Field cancerisation in colorectal cancer

By Abhilasha Patel

MA (Hons) Cantab, MB BChir, MMedEd, MRCS

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Division of Translational and Systems Medicine, Warwick Medical School, University of Warwick

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For my boys: Taran and Tilak

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Abbreviations

dCT	Delta threshold cycle
mL	millilitres
µg	micrograms
µm	Micrometres
ng	nanograms
nm	nanometres
V	Volt
AAPC	Attenuated Adenomatosis Polyposis Coli
ACF	Aberrant crypt focus
AJCC	American Joint Committee on Cancer
Akt	Protein kinase B
APC	Adenomatous Polyposis Coli
AXIN2	Axis inhibitory protein 2
β-actin	Beta-actin
BCL2	B-cell lymphoma 2
BMP3	Bone morphogenetic protein 3
BRAF	B-raf proto-oncogene
CAF	Cancer associated fibroblasts
CCL2	(C-C motif) ligand 2
CD8	Cluster of differentiation
CDKN2A	Cyclin-dependent kinase inhibitor 2A

CEA	Carcinoembryonic antigen
CGH	Comparative genomic hybridisation
CIMP	CpG island methylator phenotype
CpG	Cysteine-phosphate-guanine
CRC	Colorectal cancer
CSC	Cancer stem cell
CT	Computed tomography
CXCL2	Growth related oncogene β (GRO- β)
CXCL12	C-X-C motif ligand 12
CYP7A1	Cholesterol 7 alpha-hydroxylase
DACH1	Dachshund Family Transcription Factor 1
DALMs	Dysplasia associated lesion or mass
DCC	Deleted in colon cancer
DEG	Differentially expressed gene
DFS	Disease free survival
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial Mesenchymal Transition
Eps8	Epidermal growth factor receptor substrate 8
Erk 1/2	Extracellular signal-regulated kinase

FaMISHED	Food and Fermentation using Metagenomics in Health and Disease
FAP	Familial Adenomatous Polyposis
FGF	Fibroblast growth factor
FGF2	Fibroblast growth factor 2
FGF7	Fibroblast growth factor 7
FGF15	Fibroblast growth factor 15
FGF19	Fibroblast growth factor 19
FGF21	Fibroblast growth factor 21
FGF23	Fibroblast growth factor 23
FGFR	Fibroblast growth factor receptor
FGFR2	Fibroblast growth factor receptor 2
FGFR4	Fibroblast growth factor receptor 4
FISH	Fluorescence in situ hybridisation
FOBT	Faecal occult blood test
FRS2α	Fibroblast receptor substrate 2α
FUT2	Fucosyltransferase 2
GADD45B	Growth arrest DNA damage inducible 45, subunit B
GM-CSF	Granulocyte macrophage stimulating factor
HCC	Hepatocellular carcinoma
HIF-1α	Hypoxia-inducible factor 1α
hMLH1	MutL homolog 1
hMSH2	MutS homolog 2
hMSH6	MutS homolog 6

HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HOXB9	Homeobox B9
HS	Heparan sulfate
IBD	Inflammatory bowel disease
IBD-CRC	Colorectal cancer associated with inflammatory bowel disease
IGF	Insulin like growth factor
IL-1β	Interleukin 1β
IL-4	Interleukin 4
IL-7	Interleukin 7
IL-13	Interleukin 13
IRF-1	Interferon regulatory factor 1
IRF-2	Interferon regulatory factor 2
KGF	Keratinocyte growth factor
KRAS	Kirsten rat sarcoma virus
LIMMA	LmFT function (Bioconductor R package)
LOH	Loss of heterozygosity
LR	Local recurrence
MA	Metachronous adenoma
MAP	MYH-associated polyposis
MAPK	Mitogen activated protein kinase
MDT	Multidisciplinary team
ME1	Malic enzyme 1

ME2	Malic enzyme 2
MGMT	O-6-Methylguanine-DNA Methyltransferase
MIP2	Macrophage inhibitory protein 2
MMP	Matrix Metalloproteinase
MNM	Macroscopically normal mucosa
MP	Metachronous polyp
MSI	Microsatelite instability
MSI-H	Microsatelite stability - high
MSI-L	Microsatelite stability – low
MUC2	Mucin 2
MUC5AC	Mucin 5, subunit AC
NF-Kappa B	Nuclear factor kappa B
OS	Overall survival
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PET-CT	Positron emission tomography – computed tomography
PGE2	Prostaglandin E2
PGF	Placenta growth factor
PI3K	Phosphatidylinositol 3-kinase
PLS3	Plastin 3
PSCA	Prostate stem cell antigen
qRT-PCR	Quantitative real time polymerase chain reaction

RAF	Rapidly accelerated fibrosarcoma
RAGE	Receptor for advanced glycation end products
RAS	Rat sarcoma virus
RIPA	Radioimmunoprecipitation
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RR	Robust regression function (Bioconductor R package)
S100P	S100 calcium binding protein P
SA	Synchronous adenoma
SDF-1	Stroma derived factor 1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIR	Standardised incidence ratio
siRNA	Synthetic RNA duplex
SLC46A1	Solute carrier family 46, folate transporter, member 1
SMAD2	SMAD family member 2
SMAD4	SMAD family member 4
SP	Synchronous polyp
SR	Standardised incidence rate
STAT	Signal transducers and activators of transcription
STAT6	Signal Transducer And Activator Of Transcription **6**
TAF	Tumour associated fibroblasts
TAN	Tumour associated neutrophils

TCA	Tricarboxylic acid cycle
TCF/LEF	T-cell factor/lymphoid enhancer factor
TGF	Transforming growth factor
Th1	T helper cell response 1
TH2	T helper cell response 2
TIMP	Tissue Inhibitors of Metalloproteinases
TNF-α	Tumour necrosis factor alpha
TNM	Tumour Nodes Metastasis
TP53/p53	Tumour suppressor p53
TPPP	Tubulin Polymerization Promoting Protein
TPPP3	Tubulin Polymerization Promoting Protein 3
WNT	Wingless-type mouse mammary tumour virus integration site
UHCW	University Hospitals of Coventry and Warwickshire NHS Trust
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
ZEB	Zinc finger E-box-binding homeobox 1

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. I, Abhilasha Patel, declare that it has been composed by myself and has not been submitted in any previous application for any degree and all the research has been undertaken in accordance with University safety policy and Guidelines on Ethical Practice.

The presented work, including data generation and data analysis, was carried out by the author except in the cases outlined below:

- The micro-array chip hybridisation was commercially performed by Oxford Gene Technology
- The micro-array results were analysed in collaboration with the Department of Systems Biology, University of Warwick

List of publications

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Abstract

Colorectal cancer (CRC) continues to cause significant global health burden, despite advances in our understanding of tumour biology, the development of screening programs and increasing public awareness about the disease. Previous studies investigating CRC pathogenesis have been criticised for focussing on the tumour tissue itself. Investigators have proposed that if early biomarkers of disease are to be identified, efforts need to be undertaken in examining pre-neoplastic tissue prior to malignant transformation. Based on the field cancerisation concept, the research hypothesis was that the macroscopically normal mucosa (MNM) around colorectal cancer and polyps is biologically altered. The aims of the study were to determine if the presence of colorectal adenomas at time of cancer diagnosis was predictive of future risk of colonic neoplasia and to characterise the global gene expression profile of MNM adjacent to CRC and adenomas. A retrospective cohort study of CRC patients demonstrated that synchronous adenomas were associated with a higher risk of future adenomas at short term follow up but were not predictive of local recurrence. Thus, other more reliable biological markers of field effect need to be identified. Global gene expression profiles of MNM around cancer, polyps and in control subjects were significantly different when evaluated with micro-array. The differentially expressed genes were involved in immunity, metabolism, epithelial-mesenchymal transition and RNA transcription. CXCL2 and FGF7 were identified as being upregulated in MNM adjacent to CRC suggesting that they could be utilised as markers of field cancerisation in the colon. Further investigation demonstrated that the FGF7-FGFR2 axis was disrupted only at the tumour site with downregulation of some of its downstream targets emphasising the potential role of this signalling axis in CRC formation. Collectively, these findings support the field cancerisation concept in CRC and highlight the importance of signals released by stromal cells in facilitating epithelial growth. These genes may be utilised to develop early biomarkers of disease or could be targeted with pharmacotherapy to modulate future CRC risk.

Chapter 1: Introduction

1.1. Colorectal cancer epidemiology

Colorectal cancer is the fourth most common cancer in the UK affecting 1 in 17 people in the UK (CRUK) and more than a million people worldwide (Ferlay J *et al.*, 2013). The highest rates of cancer are found in Australia/New Zealand and the lowest in Western Africa. In Europe, it is the second most common cancer with just under half a million cases diagnosed in 2012. The incidence rate in the UK is 20th highest in males in Europe and 17th highest in females (Ferlay J *et al.*, 2013). Overall, CRC occurs more frequently in men compared to women with an overall male:female ratio of 13:10 (CRUK). Most cancers occur in the colon with just over a third (34%) in the rectum. Incidence is related to age with the majority (95%) of cancers being diagnosed in those aged above 50 yrs.

Within the UK, a north-south divide has been observed for men with the highest incidence being recorded in Scotland, Northern Ireland and north of England (Quinn *et al.*, 2005; NCIN). In comparison, the overall incidence for women does not demarcate as clearly with areas in the east and southwest also experiencing high rates of CRC.

The European age standardised incidence rates (SR) have increased by 29 % and 7 % for men and women respectively between 1975-1977 and 2009-2011 (CRUK). Some of the increase in the last decade has been the result of better screening which has detected cancers at an early stage (Jones *et al.*, 2009).

There has been a gradual improvement in CRC survival over time. The one year age standardised survival rate has increased from 46.2 % to 75.7 % between 1971-1972 and 2010-2011 (CRUK). However, despite improvements in screening and diagnosis, prognosis after CRC in the UK remains poor, particularly compared to the rest of Europe (Sant *et al.*,

2009). The age standardised 5-year survival rate in the UK is 53.6 % compared to 65.9 % and 62.6 % in Australia and Sweden respectively (Coleman *et al.*, 2011). Some of these inter-country differences have been attributed to later presentation (Woodman *et al.*, 2001) and delay in treatment, however, there may be other differences in ethnicity, dietary and lifestyle factors that could account for variation in survival.

1.2 Anatomy of the colon

The last part of the aerodigestive tract is referred to as the colon or large intestine. It consists of four sections; the ascending colon, transverse colon, descending colon and sigmoid colon. Based on its embryological origin, the right colon refers to the caecum, ascending colon and 2/3 proximal transverse colon originating from the midgut. The rest of the transverse colon, descending colon and sigmoid colon are derived from the hindgut and are collectively described as the left colon.

1.2.1. The colonic wall

The wall of the colon can be divided into four layers:

i) The innermost mucosa which consists of an epithelial layer, the lamina propria which is a connective tissue layer that contains blood vessels, nerves, lymphoid tissue to support the mucosa and the muscularis mucosa which is a layer of smooth muscle that contracts to change the shape of the lumen

ii) The submucosal region contains fat, fibrous connective tissue and larger blood vessels and nerves

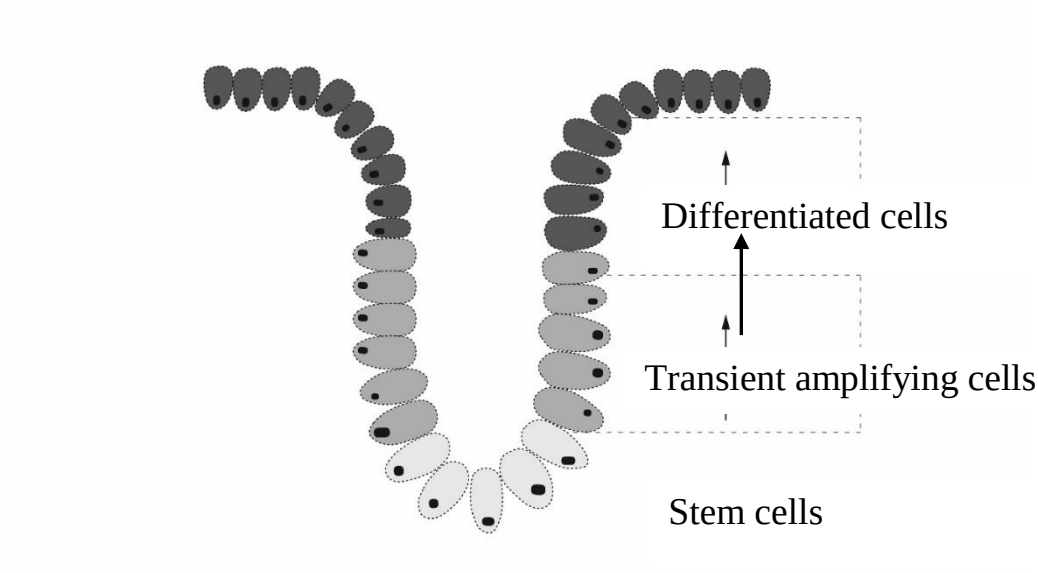
iii) The muscularis externa comprises an inner circular and outer longitudinal layer of smooth muscle fibres responsible for peristaltic activity driving digestion

iv) The serosa formed by fat and another layer of epithelial cells called mesothelium.

1.2.2. The stem cell compartment

The epithelial layer consists of a single sheet of columnar epithelial cells folded into finger like vaginations that form the functional unit of the intestine called crypts of Lieberkuhn. In the adult human colon, around 14,000 crypts per square centimeter are found and it is estimated that over 6 times 10^{14} colonocytes are produced every 5 days (Potten *et al.*, 1992; Cheng *et al.*, 1984). Each colonic crypt is shaped like a test tube and consists of 2500-5000 cells (Nooteboom *et al.*, 2010), 85-106 cells in length and 29-43 cells in circumference (Bernstein *et al.*, 2010). At the base, there are 10-20 cells designated the stem cell niche (Nicolas *et al.*, 2007; Willis *et al.*, 2008) which are pluripotent stem cells that undergo self-renewal through asymmetric division and generate a population of transit amplifying cells (Radtke & Clevers, 2005) (See figure 1.2.1). These cells proliferate and differentiate as they migrate upward along the crypt giving rise to the terminally differentiated epithelial cells found at the top of the crypt. Each stem cell can give rise to one of three differentiated epithelial cell types that include colonocytes (responsible for absorption), mucus secreting goblet cells and enteroendocrine cells.

Figure 1.2. **Crypt of Lieberkuhn** showing stem cells at base with progressive upward differentiation of cells.



Proliferation, life span and cell death are regulated to ensure that the stem cell compartment in the crypt remains constant despite this cycling process. This is achieved through Wnt signaling ligands that are produced by the mesenchymal cells of the myofibroblast lineage and are closely applied to the basal lamina that surround the crypt (Fevr *et al.*, 2007). Other factors that have been found to contribute to stem cell behavior include bone morphogenetic protein, antagonists gremlin 1 and gremlin 2 (Kosinski *et al.*, 2007), Notch signaling pathways, ephin-B1 and its receptors (Eph-B2, Eph-B3) (Batlle *et al.*, 2002; van Es & Clevers, 2005; Crosnier *et al.*, 2006).

1.2.3 Blood supply of the colon

Based on its embryological origin, the blood supply to the right colon is from the superior mesenteric artery and to the left colon is from the inferior mesenteric artery. Curative CRC surgery will aim to remove the draining lymph nodes of the tumour and therefore will require resection of the entire segment of colon that is supplied by that particular artery. Hence, a tumour of the sigmoid will require resection of the descending colon and sigmoid up to the recto-sigmoid junction with ligation of the inferior mesenteric artery at its origin.

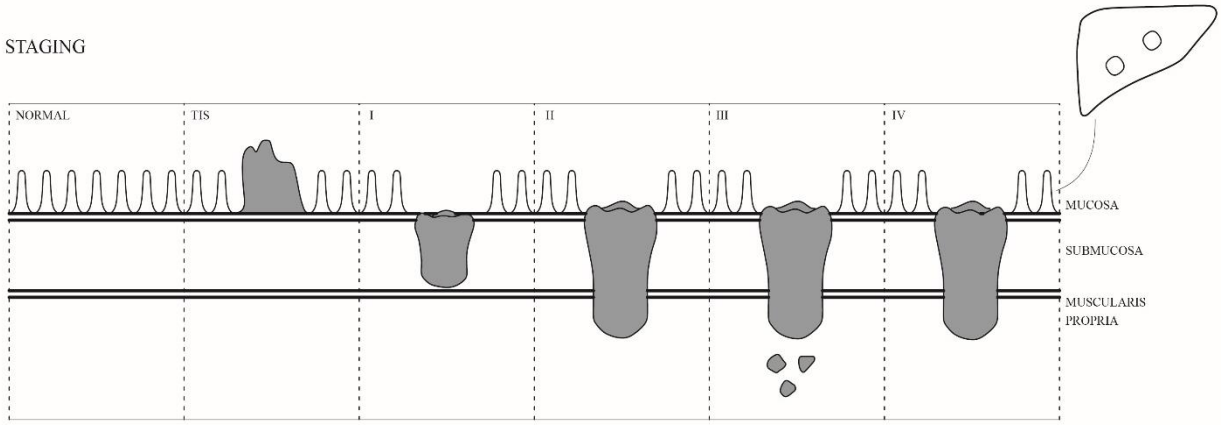
This will ensure that the entire lymphatic basin draining the tumour, which could potentially harbour metastasis, is removed.

1.3. Staging and prognosis of colorectal cancer

The pathological stage of CRC describes the disease extent both locally at the colonic site and distal spread into lymph nodes or distant organs such as the liver. In 1950, Cuthbert Dukes first described the relationship between pathological stage and rectal cancer survival (DUKES, 1950). He described 4 distinct stages of CRC based on depth of local invasion and subsequent spread into lymph nodes or distant organs. The more recent Astler-Coller (ASTLER & COLLER, 1954) and TNM staging system (Edge SB *et al.*, 2010) are based upon similar principles to that of Dukes staging, namely, description of local and distant tumour spread. TNM staging relies on 3 pieces of information –

i) T (tumour)– spread of the tumour in terms of the different layers of the colonic wall
ii) N (nodes) – spread to local or systemic lymph nodes
iii) M (metastasis) – systemic spread of tumour outside of the colon (does not include organs that are adjacent to the tumour and are directly invaded).

