and sampling of colonic mucus

Patients who were undergoing bowel resection as part of their bowel cancer treatment plan were consented as described in the research ethics committee approval form (ref: 11/NE/088) (appendix I). The patients received no bowel preparation prior to surgery. The operation proceeded as normal and once the bowel specimen had been removed and operation completed the specimen was sent to pathology as per normal clinical practice. On arrival of the resected bowel at the pathology cut-up room, the candidate was contacted and asked to attend.

The pathologists opened the colon in a fashion that did not undermine the clinical pathological

staging of the colonic specimen. The colon was opened starting from the distal resection margin. The bowel wall was cut longitudinally following the taenia coli reaching as close to the tumour site as possible. This procedure was followed from the proximal resection margin towards the tumour. The specimen was opened without washing the lumen of any stool as such a manoeuvre would flush out mucus and affect the results of the measurements. The colonic specimen was placed on a clean bench.

An area of uncontaminated (no stool) mucosa was identified and its distance from the tumour site recorded (20cm proximal to tumour). A piece of glove with a surface area of 4cm^2 was applied to the sampling area for a total of ten seconds. To ensure that the same force/pressure would be applied during sampling, a fixed weight (20g) was applied on top of the piece of glove (Figure 4.1). Care was taken to avoid any scraping movements that would result in sampling mucosa instead of mucus. Five measurements were taken at this site, circumferentially, using the same collection device (glove) as described below and the same was repeated at similar distance for the tumour in a different colonic surgical specimen.

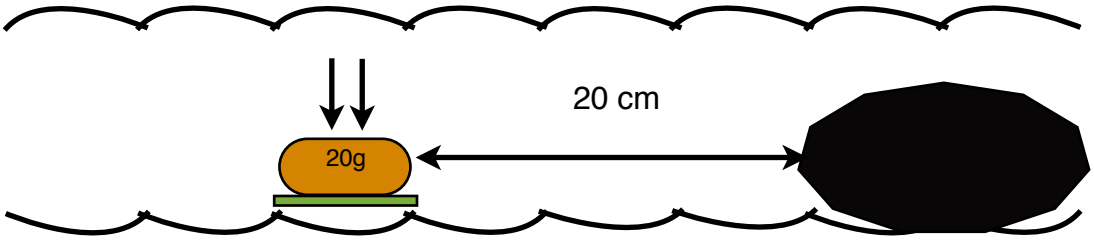

Figure 4.1: Diagram to demonstrate sampling of colonic mucus using a piece of glove (green) using a fixed weight (brown) of 20g at a set distance from tumour (black).

Following mucus sampling, the piece of glove was placed in a 15ml Falcon tube
(BD Biosciences, USA) containing 3ml of Cell Lysis Solution (Cat No 158908, Qiagen GmbH, Germany). Care was taken to ensure that the entire surface of the glove/foam was covered with cell lysis buffer. The samples were then stored at room temperature and transferred to the laboratory.

Extraction of DNA from colonic mucus samples

Principles of method

- Colonocytes in the mucus sample were lysed to release their DNA content. The lysis solution broke down the cell membrane and the nuclear membrane, releasing the cytoplasmic and nuclear contents into solution.
- RNAse was added to lyse all RNA that had been released from the cell lysis, leaving only DNA

and proteins in solution.

- Next the proteins were removed from the solution by adding protein precipitation solution containing ammonium acetate that precipitated the protein into a tight pellet.
- The DNA was in solution and by using isopropanol and glycogen it was precipitated by centrifugation, and after washing the pellet with ethanol it was rehydrated using DNA hydration solution.

Protocol (Modified Gentra Puregene Buccal Cell Kit protocol, Qiagen GmbH, Germany)

- Cell lysis was achieved by incubating the 15ml Falcon tubes with the collecting device immersed in the 3ml of cell lysis buffer, at 66 ^{o}C for one hour.
- Samples were kept at room temperature for 30 minutes to cool down and each sample was vortexed (Vortex Genie-2, Scientific Industries Inc, USA) at high speed for 20 seconds to homogenise the solution.
- 300µL of the solution was pippetted into a 1.5ml sterile micro-centrifuge tube (Eppendorf Lobind®, Germany).
- To ensure RNA free DNA 1.5µL of RNase A solution (Cat No 158924, Qiagen GmbH, Germany) was added and the micro-centrifuge tubes incubated for 15min at 37 ^{o}C. Samples were then cooled down by placing on ice for one minute.
- 100µL of protein precipitation solution (Cat No 158912, Qiagen GmbH, Germany) was added to separate proteins from DNA and vortexed at high speed for 20 seconds. Samples were then incubated in ice for 5 minutes and then centrifuged for 3 minutes at 16,000g (Eppendorf Centrifuge 5415C, Germany).
- The resultant supernatant contained DNA in solution and a tight precipitated protein pellet.
- The supernatant was then added to a clean 1.5ml micro-centrifuge tube containing 300µL of isopropanol and 0.5µL of Glycogen (Cat No 158930, Qiagen GmbH, Germany).
- The samples were mixed gently by inverting 50 times and they were then centrifuged for 5minutes at 16,000g to form a DNA pellet.
- The supernatant was carefully discarded, taking care the DNA pellet was not dislodged
- The DNA pellet was verified visually.
- 300µL of 70% ethanol was added to wash the DNA pellet and samples were centrifuged for 1minute at 16,000g.
- The supernatant was drained again taking care the DNA pellet was not dislodged and samples were allowed to air dry for 15 minutes.

- 20µL of DNA Hydration solution (Cat No 158914, Qiagen GmbH, Germany) was added and the samples vortexed for 5 seconds at medium speed and incubated at 65 ^{o}C for 1hour to hydrate the DNA.
- The samples were then stored overnight with gentle shaking using a 3D Rocking Platform STR9 (Stuart Scientific, UK).
- Samples were centrifuged briefly (Capsulefuge PMC-100 Tomy Teoh, USA) for 10seconds the next morning before measuring their DNA content using a spectrophotometer.
- The ND1000 Spectrophotometer (Nanodrop® Tech Inc, USA) was used to measure the DNA content in each sample. The Nanodrop® was cleaned before and after every sample was loaded with sterile water. The machine was calibrated by loading 1µL of ddH_2O followed by 1µL of DNA hydration solution as a blank measurement. 1µL of each sample was loaded on the Nanodrop® and its DNA concentration (ng/µL) and A260/280 ratio was recorded. Values <10ng/ µL and/or those with A260/280 ration outside 1.60-2.1 or with a spectrum graph with not a single peak were considered not to contain any DNA .

4.2.2 To establish an appropriate buffer solution to store mucus collection device following sampling of human colonic mucus, and whether it should be stored at room temperature or refrigerated.

Four buffers (described below) were studied for their properties as solvents in which to store DNA derived from rectal mucus. Storage temperature, easy availability and high DNA yield were considerations in the choice of buffer.

Buffers

Cell Lysis Buffer (Cat No 158908, Qiagen GmbH, Germany)

This buffer has been used in the preservation of mucus samples from the rectum in published studies[180, 181].

ASL Stool Lysis Buffer (Cat No 19082*, Qiagen GmbH, Germany)*

This buffer (also known as ASL Stool Wash Buffer) was chosen as it has been used in studies looking for DNA methylation markers in human stool samples[183].

RNALater Buffer (*Cat No 76106, Qiagen GmbH, Germany*)

A commonly used buffer, but as it is designed to preserve mainly RNA in preference to DNA therefore would be expected to act as a negative (or near negative) control.

Phosphate Buffered Solution (PBS)

PBS was chosen because is a commonly used biological buffer/solvent but it does not feature as a nucleic acid preservative or solvent and therefore was chosen as a negative control.

Collection of surgical specimens and sampling of colonic mucus

The surgical specimen was collected and cut up by the pathologist as described in section 4.2.1. An uncontaminated (stool free) area of the bowel with no tumour or polyps was identified. Using a

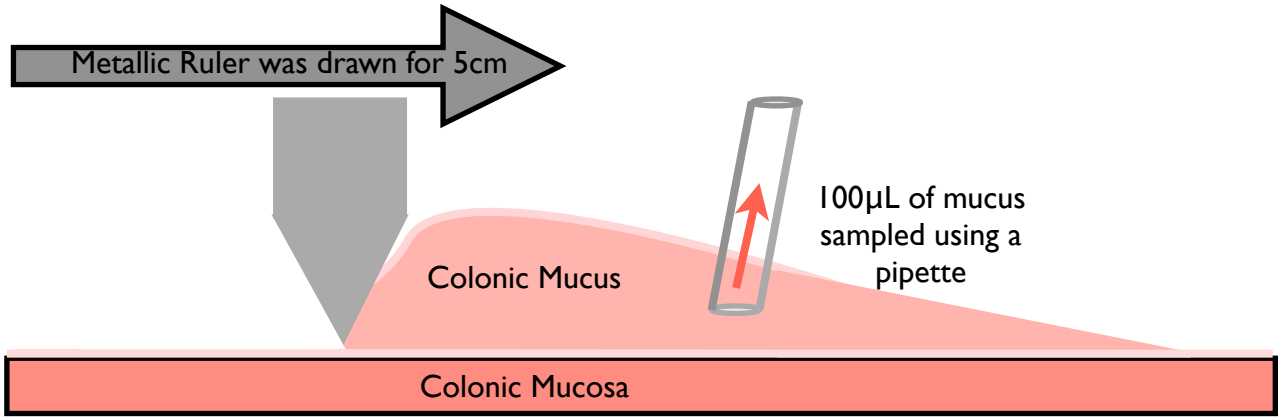

Figure 4.2: Diagram to demonstrate how a fixed volume of mucus was sampled from a surgical colonic specimen.

metallic ruler the mucus was scrapped gently over a distance of 5cm (Figure 4.2). Using a pipette 100µL of mucus was placed into four 15ml Falcon tubes (BD Biosciences, USA) each containing 3ml of one of the above four buffers. The Falcon tubes were then vortexed gently for 10 seconds to homogenise the solutions. 1.5ml from each solution was then transferred on a clean 15ml Falcon tube. The tubes were either kept in room temperature or stored in 4 ^{o}C. The DNA concentration of the 8 solutions was measured using the following protocol at 48hrs, 1 week, 2 weeks and 4 weeks after sampling.

Measurement of DNA concentration

Principle of methods

- The four main principles of the protocols are those described in 4.2.1 but here we have assumed our solutions to be tissue fluid.
- 5 measurements from each buffer solution were taken at every time point.

Protocol (Modified Gentra Puregene blood Kit body fluid protocol, Qiagen Gmbh, Germany)

- 50µL from each solution were added to a 1.5ml sterile micro-centrifuge tube (Eppendorf Lobind®, Germany) containing 250µL Cell Lysis Solution (Cat No 158908, Qiagen GmbH, Germany) and incubated for 1 hour in 66 ^{o}C.
- To ensure RNA free DNA, 1.5µL of RNase A solution (Cat No 158924, Qiagen GmbH, Germany) was added and the micro-centrifuge tubes incubated for 15 minutes at 37 ^{o}C.
- Samples were cooled by placing on ice for one minute.
- 100µL of protein precipitation solution (Cat No 158912, Qiagen GmbH, Germany) was added to separate proteins from DNA and vortexed at high speed for 20 seconds and incubated in ice for 5 minutes.
- Samples were centrifuged for 3 minutes at 16,000g (Eppendorf Centrifuge 5415C, Germany).
- The supernatant containing DNA was added in a clean 1.5ml micro-centrifuge tube containing 300µL of isopropanol and 0.5µL of Glycogen (Cat No 158930, Qiagen GmbH, Germany) and mixed gently by inverting 50 times.
- Samples were centrifuged for 5 minutes at 16,000g to form a DNA pellet.
- The supernatant was carefully discarded, taking care the DNA pellet was not dislodged. The DNA pellet was verified visually.
- 300µL of 70% ethanol was added to wash the DNA pellet and samples were centrifuged for 1 minute at 16,000g.
- The supernatant was drained again taking care the DNA pellet was not dislodged and samples were allowed to air dry for 15 minutes.
- 20µL of DNA Hydration solution (Cat No 158914, Qiagen GmbH, Germany) was added and the samples, vortexed for 5 seconds at medium speed and incubated at 65 ^{o}C for 1 hour to hydrate the DNA.
- The samples were stored overnight with gentle shaking using a 3D Rocking Platform STR9 (Stuart Scientific, UK).
- The following morning, samples were centrifuged briefly (Capsulefuge PMC-100 Tomy Teoh, USA) for 10 seconds before measuring their DNA content using a spectrophotometer.
- The ND1000 Spectrophotometer (Nanodrop® Tech Inc, USA) was used to measure the DNA content in each sample as described in section 4.2.1.

4.3 Results

4.3.1 Is it possible to perform quantitative measurements of DNA in a mucus sample taken from human surgical colonic specimens in a repeatable and reproducible method ?

Five different measurements using a 4cm^2 piece of glove as described above were taken from the same distance proximal to tumour (20cm) and DNA was isolated and measured as described in section 4.2.1. Table 4.1 summarises the results of those measurements. These results are expressed in ng/μL, referring to DNA isolated per colonic mucus sample in 20μL of DNA Hydration solution which was added to the isolated DNA pellet as described in sections 4.2.1 and 4.2.2.
The average DNA concentration was 37.7 ng/μL (95% CI 36.81-37.77, Standard deviation 0.77). The experiment was repeated at a similar distance (20cm) in another colon (Table 4.1) with a mean concentration of 25.3 ng/μL (95% CI 24.3-26.3, Standard deviation 0.81).

	Individual measurements (ng/μL)					Mean	Median	Std. Deviation	Std. Error	Confidence Interval	
Colon 1	*38.30*	*36.70*	*37.90*	*37.30*	*38.60*	*37.76*	*37.90*	*0.767*	*0.34*	*36.81*	*38.71*
Colon 2	*25.40*	*24.30*	*26.40*	*24.80*	*25.70*	*25.32*	*25.40*	*0.811*	*0.36*	*24.31*	*26.32*

Table 4.1: Individual measurements of DNA content of colonic mucus at the same distance within two different surgical colonic specimen.

4.3.2 To establish an appropriate buffer solution in which to store mucus collection device and the optimum storage temperature.

Only the samples stored in cell lysis solution had significant amounts of DNA, as shown in table 4.2 below. DNA concentrations <10ng/μL and/or those with A260/280 ratio outside 1.60-2.1 or with a spectrum graph without a single peak were considered not to contain any DNA. This is due to potential absorption at these wavelengths by fragments of nucleic acids and proteins. DNA yield of cell lysis solution increases from 34.8 ng/μL to 40.25 ng/μL over 4 weeks when stored at room temperature (figure 4.3a) and from 34.9 ng/μL to 40.3 ng/μL at 4^{o}C (figure 4.3b). The datasets for this experiment were tested using SPSS V19 (IBM Inc, USA) to assess if they were normally distributed. Shapiro-Wilk test was used as our sample size is small and p >0.05 in all groups and hence we confirmed that our data are normally distributed.

	Room Temperature				Mean	4 degrees				Mean
	<48 hrs	1 wk	2 wks	4 wks		<48 hrs	1 wk	2 wks	4 wks	
Cell Lysis Buffer	*34.80*	*38.78*	*40.22*	*40.24*	*34.9*	*34.90*	*37.90*	*39.16*	*40.30*	*38.70*
Stool Lysis Buffer	*4.50*	*6.84*	*7.56*	*8.04*	*6.70*	*5.66*	*8.22*	*8.14*	*8.94*	*7.70*
RNALater	*4.86*	*7.88*	*7.84*	*8.18*	*7.20*	*7.00*	*7.86*	*8.12*	*8.50*	*7.90*
PBS	*8.44*	*7.56*	*7.98*	*7.82*	*7.90*	*8.28*	*8.60*	*9.20*	*8.28*	*8.60*

Table 4.2: Mean DNA yield in ng/µL.

To ensure appropriate statistical tests were used in the analysis of these data, the homogeneity of variance was also checked and found to be >0.05. Hence both assumptions (normal distribution and homogenous variance) were met, the use of parametric statistical tests for analysis was appropriate.

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max	Test of Homog. of Variance
						Lower	Upper			
<48 Hours	**4C**	*5*	*34.9*	*3.88523*	*1.73753*	*30.0759*	*39.7241*	*30.10*	*39.30*	
	RT	*5*	*34.8*	*3.46338*	*1.54887*	*30.4996*	*39.1004*	*30.60*	*38.60*	*0.624*
	Total	*10*	*34.85*	*3.47027*	*1.09740*	*32.3675*	*37.3325*	*30.10*	*39.30*	
week1	**4C**	*5*	*37.9*	*2.50300*	*1.11937*	*34.7921*	*41.0079*	*34.30*	*40.40*	
	RT	*5*	*38.78*	*2.38160*	*1.06508*	*35.8229*	*41.7371*	*35.30*	*41.70*	*0.762*
	Total	*10*	*38.34*	*2.34956*	*0.74300*	*36.6592*	*40.0208*	*34.30*	*41.70*	
week2	**4C**	*5*	*39.16*	*3.21139*	*1.43618*	*35.1725*	*43.1475*	*35.70*	*43.60*	
	RT	*5*	*40.22*	*3.04828*	*1.36323*	*36.4351*	*44.0049*	*36.40*	*43.80*	*0.897*
	Total	*10*	*39.69*	*3.00424*	*0.95002*	*37.5409*	*41.8391*	*35.70*	*43.80*	
week4	**4C**	*5*	*40.3*	*2.52190*	*1.12783*	*37.1686*	*43.4314*	*37.30*	*44.30*	
	RT	*5*	*40.28*	*3.09225*	*1.38290*	*36.4405*	*44.1195*	*37.40*	*44.50*	*0.393*
	Total	*10*	*40.29*	*2.66018*	*0.84122*	*38.3870*	*42.1930*	*37.30*	*44.50*	

Table 4.3: Descriptive statistics.

A repeated measurement ANOVA was performed. The data was checked to assume that the sphericity-assumption was not violated (Mauchly's Test of sphericity) and the approximate Chi-Square value (8.394 p=0.158 for room temperature, 2.980, p=0.719 for refrigerated group) confirmed that the sphericity assumption was not violated.

In the room temperature group there was no significant difference in DNA concentration over time ($F(3,12)=2.36$ $p=0.123$). Post Hoc Bonferroni correction in the refrigerated group showed no significant difference between the mean DNA concentration at the different time points despite the

repeated measures ANOVA (F(3,12)=4.589, p=0.023) result.

Figure 4.4 summarises the mean DNA concentration over time when the cell lysis solution is stored at room temperature (blue line) or at 4^0C (green line). An independent T-Test (SPSS v19, IBM Inc USA) confirmed that there was no significant difference (t(38)=0.396 p=0.694) between the mean DNA concentration in solutions stored at room temperature and 4^0C.