Figure 1.3: **Staging of colon cancer**. Three different staging systems that have been described.



Duke's staging

Dukes' A: Invasion into but not through the bowel wall

Dukes' B: Invasion through the bowel wall penetrating the muscle layer but not involving lymph nodes

Dukes' C: Involvement of lymph nodes

Dukes' D: Widespread metastases

Astler-Coller

Stage A: Limited to mucosa

Stage B1: Extending into muscularis propria but not penetrating through it; nodes not involved

Stage B2: Penetrating through muscularis propria; nodes not involved

Stage C1: Extending into muscularis propria but not penetrating through it. Nodes involved

Stage C2: Penetrating through muscularis propria. Nodes involved

Stage D: Distant metastatic spread

TNM staging

Stage 0 - Tumour confined to mucosa (Tis)

Stage I - tumour confined to submucosa (T1) or muscularis propria (T2)

Stage II - tumour invades subserosa (T3) or adjacent organs (T4)

Stage III - any T stage with lymph node involvement

Stage IV - metastatic involvement of distant organs

Staging of CRC is not only important for planning adjuvant therapy but determines prognosis. Early stage colorectal cancer confined to the colon is associated with a 5-year survival of 93.2 % for T1-T2 lesions and 77 % for T3-T4 lesions (NCIN). In comparison, cancer that has spread to the lymph nodes or distant organs only has a 5-year survival of 47.7 % and 6.6 % respectively (NCIN). At present, only 13 % patients present with early stage disease and more than a quarter (32.8 %) have either nodal or distant metastases at time of diagnosis. Patients with liver or lung metastases that are amenable to surgical resection have a five-year survival of approximately 25% (Cady & Stone, 1991; Fong *et al.*, 1999; Kanemitsu *et al.*, 2004; Vogelsang *et al.*, 2004; Choti, 2009).

Other prognostic factors that have been described by AJCC (7th edition) (Edge SB *et al.*, 2010) include–

i) Presence of tumour deposits – these are discrete foci of tumour that are found in the pericolic, perirectal or mesenteric fat, in the absence of residual lymph node tissue but within the lymph drainage area of the primary tumour.

ii) Residual tumour at the radial or excision margin

iii) Presence of perineural invasion

iv) Microsatellite instability (MSI) – recorded as stable, MSI-low, MSI-high and not registered

v) Tumour regression grade – this describes the change in rectal tumour following neoadjuvant therapy as either no tumour viable cells present to extensive residual tumour present

vi) K-ras gene analysis as mutated K-ras is associated with poor response to anti-EGFR therapy which is given to patients with metastatic CRC

vii) 18q loss of heterozygosity (LOH) assay – this is used to determine if patients with stage II CRC require neoadjuvant therapy or not.

1.4 Risk factors for colorectal cancer

Most cases of CRC are sporadic, however, 5 % cases are associated with familial syndromes (CRUK). Risk factors for sporadic colorectal cancer include increasing age, male sex, previous colonic polyps/cancer and environmental factors such as red meat, high fat diet, low fibre intake, obesity and a sedentary lifestyle.

Familial syndromes associated with colorectal cancer include Lynch syndrome (also known as hereditary non-polyposis colorectal cancer, HNPCC), familial adenomatous polyposis (FAP) and MYH-associated polyposis (MAP). They are characterised by the presence of specific mutations (mutation in the gene APC in FAP and mutations in DNA mismatch repair genes in Lynch syndrome) that lead to earlier onset of cancer and higher risk of further lesions. Lynch syndrome and FAP are both autosomal dominant whereas MYH-associated polyposis is autosomal recessive. Aside from these syndromes, in 20 % sporadic cases there is a higher risk based on number of first-degree relatives with cancer and the underlying aetiology for this is unknown (Butterworth *et al.*, 2006; Johns & Houlston, 2001).

The other risk factor for CRC is inflammatory bowel disease. Both Ulcerative Colitis and Crohn's disease have been linked with an increased risk of CRC, particularly with increasing duration or severity of disease activity (Eaden *et al.*, 2001; Bernstein *et al.*, 2001; Canavan *et al.*, 2006; von Roon *et al.*, 2007; Laukoetter *et al.*, 2011).

1.4.1 Genetic predisposition to CRC

FAP and HNPCC account for around 5 % of all CRC (Hampel *et al.*, 2008; Bülow *et al.*, 1996) with the hamartomatous syndromes such as Peutz-Jeghers, familial juvenile polyposis

and Cowden's syndrome being associated with a further 1 % CRC. The genes that have been linked with these syndromes are shown below (Fearnhead *et al.*, 2002).

Table 1.4.: Genetic syndromes with increased colorectal cancer risk and underlying genetic defect responsible

Syndrome	Genes responsible
FAP	APC (dominant), MYH (recessive)
HNPCC	hMLH1, hMSH2, hMSH6, PMS1, PMS2, hMLH3, EXOI
Peutz-Jeghers syndrome	STK1
Juvenile polyposis	SMAD4/MADh4 or BMPR1A
Cowden disease	PTEN/MMAC1
Li-Fraumeni syndrome	p53

Modified from Arnold et al, 2005

1.4.1.1. FAP

FAP has an incidence at birth of around 1 in 8619 (Evans *et al.*, 2010). It occurs equally in both sexes and is characterised by multiple polyps (>100) in the colon and rectum. If untreated, there is 100% chance of malignancy, usually before the age of 40 years (Half *et al.*, 2009). There are three forms of FAP: classic FAP, attenuated adenomatous polyposis coli (AAPC) and MYH-associated polyposis (MAP). Classic FAP and AAPC are both autosomal dominant and are associated with a mutation in the Adenomatous Polyposis Coli gene on chromosome 5q21. In AAPC, however, the gene is partly functional which leads to fewer colonic polyps that develop at a later stage, predominantly affect the proximal colon (Knudsen *et al.*, 2003; Grover *et al.*, 2012) and confer a 70 % lifetime risk of CRC. Although FAP is usually diagnosed based on prior family history, up to 16 % cases are

associated with a *de novo* mutation in the APC gene (Evans *et al.*, 2010). The APC gene is a tumour suppressor gene that promotes apoptosis in colonic cells. It phosphorylates beta catenin leading to its ubiquitination and degradation through the proteasome pathway (Oving & Clevers, 2002). Loss of the APC protein leads to increased levels of nuclear B-catenin which is able to stimulate cell proliferation and transcriptional activation of c-myc, cyclin D1 and PPAR-delta. Interestingly, the phenotype of FAP patients depends upon the site of the truncating mutation in the APC gene (Brensinger *et al.*, 1998).

FAP is also associated with extra-intestinal manifestations such as osteomas, dental abnormalities (unerupted teeth, congenital absence of one or more teeth, supernumerary teeth, dentigerous cysts and odontomas), congenital hypertrophy of the retinal pigment epithelium (CHRPE), desmoid tumours, and extracolonic cancers (thyroid, liver, bile ducts and central nervous system) (Half *et al.*, 2009).

MYH-associated polyposis syndrome is an autosomal recessive condition characterised by a germline inactivation of a base excision repair gene called mutY homologue (Al-Tassan *et al.*, 2002; Kastrinos & Syngal, 2007). This gene is responsible for excising the 8-oxoguanine product of oxidative damage to guanine. As it is recessive, two inactive germline MYH alleles need to be inherited to develop the disease which confers an almost 100 % risk of CRC by 60 years of age (Kastrinos & Syngal, 2007). One third of individuals with more than 15 colorectal adenomas will test positive for the two common mutations Y165C and G382D. Somatic inactivation of MYH allele has not been detected in sporadic cancer.

1.4.1.2. HNPCC

HNPCC or Lynch syndrome is an autosomal dominant condition associated with defective DNA mismatch repair leading to microsatellite instability (Lynch *et al.*, 2009). HNPCC sufferers have an 80 % lifetime risk of CRC. The majority (2/3) of these cancers occur in the proximal to the splenic flexure and CRC usually occurs at an earlier age (44 yrs.) compared to the general population (63 yrs.). Accelerated carcinogenesis is seen in the colon whereby small adenomas become malignant over 2-3 years in comparison to the usual 8-10 years (Rijcken *et al.*, 2002; Rijcken *et al.*, 2008). The colorectal tumours that are encountered amongst HNPCC individuals tend to be poorly differentiated, are mucinous and associated with a lymphocytic infiltrate often described as a 'Crohn's like reaction. There is an increased risk of metachronous CRC if the initial operation was not a subtotal colectomy (Parry *et al.*, 2011). These individuals are also at increased risk of developing cancer of the endometrium (40-60 % lifetime risk), ovary (12-15%), stomach, urinary tract, pancreas, small bowel and brain (Watson *et al.*, 2008; Barrow *et al.*, 2009).

The most commonly affected genes are hMLH1 (Bronner *et al.*, 1994; Papadopoulos *et al.*, 1994) and hMSH2 (Fishel *et al.*, 1993; Leach *et al.*, 1993) which together account for 90 % of all gene mutations found in HNPCC. Other genes that have been implicated include hPMS2 (Nicolaides *et al.*, 1994) and hMSH6 (Hendriks *et al.*, 2004). These genes are involved in DNA mismatch repair and defective function usually results in microsatellite instability (Thibodeau *et al.*, 1993; de la Chapelle, 2003). Mutations in hMSH6 result in partial deficiency of mismatch repair with tumours often displaying lower levels of microsatellite instability. Clinically, this mutation is associated with an attenuated form of HNPCC with later age of onset (around 70 yrs.), however, there is a particularly elevated risk of endometrial cancer (Senter *et al.*, 2008).

Patients with HNPCC can be identified based upon the Amsterdam II criteria (Vasen *et al.*, 1999) which detail clinical aspects of the disease. However, the Revised Bethesda guidelines (Umar *et al.*, 2004), based upon pathological criteria, have been found to be more effective at discriminating HNPCC sufferers (Piñol *et al.*, 2005).

Figure 1.4: Diagnostic criteria used for HNPCC

1.4.2. Environmental risk factors

A western diet and lifestyle have been associated with an increased risk of CRC based on the geographical worldwide distribution of CRC. Furthermore, countries such as Japan, Singapore and Eastern European countries that have recently made a transition from a low-income to high-income demonstrate rising rates of CRC (Coleman *et al.*, 2011; Boyle & Langman, 2000). Migrants that move from a low risk to high risk country acquire CRC at a rate that is similar to the population of the host country (Boyle & Langman, 2000; Johnson & Lund, 2007). This has been observed in the offspring of the Japanese population that migrated to United States who have 3-4 times higher CRC incidence compared to the native Japanese.

1.4.2.1. Diet

A high fat, low fibre diet with high red meat consumption has been implicated in CRC (Bingham *et al.*, 2002; Liang & Binns, 2009). High fat content is associated with the development of bacterial flora that degrade bile salts into potentially carcinogenic N-nitroso compounds (Larsson & Wolk, 2006). A systematic review showed that high red meat consumption increased the relative risk of colonic but not rectal cancer (Magalhães *et al.*, 2012). The heme iron found in red meat and production of carcinogenic agents such as heterocyclic amines and polycyclic aromatic hydrocarbons have been proposed to be causative factors (Santarelli *et al.*, 2008; Sinha, 2002). Similarly, differences in dietary fibre intake are also associated with differing risk of CRC. A high fibre intake, particularly comprising cereal grains and wholegrains was found to be strongly associated with a reduced risk of CRC across several studies (Aune *et al.*, 2011).

1.4.2.2. Physical activity and obesity

Higher levels of physical activity have been linked to a reduced risk of CRC (Lee *et al.*, 2007; de Jong *et al.*, 2005). However, in several studies, this association appears only to exist with colonic cancers and not rectal cancers (Bazensky *et al.*, 2007; Harriss *et al.*, 2009a). The underlying mechanism behind this association is being elucidated. Moderate physical activity increases the metabolic rate, increases oxygen uptake, reduces blood pressure, reduces insulin resistance and increases gut motility (Lee *et al.*, 2007) which are believed to protect against cancer formation. Increased physical activity also protects against obesity which has also been linked to increased CRC risk (Harriss *et al.*, 2009b).

1.4.2.3. Cigarette smoking and alcohol consumption

Smoking has been linked to both formation of adenomas (Botteri *et al.*, 2008) and higher incidence of CRC (Zisman *et al.*, 2006). A review of the literature demonstrated that several parameters of cigarette smoking including quantity of cigarettes smoked, duration of smoking and number of pack years were quantitatively linked with relative risk of CRC (Liang *et al.*, 2009). This effect was more pronounced for rectal cancer in studies which examined risk according to segment of colon affected. An earlier age of onset of CRC has also been found in individuals who smoke (Zisman *et al.*, 2006; Tsong *et al.*, 2007).

Several studies have also linked increased alcohol consumption to CRC risk (Zisman *et al.*, 2006; Tsong *et al.*, 2007). Reactive metabolites that are produced in response to alcohol consumption such as acetaldehyde have been shown to be carcinogenic (Seitz & Mueller, 2015) and these effects may be potentiated by the presence of tobacco (Zisman *et al.*, 2006).

1.4.3. Inflammatory bowel disease

Inflammatory bowel disease comprises Ulcerative Colitis and Crohn's disease, both of which are associated with significant increase in risk of colorectal cancer compared to the normal population. In a meta-analysis of the incidence of CRC in UC (Eaden *et al.*, 2001), the risk of cancer was 2% after 10 years, 8 % after 20 years and 18 % after 30 years. Similar studies on the incidence of colorectal cancer in Crohn's disease show a similar risk (Maykel *et al.*, 2006; Friedman *et al.*, 2008; Seitz & Mueller, 2015), however, do not report length of duration of the disease. A subsequent meta-analysis which did adjust for duration of disease found that the SIR (standardised incidence ratio) was 2.5 and the relative risk was 4.5 (von Roon *et al.*, 2007). Therefore both Crohn's disease and UC are subject to surveillance programs when patients have had the disease for more than 10 years (Gillen *et al.*, 1994). The risk of cancer is also related to the age of onset of the disease and its extent; the greatest risk being found in patients with colitis affecting colon up to or more proximal to the hepatic flexure (Sugita *et al.*, 1991).

1.4.4. Previous colorectal cancer

Patients with CRC are at higher risk of developing cancers elsewhere in the colon. The SIR of a second cancer was higher at 1.5 compared to the general population risk (Levi *et al.*, 2013) and was related to age. The SIR was 7.5 for those below the age of 50 and declines thereafter to reach 1.0 at the age of 80 years and above. Several other studies have found similar findings (Mulder *et al.*, 2012; Liu *et al.*, 2013a). In the long-term, CRC survivors older than 50 years, elevated risk was only observed for the proximal colon with no detectable differences for rectal cancer. Amongst the patient and tumour factors that have been investigated, presence of synchronous neoplastic lesion at time of diagnosis (Ballesté *et al.*, 2007) and a proximal location of the first tumour (Gervaz *et al.*, 2005) have found to be predictive for development of subsequent metachronous lesions.

1.5. Pathogenesis of colorectal cancer

A large body of epidemiologic, clinical and pathological evidence exists to support the notion that colorectal cancer develops from precursor lesions called polyps. Histologically, there are two types of polyps: hyperplastic polyps, which contain increased number of glandular cells with decreased cytoplasmic mucus but preserved cell architecture and adenomatous polyps, which have disordered cell architecture consisting of cells that are enlarged, hyperchromatic, cigar-shaped and crowded together to form a palisade pattern. Malignant transformation involves these cells becoming invasive and migrating beyond the basement membrane. Adenomas are classified into tubular adenomas where there are branched tubules and villous adenomas that contain finger-like villi arranged in a frond. Tubulovillous adenomas contain elements of both. Increased colorectal cancer risk has previously only been associated with the presence of adenomas, however, there is emerging evidence to suggest that hyperplastic polyps in a certain setting are also pre-malignant (Jeevaratnam *et al.*, 1996; Rashid *et al.*, 2000). Risk factors that are associated with increased CRC risk in hyperplastic polyps include large polyp size (>1 cm diameter), location in the right colon, focus of adenoma within the hyperplastic polyp, presence of more than 20 hyperplastic polyps and a family history of hyperplastic polyposis syndrome (Jass, 2001). Serrated polyps may appear similar to hyperplastic polyps but have a significant associated risk of CRC as they tend to be large, are usually found in the right colon and the colonocytes in these polyps frequently contain BRAF mutations and DNA methylation (Montgomery, 2004; Wynter *et al.*, 2004).

1.5.1. Evidence to support polyps as precursors to colorectal cancer

The following clinical observations support the concept that adenomas are precursor lesions for CRC –

i) The prevalence and anatomical distribution of adenomas and CRC is similar (Morson, 1974; Muto *et al.*, 1975)

ii) Patients who have colorectal adenomas are at higher risk of developing CRC (Neugut *et al.*, 1993; Winawer *et al.*, 1993)

iii) Synchronous adenomas are found in up to one third of CRC colectomy resection specimens (Day DW & BC., 1978)

iv) Adenomatous tissue is found contiguous with CRC (Day DW & BC., 1978)

v) The risk of CRC increases with number and size of polyps (Heald & Bussey, 1975).

vi) Patients who refuse polypectomy develop CRC at a rate of 4 % after 5 years and 14 % after 10 years (Stryker *et al.*, 1987).

vii) Following curative resection, adenomas develop in 30 % patients who have CRC (McFall *et al.*, 2003; Mattar *et al.*, 2005; Moon *et al.*, 2010).