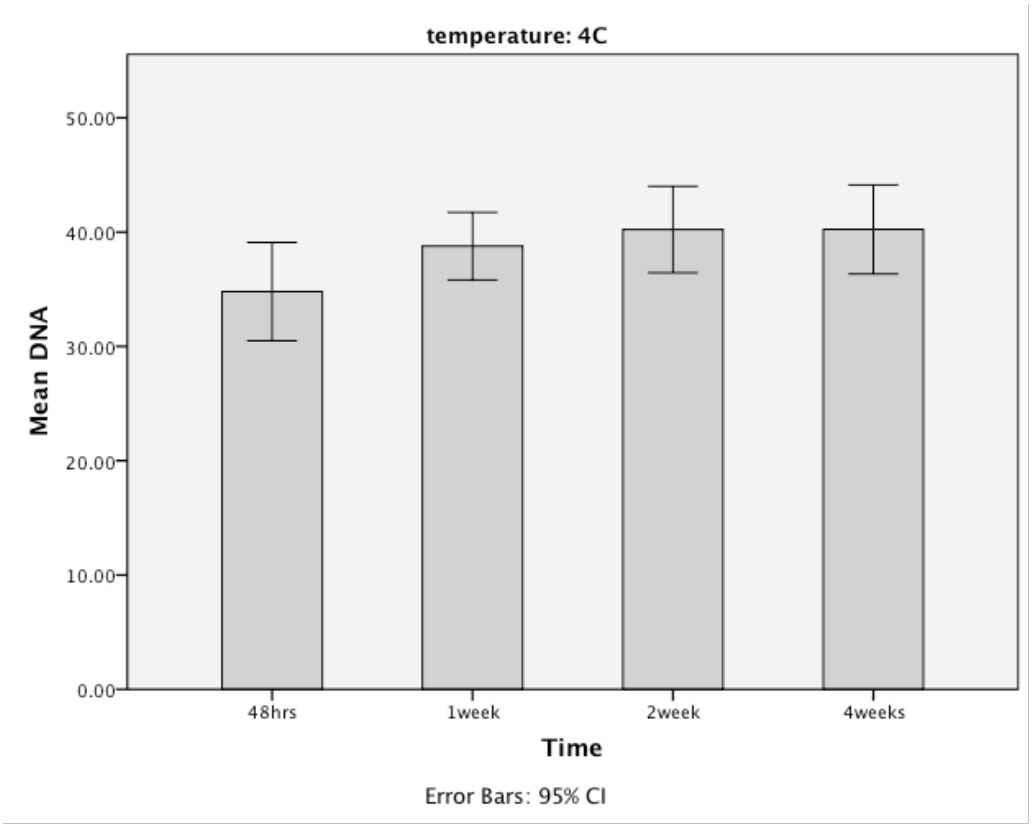

Figure 4.3a: Cell Lysis Buffer stored at 4^0C. Means are expressed in ng/μL within one standard deviation.

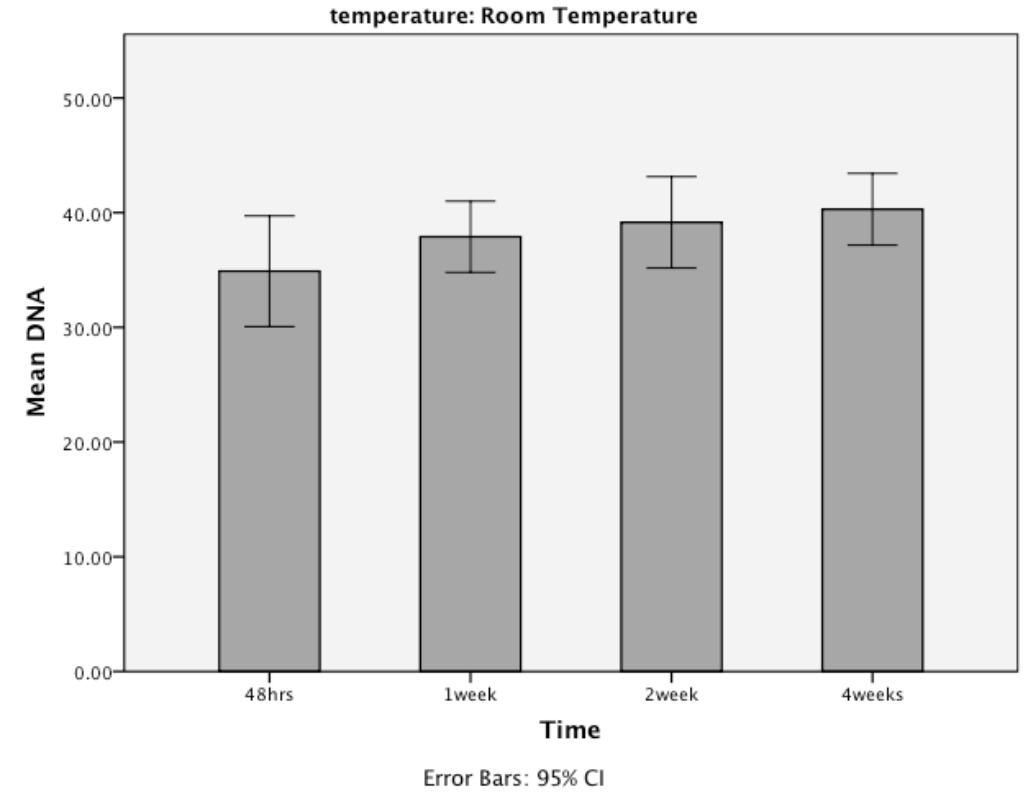

Figure 4.3b: Cell Lysis Buffer stored at Room Temperature. Means are expressed in ng/µL.

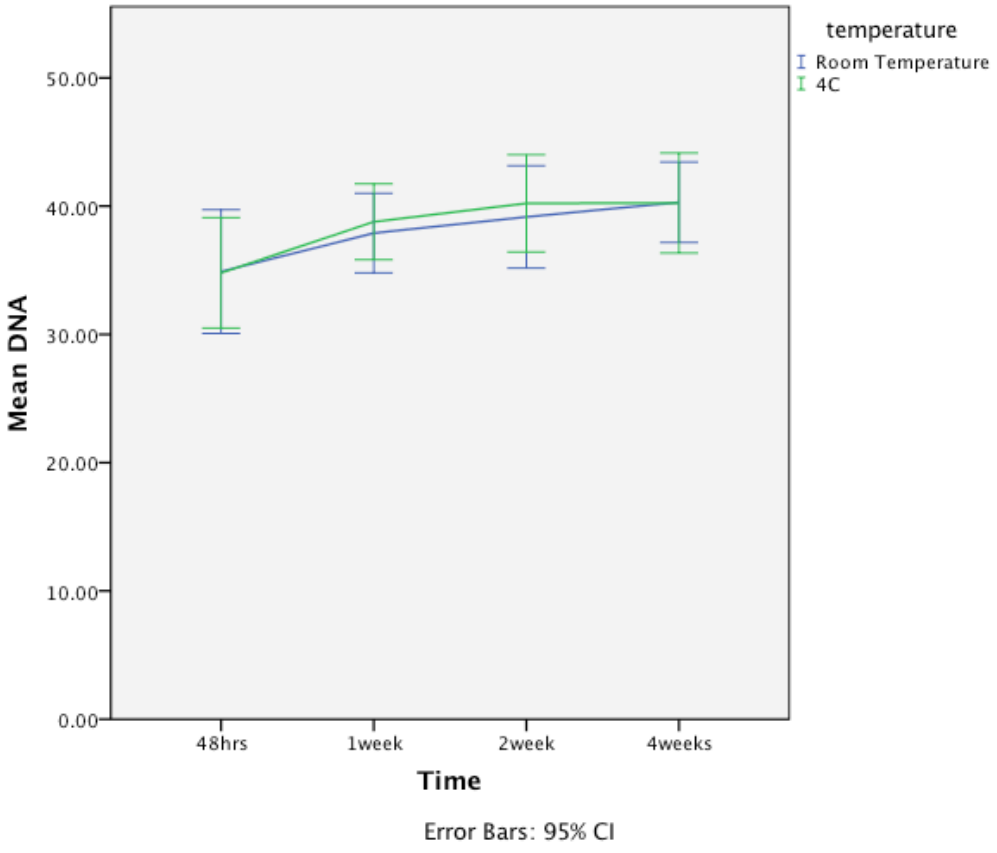

Figure 4.4: Cell Lysis Buffer mean DNA concentration in ng/µL over time stored in room temperature (blue line) and in 4^0C (green line).

4.4 Discussion

The results of the first experiment (sections 4.2.1 and 4.3.1) show that it is possible to collect and accurately measure the DNA content of colonic mucus using a $4cm^2$ piece of latex glove. In addition we took repeated measurements at a specific distance (20cm) within the colonic specimens and got consistent DNA counts indicating that DNA amount in the colonic mucosa is uniformly distributed around the circumference of the colon. When we repeated the experiment in a second colon we were able to reproduce our results. These observations are of great relevance to the experiments described later in this thesis especially in chapter 6 where we measured the DNA content of colonic mucus at set distances proximally and distally to colorectal tumour sites.

In the second experiment (sections 4.2.2. and 4.3.2) we have demonstrated that the best buffer solution to store the device used to collect mucus from the colon was cell lysis buffer. We chose to use a piece of glove as our device in this experiment because a latex based membrane device had been previously used to collect rectal mucus in previous studies[180,181]. The results of this experiment showed a clear advantage in storing the device in cell lysis buffer compared to the other three buffers. The need to immerse the collecting device into a buffer is two fold:

- This maximises the yield of DNA per device, as it allows an increased surface area of mucus and therefore colonocytes to come into contact with the buffer solution increasing the number of cells lysed compared to simply scrapping the mucus drops off the device.
- In addition as described in section 4.2 cell lysis buffer does not interact with the materials of the device. It is unclear whether glove is the optimum material to collect colonic mucus but this question is addressed in Chapter 5.

The DNA yield from collection device placed in cell lysis buffer was stable over a period of up to 1 month. In addition the storage temperature does not affect DNA yield. These two observation have significant implications for experiments described later in this thesis especially Chapter 7 where we sampled rectal mucus from symptomatic patients and had to store the samples for up to three weeks before analysis.

5. What is the best material to collect colonic mucus to assess DNA?

In the previous chapter we demonstrated that it is possible to take repeatable and accurate measurements of the DNA content of human colonic mucus. Here we will establish which collection material is best suited to this.

5.1 Objective

- To establish which collection material will yield the highest DNA content from colonic mucus.

5.2 Methodology

Three collecting devices made out of different materials will be compared:

- Cytology brush from the Gentra Puregene Buccal Cell Kit (Cat No 158867, Qiagen GmbH, Germany) was chosen as a positive control method of collecting DNA from colonic mucus as it is designed to collect saliva and buccal cells.
- A piece of glove was chosen to try and simulate in our model a latex membrane device that had been successfully used to collect rectal mucus in a published studies[180, 181].
- A polyethylene foam that has been CE marked in the EU and has been declared safe by the FDA in the USA for accidental ingestion was chosen as a possible alternative material to collect mucus from the rectum.

Preparation of collecting devices

A piece of Encore® under-glove (Ansell Healthcare Ltd) was fashioned by cutting the glove into a 1x4cm rectangle using aseptic technique to ensure no DNA decontamination. The pieces were then placed in a sterile pot for storage.

The foam sheet (40x20x5cm) provided by the manufacturer (Cat No FT-40P, Foam Techniques Ltd, UK) was cleaned with alcohol and allowed to air dry overnight. The sheet was trimmed by 0.5cm from each surface to ensure no DNA contamination had occurred from handling the foam. The inside (core) foam was then cut up into 1x4x0.5cm pieces and placed in a sterile pot. Five 1x4x0.5cm pieces of foam were randomly chosen and their surface DNA content was checked using the protocol described in section 4.2.1. No DNA was detected.

Collection of surgical specimens and sampling of colonic mucus

Twenty five surgical colonic specimens were collected and cut open as described in section 4.2.1. No oral bowel preparation was used prior to resections. For left sided colonic resections an enema was given in the morning of the operation.

Each colon was opened, an area of uncontaminated (no stool) mucosa was identified and its distance from the tumour site was recorded. A piece of glove or foam was applied to the sampling area for a total of ten seconds. To ensure that the same force/pressure would be applied during sampling, a fixed weight (20g) was applied on top of the collecting device (Figure 5.1). Care was taken to avoid any scraping movements that would result in sampling mucosa instead of mucus. Brush sampling was done by twisting the brush 720^{o} and taking care not to drag the brush along the colon, to minimise sampling mucosa instead of mucus.

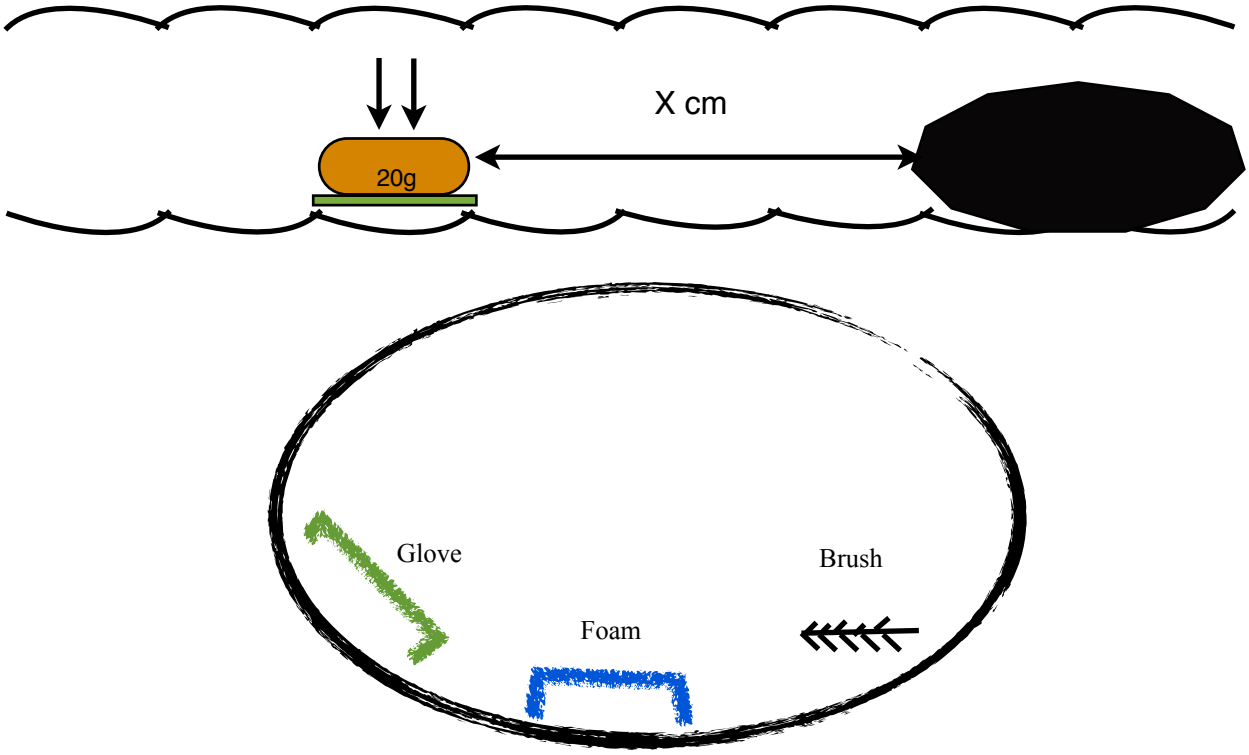

Figure 5.1: Diagram to demonstrate sampling of colonic mucus using a piece of glove (green) using a fixed weight (brown) of 20g at a set distance from tumour (black). Cross sectional diagram of colon at distance Xcm from tumour showing circumferential sampling of colonic mucus using the three different devices (glove, foam and brush)

Extraction of DNA from colonic mucus samples

Principles of method

- As described in section 4.2.1

DNA extraction was performed following the protocol described in section 4.2.1, thus providing DNA for early quantification and avoiding the need for storage.

5.3 Results

As shown in table 5.1, 118 colonic mucus samples using foam, 117 using glove and 114 using cytology brush were made using 25 colonic resection specimens. The mucus DNA content was measured as described in section 5.2. These results are expressed in ng/μL, referring to DNA isolated per colonic mucus sample in 20μL of DNA hydration solution which was added to the isolated DNA pellet as described in sections 4.2.1 and 4.2.2. The mean DNA concentration (figure 5.2) using:

- cytology brush was 236.2 ng/μL (95% CI 187.1-285.4)
- foam was 179.7 ng/μL (95% CI 113.3-246.1)
- glove was 87.7 ng/μL (95% CI 68.1-107.4).

The data were analysed using SPSS v19 (IBM Inc, USA) to test for normality (Kolmogorov-Smirnov test). DNA concentration was non-normally distributed D(120)=0.323 p=0.0001 in foam, in glove D(118)=0.238 p=0.0001 and in brush groups D(114)=0.219 p=0.0001.

We have therefore used non parametric tests to analyze and report these data. A Kruskal-Wallis test was used to compare the median DNA yield between the three groups. Median DNA yield was significantly different between the three different modalities used to sample colonic mucus H(2) =54.1 p=0.0001. A Mann-Whitney test was used as post hoc analysis to follow the Kruskal-Wallis test result above. A Bonferroni correction was used and therefore results are reported to a 0.0167 level of significance.

The median DNA concentration in the glove group (Mdn=48.5 ng/μL, IQR=75.4) was significantly lower than that in the foam group (Mdn=90.25 ng/μL, IQR=148.8) Mann-Whitney U= 4830, z=-4.237 p=0.0001 and in the brush group (Mdn=142.7 ng/μL, IQR=195.2) Mann-Whitney U=3060, z=-7.173 p=0.0001. In addition the median DNA concentration in the brush group (Mdn=142.7 ng/μL, IQR=195.2) was significantly higher than in the foam group (Mdn=90.25 ng/μL, IQR=148.8) Mann-Whitney U= 5047.5, z=-3.463 p=0.001. Results are summarised in figure 5.2a and b.

GLOVE	N=117	Mean	SD	95% CI		Median	IQR
10-15cm Distal to Tumour	*21*	*55.00*	*33.37*	*39.81*	*70.19*	*49.50*	*50.75*
5-10cm Distal to Tumour	*19*	*65.73*	*34.39*	*49.16*	*82.31*	*48.60*	*61.30*
Tumour site	*25*	*231.67*	*152.52*	*170.07*	*293.28*	*163.15*	*180.97*
5-10cm Proximal to Tumour	*10*	*52.17*	*28.07*	*32.09*	*72.25*	*35.50*	*50.38*
10-20cm Proximal to Tumour	*18*	*44.43*	*28.38*	*30.31*	*58.54*	*38.10*	*31.00*
20-40cm Proximal to Tumour	*24*	*25.12*	*20.68*	*20.68*	*29.56*	*24.90*	*10.05*
FOAM	**N=118**	**Mean**	**SD**	**95% CI**		**Median**	**IQR**
10-15cm Distal to Tumour	*22*	*100.76*	*61.52*	*73.48*	*128.04*	*95.75*	*67.57*
5-10cm Distal to Tumour	*19*	*133.16*	*91.77*	*88.92*	*177.39*	*86.40*	*146.80*
Tumour site	*25*	*500.04*	*681.30*	*230.53*	*769.55*	*262.30*	*289.70*
5-10cm Proximal to Tumour	*10*	*102.64*	*64.32*	*56.63*	*148.65*	*114.00*	*112.00*
10-20cm Proximal to Tumour	*18*	*72.52*	*39.71*	*52.76*	*92.26*	*76.35*	*64.22*
20-40cm Proximal to Tumour	*24*	*40.95*	*19.50*	*32.72*	*49.19*	*39.50*	*22.98*
BRUSH	**N=114**	**Mean**	**SD**	**95% CI**		**Median**	**IQR**
10-15cm Distal to Tumour	*20*	*136.83*	*83.99*	*97.52*	*176.13*	*135.50*	*67.05*
5-10cm Distal to Tumour	*18*	*187.68*	*118.17*	*128.92*	*246.45*	*200.50*	*178.95*
Tumour site	*25*	*575.34*	*347.46*	*437.89*	*712.79*	*523.00*	*614.30*
5-10cm Proximal to Tumour	*10*	*194.00*	*87.80*	*131.19*	*256.81*	*194.35*	*140.63*
10-20cm Proximal to Tumour	*17*	*110.58*	*61.56*	*78.93*	*142.23*	*92.10*	*65.05*
20-40cm Proximal to Tumour	*22*	*66.53*	*37.57*	*49.87*	*83.19*	*58.00*	*51.95*

Table 5.1: DNA counts (ng/μL) per modality used at different sites with the colon in relation to tumour.