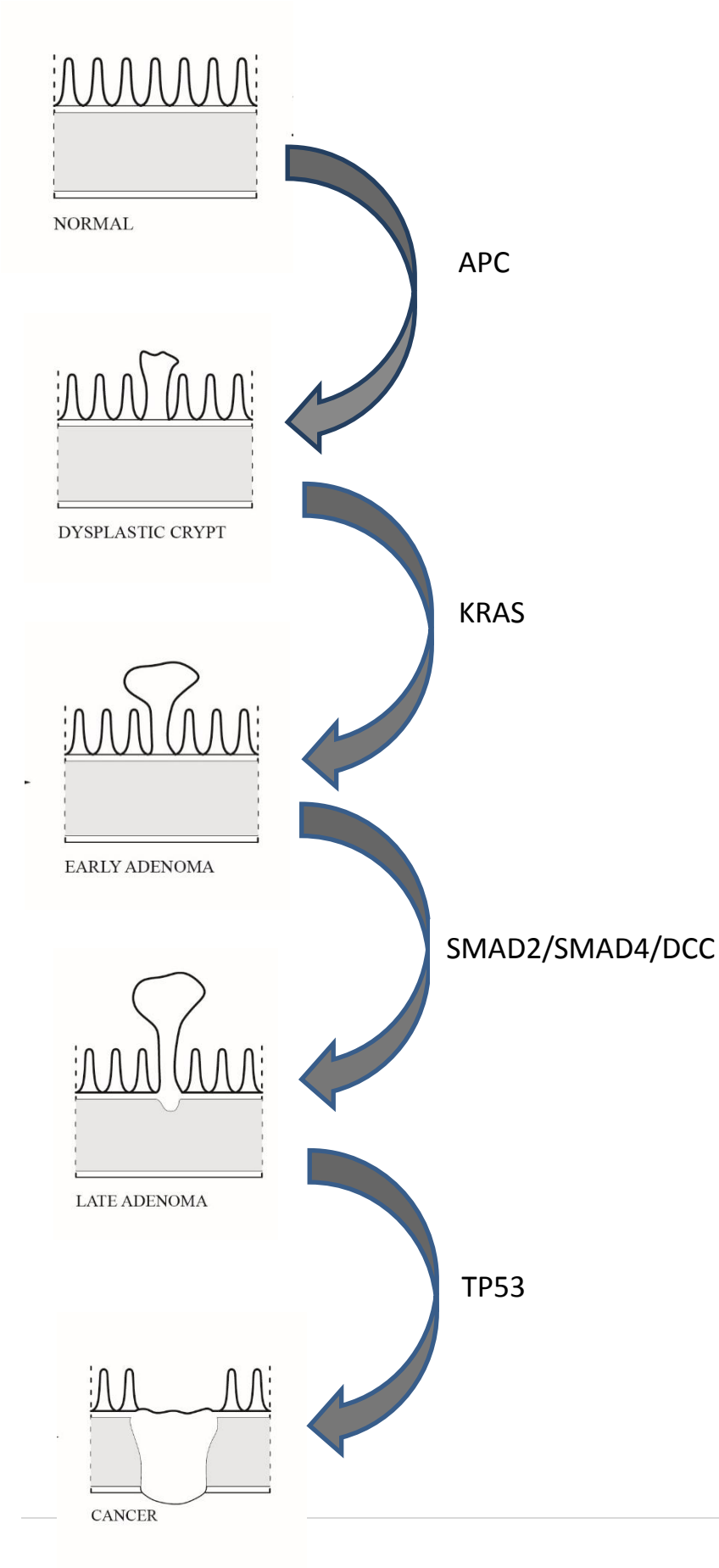
viii) Removal of adenomas results in reduced CRC incidence (Müller & Sonnenberg, 1995; O'brien *et al.*, 2004a).

The earliest lesions that lead to the development of adenomas are aberrant crypt foci (ACF) (Bird, 1987; Bird *et al.*, 1989). These consist of abnormal tube like glands, which contain cells that are resistant to apoptosis. Previous studies have utilised ACFs as a surrogate endpoint biomarker in chemoprevention trials (Raju, 2008; Corpet & Taché, 2002; Cho *et al.*, 2008), however, the relevance of ACFs in this setting remains controversial (Lance & Hamilton, 2008). Not all ACFs lead to CRC and it is unclear which attributes of ACFs predispose to further growth and development of adenomas, therefore, the use of chemical agents to reduce ACF number or size is of questionable importance.

1.5.2. The adenoma-carcinoma sequence

Based upon the body of evidence outlined above, the adenoma-carcinoma sequence was proposed. This refers to the process whereby normal mucosa becomes altered forming aberrant crypt foci, followed by adenoma of varying dysplasia and subsequently culminating in invasive CRC. The molecular changes that underpin this process were described by Vogelstein in the late 1980's (Vogelstein *et al.,* 1988). It is now well recognised that multiple genes and molecular pathways are dysregulated during CRC formation. The original description of the adenoma-carcinoma sequence refers to cancers with chromosomal instability, which includes around 80 % of all cancers (see figure below).

Figure 1.5. The adenoma-carcinoma sequence



Chromosomal instability is characterised by numerical and structural changes in chromosomes collectively referred to as aneuploidy (Muleris *et al.*, 1990; Bardi *et al.*, 1995; Thiagalingam *et al.*, 2001). In contrast, cancers with DNA mismatch repair defects (15 % of all tumours) retain a near-diploid karyotype and show normal rates of gross chromosomal aberrations (Parsons *et al.*, 1993; Eshleman *et al.*, 1998). These tumours exhibit microsatellite instability (MSI) and have distinct histological features with frameshift mutations and base pair substitutions in short tandemly repeated nucleotide sequences called microsatellites. They are similar to the tumours encountered in HNPCC but usually result from silencing of the hMLH1 gene through CpG promoter hypermethylation rather than a germline mutation. The other molecular pathway that can lead to CRC formation is CIMP (CpG island methylator phenotype) whereby tumour suppressor genes are silenced through epigenetic modification rather than gene mutation. It appears that chromosomal instability and MSI are mutually exclusive (Lengauer *et al.*, 1998). However, a subgroup of colorectal cancers with near diploid chromosomes and stable microsatellites have also been described suggesting an alternative pathway to genetic instability (Chan *et al.*, 2001).

Table 1.5: **Different molecular types of colorectal cancer** with underlying gene defects

Type of genetic instability	Type of defect	Genes involved	Phenotype
Chromosomal instability (loss of heterozygosity at multiple site)	Somatic	Loss of heterozygosity at APC, TP53, SMAD4	Characteristic of 80 to 85% of sporadic colorectal cancers, depending on stage
DNA mismatch repair defects			
HNPCC	Germline	MLH1, MSH2, MSH6 germ-line gene mutations	Multiple primary colorectal cancers, accelerated tumour progression, and increased risk of endometrial, gastric, and urothelial tumours
Sporadic CRC	Somatic	MLH1 somatic methylation	Colorectal cancer with increased risk of poor differentiation, more commonly located in right colon, less aggressive clinical behaviour than tumours without mismatch-repair deficiency
CpG island methylator phenotype	Somatic	Target loci MLH1, MINT1,MINT2 , MINT3	Characteristic of 15% of colorectal cancers, with most showing mismatch repair deficiency from loss of tumour MLH1 expression
Base excision repair defect	Germline	MYH	Development of 15 or more colorectal adenomas with increased risk of colorectal cancer

1.5.3. Chromosomal instability

Most sporadic cases have chromosomal instability characterised by an allelic imbalance at several chromosomal loci (5q, 8p, 17p and 18q) which leads to loss of tumour suppressor genes such as APC, p53 and SMAD4. There are also rare inactivating mutations of genes that are involved in maintaining chromosomal stability (Barber *et al.*, 2008). In contrast to most other cancers, amplification of gene number (Leary *et al.*, 2008) or gene rearrangement through translocation are rarely found in colorectal cancer.

The majority (around 70-80 %) of FAP and sporadic CRCs are characterised by loss of heterozygosity and bi-allelic mutations in APC (Miyoshi *et al.*, 2002; Lammi *et al.*, 2004). APC forms a 'destruction complex' in association with beta catenin, GSK-3beta and CKI kinases which triggers the phosphorylation of beta catenin in the absence of the WNT ligand. This leads to proteolytic degradation of beta catenin. In the presence of WNT, this destruction complex is inhibited which prevents beta catenin degradation, enabling it to translocate to the nucleus and activate downstream target genes through interaction with the TCF/LEF family of transcription factors (Polakis *et al.*, 1999). Constitutive activation of the WNT pathway occurs most commonly due to mutations in the APC or beta catenin genes. However, mutations in other members such as AXIN 2 have also been detected (Lammi *et al.*, 2004).

Up to 50 % of all CRCs contain an activating mutation in the KRAS2 oncogene (Bos *et al.*, 1987; Vogelstein *et al.*, 1988; Andreyev *et al.*, 2001). In its absence, 20 % of CRCs contain mutations in BRAF (Davies *et al.*, 2002; Rajagopalan *et al.*, 2002). Both play a role in the RAS-RAF-MAPK signalling pathway that modulates cell growth and survival. Although both mutations are associated with adenoma growth and progression (Vogelstein *et al.*,

1988; Rajagopalan *et al.*, 2002), there is some evidence to suggest that they may develop earlier in aberrant crypt foci (Takayama *et al.*, 2001).

Alterations in the TGFβ signal transduction pathway are associated with the progression to late adenoma/early adenocarcinoma. Most CRCs contain mutations in a component of this pathway such as TGFβ receptor 2 (Markowitz *et al.*, 1995) or SMAD2 and SMAD4 genes (Takagi *et al.*, 1996; Takagi *et al.*, 1998; Thiagalingam *et al.*, 1996) which affects angiogenesis, cell proliferation and differentiation.

The next step in the adenoma carcinoma sequence is marked by the malignant transformation from adenoma to carcinoma which is accompanied by loss of TP53, found in at least 45 % CRCs (Baker *et al.*, 1989; Delattre *et al.*, 1989; Purdie *et al.*, 1991; Iacopetta, 2003). This is a tumour suppressor gene that inhibits cell growth and stimulates cell death hence; its inactivation has far reaching consequences on the genome integrity of intestinal cells.

Additional pathways that have been implicated in CRC include those involving P13K, receptor tyrosine kinases and phosphatases (Wang *et al.*, 2004c; Samuels *et al.*, 2004; Parsons *et al.*, 2005; Bardelli *et al.*, 2003).

1.5.4. DNA mismatch repair defects

In hereditary syndromes such as HNPCC, there are mutations in DNA mismatch repair genes such as hMLH1, hMSH2 or hMSH6. These genes are responsible for recognising frameshift mutations and base pair substitutions that occur in short tandemly repeated nucleotide sequences called microsatellites during DNA replication. Loss of function of

these genes is characterised by accumulation of single nucleotide mutations and length alterations in repetitive microsatellite nucleotide sequences. Germ line defects in mismatch repair genes confer a lifetime risk of colorectal cancer of 80 % in individuals with HNPCC (Leach *et al.,* 1993; Papadopoulos *et al.,* 1994; Fishel *et al.,* 1994; Bronner *et al.,* 1994) and are associated with an accelerated course of development of colorectal cancer with invasive cancer emerging within 36 months of a normal colonoscopy.

In sporadic colorectal cancer, loss of one of the DNA repair genes, hMLH1 occurs through hypermethylation of its CpG islands, which silences the genes and leads to microsatellite instability. Defective mismatch repair function leads to the development of tumours that have a proximal location, mucinous histology, poor differentiation and lymphocytic infiltration. In sporadic cases, they are associated with older age and female sex. Genes that contain mononucleotide or dinucleotide repeat sequences such as that for TGFβ receptor 2 or BCL2-associated X protein can become inactive in individuals with mismatch repair deficiency contributing to the carcinogenesis process.

The level of microsatellite instability is usually assessed using a standard panel of five markers (BAT25, BAT26, D5S346, D2S123 and D17S250) defined by the National Cancer Institute in 1998 (Boland *et al.,* 1998). MSI-H (MSI high) is defined when two of the five markers are mutated or unstable. MSI –L (MSI low) is present when only one of the five markers is unstable. When none of the five markers are mutated, the tumour is described as MSS (microsatellite stable).

1.5.5. CpG island methylator subtype.

Loss of gene function can occur through aberrant DNA methylation, which leads to epigenetic silencing of genes. Cytosines within CpG dinucleotides are modified by DNA methylases that attach a methyl group to carbon 5 of cytosine. Under normal circumstances, cytosine methylation only occurs in areas of repetitive DNA sequences outside of exons being excluded from the CpG islands in the promoter regions of half of all genes (Issa, 2004; Tahara *et al.*, 2014). In colorectal cancer, there is an overall reduction in cytosine methylation, however, there is an increase in aberrant methylation within certain promoter associated CpG islands responsible for epigenetic gene silencing. In sporadic CRC, hypermethylation of MLH1 is responsible for loss of function of this DNA mismatch repair gene. Hypermethylation of other genes usually occurs as a collective group that has led to the term CpG island methylator phenotype (CIMP). However, the molecular mechanism that underlies CIMP or the effects of epigenetic silencing of genes other than MLH1 remain to be elucidated. There are three patterns of methylation: CIMP-high where a subgroup of genes become hypermethylated, CIMP-low with an intermediate level of hypermethylation and aberrant methylation of exon 1 of the gene encoding vimentin. The latter is not usually expressed by colonic mucosa but aberrant methylation is found in 53-83 % of patients with colorectal cancer in a pattern that is independent of CIMP (Chen *et al.*, 2005; Zou *et al.*, 2007).

1.5.6. Newer emerging theories

A number of different pathways have recently emerged that explain a higher risk of CRC in patients with particular types of adenoma.

Serrated polyposis refers to a condition whereby serrated polyps confer a high risk of CRC that develops along a different pathway to the traditional adenoma-carcinoma sequence. The current criteria for diagnosis (Snover *et al.*, 2010) include –

1. at least five serrated polyps proximal to the sigmoid colon with two or more of these being >10 mm

2. any number of serrated polyps proximal to the sigmoid colon in an individual who has a first-degree relative with serrated polyposis

3. >20 serrated polyps of any size, but distributed throughout the colon. The implied meaning of this last criterion is that the polyps are not all present in the rectum

It is associated with a five-fold increase in the risk of CRC to first degree relatives (Boparai *et al.*, 2010). Most cancers that arise in these patients are proximal (Yeoman *et al.*, 2007), however, in young onset (<50 years) individuals they are more likely to be distal (Buchanan *et al.*, 2010; Young & Jass, 2006). Somatic molecular alterations that have been associated with serrated polyposis syndrome include *BRAF* (V600E) mutation, *KRAS* (codons 12 and 13) mutations, *hMLH1* methylation, *MGMT* methylation, and CpG island methylator phenotype (CIMP) (Rashid *et al.*, 2000; O'Brien *et al.*, 2006; Chan *et al.*, 2003; O'Brien *et al.*, 2004b; Beach *et al.*, 2005). The prevalence of each mutation varies with the subtype of serrated polyp, however, in most instances, a somatic mutation in BRAF is the earliest event detected in aberrant crypt foci (Rosenberg *et al.*, 2007). The other characteristic of serrated polyposis is widespread hypermethylation of gene promoters (CIMP) (Chan *et al.*, 2002) with or without MSI (Hawkins *et al.*, 2000; Jass *et al.*, 2000). This is evident even in normal mucosa of young individuals suggesting that there is a widespread epigenetic regulatory defect that leads to premature ageing of the mucosa and confers a higher risk of developing CRC (Minoo *et al.*, 2006; Wynter *et al.*, 2004). At present, clinical management of patients with these lesions is unclear and regular surveillance colonoscopy is advised.

1.6. Screening and treatment of colorectal cancer

At present, most patients with CRC are diagnosed when they become symptomatic with a change in bowel habit, blood in the stool, abdominal mass or iron deficiency anaemia. Hence, the condition is often detected at a later pathological stage when chances of cure are less, resulting in the low observed survival rates. Consequently, population based screening programs have been introduced that aim to detect CRC in asymptomatic patients at the preneoplastic stage (adenomatous polyps) or early cancer stage. Current screening modalities rely on detecting and removing adenomas from which 80 % of CRCs are believed to arise. However, only a small proportion of these adenomas will eventually develop into a cancer, hence, screening often results in overtreatment of patients and can cause considerable morbidity to the patient.

1.6.1. Faecal occult blood test (FOBT)

In the UK, population based CRC screening commenced in 2006. The current protocol is based on the guaiac based Faecal Occult Blood Test (gFOBT) with biennial testing of individuals aged 60-74. In a systematic review, FOBT screening reduced CRC related mortality by 16 % (Hewitson *et al.*, 2007; Hewitson *et al.*, 2008). If found to be positive, individuals are offered a colonoscopy to establish a diagnosis. Results from the UK cancer screening program have shown that only 52 % of individuals invited to participate completed a FOBT and only around 50 % of those with a positive result underwent a colonoscopy. Of those attending colonoscopy, only one in 10 were diagnosed with cancer. Half of these patients had no abnormality and the remaining 40 % had polyps (Logan *et al.*, 2012). This suggests that the vast majority of those investigated by invasive tests such as colonoscopy did not have cancer. Other tests that are currently being examined include the immunochemical faecal occult blood test that has superior sensitivity with fewer samples required and less interference from animal blood in the diet (Whitlock *et al.*, 2008;

Castiglione *et al.*, 2002). However, the optimum threshold for testing is currently unknown and is being evaluated in several studies. In the UK, this test will be introduced into clinical practice in 2016.

1.6.2. Flexible sigmoidoscopy

Other approaches to CRC screening rely on more invasive tests such as flexible sigmoidoscopy or colonoscopy. As more than 60 % of cancers arise in the sigmoid colon and rectum (Lieberman *et al.*, 2000), they can be detected with a 60 cm flexible sigmoidoscope. Three large randomised trials of once only flexible sigmoidoscopy have been conducted in the UK (Atkin *et al.*, 2002), Italy (Segnan *et al.*, 2002) and Norway (Gondal *et al.*, 2003). Based on intention to treat analysis, the UK trial demonstrated a 23% reduction in CRC incidence and 31 % reduction in mortality in the intervention group (Atkin *et al.*, 2010). In the first year of follow up after sigmoidoscopy, the incidence of distal cancers is 4 % of expected which remains at 18 % expected at 4 years. In comparison to FOBT, flexible sigmoidoscopy is able to detect three times as many adenomas (Brevinge *et al.*, 1997; Segnan *et al.*, 2005). However, there are disadvantages with flexible sigmoidoscopy screening. Referral rates for colonoscopy are higher at around 5-19 % compared to the 1.2-2.1 % referral rates seen in FOBT trials. Similarly, compliance is lower than that for FOBT screening, probably as these tests are invasive and require attendance at hospital compared to FOBT tests, which can be performed at home. The overall compliance rate in the UK trial was 39 % (Atkin *et al.*, 2002) compared to the 54 % compliance rate recorded in the first round of the FOBT trial.

At present, flexible sigmoidoscopy screening is being offered in six regions in the UK that are piloting the test to determine its feasibility and cost effectiveness. There are plans to offer the test on a more universal basis across UK by 2016.

1.6.3. Colonoscopy

In the USA, the most common method of screening is colonoscopy every 10 years (Rex *et al.*, 2000; Rex & Trustees, 2004) which has been shown to have little effect on reducing rates of proximal cancers (Lakoff *et al.*, 2008; Baxter *et al.*, 2009) and can result in considerable more morbidity to the patient than flexible sigmoidoscopy as it requires full bowel preparation and sedation. There are no randomised controlled trials that demonstrate a reduction in CRC related mortality if colonoscopy is utilised for screening. Most of the evidence to support colonoscopy based screening has been extrapolated from case control studies of sigmoidoscopy. These have demonstrated an 80 % risk reduction in death from CRC in individuals who have undergone a flexible sigmoidoscopy compared to those that have not (Newcomb *et al.*, 1992; Selby *et al.*, 1993). Similarly, there is a 70 % risk reduction for CRC following negative colonoscopy which drops to 55 % at years and 28 % at 10 years (Singh *et al.*, 2006). This suggests that the benefits from a negative examination are long lasting. However, as the detection rate for advanced adenomas in the younger age group (40-49) drops to 3.5 %, screening below 50 years is not recommended in the USA (Imperiale *et al.*, 2002). The other disadvantage of colonoscopy is that up to 6 % of lesions, including polyps < 10mm can sometimes be missed. In one study, 2 % of asymptomatic patients undergoing screening had a missed polyp. Almost one quarter of these missed lesions were found within 10 cm of the anal verge and the rest were on a fold in the colon (Pickhardt *et al.*, 2004). Given the huge demand on resources, colonoscopy based screening has not been deemed cost effective in the UK and is therefore unlikely to be utilised in the future.

1.6.4. Emerging screening modalities

Current research into population based screening methods has therefore focussed on the development of non-invasive tests. Several studies have investigated DNA based tests of blood or stool (Link *et al.*, 2010; Ahlquist *et al.*, 2012; Summers *et al.*, 2013). Cologuard has

recently been developed and is being used in USA. It is a stool based test that measures KRAS mutations, aberrant NDRG4 and BMP3 methylation, and β-actin, plus a haemoglobin immunoassay. Early studies have shown that it has a sensitivity of 92 % and specificity of 87 % (Imperiale *et al.*, 2014). Its feasibility, cost effectiveness and long term impact needs to be investigated further before it can be widely adopted.

Others have used novel strategies such as evaluation of metabolomic profile of urine or faeces and breath with mass spectrometry or electronic devices that detect emitted volatile gases such as the electronic nose (Arasaradnam *et al.*, 2014; Westenbrink *et al.*, 2015). Pilot studies suggest that these technologies offer promise for the future development of more accurate screening tests.

1.7. Cancer biology

Despite considerable advances in our understanding of how CRC develops, there are a number of theoretical aspects of cancer formation that require consideration. Many different theories of carcinogenesis have been proposed which have influenced the way in which CRC pathogenesis has been explored. The fundamental concept that has been explored in greatest depth and has dominated CRC research is the somatic mutation theory (Curtis, 1965; Hahn & Weinberg, 2002) which proposes that cancer is a clonal, cell-based disease where quiescence is the default state of the cell (Soto & Sonnenschein, 2004). Consequently, investigators have focussed on characterising the genetic changes that take place in CRC in order to identify both causative agents and methods for improved diagnosis and treatment. Others have questioned this approach by challenging the very theoretical assumptions it is based on and have proposed alternative hypotheses such as the tissue organisation theory (Soto & Sonnenschein, 2011) or metabolic theory of cancer (WARBURG, 1956).

1.7.1. Multi-hit Hypothesis of cancer

The multi hit hypothesis of cancer was first described by Nordling (NORDLING, 1953) and later popularised by Knudson (Knudson, 1971). A simple mutation is not sufficient to cause cancer and it is the progressive accumulation of mutations in the cell's DNA that result in malignant growth. A three step process was described consisting of initiation, promotion and progression. Initiation refers to the process where DNA is damaged through the action of carcinogenic agents such as UV light, ionising radiation, thermal disruption or chemical sources (de Gruijl *et al.*, 2001). There are also endogenous carcinogens that cause cancer. In CRC, the colonic epithelium is exposed to numerous toxins and microbial agents found in food. Bile acids produced at high levels in individuals who have a high fat diet, damage DNA and have been identified as initiators in CRC formation (Bernstein *et al.*, 2011). Similarly, presence of macrophages and neutrophils found in inflamed colonic mucosa are a source of reactive oxygen species, which is also capable of damaging DNA (Katsurano *et al.*, 2012). There are five main types of DNA damage comprising oxidation of bases, alkylation of bases, hydrolysis of bases, bulky adduct formation and mismatch of bases during DNA replication. In the promotion phase, a promoter agent induces clonal expansion of the cell generating a group of cells that harbour mutated genes. Further mutation and genetic instability or epigenetic modulation contributes to drive the cells towards malignant transformation in the progression phases. It has been estimated that at least seven mutations are necessary for development of cancer (NORDLING, 1953). Only so called 'driver' mutations that confer selective growth advantage contribute to carcinogenesis; the remaining vast majority of mutations found in malignant cells are actually innocent bystander 'passenger' mutations that occur because of genomic dysregulation. In CRC, 15 'driver' and 60 'passenger' mutations have been described (Wood *et al.*, 2007).

The multi-hit hypothesis of cancer has developed further in the last decade. Hanahan and Weinberg described 6 attributes of cancer cells in their seminal paper on 'Hallmarks of cancer' (Hanahan & Weinberg, 2000):

i) Self –sufficiency of growth which leads to uncontrolled growth

ii) Evading growth suppressors

iii) Activating invasion and metastasis

iv) Replicative immortality (absence of senescence)

v) Induce angiogenesis

vi) Resist cell death

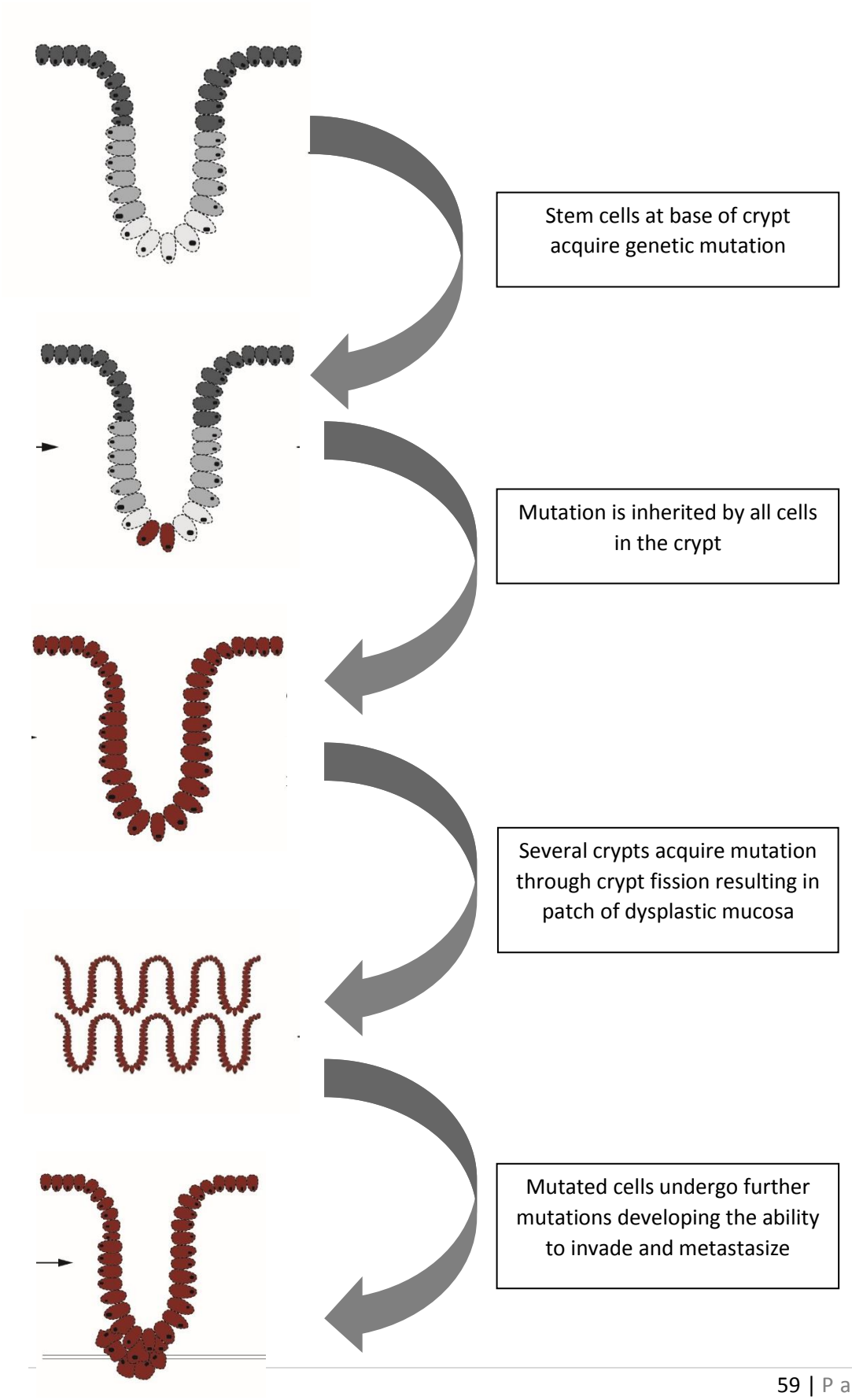
Consequently, tumours have been described as complex tissues of multiple cell types which interact with each other rather than collections of proliferating cells (Centelles, 2012). Cells need to acquire other attributes besides replicative ability to form a tumour and there are multiple signalling pathways that are disrupted in cancer formation.

Several investigators have recently challenged Knudson's two hit hypothesis. A number of experimental approaches have demonstrated haploinsufficiency in cancers whereby inactivation of one allele of a tumour suppressor gene is sufficient to cause tumour formation (Fodde & Smits, 2002). Furthermore, the dominant negative effect describes how one mutated copy of a tumour suppressor gene renders the other, wild-type copy non-functional (Willis *et al.*, 2004). Therefore, it appears that under certain circumstances, loss of function of tumour suppressor genes can arise through mutational loss or epigenetic marking of a single allele.

1.7.2. The stem cell theory

Fundamental to the concept that cancer arises from DNA damage and accumulation of mutations is the concept of the cancer stem cell theory. This proposes that the different cells found in a tumour originate from a single group of cells called cancer stem cells (CSCs) (see figure 1.7.).

Figure 1.7. **Stem cell theory** of colorectal cancer formation demonstrating how a mutation in the stem cells at the base of the crypt is acquired by daughter cells as they migrate upwards along the crypt.



CSCs are pluripotent cells capable of self-renewal that divide and give rise to the cells found in tumours. They may arise from transformation of normal adult stem cells or differentiated cells. Evidence that they arise from normal adult stem cells is the following -

i) there is a positive correlation between the risk of developing a cancer and the number of normal stem cell divisions taking place in the same tissue – this was tested across 31 cancer types and extended across 5 orders of magnitude (Tomasetti & Vogelstein, 2015).

ii) The increased incidence of cancer with age suggests that most cancers occur because cells accumulate damage. DNA is the only cellular component that can accumulate damage with age and stem cells are the only cells that can transmit DNA from the zygote to the cells we have when we die (López-Lázaro, 2015).

The mutant cells compete for space and resources. The clonal evolution theory suggests that only cells with mutations that give them a selective advantage over the other cells will survive, similar to Darwinian evolution (Nowell, 1976; Cahill *et al.*, 1999; Greaves & Maley, 2012).

Both the cancer stem cell theory and the clonal evolution theory have been applied to CRC. It has been proposed that stem cells at the bottom of a crypt acquire mutations. As these cells divide and give rise to terminally differentiated cells, the daughter cells acquire the mutations. Only cells that have a growth advantage over other cells survive and eventually the crypt contains only mutated cells. These crypts divide by crypt fission resulting in a tumour mass with cells that harbour the original mutations. It is also conceivable that due to defective DNA repair mechanisms, the cells also develop different passenger mutations, which gives rise to genetically heterogeneous populations of cells within the tumour that share similarity in the driver mutations that they have acquired. This process has also been termed bottom up histogenesis (Wright, 2000) whereby cells at the bottom of the crypt are those that are responsible in giving rise to the malignancy.

1.7.3. Top down or bottom up histogenesis

Early studies where histological examination of pre-neoplastic lesions of the colon was performed contradicted the bottom up histogenesis theory (Wright, 2000). These lesions only contained dysplastic cells at the orifices and luminal surfaces of the crypts. If the epithelial cells of the crypt were traced, there was an abrupt change to dysplastic cells midway down the crypt axis (COLE & MCKALEN, 1963; Wiebecke *et al.*, 1974; Nakamura & Kino, 1984). Furthermore, cells at the base of these crypts exhibited normal proliferation patterns whereas cells at the orifices had a similar proliferation pattern to neoplastic cells (Lipkin, 1974; Maskens, 1979; Polyak *et al.*, 1996). Several authors therefore proposed alternative theories. The stem cells that give rise to cancer cells may reside in intercryptal zones that lie between crypt orifices rather than at the base of the crypt (COLE & MCKALEN, 1963). Others suggested that the cells at the base of the crypts were genetically transformed but that the dysplastic appearance was only visible as cells migrate and become terminally differentiated (LANE & LEV, 1963; Maskens, 1979). These historical studies have led authors to investigate this phenomenon further, which has led some authors to propose a top down morphogenesis model. This was based on the observation that dysplastic cells at the top of the crypt contain genetic alterations in APC and have a neoplastic genetic expression profile (Shih *et al.*, 2001). Similar changes were not seen in the cells at the base of the crypt nor was there any evidence to suggest that these cells have a common clonal origin. The top down morphogenesis model implies that genetically altered cells are only found in the superficial portions of the mucosa and not at the base. These abnormal cells spread laterally and downward to form new crypts which connect to pre-existing crypts and eventually replace them (Shih *et al.*, 2001). Others have argued that crypt fission, as seen in monocryptal adenomas (the earliest precursor lesion to adenomas) is the main mode of adenoma progression, certainly in FAP (Chang & Whitener, 1989; Wasan *et al.*, 1998) but possibly in sporadic adenomas (Wong *et al.*, 1999). Others have suggested that these two theories may not be mutually exclusive whereby crypt fission occurs early in adenoma

formation and lateral, downward spread via the surface is a later phenomenon resulting in cancer formation.

1.7.4. Other cancer theories

1.7.4.1 Tissue Organisation theory

Several other cancer theories have been proposed that question the somatic mutation theory and cancer stem cell theory. Based on this premise, cancers are derived from a single somatic cell which acquire multiple mutations in genes that control cell proliferation and regulate the cell cycle (Alberts B *et al.*, 2004). Thus, cancer is an irreversible process that takes place at the DNA- level and the default state of the cell is assumed to be quiescent. In comparison, the proponents of the tissue organisation theory state that cancer arises through disruption of normal tissue architecture by carcinogenic agents resulting in alteration of cell-to-cell signalling and genomic instability (Soto & Sonnenschein, 2005; Soto & Sonnenschein, 2011). Hence, genetic mutations are the result rather than the cause for cancer formation. This theory also assumes that the default state of the cell is proliferation and that carcinogenesis is a reversible, curable process (Soto & Sonnenschein, 2005). Although both theories appear to have a different philosophical stance, there have been attempts to ally them by the proposition that neither process on its own is sufficient to cause a cancer to form (Rosenfeld, 2013).

1.7.4.2 Chromosomal theory

In 2011, an alternative theory was proposed to explain the genomic instability observed in cancers (Stephens *et al.*, 2011). Instead of the slow accumulation of genetic mutations, cancers arise after a single catastrophic event during which the chromosome or chromosomal region shatters into tens to hundreds of pieces. The DNA repair machinery

stitches these fragments into a mosaic pattern, giving rise to genomic instability. Cells that have acquired such genetic alteration usually undergo apoptosis, however, such remodelling is expected to give the cell a survival advantage enabling the cells to proliferate and become malignant.

1.7.4.3. Metabolic theory

Others have suggested that cancers arise due to metabolic dysfunction. Malignant, rapidly dividing cells have glycolytic rates that can be up to 200 times higher than those of normal tissues, despite the presence of oxygen (Gatenby & Gillies, 2004; Ferreira *et al.*, 2012). This has been termed the Warburg effect: there is a switch from energy needs being met by oxidative phosphorylation to aerobic glycolysis followed by lactic acid fermentation in the cytosol (Vander Heiden *et al.*, 2009). This is necessary for cancer cells to meet the energy demands when undergoing rapid rates of cell division. However, it leads to the generation and accumulation of reactive oxygen species, which induces DNA damage and contributes to cancer formation. The Warburg hypothesis stipulates that these metabolic changes are the fundamental cause for cancer formation which is also referred to as the metabolic theory of cancer (WARBURG, 1956). Others have argued that the Warburg effect is simply a consequence of the mutations that have accumulated in cancer cells rather than being a driving force for cancer formation itself (Senyilmaz & Teleman, 2015).

1.8. The role of the tumour microenvironment and supporting stroma

Several studies highlight the importance of the tumour microenvironment and the reciprocal relationship between stromal cells and epithelial cells in driving cancer formation. Chronic inflammation has been referred to as the seventh trait acquired by tumour cells (Mantovani *et al.*, 2008; Colotta *et al.*, 2009; Hanahan & Weinberg, 2011). The tumour microenvironment consists of tumour infiltrating cells (cells associated with inflammation

and immunity), vasculature, extracellular matrix and matrix-associated molecules. Transformed epithelial cells attract pro-neoplastic stromal cells that will support cell growth and help evasion of host defences enabling these cells to survive in hostile conditions.

1.8.1. Tumour infiltrating cells

Inflammation and release of soluble chemoattractants by the epithelial cells attract a number of different cells to the tumour site (Tokunaga *et al.*, 1998; Jedinak *et al.*, 2010; Zins *et al.*, 2007; Wang *et al.*, 2006b). The role of these cells in carcinogenesis appears to be dynamic. Initially they are involved in protecting against tumour formation, however, with time, the epithelial cells are able to modulate their activity to promote tumour cell proliferation, survival and metastasis (de Visser *et al.*, 2006). Some of the induced changes are reversible, however, there have been studies showing loss of heterozygosity and microsatellite instability in stromal cells suggesting that irreversible changes also occur (Ishiguro *et al.*, 2006).