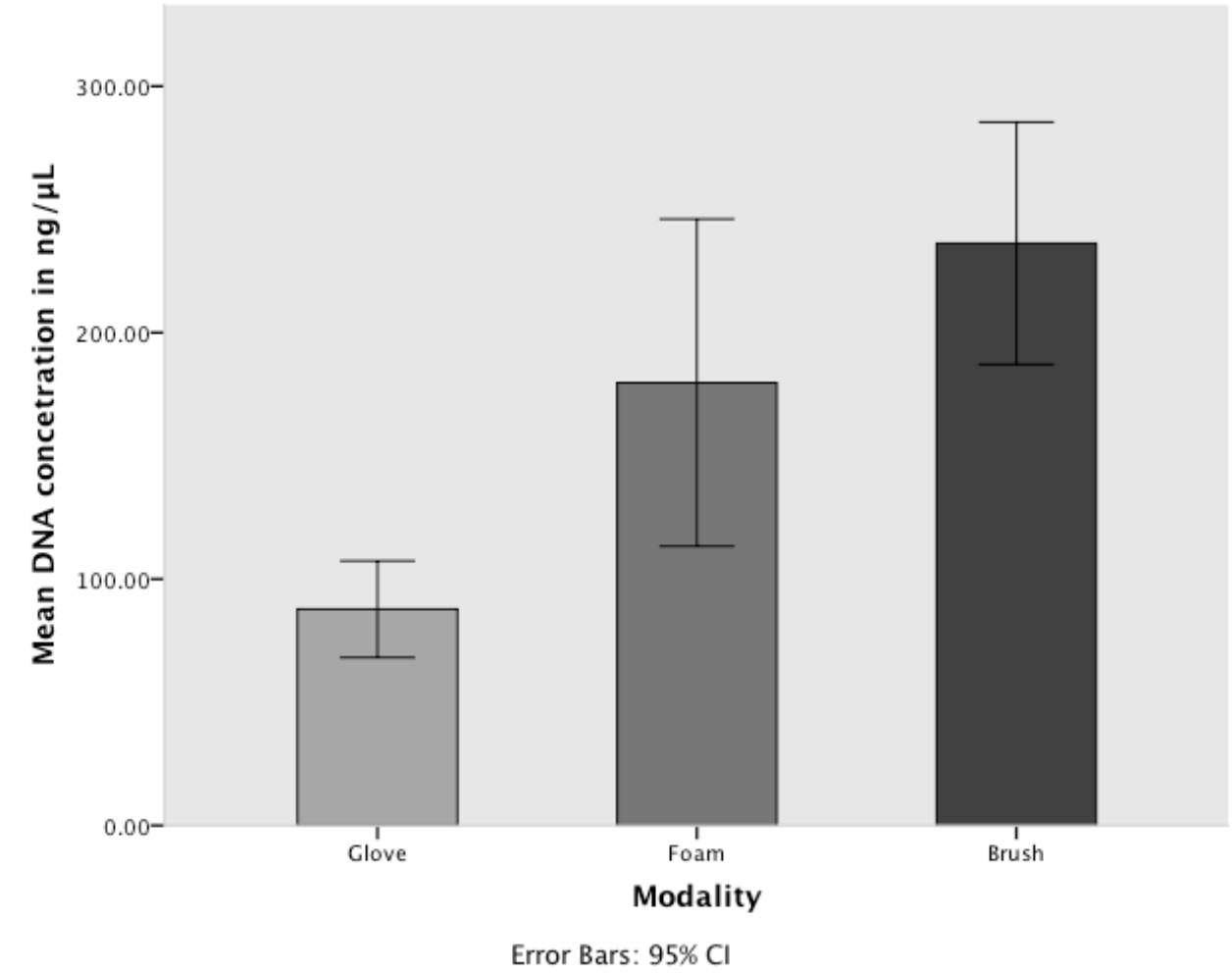

Figure 5.2a: Mean of DNA concentration in colonic mucus collected with Glove v. Foam v. Brush.

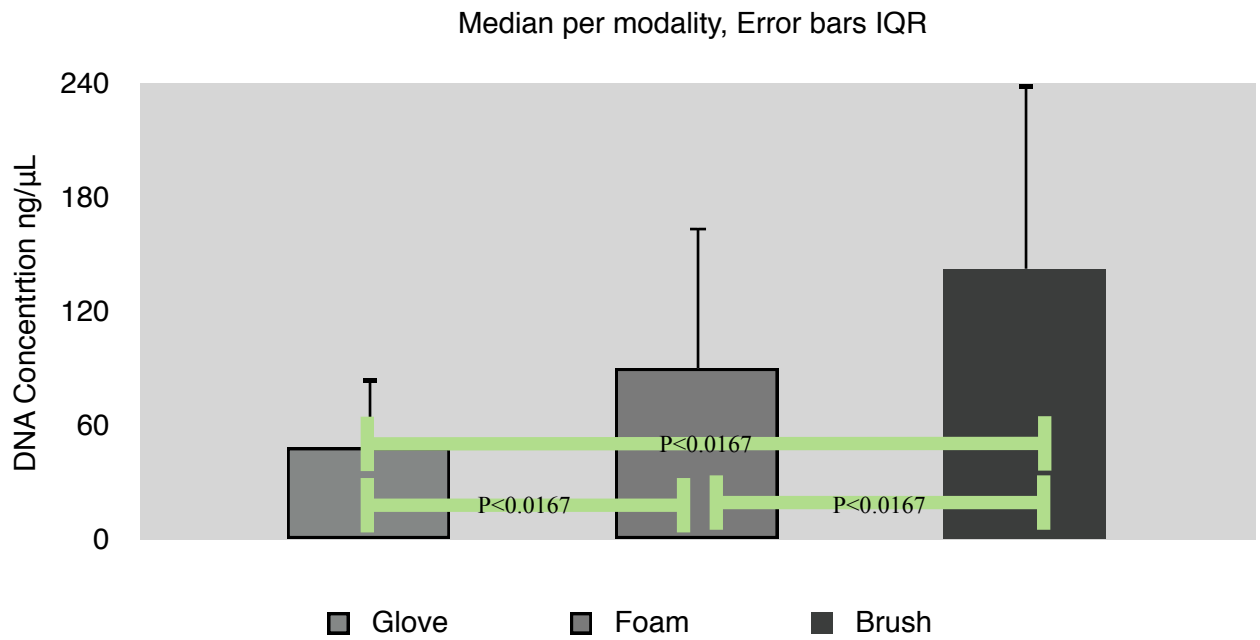

Figure 5.2b:Median of DNA concentration in colonic mucus collected with Glove v. Foam v. Brush

Since DNA counts are significantly higher at the tumour site compared to non tumour sites as later described in chapter 6 below, we have repeated the analysis by excluding the measurements taken from tumour sites. Ninety three (93) colonic mucus samples using foam, 92 using glove and 87 with brush were made. The mean DNA concentration using foam was 86.7 ng/μL (95% CI 73.0-100.4), 47.1 ng/μL (95% CI 40.7-53.4) with glove and 131.0 ng/μL (95% CI 111.4-150.6) with brush (Figure 5.3). The data in all groups were analysed for normality as described above. All data were non-normally distributed (foam D(93)=0.157, p=0.0001 glove D(92)=0.178, p=0.0001, brush D(87) =0.135, p=0.001).

A Kruskal-Wallis test was used to compare the median DNA yield between the three groups. Median DNA concentration was significantly different between the three different modalities used to sample colonic mucus at sites with no tumour H(2)=66.53 p=0.0001. Mann-Whitney test was used as post hoc analysis to follow up the Kruskal-Wallis test result above. A Bonferroni correction was used and therefore results are reported to a 0.0167 level of significance.

The median DNA concentration in the glove group (Mdn=35.7 ng/μL, IQR=40.17) was significantly lower than that in both the foam group (Mdn=60.80 ng/μL, IQR=72.4) Mann-Whitney U= 2476.5, z=-4.947 p=0.0001 and in the brush group (Mdn=102.0 ng/μL, IQR=117) Mann-Whitney U= 1279.5, z=-7.857 p=0.0001. In addition brush median DNA concentration (Mdn=102.0 ng/μL, IQR=117) was significantly higher than in the foam group (Mdn=60.80 ng/μL, IQR=72.4) Mann-Whitney U= 2739, z=-3.740 p=0.0001.

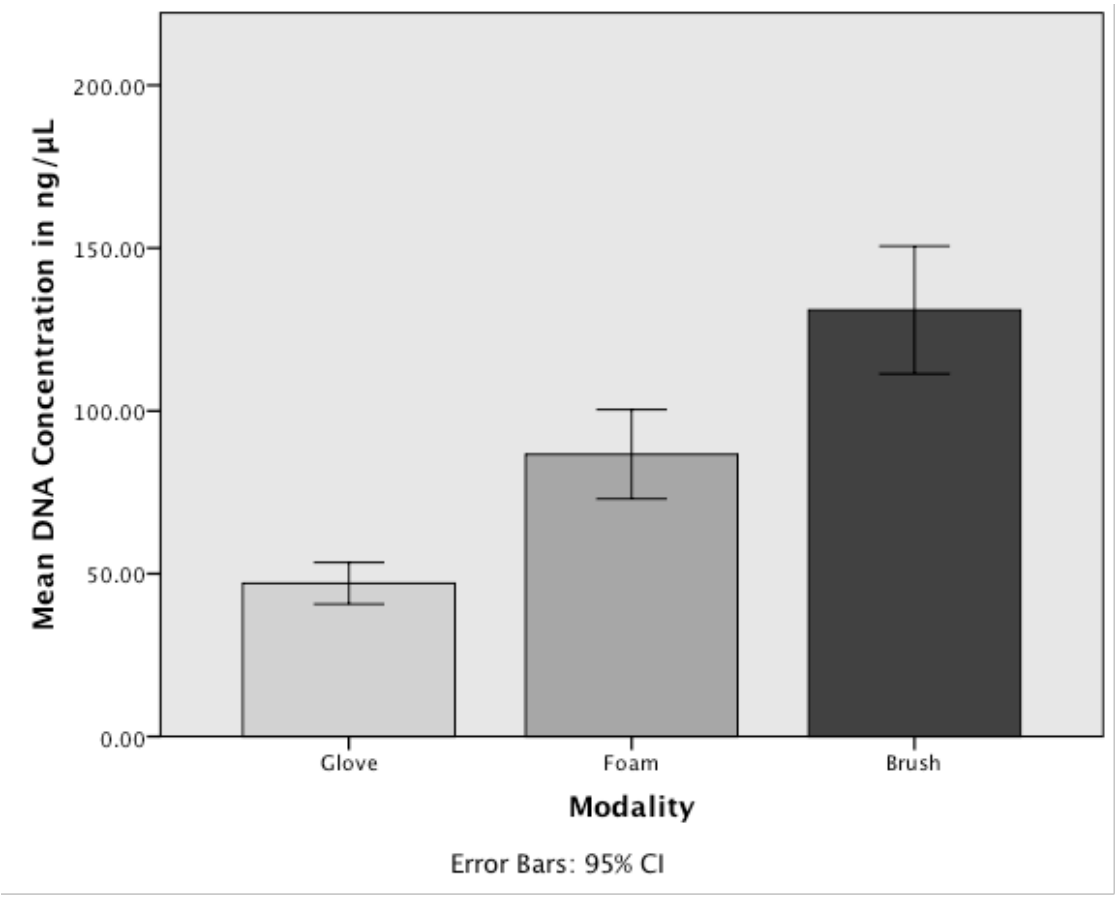

Figure 5.3a: Mean DNA concentration in ng/µL, excluding measurements taken from tumour sites.

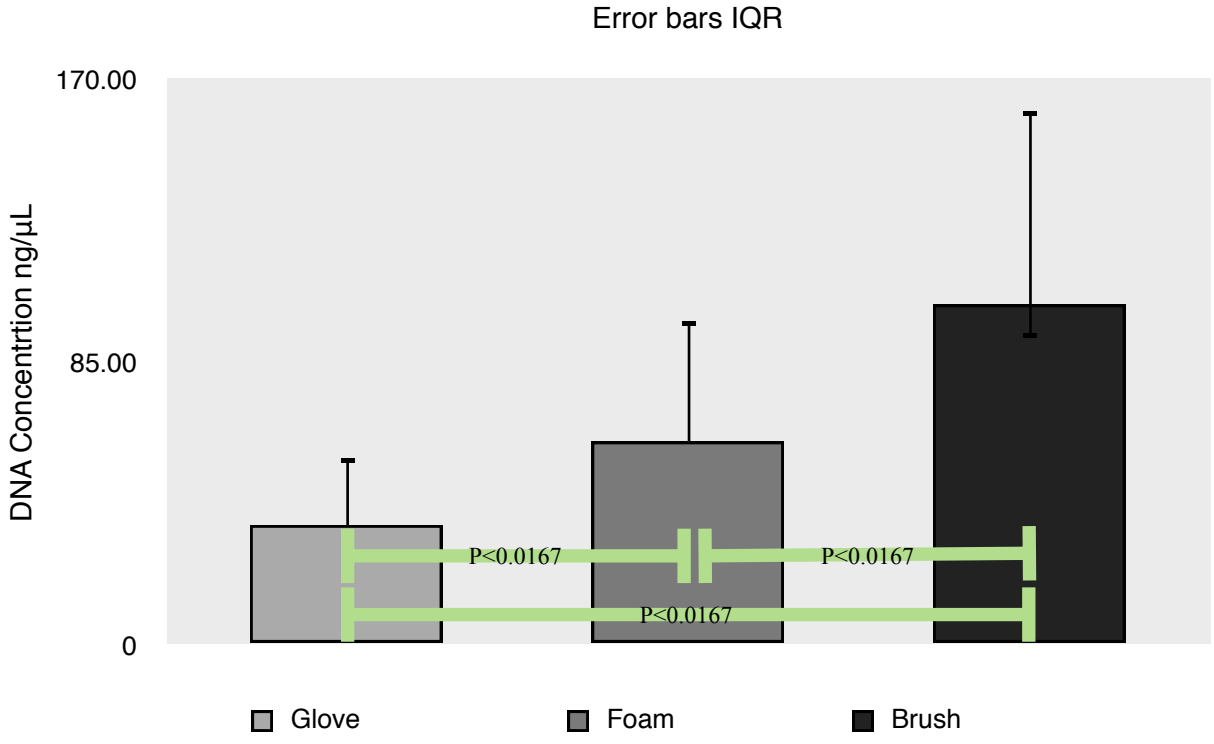

Figure 5.3b: Median DNA concentration in ng/µL, excluding measurements taken from tumour sites.

5.4 Discussion

In this chapter we aimed to establish the best material for collecting DNA from mucus in human colons. The ideal material would allow collection and also release of DNA to be analysed for the presence of biomarkers (chapter 7). A cytology brush was used as a positive control as it has been specifically designed to collect cells and DNA from buccal mucosa and is in clinical practice throughout the world. The obvious solution would be to use these brushes to sample rectal mucus from patients. Unfortunately this would not be practical for several reasons. Firstly cytology brushes have sharp ends and their use during proctoscopy could result in rectal trauma. Secondly our target DNA has been exfoliated from tumour colonocytes in sites proximal to the rectum. Cytology brushes will also collect colonocytes from the mucosa along with some DNA from the overlying mucus. As a result the DNA isolated will be mainly from rectal colonocytes instead of mucus DNA that has been exfoliated from tumour sites and has accumulated along the mucocellular layer of the colon in the rectum as described in chapter 8.

Glove was chosen as a material as it had been already successfully used in clinical studies[180, 181] to collect rectal mucus DNA. We opted for a sterile latex glove to ensure that there was no environmental DNA contamination.

A polyethylene foam was also chosen as it is a material that it can be fashioned to an atraumatic device that can be used during proctoscopy. We ensured that the pieces of foam used had no DNA contamination as described in section 5.2. Furthermore this specific foam material has been approved by FDA in USA to be safe for accidental ingestion by children and given a CE mark in the EEA.

As described in detail in section 5.3 our data in the three groups (glove, foam and brush) were not normally distributed and therefore non- parametric statistical tests were used to analyse our results.

A reason for this is that samples were collected from 25 different human colonic specimens. As described in section 1.3 there is high variability in the exfoliation rate of colonocytes between individuals and also between different tumour types. In addition exfoliation of colonocytes is affected by diet and physical activity hence there is variability of the colonocyte exfoliation rate within the same colon from one day to another.

As expected the cytology brush yielded significantly higher amounts of DNA than either foam or glove devices. This is because the cytology brush has been designed to collect mainly mucosal cells and some mucus over the mucosa mainly by its ability to detach cells and mucus by friction. As described in section 5.2 the brush was rotated over the mucocellular layer of the colon to achieve maximum friction and to act as a positive control.

On the other hand both the glove and foam devices were applied in such way as to minimise friction between the device and the mucocellular layer of the colon and to maximise collection of mucus rather than the local mucosal colonocytes. This was achieved by vertical pressure with each device over the mucocellular layer of the colon avoiding any lateral movement. This reflects the future use of the material to fashion a clinically applicable device that will collect mucus that has travelled along the mucocellular layer of the colon from proximal sites (Chapter 6 and 7).

As predicted both glove and foam yielded significantly lower amounts of DNA compared to the positive control. Interestingly foam significantly outperformed the glove device by yielding double the DNA amount compared to glove. One explanation could be that because foam has a rougher surface it was able to exert higher friction and as a result picked up some mucosal cells in addition to the overlying mucus. In addition although both glove and foam pieces were 4x1cm rectangles the effective surface area (the part of the device in actual contact with colonic tissue) of the foam is larger than the that of the of the glove (flat smooth surface of the glove compared to the rough and irregular surface of the foam). This also allows more of the foam piece to be in contact with the cell lysis buffer resulting in more colonocytes coming into contact with the buffer compared to the glove device and hence more DNA released into the buffer during storage of the collecting devices in cell lysis buffer.