Tumour associated macrophages (TAMs) have multiple roles that support tumour growth including promoting angiogenesis through release of VEGF (Barbera-Guillem *et al.*, 2002; Pollard, 2004; Sickert *et al.*, 2005), epithelial-mesenchymal transition through interaction with TGFβ (Bates *et al.*, 2004) and immunosuppression through release of cytokines that inhibit T cell activation and proliferation (Mantovani *et al.*, 1992; Baier *et al.*, 2005). In comparison, the role of neutrophils in CRC formation remains controversial. Some authors suggest that they support angiogenesis by releasing factors such as oncostatin M which stimulates production of VEGF by tumour cells (Queen *et al.*, 2005). Others have proposed that release of ROS, proteases and cytokines such as TNF-alpha and IL-1beta can kill tumour cells directly (Di Carlo *et al.*, 2001a; di Carlo *et al.*, 2001b).

Macrophages are highly plastic and can adopt a different state depending upon the micro-environment: either M1 (anti-tumour) or M2 (pro-tumour) polarisation states (Sica *et al.*, 2006; Mantovani *et al.*, 2007; Mantovani *et al.*, 2004; Van Ginderachter *et al.*, 2008). In tumours, the M2 polarisation state predominates (Mantovani *et al.*, 2008; Coussens & Werb, 2002) which is usually induced by IL-4, IL-13, immunoglobulin complexes and Toll-like receptor ligands. They are involved in scavenging debris, promoting angiogenesis, tissue remodelling and repair. In comparison, M1 macrophages participate in the Th1 response against intracellular pathogens including tumour cells. Similarly, a dual role for neutrophils may also explain the opposing results seen across studies. N1-TANs have an anti-tumour role by expressing higher levels of proinflammatory cytokines and are more cytotoxic to T cells (Scapini *et al.*, 2000). In contrast, N2-TANs do not produce pro-inflammatory cytokines but instead produce large amounts of arginase that actually inactivates T cell effector functions, disabling the host defences against tumour formation (Fridlender *et al.*, 2009).

The type, density and location of T cells is a better prognostic indicator in CRC compared to conventional histopathological factors. Although, some CD8 T cells are reactive against tumour antigens, the tumour microenvironment contains inhibitory cytokines that prevent T cell activation thus contributing to evasion of the host immune response (Waldner *et al.*, 2006).

Despite being the main cellular constituent of the reactive stroma around CRC (Kalluri & Zeisberg, 2006; Ostman & Augsten, 2009), cancer associated fibroblasts (CAFs) are poorly understood. Generally, they are believed to arise from local tissue fibroblasts or fibroblast precursors that have been stimulated by PDGF and TGFβ. In vitro studies have shown that they are able to enhance tumour cell proliferation (Nakagawa *et al.*, 2004) and in vivo, high

levels of stromal CAFs have been linked with greater potential to develop metastasis or recurrence (Henry *et al.*, 2007). CAFs have also been shown to release VEGF, FGF and CXCL12 that are pro-angiogenic thereby supporting neovascularisation. Furthermore, CAFs activate Wnt signalling through the Wnt ligands PDGF and PGE2 which leads to EMT and maintenance of the cancer stem cell phenotype (Wang *et al.*, 2004a; Wang *et al.*, 2004b; Eisinger *et al.*, 2006; Eisinger *et al.*, 2007).

1.8.2. Angiogenesis

Neovascularisation is a prerequisite for tumour growth and survival. Hypoxia of the tumour leads to activation of hypoxia-induced factor 1, which increases expression of angiogenic factors such as VEGF, bFGF and PDGF by the tumour cells. This is usually an early event in carcinogenesis and may explain how microvascular density is a predictor of survival in CRC patients (Choi *et al.*, 1998).

1.8.3. Extracellular matrix

The extracellular matrix (ECM) is a highly complex, 3-dimensional structure that not only maintains tissue integrity but regulates other cellular processes such as cell migration, differentiation and proliferation, acting as a reservoir for cytokines and growth factors. There are two components to the ECM: the basement membrane and the interstitial or stromal matrix. The basement membrane consists of thin sheets of specialised ECM that surrounds epithelial cells and separates it from the interstitial stroma. It is composed of laminin and type IV collagen with invasion by malignant cells usually requiring dissolution of the basement membrane through a complex process. The stromal matrix contains polysaccharide gels, proteoglycans and various fibrous proteins. Several alterations in the ECM constituents have been associated with CRC formation. Laminin 332 is commonly lost as a premalignant lesion becomes invasive. This parallels inactivation of Smad4 which is a positive

transcriptional regulator of all three genes that encode subunits of Laminin 332 (Zapatka *et al.*, 2007). Similarly, Syndecan 1 (a transmembrane heparan sulphate proteoglycan) is lost in human CRC and its levels have been correlated with several clinicopathological factors in CRC including TNM stage, lymph node metastasis and EMT (Hashimoto *et al.*, 2008).

Several molecules of the ECM such as fibronectin, laminin and collagen interact with integrins which are cellular receptors. Binding of integrins to these ECM molecules influences adhesion, migration and sequestration of growth factors. In CRC, increased integrin mediated ECM interactions are found in poorly differentiated cancers with disruption of this integrin mediated adhesion resulting in apoptosis and reduced PI3K activity.

The ECM is degraded by a number of different proteases including metalloproteases. The expression level of several of these metalloproteases (MMP 1, 2, 3, 7, 9 and 13) are increased in CRC and correlates with stage of disease (Zucker & Vacirca, 2004). MMP9 released by neutrophils has been associated with release of VEGF from ECM and is increased during the transition from adenoma to adenocarcinoma. MMPs are inhibited by another group of molecules called Tissue Inhibitors of metalloproteinases (TIMPs). The expression of TIMP-1 has been linked to increased resistance to chemotherapy and shorter survival time (Davidsen *et al.*, 2006; Sørensen *et al.*, 2007).

Mucins are glycosylated proteins that have been linked to CRC (Perez *et al.*, 2008).

1.8.4. Growth Factors

Communication between the stromal and epithelial cells relies on secreted factors such as growth factors and cytokines. Although growth factors are not responsible for tumour initiation, progression of the tumour beyond this stage relies upon growth factors, which play a pivotal role in clonal expansion, invasion across tissue barriers, angiogenesis and colonisation at distant sites. Often, the mutations that develop in cancer cells enable them to survive and proliferate in the absence of growth factors. However, there is also evidence to suggest that growth factors are involved in chemotherapy drug resistance suggesting that treatments where they are targeted could also be of benefit at later stages of CRC.

Several different growth factors have been implicated in CRC formation. They are compact polypeptides, which bind to transmembrane receptors with tyrosine kinase activity. Through their action on tyrosine kinase receptors, most growth factors share downstream intracellular signalling pathways. These include mitogen activated protein kinase (MAPK), the phosphatidylinositol 3-kinase (PI3K), phospholipase C-gamma and transcription factors like signal transducers and activators of transcription (STATs) or SMAD proteins.

1.8.4.1. Epidermal growth factor (EGF)

The EGF family consists of 11 growth factors (EGF, Transforming Growth Factor (TGF-α), Neuregulin (NRG 1-4), Amphiregulin, Betacellulin, Epiregulin, Heparin binding EGF (HB-EGF) and Epigen) which share a conserved EGF domain. They bind to a group of four receptor tyrosine kinases called ErbB1-4: ErbB-1 is also known as EGFR and ErbB-2 as HER-2. Mutations, amplification or dysregulation of at least one of the ErbB family members is found in > 20 % solid tumours. EGFR overexpression has been detected in up to 80 % of CRCs (LeGolvan & Resnick, 2010) and has been correlated with early tumour recurrence and extra-hepatic metastatases (Christophi *et al.*, 2008). Consequently, anti-EGFR agents have been developed and are currently used to treat metastatic cancer.

Although the benefit of single anti-EGFR treatments are marginal, when used in combination with conventional chemotherapy, there has been a positive impact on survival in patients with metastatic disease (Wanebo & Berz, 2010; Tol & Punt, 2010). As some authors have shown that this benefit is independent of EGFR expression in the primary tumour (Chung *et al.*, 2005), it is possible that EGFR expression in metastatic tissue differs from the primary tumour. Despite the use of anti-EGFR agents in clinical practice, the precise mechanism of how EGFR signalling contributes to tumour aggressiveness and metastatic spread is unknown.

1.8.4.2. Vascular endothelial growth factor (VEGF)

The VEGF family consists of five glycoproteins: VEGFA, VEGFB, VEGFC, VEGFD and PGF (placenta growth factor). Alternative exon splicing results in four isoforms: VEGF 121, 165, 189 and 206. VEGF 121 and 165 are secreted whereas VEGF189 and 206 bind to cell surface heparin like molecules. VEGFs act through one of three receptors, VEGFR 1-3. They play a role in tumour cell survival, migration, invasion and angiogenesis. In solid tumours, release of hypoxia-inducible factor 1α (HIF-1 α) (Kaur *et al.*, 2005), EGF (Niu *et al.*, 2002) and HGF (Dong *et al.*, 2001) leads to increased levels of VEGF. VEGF then induces the release of factors such as stroma derived factor 1 (SDF-1) which attracts cancer associated fibroblasts (Christophi *et al.*, 2008; Kalluri & Zeisberg, 2006). VEGF is a therapeutic target in CRC as anti-VEGF therapy has been found to be effective in the neo-adjuvant setting for metastatic CRC (Wanebo & Berz, 2010; Tol & Punt, 2010).

1.8.4.3. Transforming growth factor β (TGF- β)

There are three isoforms of TGF-β (1-3), however, the extended superfamily consists of more than 30 additional cytokines, classified into different subfamilies like bone morphogenetic proteins, activins etc. Under normal conditions, TGF-β prevents cell cycle

progression, stimulates apoptosis and differentiation. During carcinogenesis, however, genetic and epigenetic changes convert TGF-β into a tumour promoter. It activates epithelial-mesenchymal transition through activation of the canonical and non-canonical pathways. TGF- β is also able to regulate the expression of angiogenic factors such as FGF and VEGF. In CRC, the antiproliferative effects of TGF-β are lost through mutations in the genes that encode TGF-β, the type II receptor (TGF-β2) or SMAD proteins (Markowitz & Bertagnolli, 2009).

1.8.4.4. Insulin like growth factors (IGF)

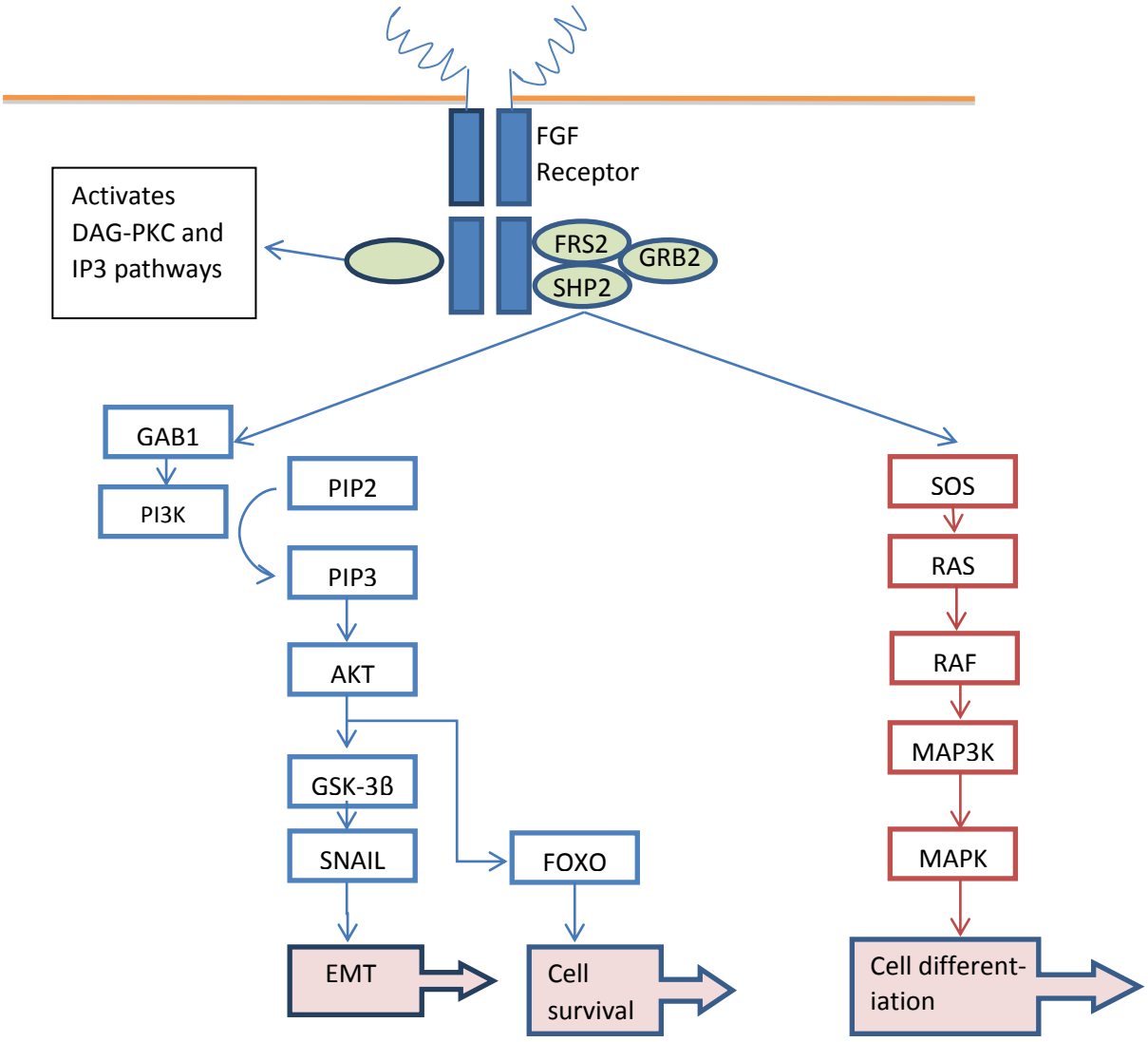
The IGF family consists of two ligands (IGF1 and IGF2), two cell surface receptors (IGF1R and IGF2R), six high affinity IGF binding proteins and IGFBP degrading enzymes. Although IGF 1 activity is not required for malignant transformation, there is evidence to suggest that it is essential for survival of transformed cells. It recruits the PI3K-AKT pathway to generate anti-apoptotic signals and is able to have an action on most cells as it is expressed in most types of tumour. High IGF-1 serum levels have been linked with high IGF-1 receptor (IGF-1R) expression in tumour cells and development of hepatic metastases. IGF-1 attracts endothelial cells and stimulates production of VEGF thus leading to increased angiogenesis and supporting tumour growth.

1.8.4.5. Fibroblast growth factors

Fibroblast growth factors (FGFs) consist of a family of 22 structurally related polypeptides that share a common core of 140 amino acids. In developmental processes, they are responsible for mesoderm induction, anterior-posterior patterning, limb development, neural induction and neural development. In mature tissues, FGFs are involved in angiogenesis, keratinocyte organisation and wound healing processes that play a key role in proliferation

and differentiation of a wide variety of cells and tissues (Ornitz & Itoh, 2015). They interact with one of four receptor tyrosine kinases (FGFR1-4) and one kinase deficient receptor, FGFR5 (Ornitz & Itoh, 2001; Sleeman *et al.*, 2001; Eswarakumar *et al.*, 2005). Binding of FGFs to its receptor results in receptor homodimerisation, autophosphorylation and recruitment of cytosolic adaptors such as fibroblast growth factor substrate 2 (FSR2) which initiates multiple signalling pathways (Eswarakumar *et al.*, 2005; Beenken & Mohammadi, 2009) (see figure 1.8.1.below). Dysregulation of this signalling axis has been shown to play a significant role in tumour development and progression of several cancers (Sugiyama *et al.*, 2010; Frullanti *et al.*, 2011; Miura *et al.*, 2012).

Figure 1.8.1. **Signalling cascades involved in FGF-FGFR interaction**



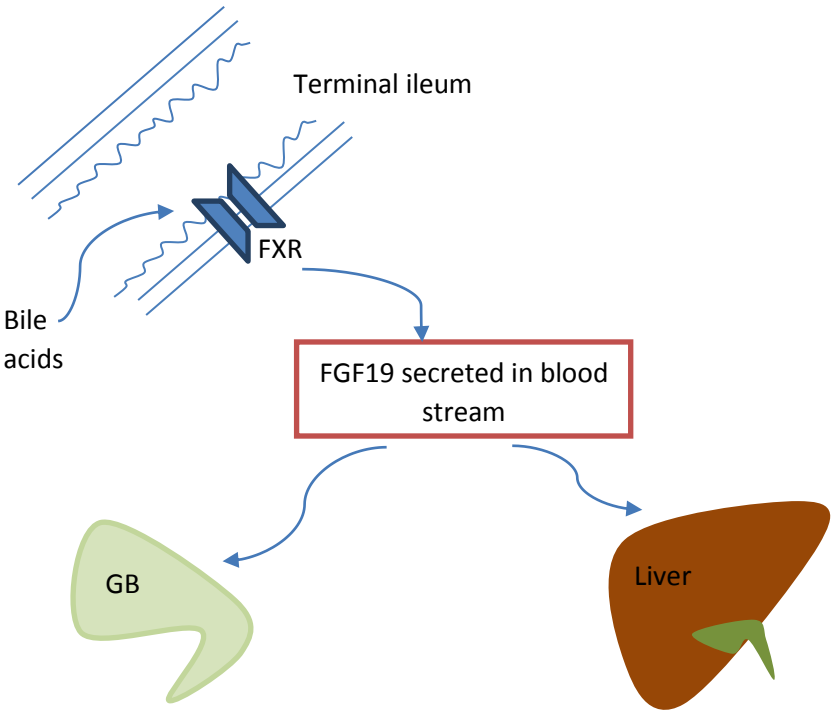
Fibroblast growth factor receptors (FGFR) 1-4 consist of three extracellular immunoglobulin-type domains (D1-D3), a single span transmembrane domain and an intracellular split tyrosine kinase domain. Alternative mRNA splicing gives rise to b and c variants of FGFR1-3 therefore there are 8 types of receptor that can be expressed at the cell surface (Eswarakumar *et al.*, 2005; Turner & Grose, 2010). FGFs bind to D2/D3domains with D3 interaction being responsible for ligand binding specificity. Heparan sulfate (HS) binding is conferred through the D2 domain and it is likely that FGFRs are permanently complexed with heparan sulfate on the cell surface (Powell *et al.*, 2002). Each FGF binds different FGFRs with differing affinity (Ornitz & Itoh, 2015). The specificity of FGFs binding to cellular targets will be influenced not only by the type of FGFRs expressed by the cell but also by the pattern of heparan sulfate binding that is generated by the cellular enzymes.