However if we look closely at the performance of the brush at distances very proximal to tumour where there is minimal DNA from the tumour as described in chapter 6, the DNA yielded by the glove is 42% of that yielded by the brush at the same distance. If we then compare this at the tumour site (potential maximum DNA yield) the glove yielded only 34% of the brush yield. This suggests that our initial assumption that the brush's high performance is due to its ability to pick up colonocytes directly from the underlying mucosa (not mucus) might not be true after all. The glove device exerts minimal friction to the mucosa and mainly picks up mucus while the brush due to its higher effective surface area (a cytology brush has many little hair like filaments) is able to pick up more mucus than the glove and that may be why it is outperforming the other two devices. Ideally we could repeat the experiment by using a brush that has a surface area of $4cm^2$ (= glove) to be able to compare like for like, but that would be extremely difficult to manufacture due to time and financial constrain.

We then compared the performance of glove and foam to that of the brush at each distance (table 5.2). The ratio of both glove or foam to brush is not similar at every distance as expected if the ability of each material to yield maximum DNA was solely reliant on its friction effect onto the mucosa.

Distance	N=118	Median Glove	Median Foam
10-15cm Distal to Tumour	*22*	*0.36*	*0.71*
5-10cm Distal to Tumour	*19*	*0.24*	*0.43*
Tumour site	*25*	*0.32*	*0.50*
5-10cm Proximal to Tumour	*10*	*0.18*	*0.58*
10-20cm Proximal to Tumour	*18*	*0.47*	*0.83*
20-40cm Proximal to Tumour	*24*	*0.42*	*0.68*

Table 5.2: DNA yield expressed as median DNA collected using glove or foam over DNA collected using brush at the same distance.

Our hypothesis states that it is possible to detect DNA from proximal sites in the colon by sampling rectal mucus in patients with colorectal cancer. As a result we repeated the analysis of the results by excluding measurements taken directly over tumour sites to try and simulate the future clinical application of such devices. Interestingly the results of the second analysis (excluding tumour sites) showed much lower yields in every category (brush, glove and foam) although relative differences remained when comparing the three devices. The lower DNA yield can be explained by the previously described high exfoliation rates seen in colorectal tumours (see section 1.3) and also due to the sharp decline in DNA amounts seen in samples taken within short distances from tumours which is described in detail in chapter 6. It is interesting that when all measurements were included foam yielded almost double the amount of DNA compared to glove, an observation which did not change when tumour sites were excluded. In addition foam yielded about 40% less DNA compared to brush in both analyses.

In conclusion, the brush, although initially used as a positive control, could indeed be the best material to collect mucus but its advantage could be simply due to its larger surface area compared to the other two devices. In addition it will be challenging to fashion an atraumatic cytology brush that can be inserted safely into the rectum. One suggestion to get around this problem, could be to fashion a cytology shaped device made of foam. At the same time foam was significantly better than the glove and once again this could only be down to its larger surface area compared to the glove. We have therefore decided that despite the fact that as described in this chapter the glove was the least efficient way to collect mucus DNA, we would use a rectal tube catheter (made of latex

and hence more similar to glove) for our clinical experiments (chapter 7). There were several reasons for this decision. It is a device regularly used by colorectal surgeons to drain/wash the rectum, it is acceptable by patients (easier to recruit patients) and therefore more likely to be approved by an ethics committee, and finally it would have been very time consuming and costly to design, test and get approval for a novel device made of foam in the shape of a brush to use in patients.

6. Is there a gradient of DNA concentration in colonic mucus from the tumour site proximally or distally?

6.1 Objectives

i. To establish the amount of DNA at tumour sites and set distances proximally and distally from the tumour site and to identify if there is a pattern or not.

6.2 Methodology

Principles of methods

• Fresh colonic specimens resected from cancer patients were cut open by a consultant pathologist

• Mucus was sampled using collecting devices as described below at the tumour site, and at distances distally (5-10 and 10-15cm) and proximally (5-10, 10-20 and 20-40cm) depending on length of the surgical specimen

• Mucus samples were taken at the proximal resection margin and along with all measurements >20 cm proximal to tumour site were, used to establish the background level of colonic DNA exfoliation

• Values at each location were expressed as the ratio to the value at the tumour site to allow comparison between measurements taken from different colons and to allow for differences in bowel preparation patients had received between right and left side bowel resections and differences in exfoliation rates by different individuals and tumours.

Collection of surgical specimens and sampling of colonic mucus

The colonic surgical specimen was collected and cut open as described in chapter 4.2.1.

Once the colon was opened, areas of uncontaminated (stool free) mucosa were identified for sampling and their distance from the tumour site recorded. A piece of foam or glove with a surface area of $4cm^2$ was applied to the sampling area for a total of ten seconds. To ensure that the same force/pressure was applied during sampling, a fixed weight (20g) was applied on top of the piece of glove. Care was taken to avoid any scraping movements that would result in sampling mucosa instead of mucus. A further sample was taken using a cytology brush as a positive control.

Following sampling mucus, the pieces of glove or foam were placed in 15ml Falcon tubes (BD Biosciences, USA) containing 3ml of Cell Lysis Solution (Cat No 158908, Qiagen Gmbh, Germany). Care was taken to ensure that the entire surface of the glove/foam was covered with cell lysis buffer (see Chapter 4). The samples were then stored at room temperature and transferred to the laboratory.

Extraction of DNA from colonic mucus samples

Principles of method

- As described in chapter 4.2.1.

DNA extraction was performed following the protocol described in section 4.2.1.

6.3 Results

The same twenty five colonic specimens were sampled as described in section 5.2. Samples were taken using both glove (n=117) and foam (n=118). The DNA amount in each sample was measured and expressed as a ratio to the DNA amount over the tumour site (DNA at distance X/ DNA at tumour site). Twenty five (25) mucus samples were taken directly over tumour sites and at specific distance ranges as summarised in table 6.1 using both modalities.

GLOVE	N=117	Mean	SD	95% CI		Median	IQR
10-15cm Distal to Tumour	*21*	*0.284*	*0.142*	*0.220*	*0.349*	*0.251*	*0.129*
5-10cm Distal to Tumour	*19*	*0.402*	*0.217*	*0.297*	*0.506*	*0.410*	*0.380*
Tumour site	*25*	*1*	-	-	-	*1*	-
5-10cm Proximal to Tumour	*10*	*0.354*	*0.216*	*0.200*	*0.509*	*0.333*	*0.261*
10-20cm Proximal to Tumour	*18*	*0.231*	*0.145*	*0.156*	*0.305*	*0.218*	*0.224*
20-40cm Proximal to Tumour	*24*	*0.140*	*0.077*	*0.107*	*0.172*	*0.118*	*0.126*
FOAM	**N=118**	**Mean**	**SD**	**95% CI**		**Median**	**IQR**
10-15cm Distal to Tumour	*22*	*0.323*	*0.153*	*0.255*	*0.391*	*0.307*	*0.213*
5-10cm Distal to Tumour	*19*	*0.459*	*0.169*	*0.378*	*0.541*	*0.510*	*0.262*
Tumour site	*25*	*1*	-	-	-	*1*	-
5-10cm Proximal to Tumour	*10*	*0.372*	*0.205*	*0.225*	*0.518*	*0.324*	*0.233*
10-20cm Proximal to Tumour	*18*	*0.246*	*0.126*	*0.182*	*0.311*	*0.231*	*0.158*
20-40cm Proximal to Tumour	*24*	*0.132*	*0.081*	*0.098*	*0.166*	*0.123*	*0.089*

Table 6.1: Ratio of DNA amount in colonic mucus at specific distances to tumour site using Glove and Foam to sample mucus from colonic surgical specimens.

The data collected from both using foam and glove were analysed using SPSS v19 (IBM Inc, USA) to test for normality (Kolmogorov-Smirnov test). In the glove group data were found to be non

normally distributed in the proximal 20-40cm (D(24)=0.192, p=0.023) but were normally distributed in the remaining groups (10-15cm distal to tumour (D(21)=0.183, p=0.066, 5-10 distal (D(19)=0.102, p=0.200), 5-10 proximal (D(10)=0.215, p=0.200), 10-20cm proximal (D(17)=0.143, p=0.200)). Lavene's test (F(4,86)=4.030, p=0.005) showed that the homogeneity of variance was violated in these data and therefore, nonparametric tests were used to analyse and report the results of this experiment. Similarly the data in the foam group were also parametric (10-15 distal D(22) =0.167, p=0.111, 5-10 distal (D(19)=0.145, p=0.200), 5-10 proximal (D(10)=0.226, p=0.161), 10-20cm proximal (D(17)=0.167, p=0.200), 20-40cm (D(24)=0.139, p=0.200)) but Lavene's test (F (4,87)=3.768, p=0.007) showed that the homogeneity of variance was violated in these data hence non-parametric tests were used again.

Figure 6.1 summarises the ratio of DNA measured at the set distances to DNA at tumour site.

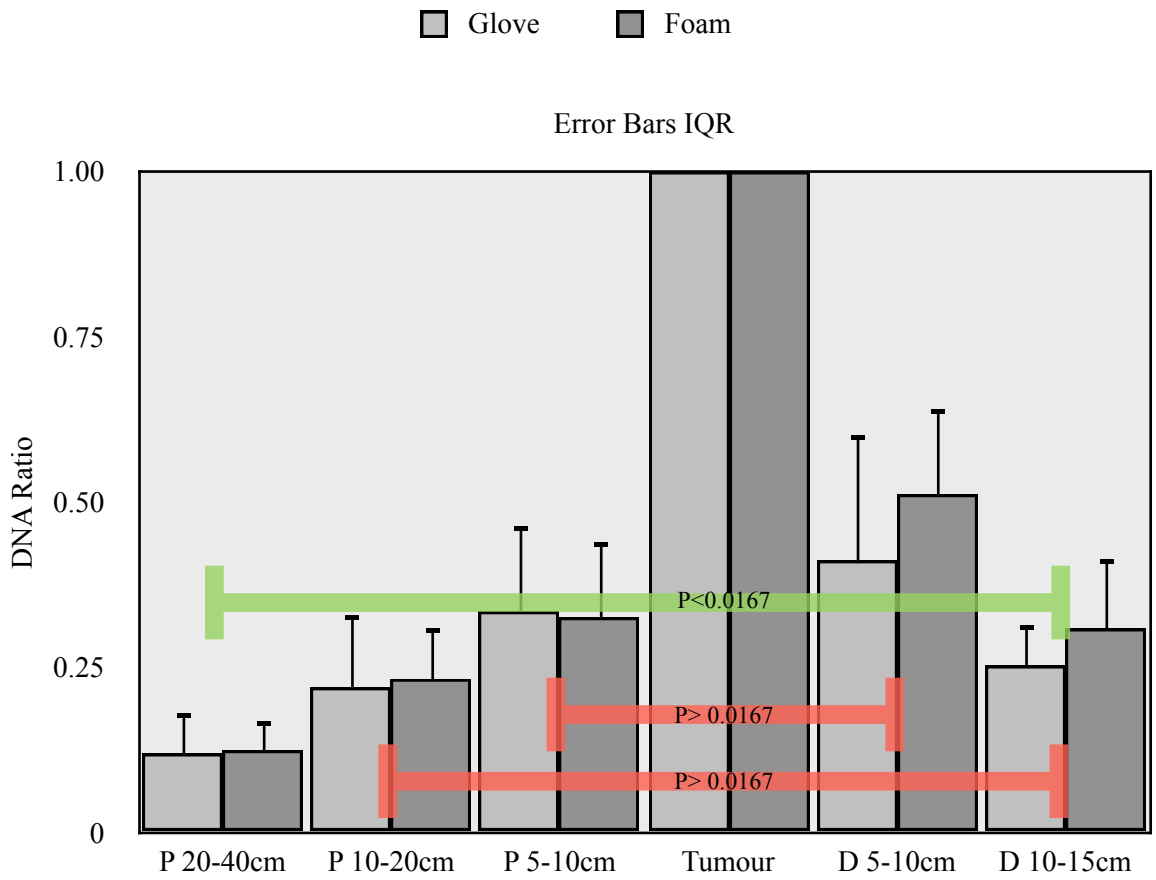

Figure 6.1: Median of Ratio of DNA measured at each distance / DNA at the tumour site. D=distal to tumour, P=proximal to tumour.

A Kruskal-Wallis test was used to compare the median ratios at these distances which were found to be significantly different both when using glove (H(4)=28.307 p=0.0001) and foam (H(4)=40.440 p=0.0001) to collect the mucus. Three Mann-Whitney tests were used as post hoc tests with a

Bonferronni correction to compare median values between specific distances. Of note, as three post hoc tests were performed the results were reported to a 0.0167 level of significance[182]. When mucus was collected with glove, the median ratio at a distance of 10-15cm distal to tumour site (Mdn=0.251 IQR=0.129) was significantly higher than that at a distance more than 20cm proximal to the tumour site (Mdn=0.118 IQR=0.126) Mann-Whitney U= 77.0, z=-3.982 p=0.0001 (<0.0167). The median ratio at a distance of 10-15cm distal to tumour site (Mdn=0.251 IQR=0.129) was not significantly higher than that at a distance 10-20cm proximal to the tumour site (Mdn=0.218 IQR=0.224) Mann-Whitney U= 135.00, z=-1.277 p=0.209 (>0.0167). At the same time, the median ratio at a distance 5 to 10cm distal to tumour site (Mdn=0.410 IQR=0.380) was not significantly higher than the same distance proximal to the tumour site (Mdn=0.333 IQR=0.261) Mann-Whitney U= 80.5, z=-0.666 p=0.512 (>0.0167).

When mucus was collected with foam a similar pattern of DNA ratio was observed. The median ratio at a distance of 10-15cm distal to tumour site (Mdn=0.307 IQR=0.213) was significantly higher than that at a distance more than 20cm proximal to the tumour site (Mdn=0.123 IQR=0.089) Mann-Whitney U= 61.5, z=-4.455 p=0.0001 (<0.0167) while it was not significantly higher than that at a distance 10 to 20cm proximal to the tumour site (Mdn=0.231 IQR=0.158) Mann-Whitney U= 142.0, z=-1.275 p=0.210 (>0.0167). The median ratio at a distance 5 to 10cm distal to tumour site (Mdn=0.510 IQR=0.262) was not significantly higher than the same distance proximal to the tumour site (Mdn=0.324 IQR=0.233) Mann-Whitney U= 65.0, z=-1.377 p=0.179 (>0.0167)

Table 6.2 summarises the results of this analysis.

	Distal 10-15cm v. Proximal 10-20cm	**Distal 5-10cm v. Proximal 5-10cm**	**Distal 10-15cm v. Proximal 20-40cm**
Glove	*Not Significant*	*Not Significant*	Significant
Foam	*Not Significant*	*Not Significant*	Significant

Table 6.2: Comparing median DNA ratio between three distances. Summary of statistical analysis. Significant=p<0.0167, Non significant p>0.0167.

As described in section 6.2, measurements taken more than 20cm proximally from the tumour site can be assumed to represent the background DNA value of each colon.

It is interesting to repeat the analysis by grouping all measurements taken by glove distally to the tumour and compare them with those taken proximally and those taken from the background colon.

Twenty seven (27) measurements were taken from proximal sites, 40 from distal, 24 from background sites using glove and 27, 41 and 24 respectively using foam. Table 6.3 summarises the results of these measurements.

	Background				Proximal				Distal			
Modality	Mean	Median	SD	IQR	Mean	Median	SD	IQR	Mean	Median	SD	IQR
Glove	0.140	0.118	0.07	0.13	0.276	0.25	0.18	0.24	0.340	0.29	0.19	0.27
Foam	0.132	0.123	0.08	0.09	0.293	0.240	0.17	0.18	0.386	0.393	0.17	0.35

Table 6.3: Ratio of DNA amount in colonic mucus at proximal, distal and background sites using glove and foam to sample mucus from colonic surgical specimens.

The data collected both using foam and glove were analysed using SPSS v19 (IBM Inc, USA) to test for normality (Kolmogorov-Smirnov test). Foam data (Proximal, $D(27)=0.142$, $p=0.173$, Distal $D(41)=0.126$, $p=0.102$, Background $D(24)=0.139$, $p=0.200$) were parametric but once again Lavene's test ($F(2,89)=7.277$, $p=0.001$) showed that the homogeneity of variance was violated in these data. The Glove data were similar and again non-parametric tests were used to analyse and report the results in both foam and glove data.

Figure 6.3 summarises the ratio of DNA at distal, proximal and background sites to tumour site using glove and foam.

A Kruskal-Wallis test was used to compare the median ratio at distal, proximal and background sites which were found to be significantly different both when using glove ($H(2)=23.888$ $p=0.0001$) and foam ($H(2)=34.384$ $p=0.0001$) to collect the mucus. Three Mann-Whitney tests were used as post-hoc tests with a Bonferronni correction to compare median values between specific distances. Of note, as three post hoc tests were performed the results were reported to a 0.0167 level of significance[181].

When using a glove the distal median ratio (Mdn=0.290 IQR=0.265) was significantly higher than that at the background site (Mdn=0.118 IQR=0.126) Mann-Whitney $U= 132.0$, $z=-4.827$ $p=0.0001$ (<0.0167). The proximal median ratio (Mdn=0.250 IQR=0.246) was also significant when compared against the background site Mann-Whitney $U= 152.0$, $z=-3.246$ $p=0.001$ (<0.0167). Interestingly the Proximal median ratio (Mdn=0.250 IQR=0.246) was not significantly lower than the distal ratio (Mdn=0.290 IQR=0.265) Mann-Whitney $U= 429.5$, $z=-1.413$ $p=0.158$ (>0.0167).

In the foam group the results were similar, and again both proximal (Mdn=0.240 IQR=0.184) and distal median ratio (Mdn=0.393 IQR=0.350) were significantly different when compared to the

background median ratio (Mdn=0.123 IQR=0.089), Mann-Whitney U= 108.0, z=-4.077 p=0.0001 (<0.0167) and Mann-Whitney U= 82.5, z=-5.568 p=0.0001 (<0.0167) respectively.

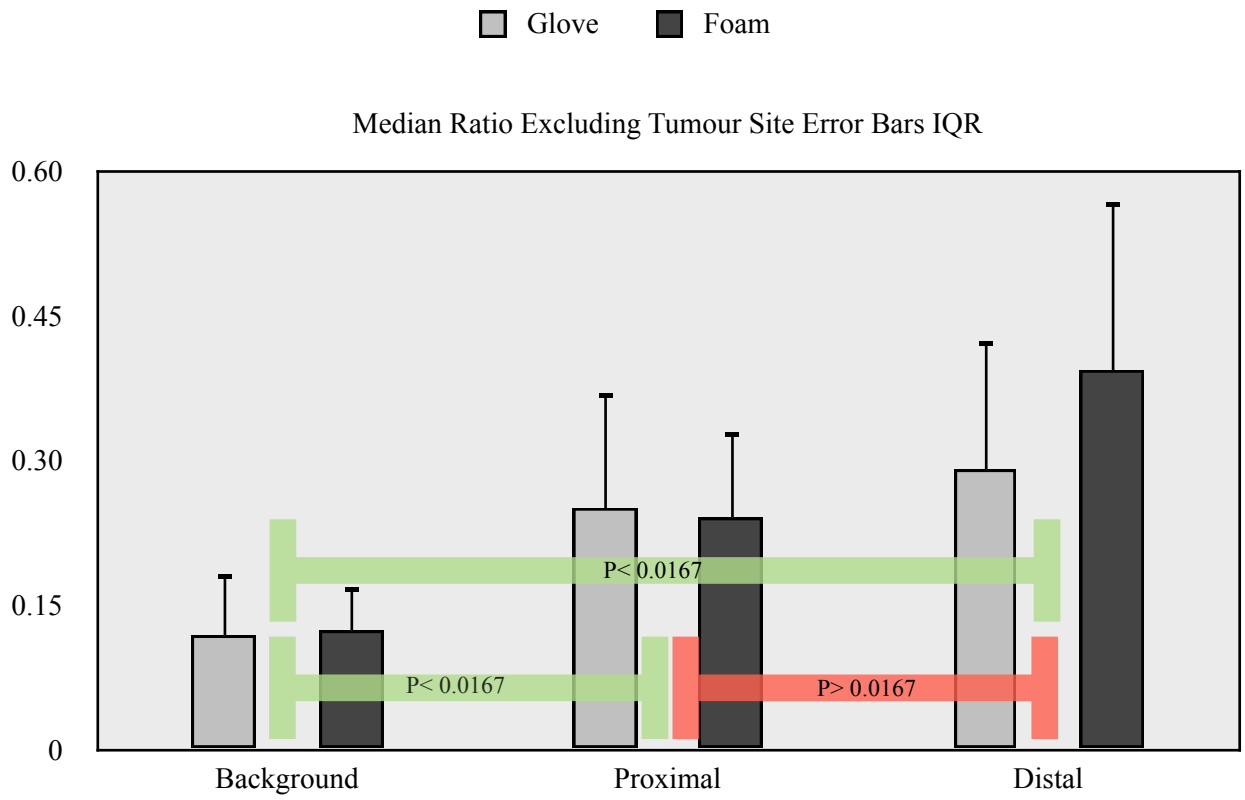

Figure 6.2: Median ratio of DNA at distal, proximal and background sites to tumour site using glove and foam.

As in the glove group, the foam median proximal and distal ratio were not significantly different Mann-Whitney U= 386, z=-2.100 p=0.036 (>0.0167). Table 6.4 summarises the statistical analysis.

	Distal v. Background	**Proximal v. Background**	**Distal v. Proximal**
Glove	Significant	Significant	*Non Significant*
Foam	Significant	Significant	*Non Significant*

Table 6.4: Comparing median DNA ratio between three grouped sites. Summary of statistical analysis. Significant=p<0.0167, Non significant=p>0.0167.

6.4 Discussion

The mucus layer over the tumour site in this study demonstrated significantly higher counts of DNA compared to both adjacent and distant sites along the colon. This is in agreement with published data[160]. The amounts of DNA found in non tumour site was several orders of magnitude lower compared to the tumour site even at distances within 5cm from the tumour site. Interestingly there is no significant difference in the amount of DNA found in the mucus between distal and proximal sites in relation to tumour within the colon when the measurements are taken within 15-20cm. This implies a 'volcano' effect of cell exfoliation from tumour sites, with the tumour being the 'crater' and DNA being the 'lava' being 'expelled' (exfoliated) and distributed evenly around the tumour site. However, in this study when distal sites to tumour (up to 15cm) were compared to sites more than 20cm proximal to the tumour there was a significant increase in DNA counts. This points to the conclusion that at these distances the above 'volcano' effect stops and the DNA found represents colonocyte DNA exfoliation as part of the physiological regeneration of human colonic mucosa. When we grouped together the measurements from all distal sites and compared them to background DNA counts, they were significantly higher as were the grouped proximal sites. When we compared the grouped proximal to the grouped distal sites, again there seem to be no difference, indicating that there is a 'volcano' effect of DNA exfoliation around the tumour.

Unfortunately the maximum distances from the tumour sites were limited to the macroscopic clearance of our 25 surgical specimens, with 15cm and 40cm being the maximum distal and proximal resection margins respectively. These maximum distances were related to whether the specimen was from a right colectomy (specimens with longer distal distances but lower proximal distance) or a left colectomy (specimens with longer proximal and shorter distal distances). Ideally we would prefer to have taken measurements along the entire length of the colon in each specimen but that would only have been possible if the patients underwent a total colectomy. This operation is usually indicated for synchronous tumours or in patients with polyposis syndromes, both of which would have different exfoliation characteristics to a single site tumour.

It will be interesting to see if there is indeed an increased accumulation of DNA at the rectum of cancer patients. Two recent studies[180, 181] have showed this effect, namely accumulation of higher than normal DNA in the rectal mucus of patients with colonic cancers. In chapter 7 we measure and analyse DNA from samples taken from rectal mucus of patients with cancer.

7. Can we use DNA isolated from rectal mucus to detect colorectal cancer?

7.1 Objectives

i. To identify if there is a significant difference in the amount of DNA extracted from sampled rectal mucus between patients with neoplasia and those without.

ii. To identify when a patient has CRC by using a three markers methylation panel assay on DNA isolated from rectal mucus in symptomatic patients referred to a colorectal clinic.

7.2 Methodology

7.2.1 Comparing DNA content in rectal mucus samples from symptomatic colorectal patients.

Patient recruitment, consent procedure and sampling rectal mucus

The following studies have been approved by the National Research Ethics Committee South Central Oxford C (ref 11/SC/0305) and the Oxford University Hospitals NHS Trust Research and Development department (ref PID 6425).

Participants were identified following referral by their General Practitioner to specialist outpatient clinics (colorectal and gastroenterology) or the ward for investigation of bowel symptoms suspicious of bowel cancer. The patients were sent details of the study (for patient information sheet and invitation letter see Appendix I) prior to attending the clinic by the study team. At the clinic patients were given the opportunity to discuss any aspects of the study and sign the consent form. A digital rectal examination was performed to identify any palpable tumour/polyps and if present, the patient was excluded. Proctoscopy using a standard proctoscope was performed and a rectal catheter (30Fr, PTFE Coated Latex, C.R Bard Inc, USA) was inserted into the rectum. The balloon was then inflated using air to 40ml so as to touch the lower rectal wall, then deflated and retracted avoiding touching the inside wall of the proctoscope to reduce trauma to the mucosa. The lower 10cm of the rectal catheter was then immersed in a 15ml Falcon tube (BD Biosciences, USA) containing 3ml of Cell Lysis Solution (Cat No 158908, Qiagen Gmbh, Germany). Following sample collection patients underwent the standard investigations for their symptoms, namely colonoscopy

or CT colonoscopy. Their final diagnosis (neoplasia, benign disease or normal investigation) was recorded. DNA extracted from the rectal catheter was measured using the following protocol.

Extraction and measurement of DNA from patient rectal mucus samples

Principles of method

- The four main principles of the protocol are those described in 4.2.1.

Protocol (Modified Gentra Puregene Kit body fluid protocol, Qiagen Gmbh, Germany)

- Samples were incubated for 1 hour at 66 ^{o}C.
- To maximise yield 90µL of Puregene Proteinase K (Cat no 158918, Qiagen Gmbh, Germany) was added.
- To ensure RNA free DNA 90µL of RNase A solution (Cat No 158924, Qiagen Gmbh, Germany) was added and Falcon tubes incubated for 15min at 37 ^{o}C. Samples were then cooled down by placing on ice for five minutes.
- 6mL of protein precipitation solution (Cat No 158912, Qiagen Gmbh, Germany), to separate proteins from DNA, was added and Vortex at high speed for 20 seconds. Samples were then incubated in ice for 5 minutes and then centrifuged for 10min at 2,000g.
- The resultant supernatant containing DNA added to a clean 50ml Falcon tube containing 18ml of isopropanol and 30µL of Glycogen (Cat No 158930, Qiagen Gmbh, Germany). The samples were mixed gently by inverting 50 times and incubated at room temperature for 5 minutes.
- They were centrifuged for 10min at 2,000g to form a DNA pellet. The supernatant was carefully discarded, taking care the DNA pellet was not dislodged. Visual verification of the DNA pellet was done.
- 18ml of 70% ethanol was added to wash the DNA pellet and samples were centrifuged for 1min at 2,000g.
- The supernatant was drained again taking care the DNA pellet was not dislodged and samples were allowed to air dry for 15minutes.
- 300µL of DNA Hydration solution (Cat No 158914, Qiagen Gmbh, Germany) was added and the sample vortexed for 5 seconds at medium speed and incubated at 65 ^{o}C for 1hour to hydrate the DNA.
- The samples were then stored overnight with gentle shaking using a 3D Rocking Platform STR9 (Stuart Scientific, UK).
- Samples were centrifuged briefly (Capsulefuge PMC-100 Tomy Teoh, USA) for a few seconds

the next morning, before measuring their DNA content using a spectrophotometer.

The ND1000 Spectrophotometer (Nanodrop® Tech Inc, USA) was used to measure the DNA concentration in each sample as described in section 4.2.1. Using the concentration value of DNA per sample we calculated its content and expressed the results as total DNA isolated in ng from each patient.

7.2.2 Using a three biomarker panel assay to diagnose CRC from DNA isolated form rectal mucus in symptomatic colorectal patients.

The methylation markers

Numerous biomarkers have been described[123-126]. For the purposes of this project three methylation markers were chosen due to a combination of scientific evidence and availability from collaborating teams. The following three methylation markers have been reviewed recently[183-185] and appear to be promising candidates for CRC detection in stool DNA and have showed promising results in early studies with high sensitivity and specificities in both CRC tissue and stool based studies (summarised below for each marker).

N-Myc Downstream Regulated Gene 4 (NDRG4)

NDRG4 is a potential tumour suppression gene which has been identified in a variety of tumours and cancer cell lines. In 2009 Melotte et al[183] also demonstrated that the *NDRG4* promoter was methylated in CRC. It was present in 86% and 70% of two independent series of mucosa samples from CRC tissue, while present in only 4% of non CRC mucosa. Stool samples from 75 CRC patients and healthy matched controls were tested for methylation of *NDRG4* using quantitative methylation-specific PCR with a sensitivity of 53% and specificity of 100%. In addition, although CpG methylation is more commonly associated with more proximal tumours, *NDRG4* was found to be methylated to similar levels in proximal (mostly MSI) tumours and distal (mostly CIN) tumours. This may make a useful biomarker for both MSI and CIN CRC.

Tissue Factor Pathway Inhibitor 2 (TFPI2)

TFPI2 is a Kunitz-type serine proteinase inhibitor that protects the extracellular matrix of cancer cells from degradation and inhibits in vitro colony formation and proliferation[184,185]. It is thought that loss of TFPI2 function could predispose cells toward a pro invasive program, consistent with an important role for this protein in later stages of carcinogenesis. Glockner *et al*[186] in 2009

demonstrated methylation of *TFPI2* in 99% of CRC tissue. Stool samples from CRC and healthy controls were tested for methylated *TFPI2* showing sensitivity of 75%-89% and specificity of 79-93%.

GATA Binding Protein 4 (GATA4)

GATA4 is involved in development of the gastrointestinal tract. Hellebrekers *et al*[187] in 2009 demonstrated that *GATA4* had tumour suppressor properties in CRC and was methylated in 70% of CRC tissues but only in 6% of normal control mucosa from healthy non cancer individuals. When testing stool DNA from 75 known CRCs and 75 healthy individuals for methylation of *GATA4* they demonstrated sensitivity of 71% and specificity of 84%.

DNA was extracted as described above (section 7.2.1) from rectal mucus collected from symptomatic colorectal patients. Samples were blinded and then handed over to an experienced laboratory technician (Dr Kim A.D. Wouters) working with Dr Manon Van Engeland in GROW–School for Oncology and Developmental Biology, Maastricht University Medical Centre, The Netherlands.

The protocol for the methylation assay is described in detail in Hellebrekers *et al*[187] and Melotte *et al*[183]. The major steps in this assay are sodium bisulfite conversion, sequencing, and quantitative methylation-specific PCR. A positive assay was defined as more than two markers methylated. The final diagnosis for each patient was recorded as described above (section 7.2.1).

7.3 Results

7.3.1 Comparing DNA counts in rectal mucus samples from symptomatic colorectal patients.

Fifty eight (58) patients consented to rectal sampling using the method described above. Twenty four (24) were women. All patients were sampled prior to receiving any laxatives or bowel preparation. Fifty seven patients received colonoscopy as their method of investigating their colorectal symptoms. One patient had a flexible sigmoidoscopy and a CTC in view of co-morbidities and age, which were both reported as normal. Patients presented with bleeding per rectum (22), iron deficiency anaemia (11) and change in bowel habit (25). Thirty nine (39) were eventually diagnosed with colorectal cancer (32 had tumours in the left colon), 4 patients had

benign polyps (with low grade dysplasia adenomas <1cm) and 15 had no colonic disease at all. Twenty patients were within the target age group (60-69) for CRC screening in England, 5 of which were diagnosed with cancer. None of the twenty eligible patients had participated in a screening cycle prior to clinic attendance. Only one patient diagnosed with cancer had a mucinous adenocarcinoma on final histology while the remainder had conventional adenocarcinomas. Table 7.1 summarises the two groups (benign and CRC) of patients while figure 7.1 shows the median DNA isolated from each group of patients.

	Benign	**Cancer**
Sex	Men=12 Women=7	Men=22 Women=17
Screening Age (60-69)	5	14
Site of lesion	N/A	Left=32 Right=7
Dukes Staging	N/A	A=3 B=15 C=21

Table 7.1: Summary of demographics, lesion site and staging in patients with and without colorectal cancer.

The data sets from both groups were tested using SPSS v19 (IBM Inc, USA) to test for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Lavene's test). Benign data were non parametric (benign, $D(19)=0.173, p=.0137$) while cancer data were parametric (cancer, $D(39)=0.192$, $p=0.001$) but the homogeneity of variance was violated in these dataset ($F(2,56)=18.270$, $p=0.0001$) and therefore non parametric statistical analysis was performed.

The patients diagnosed with cancer had significantly higher amounts of DNA in their rectal mucus 1247000.0 ng compared to those with benign disease 33575.0 ng, Mann-Whitney $U= 212$, $z=-2.626$ $p=0.009$. The CRC patients were further analysed using Mann-Whitney tests to look for any differences in the DNA amounts isolated between patients with right v left colonic lesions, men v women, patients with different Dukes staging or patients within the screening target age or not. Because of small numbers, patients with Dukes A and B were grouped together and compared to those with Dukes C. Table 7.2 summarises the above analysis.

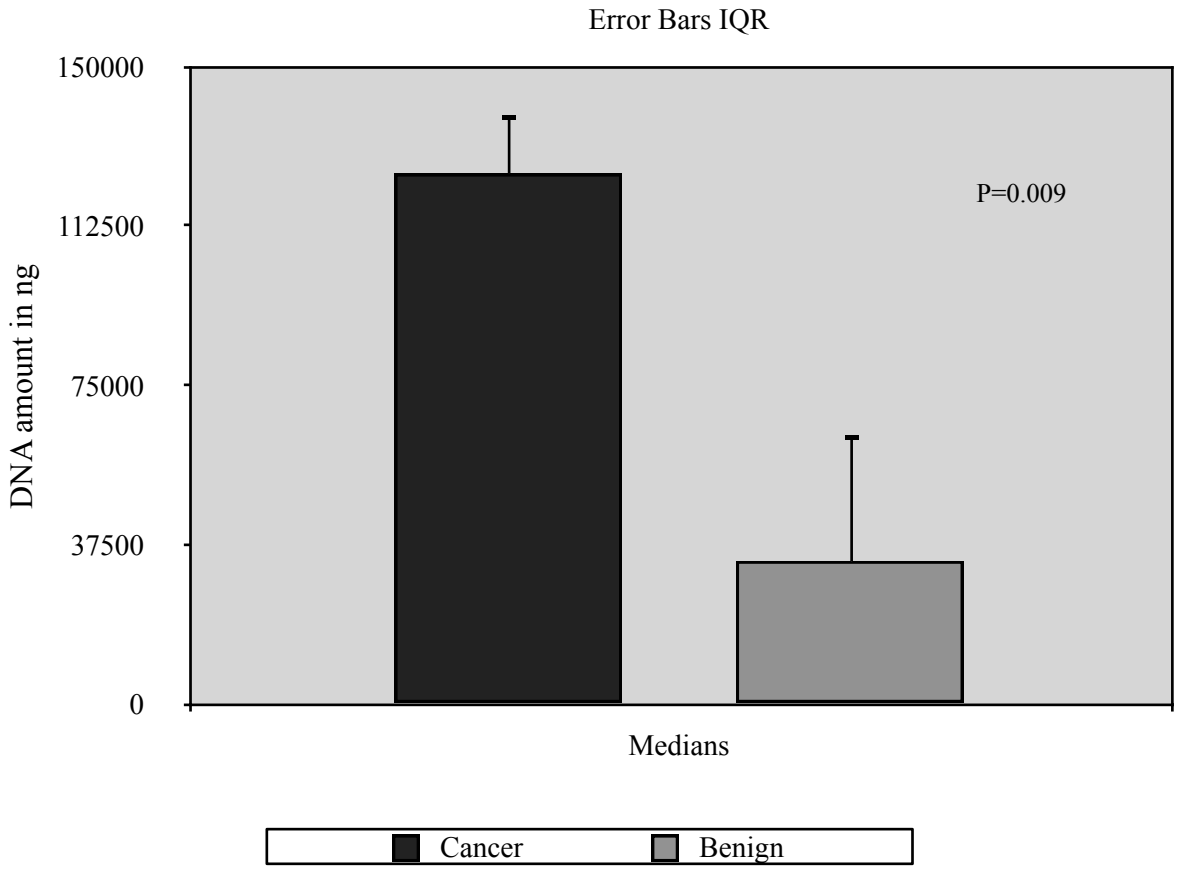

Figure 7.1: Medians of DNA amount collected from rectal mucus from symptomatic patients with a diagnosis of cancer and not.

Finally, median DNA isolated from left colonic lesions (Median=119,575ng) was significantly higher compared to the median DNA amount from benign patients, Mann-Whitney U= 164, z=-2.934 p=0.003. Interestingly this was not the case for tumours on the right side of the colon Mann-Whitney U= 48, z=-1.217 p=0.240.

Cancer			
Sex	Men N=22	Women N=17	
	Median=126,871 (IQR=307,960)	Median=108,121 (IQR=271,577.5)	P=0.590
Age	Screening Age (60-69) N=14	Non-Screening Age N=25	
	Median=227,189.1(IQR=292,387.8)	Median=108,733.83 (IQR=272,677.5)	P=0.251
Site	Left Colon N=32	Right Colon N=7	
	Median=119,575.0 (IQR=233,720)	Median=339,600.0 (IQR=582032)	P=0.608
Staging	Dukes A+B N=18	Dukes C N=21	
	Median=126,871 (IQR 218,533)	Median=108,121 (IQR 324,625)	P=0.922

Table 7.2: Median DNA in ng isolated from rectal mucus from symptomatic colorectal patients diagnosed with cancer.