Most FGFs are only capable of a paracrine action as they are secreted proteins that bind heparan sulfates and therefore are usually caught up in the extracellular matrix of tissues that contain heparan sulfate proteoglycans (Itoh & Ornitz, 2011). In comparison, members of the FGF19 subfamily (FGF15, FGF19, FGF21, and FGF23) have poor affinity with heparan sulphate and therefore are able to diffuse through the HS-rich extracellular matrix to enter into the bloodstream (Goetz *et al.*, 2007). This enables them to have an endocrine action such as is seen with FGF15/19 which is produced by intestinal cells but acts upon FGFR4 expressed in the liver to downregulate CYP7A1 in the bile acid synthesis pathway (Jones, 2012). Similarly, FGF23 is produced in the bone but acts upon FGFR1 expressing kidney cells to regulate vitamin D synthesis and maintain phosphate homeostasis (Razzaque, 2009).

Furthermore, the FGF19 subfamily members have poor affinity for their cognate FGF receptors and cannot bind to them and activate them in a solely HS-dependent fashion. Therefore, they require members of the Klotho family of proteins for high affinity receptor binding (Kurosu & Kuro-O, 2009). There are different types of Klotho proteins that interact with FGFs: α-klotho is the cofactor for FGF23, β-klotho for FGF15/19 and FGF21 (Kurosu *et al.*, 2006; Kurosu *et al.*, 2007; Urakawa *et al.*, 2006; Ogawa *et al.*, 2007; Wu *et al.*, 2007b). Despite having widespread tissue distribution, sites of action of the endocrine FGFs are limited by the distribution of klotho proteins (Fon Tacer *et al.*, 2010).

Aberrant FGF signalling is found in many different cancers including cancer of the prostate, breast, lung, bladder and colon (Brooks *et al.*, 2012; Turner & Grose, 2010). The most widely studied FGF in CRC is FGF2 which has a synergistic action with VEGF on angiogenesis and has been shown to have prognostic relevance (Elagoz *et al.*, 2006). Other fibroblast growth factors that have been implicated in CRC include FGF19 and FGF7. As alluded to earlier, FGF19 belongs to the endocrine family of FGFs and usually plays a role in bile acid, protein and glucose metabolism (see figure 1.8.2.).

Figure 1.8.2. The physiological roles of FGF19



- Increases cAMP levels which increase gallbladder filling

- Inhibits CYP7A1 reducing bile acid synthesis
- Blocks CREB phosphorylation which reduces gluconeogenesis
- Stimulates glycogen and protein synthesis through Erk pathway

FGF19 has been implicated in a number of cancers, particularly hepatocellular carcinoma (French *et al.*, 2012). FGF19 transgenic mice develop HCC and liver dysplasia by 10-12 months of age (Nicholes *et al.*, 2002). FGF19 appears to interact with FGFR4 in driving HCC formation. Blocking the action of FGFR4 using an antibody (LD1) prevented interaction of FGF19 with FGFR4. This reduced colony formation and proliferation *in vitro* and was shown to decrease tumour growth in a preclinical model of liver cancer *in vivo* (French *et al.*, 2012). β-klotho has also been shown to play a role in HCC carcinogenesis. Beta klotho expression is increased in HCC tumour tissue compared to non-tumour tissue and a greater than 2 fold increase in its expression is associated with the development of multiple tumours (Poh *et al.*, 2012). This suggests that it is not only the action of FGF19 that

is important but that there is concurrent increase in the expression of its receptor and cofactor that contribute to HCC formation.

Previous analysis suggests that FGF19 may also play a role in colorectal cancer by acting on the wnt signalling pathway. Ectopic expression of FGF19 in transgenic mice results in development of HCC and these liver tumours contain neoplastic cells with nuclear localisation of beta-catenin indicative of activation. Co-activation of Wnt and FGF signalling pathways in tumours leads to a more malignant phenotype and inhibition of beta catenin using siRNA completely abolishes FGF19 expression in HCT116 cells suggesting that beta-catenin influences FGF19 expression (Katoh, 2006).

Besides the direct action of FGF19 expression on tumour growth, modifications of its receptor, FGFR4 have also been linked to carcinogenesis. Reduced expression of FGFR4 leads to upregulation of E-cadherin and downregulation of other epithelial-mesenchymal transition (EMT) mediators such as SNAIL, TWIST and ZEB. E-cadherin loss has been recognised as one of the central events in EMT as the study noted that such a change led to an accompanied concurrent reduction in tumour growth both *in vitro* and *in vivo* models. The *in vivo* tumours that were produced from FGFR4 silenced cells were not only smaller, but consisted of a whitish aspect indicative of deficiency in angiogenesis (Peláez-García *et al.*, 2013). FGFR4 may also play a role in crosstalk between tumour associated fibroblasts (TAF) that govern EMT and the tumour tissue itself. Upregulation of FGFR4 was observed in CRC cells that were co-cultured with TAFs (Liu *et al.*, 2013b). This study demonstrated that inhibition of FGFR4 reduced TAF-induced signalling cascades including FRS2 and ERK phosphorylation. Using both *in vitro* and *in vivo* models, suppression of FGFR4 was able to reverse TAF-induced migration and invasion of CRC cells. The concept that was proposed suggested that TAFs produce CCL2 which induces FGFR4 expression. FGFR4 overexpression leads to phosphorylation of beta catenin which translocates to the nucleus

and initiates expression of SNAIL. This represses expression of E-cadherin, which leads to induction of EMT in CRC cells.

In contrast to FGF19, the studies investigating FGF7 and FGFR2b expression in cancer have yielded conflicting results. Fibroblast growth factor 7 (FGF7), also known as keratinocyte growth factor, is produced by cells of mesenchymal origin (Finch *et al.*, 1989; Rubin *et al.*, 1989) and acts on epithelial cells through its interaction with a specific isoform of the FGF receptor, FGFR2b (Miki *et al.*, 1991). Generally, KGF is not expressed in epithelial cancer cell lines (Dahiya *et al.*, 1996; Iida *et al.*, 1994; Knerer *et al.*, 1998) except in a few cell lines from pancreatic and breast cancer (Bansal *et al.*, 1997; Siddiqi *et al.*, 1995). In comparison, tumour tissue usually expresses KGF. However, there is disagreement across the literature with regards to whether KGF expression levels are higher (Siddiqi *et al.*, 1995; Watanabe *et al.*, 2000) or lower (Knerer *et al.*, 1998) in tumour tissue compared with paired normal mucosa. Despite these differences, studies where in situ hybridisation is used to locate cells expressing KGF have shown that it is usually expressed by stromal cells lying in close proximity to cancer cells. Hence, cancer cells probably induce KGF production which stimulates further epithelial cell proliferation.

FGFR2b is expressed in cancer cell lines including those of the breast, colon, stomach, oesophagus, stomach, pancreas, prostate and oral mucosa. There is similar disagreement in the literature with regards to whether expression levels are higher or lower in cancer tissue. This is further complicated by the possibility of class switch. Reduced FGFR2b expression has been associated with increased FGFR2IIIc expression, which confers a more malignant phenotype and has been related to progression of prostate cancer and EMT in bladder cancer cells (Carstens *et al.*, 1997; Chaffer *et al.*, 2006; Oltean *et al.*, 2006). Conversely, FGFR2IIIb and FGF7 have been shown to increase VEGF-A expression and are associated with a poor prognosis in pancreatic cancer (Cho *et al.*, 2007). Therefore, in certain

situations, FGFR2b actually helps to maintain differentiation, inhibit tumour growth and tumour invasion and in others, it promotes venous invasion and tumour angiogenesis leading to a more malignant form of cancer.

1.9 Genetic profiling in CRC

Genetic profiling refers to the process whereby a disease state is classified by a set of genetic lesions that are consistently found across individuals with similar clinical disease and tumour behaviour. It has the potential to identify patients with disease from healthy individuals but also sub classify individuals believed to have the same disease based upon pathological factors or response to treatment. As a consequence, it can be utilised to screen amongst asymptomatic individuals and identify those at most risk of cancer. It can also be translated to develop individual targeted molecular therapy where only agents known to act upon the specific molecular subtype of cancer are given the therapy. The molecular characteristics that are utilised to perform genetic profiling include differences at the -

i) chromosomal level (karyotype)

ii) nucleotide level (point mutations or variation of sequences)

iii) nucleotide-modification level (epigenetic features).

1.9.1. Techniques for genetic profiling

Multiple techniques have been previously employed to perform genetic profiling. Conventionally, genetic analysis of patients with inherited syndromes with linkage analysis has been effective at elucidating the genetic alterations that occur in diseases such as CRC (Leppert *et al.*, 1990; Lynch & de la Chapelle, 2003). This technique was effective at identifying mutations in APC as the causative agent for FAP predisposition and highlighted defective DNA mismatch repair genes in HNPCC.

Cytogenetics refers to 'gross' chromosomal analysis in which large deletions and initial gene mapping is performed. This includes techniques such as G-band analysis, fluorescence in situ hybridisation (FISH) and comparative genomic hybridisation (CGH). Based on the analysis of 17p and 18q loci respectively, this approach discovered p53 and DCC in CRC (Baker *et al.,* 1989; Fearon *et al.,* 1990; Jen *et al.,* 1994). Cytogenetic evaluation has identified two distinct modes of genetic instability in CRC, namely the microsatellite instability pathway and chromosomal instability pathway (Lindblom, 2001).

Classical molecular analysis relies upon identification of mutations in individual genes that then have to be associated with a specific disease state, have to satisfy the rules of causality (as expressed by Koch) and be attributed to some clinicopathological feature of the disease process such as diagnosis, prognosis or response to therapy. These techniques were effective at elucidating the molecular changes that underpin the adenoma –carcinoma sequence as described by Vogelstein (Vogelstein *et al.,* 1988). The methods used to investigate multiple genes in this manner include serial analysis of gene expression, micro-array and polymerase chain reaction (PCR).

1.9.2. Micro-array analysis

Microarray technology has been utilised to investigate several aspects of CRC (Nannini *et al.,* 2009). Studies have investigated the carcinogenesis process itself by evaluating the differences in gene expression between cancer tissue and control tissue. Genetic profiling using micro-array methods has also enabled identification of genes that indicate a better prognosis or can be utilised to determine if individuals will respond to chemotherapy agents. The identification of genes or pathways in this way can contribute to the development of biomarkers that detect disease, predict prognosis or determine outcome following treatment.

Previous investigators have demonstrated that it is possible to cluster individuals with cancer, polyps and control based on gene expression profile of often a small number of genes (Notterman *et al.*, 2001; Kitahara *et al.*, 2001; Grade *et al.*, 2007). Furthermore, studies have clearly identified that there is considerable molecular heterogeneity within CRC patients that may explain the observed differences in prognosis and response to treatment that cannot be justified based on existing clinicopathological features (Groene *et al.*, 2006; Perez-Villamil *et al.*, 2012). Common processes that have been found to be dysregulated in CRC across the studies include cell adhesion, cell communication, cell cycle regulation, cellular and nuclei acid metabolism and cellular response to external stimuli. Although microarray methods have been successful in this manner, it is important to realise that there are some limitations to this approach.

There is little agreement in the genes that are identified in different studies and often inconsistencies in the direction in which the change occurs. The authors of a recent review propose that this may be explained by the use of different sample collection techniques or different type of omics platform (Chan *et al.*, 2008). There is no standardisation of microarrays with some investigators custom making their chip and others using a commercially available, though more expensive, chip. Similarly, there is no standardisation of analytical techniques that are used for subsequent data analysis leading to variation in the conclusions that are reached. Despite some of these shortcomings, microarray profiling has led to the development of multiple gene marker panels that identify individuals most likely to benefit from adjuvant therapy. Examples include Mammaprint (Agendia, Amsterdam, Netherlands) and Oncotype Dx (Genomic Health, Redwood City, California) (Slodkowska & Ross, 2009; Gray *et al.*, 2011).

1.10. Field cancerisation

Most of the studies that have been conducted in colorectal cancer have focused on describing the molecular changes found in cancer tissue itself and using these to develop potential biomarkers for disease detection. Some investigators have argued that if one is investigating early biomarkers of disease, the focus should be on the disease process rather than the disease itself (Dakubo *et al.,* 2007; Rubin, 2011). Therefore, using molecular markers that are known to be mutated in CRC can lead to the identification of potential targets that represent late changes in the disease process. Field cancerisation offers a different approach to identify pathological changes that occur early on in the cancer process. It refers to the concept that the genetic and environmental factors linked with colorectal cancer inflict a diffuse field of injury that provides 'fertile' background upon which further genetic and epigenetic events give rise to cancer. Therefore, the initial genetic or environmental insult that preconditioned the colonic mucosa to cancer should not only be detectable at the primary tumour site but should also be found throughout the entire colon. These early changes do not give rise to a detectable difference in histological appearance of the cells and therefore this process has been described as (Dakubo *et al.*, 2007) –

"the process whereby cells in a particular tissue or organ are transformed, such that genetically altered but histologically normal appearing cells predate the development of neoplasia or coexist with malignant cells, irrespective of clonality".

The concept of field cancerisation was first introduced by Slaughter in 1953 (SLAUGHTER *et al.*, 1953) based upon a number of observations in head and neck squamous cell cancer –

i) Development of cancer in multifocal areas of precancerous change

ii) Abnormal tissue surrounds cancer

iii) Persistence of abnormal tissue after surgery may explain the local recurrence of tumour at the same site

Since then, field cancerisation has been described in the colon as the 'process whereby colonic epithelial cells acquire pro-tumourigenic mutations that are insufficient to cause morphological change but which predispose to tumour. It is the clonal expansion of these mutant cells that results in large 'patches' or fields of tissue that are primed to become neoplastic' (Luo *et al.*, 2014).

The ability to detect the early changes characteristic of field cancerisation could enable risk stratification of both asymptomatic patients in the context of screening and aid development of more accurate post cancer surveillance programs. Furthermore, at present, chemoprevention trials investigating the use of pharmacotherapy in modulating a patient's risk of cancer rely upon the use the incidence of adenoma as a surrogate marker of colorectal cancer. Consequently, a long period of follow up, often spanning many years, is required reflecting the length of time it can take to form an adenoma. If an early marker of colorectal cancer was identified, its use in such trials could considerably shorten the follow up period required to determine efficacy.

1.10.1 Field defect in inflammatory bowel disease

In a similar manner to sporadic CRC, IBD-associated CRC also follows a multistep process where inflamed, regenerative epithelium changes to hyperplastic epithelium, flat dysplasia and eventually invasive adenocarcinoma (Riddell *et al.*, 1983). Current screening of these patients relies upon the identification of dysplastic lesions. Low-grade dysplasia is associated with a risk of CRC of around 20 %, which warrants further surveillance (Woolrich *et al.*, 1992; Lennard-Jones *et al.*, 1983; Levin *et al.*, 1991). However, the presence of high-grade dysplasia is indicative of a higher probability of coexisting cancer and progression to carcinoma (around 60 %) which necessitates colectomy (Bernstein *et al.*, 1994; Connell *et al.*, 1994). IBD associated neoplasia can therefore be used to risk stratify

patients and is indicative of colonic mucosa that had been preconditioned to form neoplasia. As such, the field cancerisation theory has also been described for IBD as

'*the formation of a histologically indistinguishable area of clonally derived, mutant cells within the inflamed segment of intestinal tract in IBD'* (Graham *et al.*, 2011).

Molecular studies have found evidence to support a field defect in IBD. The same mutation spectrum in either, TP53 and KRAS is seen in entire neoplastic lesions as well as non-dysplastic crypts, suggesting that these mutant clones are involved in priming the colon for cancer formation (Leedham *et al.*, 2009; Graham *et al.*, 2011). Further characterisation of this field defect is necessary to aid risk stratification of these patients. Nevertheless, it highlights that once dysplasia is identified in these patients, the entire colon is at risk of developing cancer and warrants surgical removal.

1.10.2. Mechanism for field cancerisation

Several different mechanisms for how field cancerisation occurs in colorectal cancer have been proposed (see figure 1.10. below). Based on the stem cell theory, a field defect may occur when a epimutation or mutation occur in stem cells giving it reproductive advantage so that it generates clonal descendants that outcompete neighbouring stem cells. These stem cells replace all the stem cells in the crypt and through crypt fission, a patch of mucosa containing several crypts with cells containing mutations is created. As further mutations develop, a second patch of altered crypts is created within the first patch (Bernstein *et al.*, 2013). Eventually, a cell is generated that is capable of invasion and malignant growth. Others have postulated that the widespread changes in methylation of genes found at distant sites along the colon in cancer patients are explained by dietary exposure to vitamin B and

folate which alter the methylation state of macroscopically normal mucosa (Luo *et al.*, 2014). In contrast, there have been antagonists of field cancerisation who argue that these changes occur in response to the tumour itself and do not predate malignant transformation (Kuniyasu *et al.*, 2000).

However, it has emerged that 'more than 80 % of somatic mutations found in the mutator phenotype of colorectal cancer occur before the onset of clonal expansion' (Tsao *et al.*, 2000) and may be indicative of an underlying field defect. Furthermore, synchronous cancers in the same individual have been shown to share similar epigenetic changes (Nosho *et al.*, 2009) suggesting that they may arise from the same mutated clone. There are three theories that have been proposed to explain the common clonal origin of multiple primary tumours (Braakhuis *et al.*, 2003) –

i) Single cells or small clusters of cells migrate through the submucosa

ii) Cells are shed in the lumen of an organ at one place and grow at another which has been used as a possible theory in oral cancer (Califano *et al.*, 1999) or bladder cancer (Bedi *et al.*, 1996).