7.3.1 Using a three biomarker panel assay to diagnose CRC from DNA isolated from rectal mucus in symptomatic colorectal patients.

The panel of biomarkers was positive in 48 patients (83%). In the majority (35/48) of these positive tests all three markers were methylated (73%). Thirty five patients were correctly identified as having cancer by the biomarker panel assay while 5 cases of cancer had a negative test. Out of the missed cancer cases three had only one methylated marker positive (*TFPI2)* while the remaining two showed no methylation at all. In addition there were thirteen patients that had positive tests but had no cancer, the majority (11) having all three markers methylated. Overall, there were 35 true positives, 5 true negatives, 13 false positives and 5 false negatives. The sensitivity of the three marker test was 87.5% with a specificity of 27.5%. SPSS v19 (IBM Inc, USA) was used to calculate the Kappa measure of agreement[188] along with likelihood ratios and positive and negative predictive values. A slight measure of agreement (K=0.174, p=0.154) was shown between the three marker panel and standard diagnostic tests for CRC . The positive likelihood ratio +LR was 1.21

while negative likelihood ration -LR was 0.45 with a positive predictive value of 72.9% and negative prediction value of 50%.

Marker	Sensitivity	Specificity	PPV	NPV
NDRG4[182]	72.5%	27.8%	69%	80%
TFPI2[183]	92.5%	11.1%	69.8%	40%
GATA4[184]	72.5%	33.3%	72.9%	33%
All three	87.5%	27.5%	72.9%	50%

Table 7.3: Summary of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of DNA methylation assay on DNA isolated from rectal mucus.

The performance of each marker alone was also analysed. *NDRG4* identified correctly 29 patients with cancer (true positives), 5 without cancer (true negative), 13 patients had methylated *NDGR4* but had no cancer (false positives) and 11 patients had un-methylated *NDGR4* but actually had cancer (false negatives). The overall sensitivity was 72.5%, specificity of 27.8% with a slight measure of agreement (K=0.003, p=0.983). PPV was 69%, NNV= 80% and a +LR=1 and LR=0.09. Twenty nine patients were correctly diagnosed with cancer by *GATA4* and 6 were identified as normal. Eleven cases were identified as having cancer but actually had no CRC, while 12 patients were identified as normal but actually had CRC. The sensitivity was 72.5%, specificity of 33.3% with moderate measure of agreement (K=0.59 p=0.652). PPV was 72.9%, NNV= 33% and a +LR=1.1 while -LR=0.83. Finally when using *TFPI2* alone we identified correctly 37 cancers, missed 3, while 2 patients were correctly cleared of CRC and 16 had cancer while the marker was un-methylated. Sensitivity for *TFPI2* was 92.5, specificity 11.1%, with a slight measure of agreement (K=0.045, p=0.650). PPV was 69.8%, NNV= 40% and positive LR of 1.1 and negative LR of 0.68. Table 7.3 summarises and compares sensitivity, specificity PPV and NVP between the three markers and the panel.

7.4 Discussion

The idea of measuring DNA from exfoliated colonocytes in stool as a screening methods has been previously described[187-189]. Two recent studies[180, 181] have also used rectal mucus instead of stool. We showed higher DNA amounts in patients with CRC in agreement with the above two studies. Mahadavan *et al*[191] in 2012 published a large cohort study where 828 patients with symptom of colorectal disease had rectal mucus samples taken and their DNA concentration measured. 10% of those patients were diagnosed with CRC (colonoscopy, CTC or barium enema). The DNA concentration was significantly higher in patients with CRC. In addition, studies have reported secondary distal tumours[177-179] in colorectal cancer patients, via re-implantation of viable tumour cells at sites distal to the tumour. Our findings, the accumulation of DNA in rectal mucosa in cancer patients along with the higher DNA counts found at distal sites up to15cm from the tumour site compared to background sites (chapter 6) and the well described secondary distal metachronus tumours, suggest that there is DNA transported along the MCL in such patients. As previously described[180, 181] staging of CRC does not affect the amount of DNA isolated from the rectal mucus of such patients. Our findings supports that observation as we found no difference in the amount of DNA isolated from patients with Dukes stage A or B compared to C.

In our study there was no significant difference in the amount of DNA isolated from patients with left compared to right lesions which contradicts both of these studies[180, 181] which demonstrated higher counts of DNA in rectal mucus samples from patients with lesions distal to the splenic flexure. However, we too have demonstrated that patients with tumours in the left colon have higher DNA amounts in their rectal mucus compared to controls. Right sided tumours gave rise to an increased amount rectal mucus DNA compared to controls which did not reach statistical significance, in agreement with Loktionov *et al*[180]. One explanation for the difference in DNA detected between right tumours v controls and left tumours v controls is that this could reflect the differences in the biological and oncological characteristics of right and left tumours (MSI v CIN) as previously described[192]. It is possible to suggest that most of the colonocytes exfoliated by right sided tumours have died by the time they reach the rectum as they have a much longer distance to travel on the MCL. As described in section 1.3, exfoliating cancer colonocytes have certain survival advantages (eg loss of anoikis) compared to normal colonocytes that allow them to survive for longer in the colonic lumen. This ‘extra survival time’ could potentially expire by the time such cells reach the rectum if have originated from the right colon, given the longer distance they need to travel. Normal colonic transit times may however be impaired in patients with CRC due to obstructive symptoms or excessive secretion of mucus causing diarrhoea. Further detailed work

would need to be done in order to determine the inter-relation of these factors.

In both of the previously published studies, the authors proposed using quantitative DNA assays as a screening method for colorectal cancer. However to do that, a cut off value of DNA detected needs to be defined above which such a test will be positive. However we have found in our study there was large variation in the amount of DNA detected in both patients with CRC (range 2455-923,000ng) and those without (range 170-152,000ng). In the two previously studies a cut off value was calculated using statistical methodology. Interestingly Mahadavan *et al*[191] *in* 2012 used the cut off DNA values suggested by earlier studies[180, 181] along with other parameters suggestive of CRC such as lower GI bleeding, positive FOBT, age, CEA and male sex in a large cohort study to try to diagnose CRC. They concluded that DNA amount on its own was unreliable to diagnose CRC. In 2013 Bajwa *et al*[193] used rectal mucus DNA quantitate analysis in 467 patients referred with colorectal symptoms. One of the aims of the study was to define a cut off value of total DNA concentration that will attain a specificity of at least 60%. They proposed a value of >1.4mg/l a value different from all three previously published values, highlighting the challenges of DNA quantitative analysis alone as a screening tool for CRC. It is clear from the above that even by using a large number of patients quantitative DNA analysis form rectal mucus is not sufficient to reliably diagnose colorectal neoplasia.

We used the DNA isolated from the rectal mucus of symptomatic patients to test for presence of methylated biomarkers as described above. Our three methylation marker panel test was good in identifying CRC with a sensitivity of 87.5%. Unfortunately we observed a very high rate of false positives in our control group of symptomatic patients, resulting in an unacceptably low specificity of 27.5%. When the performance of each of the markers was analysed individually, we observed again good sensitivity but unfortunately low specificities, reflecting the high false positive rates. The low sensitivity contradicts the published performance[183,186,187] of these markers in DNA isolated from stool from cancer patients and healthy volunteers. Reasons for the higher then expected false positives observed in our study could be due to differences in our patient's demographics and those of the published study populations, or related to the assay used or due to logistical problems.

Our patients were undiagnosed and symptomatic and therefore were referred to a colorectal clinic for urgent investigation. The populations of the three previously published studies were already known to have CRC or not and the control groups were healthy volunteers. In the *NDGR4*[183] and *TFPI2*[186] studies the authors noted a significant difference in age between the CRC and control groups (younger controls compared to CRC patients). In our study the two groups were matched for

age (no significant difference, $p<0.005$). Background aberrant methylation in normal colonic mucosa increases with age[29] and therefore we would expect a slightly higher prevalence of methylation markers in healthy colonic mucosa as age increases. However this age related increase is unlikely to be enough to explain the very high positive false positive results we have observed in our study. It is unlikely that the above differences (age and symptomatic v asymptomatic) in the two populations fully explains the low specificity we have observed. The above previously published[183,186,187] studies validated their methylation assay using a statistical model to identify a cut off value to consider the marker as positively methylated to achieve acceptable sensitivity and specificity. To do so they used training sets of DNA isolated from the stool samples of CRC patients and healthy individuals. It is possible that the methylation cut off value might be different for DNA isolated from mucus. In other words it is plausible that the value at which methylation is positive might be higher for DNA isolated from mucus compared to stool due to more pure/clean DNA isolated. To isolate DNA from stool, a full motion of stool is used which is then processed to isolate human DNA (about 1% of total DNA isolated) and to neutralise PCR inhibitors. In contrast, in our method of collection, only a very tiny amount of rectal mucus is required and a very simple process (Chapter 4) is used to isolate large amounts of human DNA. If we assume that a higher threshold for methylation positivity is required then that could explain our low specificity as well as our slightly higher sensitivity rates. A validation study where a threshold is calculated for DNA isolated from mucus will confirm or reject this explanation. Finally technical or logistical mistakes are very unlikely as the methylation assays were performed in the laboratory that published two[183,186] out of the three studies on these markers, by a very experienced scientist who was co-author in both previous publications. A mislabelling mistake is also unlikely as the samples were labelled at the time of collection and DNA in each sample was isolated and its concentration measured in the UK and then sent to the The Netherlands. The quantitive DNA results (section 7.3.1) suggest that there was no mislabeling between CRC and controls and in addition the results from The Netherlands were reported using the original UK samples identification system.

In conclusion our three methylation marker panel has not performed as expected and was not able to reliable identify and exclude CRC in our study population. Further work (see chapter 8) will identify if this is due to the sampling technique or the markers used.

8. Conclusion and future work

Most current early detection and screening strategies for CRC use stool samples to look for blood DNA or other biomarkers. It is a fairly novel concept to use rectal mucus as the material of choice. This project has explored the feasibility of obtaining reliable rectal mucus samples in order to obtain DNA and look for biomarkers. There are several major problems which will need to be overcome in order to translate this concept into a clinical tool. Firstly, rectal mucus may be contaminated with stool, blood and or rectal mucosa tissue and therefore obtaining rectal mucus may be no better than directly sampling the mucosa, stool or blood. Secondly, rectal mucus may contain DNA from various sources including exfoliated colonocytes, blood, bacteria, ingested materials or contamination from collecting devices.

Exfoliated colonocytes or tissue and blood dislodged from the surface of a tumour may all be useful in contributing to the DNA extracted from rectal mucus for diagnostic purposes. Blood or mucosa from the normal rectal epithelium dislodged by trauma from the collecting device may confound the results of such tests. DNA from non human sources does not have the same light absorption properties as human DNA and therefore specimens contaminated with significant amounts of these types of DNA would be excluded from further analysis when the purity of DNA samples was tested.

Collection and storage of rectal mucus

In order to test our hypothesis we first needed to prove that it was possible to collect, safely store and accurately measure the DNA content of colonic mucus. The result of our first set of experiments (chapter 4) showed that it is possible to reliably use a piece of latex glove to sample colonic mucus and extract its DNA content. The results were reproducible when we repeated the experiment. In addition we took repeated measurements at a specific location within the colonic specimens and got consistent DNA counts indicating that the total amount of DNA in the colonic mucosa is uniformly distributed around the circumference of the colon.

The total DNA extracted from rectal mucus may include DNA from several sources (see above) as well as the colonocytes exfoliated from the surface mucosa. It would be useful to perform cytological examination of mucus smears in parallel with DNA assay from the same mucus sample in order to check that at least some of the DNA obtained originated in colonocytes.

We also demonstrated that cell lysis buffer should be used to store our collecting device prior to DNA extraction. These results allowed us to proceed to the next stage of the project to test our hypothesis.

Collection Device

The next step was to identify which material was the best for yielding the highest DNA counts in mucus sampled from fresh colonic specimens excised from patients with CRC. The reasons why the three materials were chosen have been described earlier in chapter 5, but in summary a cytology brush was used as positive control, a piece of latex glove as it would simulate a device previously used in published studies[180, 181] to sample rectal mucus and a piece of foam as potential novel material. Ideally it would have been useful to use the same devices as described in Loktionov's seminal papers[180, 181]. However unfortunately this device was not commercially available during the course of this project

As predicted both glove and foam yielded significantly lower amounts of DNA compared to the positive control. Interestingly foam significantly out performed the glove device by yielding double the DNA amount compared to glove. We proposed that these observations were due to the differences in the effective surface of each material (surface area of brush>foam>glove) and not as a result of increased friction to the underlying mucosa and hence possible direct sampling of colonocytes). Our hypothesis states that it is possible to detect DNA from proximal sites in the colon by sampling rectal mucus in patients with colorectal cancer. As a result we repeated the analysis of the results by excluding measurements taken directly over tumour sites to try and simulate the future clinical application of such devices. The performance of each material was comparable to our initial analysis although all three materials yielded less DNA than expected compared to the initial analysis.

In conclusion the brush although initially used as a positive control, could indeed be the best material to collect mucus. Foam was almost twice as good as glove in yielding DNA from colonic mucus samples. This advantage could be simply due to the larger surface area of a brush and piece of foam compared to a piece of glove.

Despite the fact that glove was the least efficient way to collect mucus DNA, we decided to use a rectal tube catheter (made of latex and hence more similar to glove) for our clinical experiments (chapter 7). There are several reasons for this decision. It is a device regularly used by colorectal surgeons to drain/wash the rectum, it is acceptable to patients (easier to recruit patients) and therefore more likely to be approved by the ethics committee, and finally it would be very time consuming and costly to design, test and get approval for a novel device made of foam in the shape of a brush to use in patients. At the same time we used foam and glove to try an establish if there is

a gradient of DNA concentration in colonic mucus from the tumour site proximally or distally, as an internal control.

DNA gradient in colorectal mucus

This study has demonstrated that there is a significantly higher count of DNA in the mucus over the tumour site compared to both adjacent and distant sites along the colon. Interestingly there is no significant difference in the amount of DNA found in the mucus within 15-20cm either proximal or distal to the tumour. This observation implies a 'volcano' effect of cell exfoliation from tumours as described in chapter 6.

DNA counts were significantly higher at sites distal to the tumour compared to background DNA counts taken at >20cm proximal to the tumour. In addition, as detailed in chapter 7, we found that DNA counts were higher in rectal mucus of patients with colorectal cancer compared to control patients. These findings, along with the previously described phenomenon of distal seeding of metachronus tumours[177-179], suggest that DNA is transported along the MCL in CRC patients. As discussed in chapter 6 there are some limitations in our experimental model as the maximum proximal and distal distances from tumour were dictated by the length of the resected specimen. Ideally we would have preferred to have taken samples along the entire length of the colon in each patient but that would only have been possible if the patients underwent a total colectomy. This operation is only indicated for synchronous tumours, in patients with polyposis syndromes or cancers arising in inflammatory bowel disease, all of which would have different exfoliation characteristics to a single site tumour.

This study is based upon the assumption that colorectal cancers shed cells containing DNA that can be extracted from rectal mucus, and that a higher total DNA count obtained from rectal mucus may indicate the presence of a proximal tumour. However, there is no evidence to confirm or refute the hypothesis that normal individuals may have a gradient of DNA concentrations in the MCL from proximal to distal colon, with the highest level in their rectal mucus due to normal exfoliation of intestinal mucosa. Therefore it may be more appropriate to consider the relative amounts of DNA from proximal to distal in an individual colon or between CRC patients and controls at a given site such as the rectum, rather than absolute amounts of DNA extracted.

Rectal mucus DNA yield in CRC

If rectal mucus is ever to be used as a screening or early diagnosis sample, it is necessary to show that either the absolute or relative amounts of rectal mucus DNA are significantly different between CRC patients and controls. We demonstrated higher rectal mucus DNA in CRC compared to controls, which was not affected by tumour staging or age of the patients. However the site of the tumour did influence rectal DNA counts. Left sided tumours had significantly higher rectal mucus DNA counts compared to controls. These observations were in agreement with published studies[180,181]. For right sided tumours there was an increased level of rectal mucus DNA compared to controls but this did not reach statistical significance. These finding may be due to the relatively small number of cases in this study and a larger population would need to be studied in order to confirm or refute this.

The majority of cancers arise via the adenoma carcinoma sequence and current screening programs aim to detect adenomas which can be removed in preference to established cancers. Due to the small number of cases in this study, we did not attempt to distinguish between adenoma and other non malignant colorectal disorders. We performed a proof of principle study to try to distinguish between cancer and non cancer patients. We also analysed Dukes C cancers separately to the earlier stages. This project has shown that although there was a significant difference in the amount of rectal mucus DNA between CRC and non CRC samples, there was no significant difference between the different Dukes stages.

As discussed in chapter 7, there have been previous attempts to identify a cut off value of rectal mucus DNA which would distinguish between CRC and controls. This would have been an ideal simple screening method. However, there is high variation in the amounts of DNA isolated from rectal mucus, an observation we also confirmed. Two recent studies[191, 193] attempted to establish a way of combining quantitative DNA assays using rectal mucus sampling along with other clinical parameters to produce a predictive score. These concluded that due to the variation in quantitative DNA assays, such scores were not a reliable method for CRC screening.

Since total DNA assays are not a useful screening tool, it may be possible that biomarkers in the rectal mucus DNA could be used to distinguish between CRC and controls.

Methylation markers

A small number of groups worldwide have studied methylation markers in stool based tests[123,124,183,186,187]. However to our knowledge no group has studied these markers in rectal mucus. This project has therefore collaborated with the Maastricht group who performed some of the

experimental work described in chapter 7. The three methylation marker panel test was good at identifying CRC with a sensitivity of 87.5%. Unfortunately we observed a very high rate of false positives in our control group, resulting in an unacceptably low specificity of 27.5%. When the performance of each of the markers was analysed individually, we again observed good sensitivity but low specificities, reflecting the high false positive rates. The low specificity in our study contradicts the published performance[183,186,187] of these markers in DNA isolated from stool in cancer patients and healthy volunteers. We concluded that our three methylation marker panel has not performed as expected and was not able to reliable identify and exclude CRC in our study population. The very high false positive rates we observed amy be due to the fact that our control population was symptomatic colorectal clinic patients rather healthy volunteers. Non malignant colorectal disorders in this group may have confounded the results. Secondly we have assumed that the DNA we have isolated from the rectal mucus comes directly from the tumour, but exfoliated colonocytes from other areas of the colon along with blood and contaminates may have contributed to the specific methylation profile obtained in our samples. In addition, the high positive rates (resulting in marginally higher sensitivity and significantly lower specificity) we observed in our study compared to the previously published studies[183,186,187] could be explained by the way the methylation assay was calibrated using DNA extracted from stool instead of mucus as discussed in chapter 7, i.e. the methylation threshold might need to be raised for DNA isolated from mucus compared to that used for stool.

In summary, we have shown that it is possible to sample and measure the DNA content of colonic mucus and that the best material to do so would be a foam based device in a shape that would be clinically applicable (patient compliant, atraumatic etc) and that maximises the effective surface in contact with colonic mucus. There is a ‘volcano’ DNA exfoliation from tumours with DNA accumulating distally in the rectum of CRC patients which can be sampled and isolated using a latex ballon device during standard proctoscopy. Unfortunately the three methylation marker panel we used was not able to reliably distinguish between CRC and controls in our study population. Further work (see below) will answer whether this failure was due to sampling technique or marker limitations.

Future work

This study has opened up numerous areas for future scientific and clinical research.

It would be useful to establish the presence or absence of a gradient for the DNA in the MCL in different sections of the colon in healthy individuals. This would give all future studies a reference point.

It would be interesting to use DNA from CRC tissue blocks in combination with matched rectal mucus DNA samples, to test for the presence of common genetic markers (e.g. Mutant *APC, p53* and *KRAS*, as the three most commonly found genetic markers in CRC) to confirm a correlation. If the two sets of DNA express the same markers, it will indicate that the DNA sampled in the rectum had originated from the tumour and therefore sampling rectal mucus is a technique worth exploring further as a method of CRC screening.

Similarly methylation markers could be compared between matched CRC tissue and rectal mucus specimens. Numerous methylation markers have been described and future may identify more appropriate markers to use in early colorectal neoplasia.

Due to differential DNA yield between stool samples and rectal mucus samples, the methylation assays conditions and thresholds may need to be modified in order to obtain significant results. Large sample populations will be required in order to determine these parameters.

If these scientific studies show promising results the next step will be to develop a device to sample rectal mucus which is well tolerated by patients and easy to use in clinic or at home. A device with a foam surface on a proctological instrument or catheter base would appear to be the most likely design but several prototypes will need to be tested.

Although early detection of CRC is useful, screening ideally identifies adenoma prior to malignant transformation. Therefore large studies in conjunction with the current National Bowel Screening program should be undertaken in order to obtain enough patients with various stages of adenoma and to identify whether progressive genetic changes can be identified in their rectal mucus DNA.

9. References

1. Cancer Statistics registrations: registrations of cancer diagnosed in 2008, UK. http://publications.cancerresearchuk.org/downloads/Product/CS_REPORT_WORLD.pdfhttp://www.isdscotland.org/isd/183.htmlhttp://www.wales.nhs.uk/sites3/page.cfm?orgid=242&pid=35385http://www.qub.ac.uk/research-centres/nicr/Data/OnlineStatistics/, Accessed July 2011.
2. International Agency for Research on Cancer website www.iarc.fr, Accessed July 2011.
3. Coleman M, Esteve J, Damiecki P, et al.Trends in Cancer Incidence and Mortality. IARC Scientific Publications.Vol. 121. Lyon: IARC, 1993.
4. Bray F, Atkin W. International cancer patterns in men: geographical and temporal variations in cancer risk and the role of gender. jmhg. 2004;1:38–46
5. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. CA Cancer J Clin. 2008 Mar-Apr; 58(2):71-96. Epub 2008 Feb 20.
6. Chlebowski RT, Wactawski-Wende J, Ritenbaugh C, et al.Estrogen plus progestin and colorectal cancer in postmenopausal women. N Engl J Med. 2004 Mar 4;350(10):991-1004.
7. Rostom A, Dubé C, Lewin G, et al. Nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 inhibitors for primary prevention of colorectal cancer: a systematic review prepared for the U.S. Preventive Services Task Force. Ann Intern Med. 2007 Mar 6;146(5):376-89.
8. International Agency for Research on Cancer website: www.iarc.fr/en/publications/pdfs-online/epi/sp160/CI5vol9-A.pdf Accessed July 2011
9. Office of National Statistics website www.statistics.gov.uk Accessed July 2011
10. Fernandez E, La Vecchia C, González JR, et al. Converging patterns of colorectal cancer mortality in Europe. Eur J Cancer. 2005 Feb;41(3):430-7.
11. Vogelstein B, Fearon ER, Hamilton SR, et al. Genetic alterations during colorectal-tumor development. N Engl J Med. 1988 Sep 1;319(9):525-32.
12. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell. 1990 Jun 1;61 (5):759-67.
13. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. Nature. 1998 Dec 17;396(6712):643-9.
14. Pino MS, Chung DC.The chromosomal instability pathway in colon cancer. Gastroenterology. 2010 Jun;138(6):2059-72.
15. Miyaki M, Konishi M, Kikuchi-Yanoshita R, et al. Characteristics of somatic mutation of the adenomatous polyposis coli gene in colorectal tumors.Cancer Res. 1994 Jun 1;54(11): 3011-20.

16. Cottrell S, Bicknell D, Kaklamanis L et al. Molecular analysis of APC mutations in familial adenomatous polyposis and sporadic colon carcinomas. Lancet. 1992 Sep 12;340(8820): 626-30.
17. Powell SM, Zilz N, Beazer-Barclay Y et al. APC mutations occur early during colorectal tumorigenesis. Nature. 1992 Sep 17;359(6392):235-7.
18. Esteller M, Sparks A, Toyota M et al. Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. Cancer Res. 2000 Aug 15;60(16):4366-71.
19. Santini D, Loupakis F, Vincenzi B, et al.High concordance of KRAS status between primary colorectal tumors and related metastatic sites: implications for clinical practice. Oncologist 2008;13:1270-1275
20. Leslie A, Carey FA, Pratt NR, et al. The colorectal adenoma-carcinoma sequence. Br J Surg. 2002 Jul;89(7):845-60.
21. Eberhart CE, Coffey RJ, Radhika A, et al. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas.Gastroenterology. 1994 Oct;107(4):1183-8.
22. Greenhough A, Smartt HJ, Moore AE, et al.The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. Carcinogenesis. 2009 Mar;30(3):377-86
23. Ionov Y, Peinado MA, Malkhosyan S, et al.Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis.Nature. 1993 Jun 10;363 (6429):558-61.
24. Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. Science. 1993 May 7;260(5109):816-9.
25. Boland CR, Goel A. Microsatellite instability in colorectal cancer.Gastroenterology. 2010 Jun;138(6):2073-2087.
26. Kane MF, Loda M, Gaida GM, et al.Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines.Cancer Res. 1997 Mar 1;57(5):808-11.
27. Wang L, Cunningham JM, Winters JL, et al. BRAF mutations in colon cancer are not likely attributable to defective DNA mismatch repair. Cancer Res. 2003 Sep 1;63(17):5209-12.
28. Boland CR, Thibodeau SN, Hamilton SR, et al.A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of

international criteria for the determination of microsatellite instability in colorectal cancer.Cancer Res. 1998 Nov 15;58(22):5248-57.

29. Kim MS, Lee J, Sidransky D. DNA methylation markers in colorectal cancer. Cancer Metastasis Rev. 2010 Mar;29(1):181-206
30. Toyota M, Ahuja N, Ohe-Toyota M, et al. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci U S A. 1999 Jul 20;96(15):8681-6.
31. Shen L, Toyota M, Kondo Y, et al. Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. Proc Natl Acad Sci U S A. 2007 Nov 20;104(47): 18654-9.
32. Yagi K, Akagi K, Hayashi H, et al. Three DNA methylation epigenotypes in human colorectal cancer. Clin Cancer Res. 2010 Jan 1;16(1):21-33.
33. Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology. 2007 Jan;50(1):113-30.
34. Cheng YW, Pincas H, Bacolod MD, et al.CpG island methylator phenotype associates with low-degree chromosomal abnormalities in colorectal cancer. Clin Cancer Res. 2008 Oct 1;14(19):6005-13.
35. Issa JP. Colon cancer: it's CIN or CIMP. Clin Cancer Res. 2008 Oct 1;14(19):5939-40.
36. Suzuki K, Suzuki I, Leodolter A, et al. Global DNA demethylation in gastrointestinal cancer is age dependent and precedes genomic damage. Cancer Cell. 2006 Mar;9(3):199-207.
37. Lichtenstein P, Holm NV, Verkasalo PK, et al. Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. N Engl J Med. 2000 Jul 13;343(2):78-85.
38. Grady WM.Genetic testing for high-risk colon cancer patients.Gastroenterology. 2003 May; 124(6):1574-94.
39. Jasperson KW, Tuohy TM, Neklason DW, et al. Hereditary and familial colon cancer. Gastroenterology. 2010 Jun;138(6):2044-58.
40. Lynch HT, Shaw MW, Magnuson CW, et al. Hereditary factors in cancer. Study of two large midwestern kindreds. Arch Intern Med. 1966 Feb;117(2):206-12.
41. Hampel H, Frankel WL, Martin E, et al. Feasibility of screening for Lynch syndrome among patients with colorectal cancer. J Clin Oncol. 2008 Dec 10;26(35):5783-8. doi: 10.1200/ JCO.2008.17.5950.

42. Hampel H, Frankel W, Panescu J, et al. Screening for Lynch syndrome (hereditary nonpolyposis colorectal cancer) among endometrial cancer patients. Cancer Res. 2006 Aug 1;66(15):7810-7.
43. Stoffel E, Mukherjee B, Raymond VM, et al.Calculation of risk of colorectal and endometrial cancer among patients with Lynch syndrome. Gastroenterology. 2009 Nov;137 (5):1621-7.
44. Aarnio M, Sankila R, Pukkala E, et al. Cancer risk in mutation carriers of DNA-mismatch-repair genes. Int J Cancer. 1999 Apr 12;81(2):214-8.
45. Vasen HF, Watson P, Mecklin JP, et al. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. Gastroenterology. 1999 Jun;116(6):1453-6.
46. Lindor NM, Rabe K, Petersen GM, et al. Lower cancer incidence in Amsterdam-I criteria families without mismatch repair deficiency: familial colorectal cancer type X. JAMA. 2005 Apr 27;293(16):1979-85.
47. Llor X, Pons E, Xicola RM, et al. Differential features of colorectal cancers fulfilling Amsterdam criteria without involvement of the mutator pathway. Clin Cancer Res. 2005 Oct 15;11(20):7304-10.
48. Umar A, Boland CR, Terdiman JP, et al. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst. 2004 Feb 18;96(4):261-8.
49. Burt RW, Leppert MF, Slattery ML, et al. Genetic testing and phenotype in a large kindred with attenuated familial adenomatous polyposis. Gastroenterology. 2004 Aug;127(2): 444-51.
50. Nieuwenhuis MH, Vasen HF. Correlations between mutation site in APC and phenotype of familial adenomatous polyposis (FAP): a review of the literature. Crit Rev Oncol Hematol. 2007 Feb;61(2):153-61.
51. Knudsen AL, Bisgaard ML, Bülow S. Attenuated familial adenomatous polyposis (AFAP). A review of the literature. Fam Cancer. 2003;2(1):43-55.
52. Burt RW. Gastric fundic gland polyps. Gastroenterology. 2003 Nov;125(5):1462-9.
53. Bülow S, Björk J, Christensen IJ, et al. Duodenal adenomatosis in familial adenomatous polyposis. Gut. 2004 Mar;53(3):381-6.

54. Speake D, Evans DG, Lalloo F, et al. Desmoid tumours in patients with familial adenomatous polyposis and desmoid region adenomatous polyposis coli mutations. Br J Surg. 2007 Aug;94(8):1009-13.
55. Lubbe SJ, Di Bernardo MC, Chandler IP, et al. Clinical implications of the colorectal cancer risk associated with MUTYH mutation. J Clin Oncol. 2009 Aug 20;27(24):3975-80.
56. Boparai KS, Dekker E, Van Eeden S, et al. Hyperplastic polyps and sessile serrated adenomas as a phenotypic expression of MYH-associated polyposis. Gastroenterology. 2008 Dec;135(6):2014-8.
57. McGraw-Hill Concise Dictionary of Modern Medicine. © 2002 by The McGraw-Hill Companies, Inc.
58. Schreibman IR, Baker M, Amos C, et al. The hamartomatous polyposis syndromes: a clinical and molecular review. Am J Gastroenterol. 2005 Feb;100(2):476-90.
59. McGarrity TJ, Amos C. Peutz-Jeghers syndrome: clinicopathology and molecular alterations. Cell Mol Life Sci. 2006 Sep;63(18):2135-44.
60. Gammon A, Jasperson K, Kohlmann W, et al. Hamartomatous polyposis syndromes. Best Pract Res Clin Gastroenterol. 2009;23(2):219-31.
61. Brosens LA, van Hattem A, Hylind LM, et al. Risk of colorectal cancer in juvenile polyposis. Gut. 2007 Jul;56(7):965-7.
62. Johns LE, Houlston RS. A systematic review and meta-analysis of familial colorectal cancer risk. Am J Gastroenterol. 2001 Oct;96(10):2992-3003.
63. Tenesa A, Dunlop MG. New insights into the aetiology of colorectal cancer from genome-wide association studies. Nat Rev Genet. 2009 Jun;10(6):353-8.
64. Burkitt DP. Related disease--related cause? Lancet. 1969 Dec 6;2(7632):1229-31.
65. Trock B, Lanza E, Greenwald P. Dietary fiber, vegetables, and colon cancer: critical review and meta-analyses of the epidemiologic evidence. J Natl Cancer Inst. 1990 Apr 18;82(8): 650-61.
66. Howe GR, Benito E, Castelleto R, et al. Dietary intake of fiber and decreased risk of cancers of the colon and rectum: evidence from the combined analysis of 13 case-control studies. J Natl Cancer Inst. 1992 Dec 16;84(24):1887-96.
67. Park Y, Hunter DJ, Spiegelman D, et al. Dietary fiber intake and risk of colorectal cancer: a pooled analysis of prospective cohort studies. JAMA. 2005 Dec 14;294(22):2849-57.

68. van Duijnhoven FJ, Bueno-De-Mesquita HB, Ferrari P, et al. Fruit, vegetables, and colorectal cancer risk: the European Prospective Investigation into Cancer and Nutrition. Am J Clin Nutr. 2009 May;89(5):1441-52.
69. Schatzkin A, Lanza E, Corle D, et al. Lack of effect of a low-fat, high-fiber diet on the recurrence of colorectal adenomas. Polyp Prevention Trial Study Group. N Engl J Med. 2000 Apr 20;342(16):1149-55.
70. Alberts DS, Martínez ME, Roe DJ, et al. Lack of effect of a high-fiber cereal supplement on the recurrence of colorectal adenomas. Phoenix Colon Cancer Prevention Physicians' Network. N Engl J Med. 2000 Apr 20;342(16):1156-62.
71. Bonithon-Kopp C, Kronborg O, Giacosa A, et al. Calcium and fibre supplementation in prevention of colorectal adenoma recurrence: a randomised intervention trial. European Cancer Prevention Organisation Study Group. Lancet. 2000 Oct 14;356(9238):1300-6.
72. Norat T, Bingham S, Ferrari P, et al. Meat, fish, and colorectal cancer risk: the European Prospective Investigation into cancer and nutrition. J Natl Cancer Inst. 2005 Jun 15;97(12): 906-16.
73. Chan AT, Giovannucci EL. Primary prevention of colorectal cancer. Gastroenterology. 2010 Jun;138(6):2029-2043.
74. Gnagnarella P, Gandini S, La Vecchia C, et al. Glycemic index, glycemic load, and cancer risk: a meta-analysis. Am J Clin Nutr. 2008 Jun;87(6):1793-801.
75. Higginbotham S, Zhang ZF, Lee IM, et al. Dietary glycemic load and risk of colorectal cancer in the Women's Health Study. J Natl Cancer Inst. 2004 Feb 4;96(3):229-33.
76. Michaud DS, Fuchs CS, Liu S, et al. Dietary glycemic load, carbohydrate, sugar, and colorectal cancer risk in men and women. Cancer Epidemiol Biomarkers Prev. 2005 Jan;14 (1):138-47.
77. Giovannucci E, Stampfer MJ, Colditz GA, et al. Folate, methionine, and alcohol intake and risk of colorectal adenoma. J Natl Cancer Inst. 1993 Jun 2;85(11):875-84.
78. Ferrari P, Jenab M, Norat, et al. Lifetime and baseline alcohol intake and risk of colon and rectal cancers in the European prospective investigation into cancer and nutrition (EPIC). Int J Cancer. 2007 Nov 1;121(9):2065-72.
79. Choi SW, Stickel F, Baik HW, et al. Chronic alcohol consumption induces genomic but not p53-specific DNA hypomethylation in rat colon. J Nutr. 1999 Nov;129(11):1945-50.
80. Kune GA, Kune S, Read A, et al. Colorectal polyps, diet, alcohol, and family history of colorectal cancer: a case-control study. Nutr Cancer. 1991;16(1):25-30.

81. Chao A, Thun MJ, Jacobs EJ, et al. Cigarette smoking and colorectal cancer mortality in the cancer prevention study II. J Natl Cancer Inst. 2000 Dec 6;92(23):1888-96.
82. Heineman EF, Zahm SH, McLaughlin JK, et al. Increased risk of colorectal cancer among smokers: results of a 26-year follow-up of US veterans and a review. Int J Cancer. 1994 Dec 15;59(6):728-38.
83. Newcomb PA, Storer BE, Marcus PM. Cigarette smoking in relation to risk of large bowel cancer in women. Cancer Res. 1995 Nov 1;55(21):4906-9.
84. Hannan LM, Jacobs EJ, Thun MJ. The association between cigarette smoking and risk of colorectal cancer in a large prospective cohort from the United States. Cancer Epidemiol Biomarkers Prev. 2009 Dec;18(12):3362-7.
85. Tsoi KK, Pau CY, Wu WK, et al. Cigarette smoking and the risk of colorectal cancer: a meta-analysis of prospective cohort studies. Clin Gastroenterol Hepatol. 2009 Jun;7(6): 682-688.
86. Liang PS, Chen TY, Giovannucci E. Cigarette smoking and colorectal cancer incidence and mortality: systematic review and meta-analysis. Int J Cancer. 2009 May 15;124(10): 2406-15.
87. International Agency for Research on Cancer http://monographs.iarc.fr/ENG/Monographs/vol100E/mono100E-6.pdf, Accessed August 2011
88. Larsson SC, Wolk A. Obesity and colon and rectal cancer risk: a meta-analysis of prospective studies. Am J Clin Nutr. 2007 Sep;86(3):556-65.
89. Giovannucci E, Colditz GA, Stampfer MJ, et al. Physical activity, obesity, and risk of colorectal adenoma in women (United States). Cancer Causes Control. 1996 Mar;7(2): 253-63.
90. Giovannucci E. Insulin and colon cancer. Cancer Causes Control. 1995 Mar;6(2):164-79.
91. Jenab M, Riboli E, Cleveland RJ, et al. Serum C-peptide, IGFBP-1 and IGFBP-2 and risk of colon and rectal cancers in the European Prospective Investigation into Cancer and Nutrition. Int J Cancer. 2007 Jul 15;121(2):368-76.
92. Limburg PJ, Vierkant RA, Fredericksen ZS, et al. Clinically confirmed type 2 diabetes mellitus and colorectal cancer risk: a population-based, retrospective cohort study. Am J Gastroenterol. 2006 Aug;101(8):1872-9.
93. Limburg PJ, Anderson KE, Johnson TW et al. Diabetes Mellitus and sub site specific CRC risk.. Cancer Epidemiol Biomarkers Prev 2005;14:133-137.