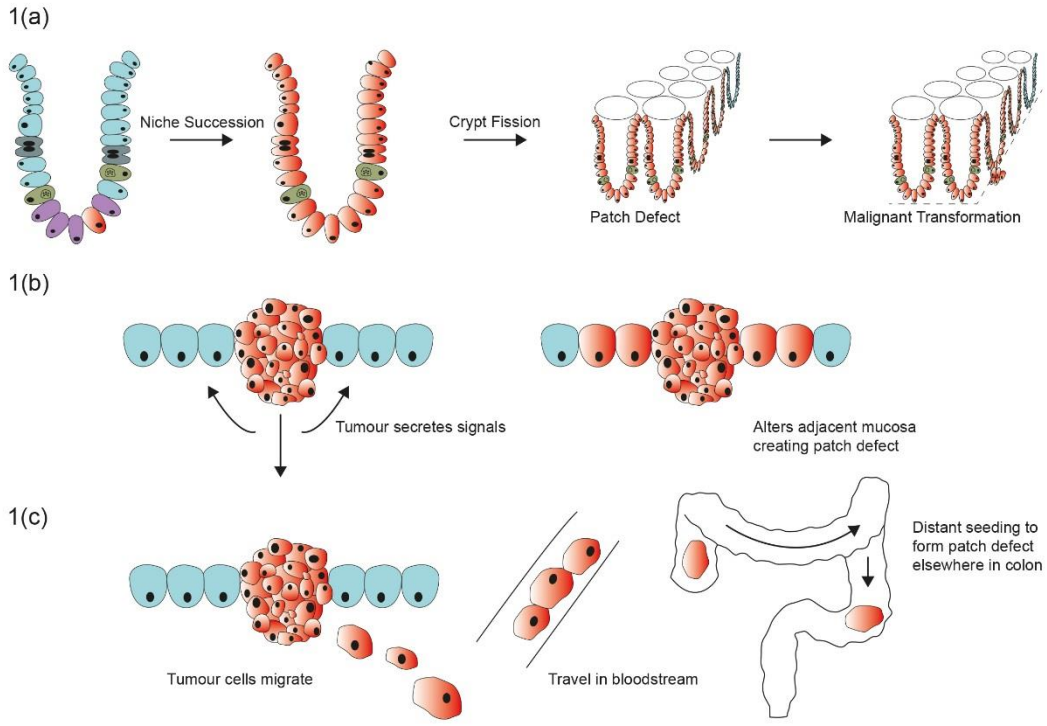
iii) There is a large contiguous genetically altered field in the epithelium in which multiple clonally related neoplastic lesions develop.

Figure 1.10. Schematic representation of proposed mechanisms for formation of field defect

1(a): A mutation or epigenetic alteration in a stem cell (depicted in red) is inherited by all cells within the crypt through niche succession. Crypt fission results in several crypts becoming biologically altered creating a patch defect. Further mutation within this field of altered mucosa leads to malignant transformation.

1(b): Tumour secretes chemical signals that alter the adjacent mucosa resulting in a field defect

1(c): Malignant cells shed from a tumour travel in the bloodstream and seed in a distant site rendering the mucosa susceptible to malignant transformation



1.10.3. Field cancerisation in CRC

There is emerging molecular evidence to suggest that genetic changes seen in cancer tissue can be detected in adjacent macroscopically normal appearing mucosa (MNM) of the colon (Shen 2005, Chai 2009, Facista 2012, us 2015) lending support to the field cancerisation theory. However, clarity regarding which cellular processes and genes are dysregulated in the adjacent MNM are lacking. Furthermore, although some of the early studies attempting to translate this concept into clinical practice are promising, the utility of field cancerisation in a clinical setting needs further evaluation.

1.11. Summary

Colorectal cancer prognosis remains poor as it is usually diagnosed at a late stage when patients present with symptoms. Despite considerable efforts to develop screening programs to detect disease at an early stage in asymptomatic individuals, current tests lack specificity, are invasive or poorly tolerated by the general population. There have been considerable advances in our understanding of colorectal cancer formation, however, there is a relative paucity of translation of these findings into the clinical arena. Some have criticised investigators on focussing on the tumour tissue itself which has already undergone malignant transformation and therefore harbours biological changes that occur relatively late in the cancer pathway. Instead, a different perspective is required where the early changes that occur prior to malignant transformation are identified. Field cancerisation offers such an approach as it eludes that the 'normal' mucosa adjacent to cancer contains early biological changes that contribute to cancer formation. Characterisation of this field 'defect' could thus enable better screening tests to be developed and would facilitate more accurate estimation of future neoplastic risk compared to existing modalities which rely upon detection of polyps. In addition, individuals deemed to be at high risk could undergo targeted pharmacotherapy, which would modulate their risk of colorectal cancer prior to the development of any histological abnormality in their colon.

1.12. Research hypothesis, aims and objectives.

1.12.1. Research Hypothesis

The present research hypothesis is-

'The macroscopically normal mucosa adjacent to polyps and cancers differs from that in control subjects therefore field cancerisation takes place in the colon.'

1.12.2. Research aims

The research aims are –

i) to determine if colorectal adenomas are reliable markers of field effect in the colon

ii) to characterise the global gene expression profile of macroscopically normal mucosa (MNM) adjacent to cancer, adenomas and in control subjects

iii) to investigate the role of fibroblast growth factors in field cancerisation and CRC formation

1.12.3. Research objectives

Although polyps are well established as a precursor lesion for CRC, evidence to support their role in identifying individuals with a field defect are lacking. Thus, the first chapter will investigate the utility of polyps as a biomarker of field cancerisation in CRC. The assumption being that CRC survivors that develop polyps in the remaining bowel after surgery are more likely to have a diffuse field of altered mucosa and therefore are at higher risk of both polyp and cancer recurrence. Similarly, those with synchronous polyps at time of diagnosis are also more likely to harbour a field defect and therefore would be expected to exhibit poorer survival.

In the next chapter, the global gene expression profile of the macroscopically normal mucosa around cancer or polyp will be compared to that found in control subjects using micro-array technology. The findings from this study will help to identify important genes that are dysregulated and this will then be validated in the subsequent chapter.

In the final chapter, the role of fibroblast growth factor 7 and 19 will be investigated to determine the contribution of stromal elements to field cancerisation. As most of the studies investigating field cancerisation have focussed on changes occurring in the epithelial cells themselves, it will be important to evaluate how growth factors released by stromal cells precondition the mucosa to CRC formation.

Section 2: Subjects, Materials and Methods

2.1. Ethical approval

This was part of a prospective NIHR observational cohort study (FaMISHED- Food and Fermentation using Metagenomics in Health and Disease designed to evaluate the role of biomarkers in gastrointestinal diseases, including colorectal cancer. Ethical approval was granted by Coventry and Warwick Local Research Ethics Committee (ref MREC ref no 09/H1211/38) and University Hospital Coventry & Warwickshire Research & Development division. Funding was obtained from various sources including the Bowel Disease Research Foundation and the Colorectal Cancer Research Fund at University Hospitals of Coventry and Warwickshire NHS Trust

All participants provided written informed consent in accordance with the Declaration of Helsinki.

2.2. Setting and participants

Subjects were either recruited at time of endoscopy (both screening and symptomatic) or at time of surgery. Written informed consent was gained.

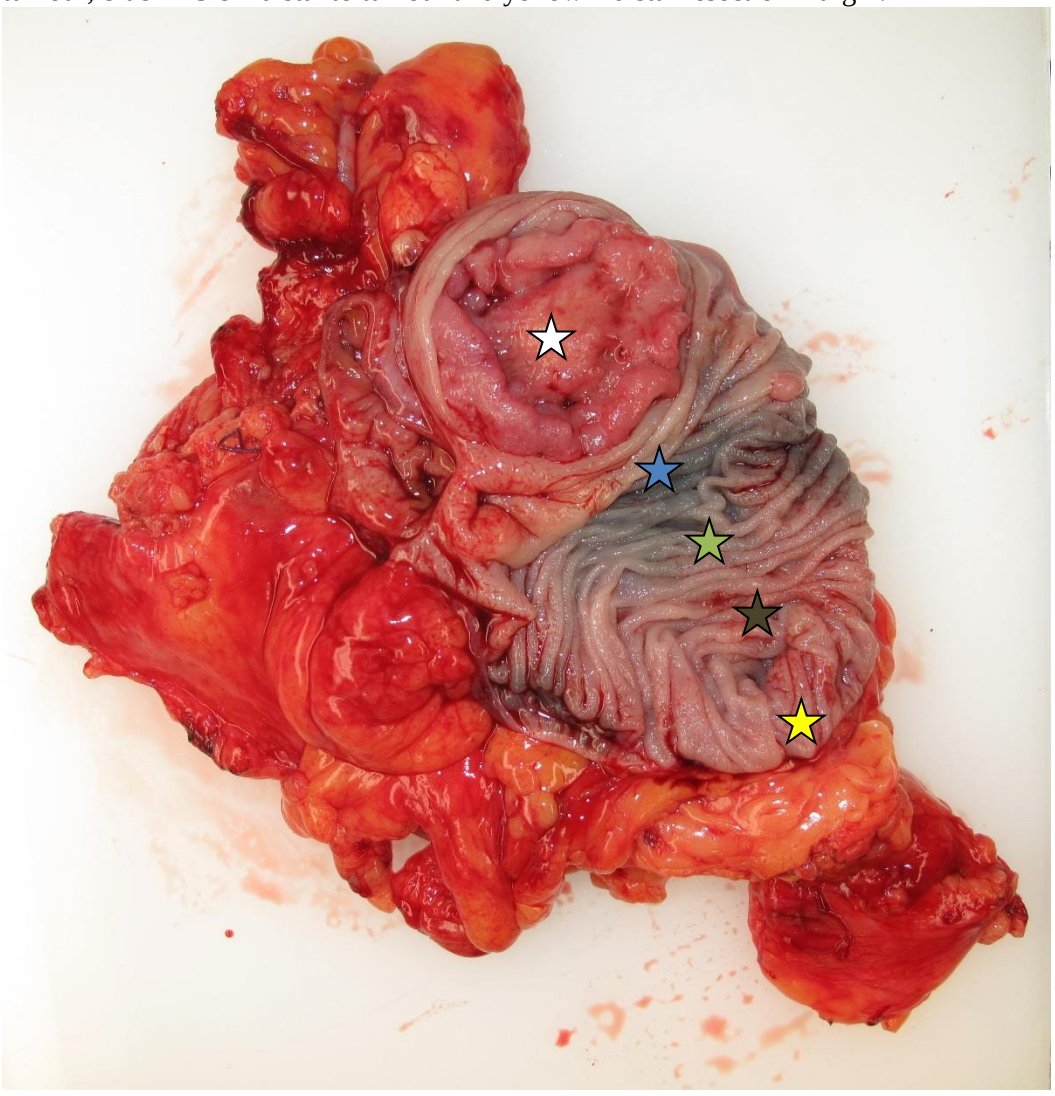
2.2.1. At endoscopy

Mucosal pinch biopsies were taken from the caecum and rectum in all subjects. Participants who were diagnosed with polyps or a cancer also underwent biopsy of macroscopically normal mucosa (MNM) within 1 cm of the lesion. This was classified as MNM adjacent to polyp/cancer. Although biopsies were taken from cancer tissue itself, this was not possible for polyps therefore the values of gene expression given under the name ‘polyp’ refer to tissue adjacent to the polyp rather than the polyp tissue itself. Polyps that have been removed endoscopically require histopathological analysis to determine there are no cancerous elements, hence, it was not possible to use this tissue for research purposes.

2.2.2. At surgery

The colectomy specimen was collected after bowel resection and taken to the pathology department. In conjunction with the histopathologist who would be examining the specimen, the specimen was opened and mucosal biopsies were taken from the areas outlined in figure 2.2. The sample taken from the most distant margin to the tumour was utilised for analysis as the nearer margin may contain altered mucosa through a field defect. In a subset of patients, serial biopsies were taken at 3cm, 5 cm and 10 cm from the tumour edge. Patients who had undergone neoadjuvant chemoradiotherapy were excluded from the study as this has been shown to alter tumour biology and subsequent clinical outcome (Rödel *et al.*, 2005).

Figure 2.2. **Sampling sites.** Photo of right hemicolectomy specimen showing site of sampling marked with crosses (white – tumour, blue – adjacent MNM, green- 3 cm distal to tumour, black – 5 cm distal to tumour and yellow – distal resection margin.



2.2.3. Sample processing

Tissue samples were taken for three purposes; for RNA analysis, for protein analysis and for immunohistochemistry. Hence, each biopsy was divided into three parts and stored in RNA later (Fisher Scientific, UK), formalin or snap frozen using liquid nitrogen. All samples were stored at -80 ^{o}C for long term storage.

2.2.4. Collection of blood samples

Blood samples were taken at time of endoscopy or on the day of surgery. In a subset of patients, samples were collected after overnight fast on the morning of surgery/endoscopy for the measurement of FGF19 which has both a diurnal variation and responds to oral intake (Lundåsen *et al.*, 2006; Reiche *et al.*, 2010). Samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes and thrombin based clot serum separator tubes (serum collection). Samples were spun at 25000 g for 15 mins and the supernatant was aliquoted for serum and plasma analysis. These samples were stored at -80 ^{o}C for long term storage.

2.2.5. Study design

For the purposes of gene expression analysis, patients with cancer were age and sex matched with control subjects. Cancer patients with right sided tumours or polyps (caecum to mid transverse) were matched to caecal samples taken from control subjects and patients with left sided tumours/polyps (distal to mid transverse) were matched with rectal samples from control subjects. This is because initial analysis revealed differences in the genes being measured in the right colon compared to the left colon.

2.3. Micro-array methods

2.3.1 Participants

Subjects (n=16) were recruited into the study from December 2010 to April 2011. Informed consent was obtained from all patients. Mucosal pinch biopsies were taken at time of endoscopy from macroscopically normal mucosa within 1 cm of polyp (n=6) or cancer (n=5). In healthy controls (n=5), biopsies were taken from the caecum and rectum. Diagnosis was confirmed by histology and patients were divided into three groups: control, polyp and cancer. The clinico-pathological characteristics of these patients are given in table 2.3. All tissue specimens were taken and immediately placed in Qiazol prior to being frozen and stored at -80 deg C.

Table 2.3: Demographic and clinical details of patients that were included in the microarray study. The reference numbers refer to each individual patient in the three groups. Patient (Adc-5) did not undergo surgical resection therefore there is no TNM staging available for this patient.

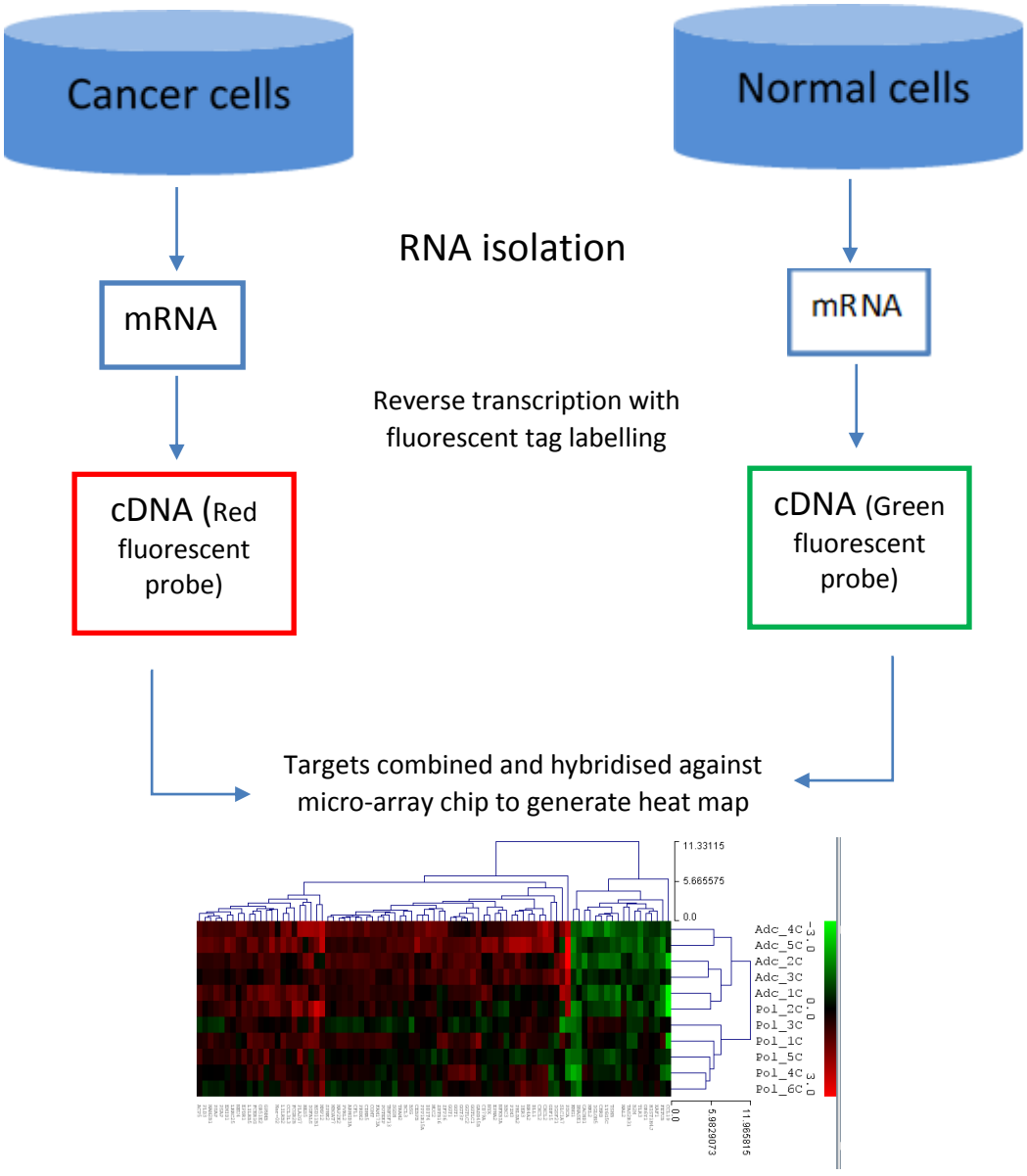
Sample type	Reference Number	Gender	Age	Indication/clinical details	Histology
Control	C1	M	82	Change in bowel habit	Normal
	C2	M	80	Acute diarrhoea	Normal
	C3	M	75	Change in bowel habit	Normal
	C4	F	67	Abdo pain, PR bleeding	Not done
	C5	F	83	Microcytic anaemia	Not done
Polyp	Pol-1	F	60	4 mm sigmoid sessile	TVA + LGD
	Pol-2	M	79	3 mm sigmoid sessile	TA + LGD
	Pol-3	F	71	4 mm sigmoid sessile	TVA + LGD
	Pol-4	M	61	3 mm transverse sessile	Hyperplastic
	Pol-5	M	53	Small rectal polyp	TA + LGD
	Pol-6	F	77	5 mm splenic pedunculated	TA + LGD
Cancer	Adc-1	M	53	Rectal	T3N0M0
	Adc-2	M	62	Rectal	T2N0M0
	Adc-3	M	66	Polyp cancer sigmoid	T1
	Adc-4	M	72	Hepatic flexure	T3N0M0
	Adc-5	F	81	Rectal	Poorly diff cancer

Abdo pain=abdominal pain, TVA – tubulovillous adenoma, TA – tubular adenoma, LGD – low grade dysplasia, Poorly diff = poorly differentiated cancer. TNM staging used for cancers.