94. La Vecchia C, Negri E, Decarli A, et al. Diabetes mellitus and CRC risk Cancer Epidemiol Biomarkers Prev 1997;6:1007-1010.
95. Thun MJ, Calle EE, Namboodiri MM, et al. Risk factors for fatal colon cancer in a large prospective study. J Natl Cancer Inst. 1992 Oct 7;84(19):1491-500.
96. Slattery ML, Potter J, Caan B, et al. Energy balance and colon cancer--beyond physical activity. Cancer Res. 1997 Jan 1;57(1):75-80.
97. Wolin KY, Yan Y, Colditz GA, et al. Physical activity and colon cancer prevention: a meta-analysis. Br J Cancer. 2009 Feb 24;100(4):611-6.
98. Dowse GK, Zimmet PZ, Gareeboo H, et al. Abdominal obesity and physical inactivity as risk factors for NIDDM and impaired glucose tolerance in Indian, Creole, and Chinese Mauritians. Diabetes Care. 1991 Apr;14(4):271-82.
99. Regensteiner JG, Mayer EJ, Shetterly SM, et al. Relationship between habitual physical activity and insulin levels among nondiabetic men and women. San Luis Valley Diabetes Study. Diabetes Care. 1991 Nov;14(11):1066-74.
100. Flossmann E, Rothwell PM. Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies. Lancet. 2007 May 12;369 (9573):1603-13.
101. Rothwell PM, Wilson M, Elwin CE, et al. Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials. Lancet. 2010 Nov 20;376(9754):1741-50.
102. Rothwell PM, Fowkes FG, Belch JF, et al. Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. Lancet. 2011 Jan 1;377(9759):31-41.
103. Dubois RN. Nonsteroidal antiinflammatory drugs, cyclooxygenase-2, and colorectal cancer prevention. Curr Opin Gastroenterol. 2001 Jan;17(1):65-71.
104. Eberhart CE, Coffey RJ, Radhika A, et al. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology. 1994 Oct;107(4):1183-8.
105. Cuzick J, Otto F, Baron JA, et al. Aspirin and non-steroidal anti-inflammatory drugs for cancer prevention: an international consensus statement. Lancet Oncol. 2009 May;10(5): 501-7.

106.Bipat S, Glas AS, Slors FJ, et al. Rectal cancer: local staging and assessment of lymph node involvement with endoluminal US, CT, and MR imaging--a meta-analysis. Radiology. 2004 Sep;232(3):773-83.

107.Dukes CE: Cancer of the rectum: An analysis of 1000 cases. J Pathol 50:527-539, 1940

108.www.ncin.org.uk/publications/data_briefings/colorectal_cancer_survival_by_stage.aspx Accessed July 2011

109.AJCC Cancer Staging Manual (seventh ed.). https://cancerstaging.org/references-tools/quickreferences/Pages/default.aspx.

110.Gunderson LL, Jessup JM, Sargent DJ, et al. Revised TN categorization for colon cancer based on national survival outcomes data. J Clin Oncol. 2010 Jan 10;28(2):264-71.

111.Gunderson LL, Jessup JM, Sargent DJ, et al. Revised tumor and node categorization for rectal cancer based on surveillance, epidemiology, and end results and rectal pooled analysis outcomes. J Clin Oncol. 2010 Jan 10;28(2):256-63.

112.Commission on Chronic Illness (1957) Chronic illness in the United States: Volume I. Prevention of chronic illness, Cambridge, Mass., Harvard University Press, p. 45

113. Wilson JMG, Jungner G. (1968) Principles and practice of screening for disease (large pdf) http://whqlibdoc.who.int/php/WHO_PHP_34.pdf accessed December 2011

114.Whitlock EP, Lin JS, Liles E, et al. Screening for colorectal cancer: a targeted, updated systematic review for the U.S. Preventive Services Task Force. Ann Intern Med. 2008 Nov 4;149(9):638-58.

115.Hewitson P, Glasziou PP, Irwig L, et al. Screening for colorectal cancer using the faecal occult blood test, Hemoccult. The Cochrane Library http://onlinelibrary.wiley.com/store/10.1002/14651858.CD001216.pub2/asset/CD001216.pdfv=1&t=hp8y5k7c&s=4555ac1faf7ed0f1a6f3431f4914dc1423d92181 Accessed February 2012

116.Hewitson P, Glasziou P, Watson E, et al. Cochrane systematic review of colorectal cancer screening using the fecal occult blood test(hemoccult): an update. Am J Gastroenterol. 2008 Jun;103(6):1541-9

117.Morikawa T, Kato J, Yamaji Y, et al. Sensitivity of immunochemical fecal occult blood test to small colorectal adenomas. Am J Gastroenterol. 2007 Oct;102(10):2259-64.

118.van Rossum LG, van Rijn AF, Laheij RJ, et al. Random comparison of guaiac and immunochemical fecal occult blood tests for colorectal cancer in a screening population. Gastroenterology. 2008 Jul;135(1):82-90.

119.Hol L, van Leerdam ME, van Ballegooijen M, et al. Screening For Colorectal Cancer; Randomised Trial Comparing Guaiac-Based And Immunochemical Faecal Occult Blood Testing And Flexible Sigmoidoscopy.Gut. 2010 Jan;59(1):62-8.

120.Wood LD, Parsons DW, Jones S, et al. The genomic landscapes of human breast and colorectal cancers. Science 2007;318: 1108–1113.

121.Sjoblom T, Jones S, Wood LD, et al. The consensus coding sequences of human breast and colorectal cancers. Science 2006;314:268–274.

122.Sidransky D, Tokino T, Hamilton SR, et al. Identification of ras oncogene mutations in the stool of patients with curable colorectal tumors. Science 1992;256:102–105.

123.Imperiale Ransohoff DF, Itzkowitz SH, et al. Fecal DNA versus fecal occult blood for colorectal-cancer screening in an average-risk population.
N Engl J Med 2004;351:2704–2714.

124.Ahlquist DA Sargent DJ, Loprinzi CL, et al. Stool DNA and occult blood testing for screen detection of colorectal neoplasia. Ann Intern Med 2008;149:441.

125.Levin B, Lieberman DA, McFarland B, et al. Screening and Surveillance for the Early Detection of Colorectal Cancer and Adenomatous Polyps, 2008: A Joint Guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. CA Cancer J Clin 2008;58:130–160.

126.Rex DK, Johnson DA, Anderson JC, et al. American College of Gastroenterology guidelines for colorectal cancer screening 2009 [corrected]. Am J Gastroenterol 2009;104:739–750.

127.Atkin WS, Edwards R, Kralj-Hans I, et al. Once-only flexible sigmoidoscopy screening in prevention of colorectal cancer: a multicentre randomised controlled trial. Lancet. 2010 May 8;375(9726):1624-33.

128.Hoff G, Grotmol T, Skovlund E, et al. Risk of colorectal cancer seven years after flexible sigmoidoscopy screening: randomised controlled trial. BMJ. 2009;338:b1846.

129.Segnan N, Senore C, Andreoni B, et al. Baseline findings of the Italian multicenter randomized controlled trial of "once-only sigmoidoscopy"--SCORE. J Natl Cancer Inst. 2002 Dec 4;94(23):1763-72.

130.Weissfeld JL, Schoen RE, Pinsky PF, et al. Flexible sigmoidoscopy in the PLCO cancer screening trial: results from the baseline screening examination of a randomized trial. J Natl Cancer Inst. 2005 Jul 6;97(13):989-97.

131.Levin TR, Farraye FA, Schoen RE, et al. Qualifying the technical performance of screening flexible sigmoidoscopy:recommendations of an international multi-society task group. Gut. 2005Jun;54(6):807-13.

132.Nelson DB, McQuaid KR, Bond JH, et al. Procedural success and complications of large-scale screening colonoscopy. Gastrointest Endosc. 2002 Mar;55(3):307-14.

133.Regula J, Rupinski M, Kraszewska E, et al. Colonoscopy in colorectal-cancer screening for detection of advanced neoplasia. N Engl J Med. 2006 Nov 2;355(18):1863-72.

134.NHSBCSP Quility assurance for colonoscopy Nov 2011http://www.cancerscreening.nhs.uk/bowel/publications/nhsbcsp06.pdf Accessed March 2012

135.van Rijn JC, Reitsma JB, Stoker J, et al. Polyp miss rate determined by tandem colonoscopy: a systematic review. Am J Gastroenterol. 2006 Feb;101(2):343-50.

136.Rex DK, Petrini JL, Baron TH, Chak A, Cohen J, Deal SE, et al. Quality indicators for colonoscopy. Gastrointest Endosc. 2006 Apr;63(4 Suppl):S16-S28.

137.Brenner H, Chang-Claude J, Seiler CM, et al. Long-term risk of colorectal cancer after negative colonoscopy.J Clin Oncol. 2011 Oct 1;29(28):3761-7.

138.Singh H, Turner D, Xue L, et al. Risk of developing colorectal cancer following a negative colonoscopy examination: evidence for a 10-year interval between colonoscopies. JAMA. 2006 May 24;295(20):2366-73.

139.Brenner H, Chang-Claude J, Seiler CM, Set al. Does a negative screening colonoscopy ever need to be repeated? Gut. 2006 Aug;55(8):1145-50.

140.Baxter MN Goldwasser MA Pazsat LF et al Association of colonoscopy and death from CRC a population based case control study. Ann Intern Med 2009;150:1-8.

141.Kim DH, Pickhardt PJ, Taylor AJ, et al. CT colonography versus colonoscopy for the detection of advanced neoplasia. N Engl J Med. 2007 Oct 4;357(14):1403-12.

142.American College of Radiology . ACR practice guideline for the performance of computed tomography (CT) colonography in adults. Practice Guidelines and Technical Standards; Reston, VA: 2009. http://www.acr.org/~/media/ACR/Documents/PGTS/guidelines/CT_Colonography.pdf Accessed September 2012.

143.Pickhardt PJ, Choi JR, Hwang I, et al. Computed tomographic virtual colonoscopy to screen for colorectal neoplasia in asymptomatic adults. N Engl J Med. 2003 Dec 4;349(23): 2191-200.

144.Johnson CD, Chen MH, Toledano AY, et al. Accuracy of CT colonography for detection of large adenomas and cancers. N Engl J Med. 2008 Sep 18;359(12):1207-17.

145.Cotton PB Durkalski VL, Pineau BC, et al CT Colonography a multicentre comparison with standard colonoscopy for detection of colorectal neoplasm JAMA 2004;291:1713-1719.

146.Lieberman DA Moravec M Holub J et al Polyp size and advanced histology in patients undergoing colonoscopy screening implications for CT Colonography Gastroenterology 2008;135:1100-1105.

147.Evaluation of English Bowel (Colorectal) Cancer Screening Pilot. First Round Pilot report: http://www.cancerscreening.nhs.uk/bowel/finalreport.pdf Accessed September 2012

148.Evaluation of English Bowel (Colorectal) Cancer Screening Pilot. Second Round Pilot report: http://www.cancerscreening.nhs.uk/bowel/pilot-2nd-round-evaluation.pdf Accessed September 2012

149.Bowel Screening Wales Website. http://www.wales.nhs.uk/sites3/home.cfm?orgid=747 Accessed September 2012

150. Scottish Bowel Screening Programme website http://www.bowelscreening.scot.nhs.uk/ Accessed September 2012

151.http://www.cancerscreening.hscni.net/2008.htm Accessed May 2014

152.Radtke F, Clevers H. Self-renewal and cancer of the gut: two sides of a coin. Science 2005; 307: 1904-9.

153.Brittan M, Wright NA. Stem cell in gastrointestinal structure and neoplastic development. Gut 2004; 53: 899-910.

154.Eastwood GL. Gastrointestinal epithelial renewal. Gastroenterology 1977;72: 962-75.

155.Lamprecht SA, Lipkin M. Migrating colonic crypt epithelial cells: primary targets for transformation. Carcinogenesis 2002; 23: 1777-80.

156.Nair P, Lagerholm S, Dutta S, Set al. Coprocytobiology: on the nature of cellular elements from stools in the pathophysiology of colonic disease. J Clin Gastroenterol 2003; 36 (Suppl 5): S84-S93.

157.Kamra A, Kessie G, Chen JH, et al. Exfoliated colonic epithelial cells: surrogate targets for evaluation of bioactive food components in cancer prevention. J Nutr 2005; 135: 2719-22.

158.Frisch SM, Screaton RA Anoikis mechanisms. Curr Opin Cell Biol 2001;13:555-562.

159.Shanmugathasan M, Jothy S. Apoptosis, anoikis and their relevance to the pathobiology of colon cancer. Pathol Int 2000; 50: 273-9.

160.Ahlquist DA, Harrington JJ, Burgart LJ, et al. Morphometric analysis of the “mucocellular layer” overlying colorectal cancer and normal mucosa: relevance to exfoliation and stool screening. Hum Pathol 2000; 31: 51-7.

161.Renehan AG, Bach SP, Potten CS. The relevance of apoptosis for cellular homeostasis and tumorigenesis in the intestine. Can J Gastroenterol 2001; 15: 166-76.

162.Osborn NK, Ahlquist DA. Stool screening for colorectal cancer: molecular approaches. Gastroenterology. 2005 Jan;128(1):192-206.

163.Barkla DH, Gibson PR. The fate of epithelial cells in the human large intestine. Pathology 1999; 31: 230-8.

164.Bjerknes M, Cheng H, Hay K, et al. APC mutation and the crypt cycle in murine and human intestine. Am J Pathol. 1997 Mar; 150(3):833-9

165.Wasan HS, Park HS, Liu KC, et al. APC in the regulation of intestinal crypt fission.J Pathol. 1998 Jul;185(3):246-55.

166.Wong WM, Mandir N, Goodlad RA, et al. Histogenesis of human colorectal adenomas and hyperplastic polyps: the role of cell proliferation and crypt fission. Gut 2002; 50: 212-7.

167.Wiebecke B, Brandts A, Eder M, et al Proliferation and morphogenesis of hyperplastic adenomatous and villous polyps of the human colon. Arch A Pathol Anat Histol. 1974;364:35–49.

168.Nakamura S, Kino I. Morphogenesis of minute adenomas in familial polyposis coli. Natl Cancer Inst. 1984;73:41–49.

169.Shih IM, Wang TL, Traverso G, et al. Top-down morphogenesis of colorectal tumors Proc Natl Acad Sci USA 2001; 98: 2640-5.

170.Lowe SW, Lin AW. Apoptosis in cancer. Carcinogenesis 2000; 21: 485-95.

171.Brown JM, Attardi LD. The role of apoptosis in cancer development and treatment response. Nat Rev Cancer 2005; 5: 231-7.

172.Haier J, Nicolson GL. The role of tumor cell adhesion as an important factor in formation of distant colorectal metastasis. Dis Colon Rectum 2001; 44: 876-84.

173.Christofori G. Changing neighbors, changing behaviour: cell adhesion molecule-mediated signaling during tumour progression. EMBO J 2003; 22: 2318-23.

174.Bogenrieder T, Herlyn M. Axis of evil: molecular mechanisms of cancer metastasis. Oncogene 2003; 22: 6524-36.

175.Loktionov A, O'Neill IK, Silvester KR, et al. Quantitation of DNA from exfoliated colonocytes isolated from human stool surface as a novel noninvasive screening test for colorectal cancer. Clin Cancer Res 1998; 4: 337-42.

176.Klaassen CH, Jeunink MA, Prinsen CF, et al. Quantitation of human DNA in feces as a diagnostic test for the presence of colorectal cancer. Clin Chem 2003; 49: 2112-3.

177.Wind P, Douard R, Poupardin E, et al. Anal implantation of exfoliated tumor cells from a rectal adenocarcinoma after colorectal stapled anastomosis. Eur J Surg 1999; 165: 905-6.

178.Baig MK, Stebbing JF & Marks CG. Anal canal metastases from left sided colorectal cancer. Colorectal Dis 2002; 4: 371-2.

179.Maeda K, Maruta M, Hanai T, et al. Irrigation volume determines the efficacy of "rectal washout". Dis Colon Rectum 2004; 47: 1706-10.

180.Loktionov A, Ferrett CG, Gibson JJ, et al. A case-control study of colorectal cancer detection by quantification of DNA isolated from directly collected exfoliated colonocytes. Int J Cancer. 2010 Apr 15;126(8):1910-9.

181.Loktionov A, Bandaletova T, Llewelyn AH, et al. Colorectal cancer detection by measuring DNA from exfoliated colonocytes obtained by direct contact with rectal mucosa. Int J Oncol. 2009 Feb;34(2):301-11.

182.Field A. Discovering Statistics Using SPSS. 3rd Edition. SAGE Publications, CA USA

183.Melotte V, Lentjes MHFM, van den Bosch SM, et al. N-Myc Downstream-Regulated Gene 4 (NDRG4): A Candidate Tumor Suppressor Gene and Potential Biomarker for Colorectal Cancer J Natl Cancer Inst 2009;101: 916 – 927.

184.Wong CM, Ng YL, Lee JM, et al. Tissue factor pathway inhibitor-2 as a frequently silenced tumor suppressor gene in hepatocellular carcinoma. Hepatology 2007;45:1129–38.

185.Sato N, Parker AR, Fukushima N, et al. Epigenetic inactivation of TFPI-2 as a common mechanism associated with growth and invasion of pancreatic ductal adenocarcinoma. Oncogene 2005;24:850–8.

186.Glockner SC, Dhir M, Mi Yi J, et al. Methylation of TFPI2 in Stool DNA: A Potential Novel Biomarker for the Detection of Colorectal Cancer. Cancer Res 2009;69(11):4691–9.Cancer Res 2009;69(11):4691–9.

187.Hellebrekers DM, Lentjes, MH, van den Bosch SM, Melotte V, et al. (2009). GATA4 and GATA5 are potential tumor suppressors and biomarkers in colorectal cancer. Clinical Cancer Research, 15(12), 3990–3997.

188.Viera AJ, Garrett JM.Understanding interobserver agreement: the kappa statistic. Fam Med. 2005 May;37(5):360-3.

189.Loktionov A, O'Neill IK, Silvester KR,et al. Quantitation of DNA from exfoliated colonocytes isolated from human stool surface as a novel noninvasive screening test for colorectal cancer. Clin Cancer Res 4: 337-342, 1998.

190.Onouchi S, Matsushita H, Moriya Y, et al. New method for colorectal cancer diagnosis based on SSCP analysis of DNA from exfoliated colonocytes in naturally evacuated feces. Anticancer Res. 2008 Jan-Feb;28(1A):145-50.

191.Mahadavan L, Loktionov A, Daniels IR, et al. Exfoliated colonocyte DNA levels and clinical features in the diagnosis of colorectal cancer: a cohort study in patients referred for investigation. Colorectal Dis. 2012 Mar;14(3):306-13.

192.Gervaz P, Bucher P, Morel P. Two colons – two cancers: paradigm shift and clinical implications. J Surg Oncol 2004;88:261–66.

193.Bajwa AA, Peck J, Loktionov A, et al. DNA quantification of exfoliated colonocytes as a novel screening tool for colorectal cancer. Eur J Surg Oncol. 2013 Dec;39(12):1423-7.