2.3.2. Experimental design

The SurePrint G3 Human Gene Expression 8x60K Gene Expression array was utilized. This consists of 62, 976 probes of which 42, 405 are unique. Agilent have developed an inkjet method which prints oligonucleotides onto the surface of a glass slide similar to a colour printer where the four ink colours are replaced with the nucleotides ATCG. Based on oligonucleotides of 60 bases in length, differences in gene expression between samples were assessed in a 2 channel micro-array experiment (see fig 2.3.).

Figure 2.3. Two channel microarray experiment



2.3.3. Isolation, purification and quantification of RNA

RNA was extracted from the mucosal biopsy tissue (approximately 0.2 mg) using Qiazol (Qiagen, UK) and total RNA was extracted using a spin column (RNeasy Mini Tissue Kit: Qiagen, UK) according to manufacturer's instructions. Quantification was performed using a spectrophotometer (Nanodrop ND-1000, Labtech, UK) by measuring absorbance at 260 nm wavelength.

2.3.4. Genechip micro-array assay method

All experiments were performed in triplicate. Total RNA samples were quality checked on an Agilent Bioanalyser 1200 (Agilent Technologies Inc, Santa Clara, CA, USA) and only RNA Integrity numbers between 6.5 and 9.8 were selected. The RNA concentrations were normalised to 100ng in 1.5µL and the Low Input QuickAmp Labelling Kit Protocol (Agilent Technologies) was followed according to manufacturer's instructions. This uses a T7 RNA Polymerase to simultaneously amplify target material and incorporate cyanine 3 or cyanine 5 labelled CTP. A universal RNA reference was labelled with Cyanine 5-CTP. The samples were labelled in randomised batches with Cyanine 3-CTP and yields and specific activities were tested to ensure they were in the recommended ranges. The labelled cRNAs were hybridised to SurePrint G3 Human Gene Expression 8x60K Gene Expression arrays (AMADID 028004; Agilent Technologies) according to manufacturer's instructions. The arrays were washed in Gene Expression Wash Buffer 1 and Gene Expression Wash Buffer 2 and immediately scanned in an Agilent GA2565CA scanner at 2µm resolution. The data were extracted using Feature Extraction v10.7.3.1 with default settings appropriate to the array design.

2.3.5. Micro-array data analysis

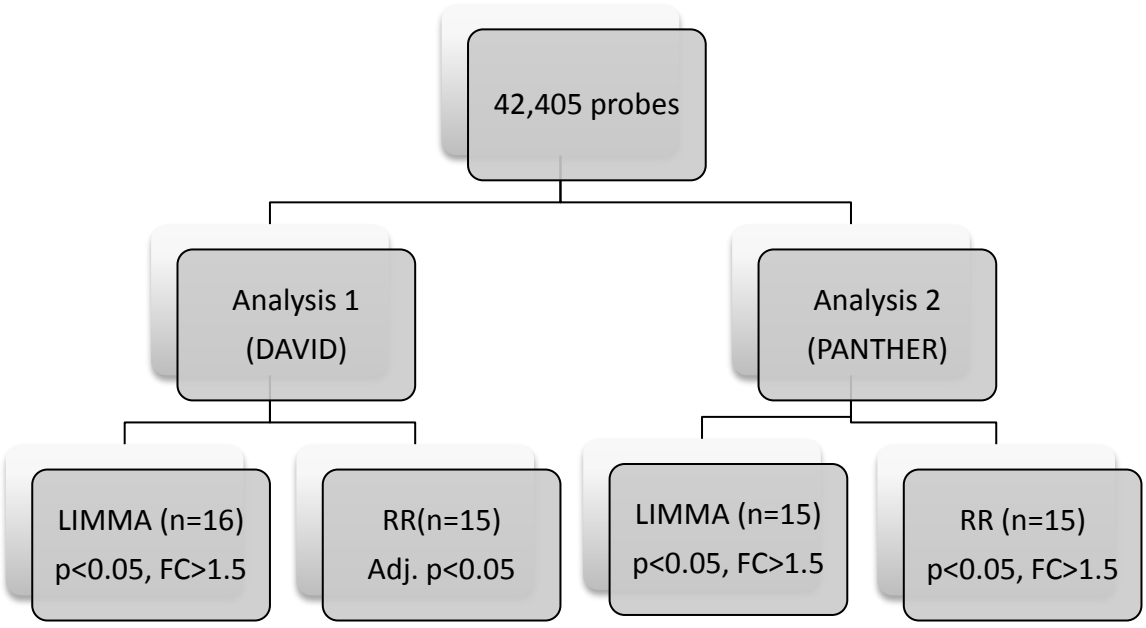
Bioinformatic software was used to convert the output images into data for further analysis. Data normalisation was performed both within array (Lowess) and between array (Aquantile) using the Agilent microarray scanner. Summarisation was performed as despite more than 62,000 probes, only 42, 405 probes were unique. The Bioconductor R package was used for statistical analysis. In the lmFit function, either 'ls' (LIMMA) or 'robust' (RR) were used for the two different types of data analysis to identify differentially expressed genes (DEGs). Previous studies have utilised a similar approach with multiple statistical methods to reduce the likelihood of false positives (Xu *et al.*, 2013). For the functional analysis, genes found with either statistical method were used as a larger pool of genes would more likely represent the cellular processes that were dysregulated. Furthermore, two different types of analysis were undertaken (Figure 1). In the first analysis, functional analysis was performed using DAVID software (Huang *et al.*, 2009a). Using DAVID software, the genes were classified into biological functional terms. The enrichment ratio of genes in each category was calculated as a means of determining how prevalent these genes were compared to the background levels of expression. An enrichment ratio of greater than 1.33 was considered to be significant.

In the second analysis, functional analysis was undertaken using PANTHER software (Mi *et al.*, 2013). Three separate analyses were undertaken; cellular components, molecular function and biological process. A statistical overrepresentation test was performed using the DEGs in each category to identify biological processes that were overrepresented in the DEGs list compared to the background level of gene expression. A p value of <0.05 was considered significant.

Although both methods compare DEGs with background levels of gene expression, the way in which the genes are clustered differs. The first analysis clusters genes according to biological process only whereas the second analysis used molecular function and cellular

component. This enhanced the understanding of which cellular compartments were affected along the adenoma-carcinoma sequence and enabled linkage of this to molecular/biological function.

Figure 2.4: Analysis of micro-array data showing thresholds used for each statistical method and details of the functional analysis. FC is fold change difference between the groups being examined.



2.4. Gene expression

2.4.1. RNA extraction and processing

RNA was extracted from mucosal biopsy tissue stored in RNA later (approximately 0.2 mg). This was homogenised in 1 mL Qiazol (Qiagen, UK) and total RNA was extracted using a column based isolation method (RNeasy Mini Tissue Kit: Qiagen, UK) according to manufacturer's instructions. This gave 30 µL of RNA. A DNase I Kit (Sigma) was utilised to remove possible genomic DNA. 3.5 µL (1000 U/mL) DNase I digestion enzyme and 3.5 µL reaction buffer were added for 15 minutes at room temperature followed by 3.5 µL stop solution (50mM EDTA). The solution was centrifuged for 8 seconds and heated to 70ºC for 10 minutes then chilled on ice. Quantification was performed using a spectrophotometer (Nanodrop, Labtech) at absorbance of 260 nm using 1.5 µL sample in duplicates. The ratio between absorbance at 260/280 nm and at 260/230 nm were measured to determine RNA purity. RNA with a value between 1.8 and 2.1 was deemed appropriate for use.

RNA integrity was also evaluated using agarose gel electrophoresis with ethidium bromide. A 1.5 % agarose gel was made by adding 1.5 g agarose to 150 mL 1x TAE (Tris base, acetic acid and EDTA). The agarose was dissolved by heating for 60 seconds in a 900-watt microwave. The solution was cooled before adding 4 µL 1 µg/ml ethidium bromide. After the gel had set, eight lanes with 2 µL RNA were loaded. 1x TAE was used as a buffer during the 60-minute electrophoresis at 100 V. The gel was viewed under UV light to show ethidium bromide incorporated DNA and images were taken (ChemiGenius). If there were two sharp distinct bands representing 18S and 28S, the RNA was considered to be of acceptable integrity for use.

2.4.2. Complimentary DNA (cDNA) synthesis

cDNA was synthesised using a Bioline kit (#BIO-65026). Based on quantification as outlined above, 250 ng RNA per sample was used to make complimentary DNA (cDNA). The following were added to a 200 µL sterile micro centrifuge tube –

- RNA (250 ng)
- 1 µL random hexamers 50-250 ng (Bioline, UK)
- 1 µL 10 mM dNTP mix (Invitrogen, UK).
- RNase free water

to a total volume of 10 µL.

Samples were vortexed, spun briefly and heated to 70 ºC for 5 minutes and chilled on ice for 2 minutes. Samples were mixed with 10 µL reverse transcription master mix (4µL of 5X reaction buffer, 1µL RNase inhibitor, 0.5 µL reverse transcriptase (200u/µL) made up to 10µL by adding RNase free water) giving a total volume of 20 µL. Each sample was mixed thoroughly, briefly centrifuged and incubated at room temperature for 5 minutes. The samples were transferred to a thermocycler (Biorad, UK). This enabled the samples to be heated for 37 ºC for 5 mins, 42 ºC for 55 minutes and 70 ºC for 15 minutes to make cDNA which was stored at -20 ºC until use.

2.4.3. Quantitative real-time Polymerase Chain Reaction (qRT-PCR).

This was carried out using an ABI 7500 standard Sequence Detection System (Applied Biosystems, UK). Reactions were prepared to 25 µL volumes in a 96 well plate, each containing the following –

- Taqman universal PCR mastermix 12.5 µL (Applied Biosystems, UK)
- cDNA 1 µL
- Commercially available Taqman™ Gene Expression Assay (Applied Biosystems, UK).

Samples were processed in triplicate and a housekeeping gene 18 S (ribosomal RNA) was be used as an endogenous control in each reaction. Reactions were carried out at 50 ^{o}C for 2 minutes, 95 ^{o}C for 10 minutes and then 40-44 cycles of 95 ^{o}C for 15 seconds, followed by 60 ^{o}C for 1 minute. Pre-designed Taqman primers were used (Applied Biosystems, UK, FGF7 (Hs00940253_m1); FGF19 (Hs00192780_m1); FGFR2 (Hs01552918_m1); FGFR4 (Hs01106908_m1); CXCL2 (Hs00601975_m1), FUT2 (Hs00704693_s1), MUC2 (Hs00894043_g1), MUC5AC (Hs00873651_mH), GADD45B (Hs00169587_m1), S100P (Hs00195584_m1), SLC46A1 (Hs00560565_m1) and PSCA (Hs04177224_g1). All reactions were multiplexed so that both the target gene primer and housekeeping gene primer were included in the same well. Each sample was processed in triplicate. A $^{\Delta}$Ct value was determined for each well by subtracting the Ct value of the housekeeping gene from that of the target gene. The mean $^{\Delta}$Ct was calculated for each sample by taking the average of the triplicates. All statistical analysis was performed using the inverse of the $^{\Delta}$Ct which is a measure of level of gene expression. Relative fold change difference in gene expression was calculating by $2^{-\Delta\Delta Ct}$ where $^{\Delta\Delta}$Ct is $^{\Delta}$Ct (control sample) subtracted from $^{\Delta}$Ct (test sample, that is cancer or polyp).

2.5. Protein expression

2.5.1. Protein extraction

Snap frozen colonic samples were homogenised and re-suspended in 1 mL of protein lysis buffer. The latter consisted of 5mL of 1x radioimmunoprecipitation (Tripathi *et al.*) (Millipore, UK) with 100µL of dissolved protease and phosphatase inhibitors (2 Roche Complete Mini protease inhibitor cocktail tablets with 8 mg sodium fluoride (Fisher Scientific) and 20 mg sodium vanadate (Acros Organics) in 2 mL 1x RIPA. Protein concentration was measured using Bio-Rad detergent compatible protein assay kit (Bio-Rad Laboratories, CA). A standard curve of absorbance against protein concentration was created using a series of known dilutions of bovine serum albumin (BSA, fraction V, Sigma, UK) (2

µg/µL) in the range 0 to 6 µg/µL. The absorbance of both samples and standards was measured at a wavelength of 595 nm, using a spectrophotometer (Tecan, UK), allowing the protein concentration (µg/µL) of samples to be determined.

2.5.2. Western blot analysis

This was conducted in a manner similar to previously described (Alhusaini, *et al.*, 2010).

25 – 30 µg of protein was loaded onto a 7.5 – 10% polyacrylamide gel (Geneflow Ltd, Fradley, UK), under reducing conditions. Proteins were resolved by SDS-PAGE at 100V for 60 – 90 minutes and then transferred to Immobilon-P transfer membranes 0.45 µm pore size (Fisher Scientific, UK). The membrane was blocked for 60 minutes in 0.2 % I-Block PBS-tween (PBST) and was then incubated in primary rabbit-derived antibody diluted in 0.2 % I-Block PBST (FRS2 1:1000, Abcam, UK; pFRS2 – 1:250; pErk 1:2000, Erk 1:2000, pAKT 1:1000 and AKT 1: 1000; β-actin 1:70,000 (Cell Signalling, UK) at 4ºC overnight. Equal protein loading was determined by measuring β-actin expression. Membranes were washed three times for ten minutes in PBST and were then incubated in anti-rabbit IgG (whole molecule), horseradish peroxidase antibody produced in goat, IgG fraction of antiserum, buffered aqueous solution (Sigma #A9169). A chemiluminescent detection system ECL/ECL^{+} (GE Healthcare, UK) enabled visualization following exposure on hyperfilm MP (Fisher Scientific, UK). Intensity was determined using densitometry (GeneTool software, Syngene, UK) and expression values obtained were normalised against those of β-actin.

2.6. Serum analysis

Serum concentrations of FGF7 and FGF19 were measured using Quantikine ELISA FGF19 kit (intra-assay CV – 3.6-6.4%) and inter-assay CV – 4.5-5.5%) (R&D systems, UK) and Quantikine ELISA FGF7 kit (intra-assay CV – 3-3.5% and inter-assay CV 5.2-7.7%) (R&D systems, UK) according to manufacturer's instructions. A standard curve was created using serial dilutions of FGF19 standard or FGF7 standard (R&D systems, UK). 100 µL serum

was utilised in each reaction and the assay was performed in duplicates. The absorbance of samples and standards was measured at a wavelength of 570 nm, using a spectrophotometer (Tecan, UK) which enabled the concentration (pg/mL) of the samples to be determined by comparison with the standard curve.

Serum concentration of CXCL2 was measured using the MIP2 (CXCL2) Human Simple Step ELISA kit (intra-assay CV – 2.8 % and inter-assay CV – 3.5 %) (Abcam, UK - ab184862). A standard curve was created using serial dilutions of CXCL2 in duplicates. Serum samples were diluted at a ratio of 1:16 and 50 µL diluted serum was utilised in each reaction. The assay was performed in duplicates and the absorbance was measured at a wavelength of 450 nm using a spectrophotometer (Tecan, UK). The serum CXCL2 concentration was calculated based on the standard curve and dilution ratio.

2.7. Statistical analysis

Statistical analysis was undertaken using Microsoft Excel and IBM SPSS Statistics 21.0. Parametric data were expressed as mean ± SEM and comparisons between groups have been made using the unpaired two-tailed Student's t test and within groups, using the paired sample t test. Non-parametric data have been described using median ± interquartile range. Multivariate logistic regression was utilised to determine which variables, including level of gene expression, were predictive of cancer in subjects. A multivariate linear regression model was utilised to determine patient and pathological factors that affect serum concentration of the proteins measured. A p value < 0.05 was considered as statistically significant and significance levels have been indicated as follows; *P<0.05, **P<0.01, ***P <0.001.

Chapter 3: Colonic polyps are biomarkers of field cancerisation in colorectal cancer

3.1 Introduction, aims and objectives

3.1.1 Introduction

A large body of epidemiological, post mortem, clinical and molecular evidence exists to support the notion that adenomas are the precursors to colorectal cancer (Muto *et al.*, 1975; Stryker *et al.*, 1987; Vogelstein *et al.*, 1988; Winawer *et al.*, 1993; Olsen *et al.*, 1988). A single adenoma develops into a cancer at a rate of approximately 0.25 % to 1 % per year (Eide, 1986; Otchy *et al.*, 1996; Morson & Bussey, 1985; Wilson & Lightwood, 2001). The exact risk of progression into cancer varies with the number of polyps (Winawer *et al.*, 1993; Atkin *et al.*, 1992), size (Muto *et al.*, 1975; Stryker *et al.*, 1987; Eide, 1986), histology (Eide, 1986; Yang *et al.*, 1998), age and sex (Jensen *et al.*, 1996; van Stolk *et al.*, 1998; Noshirwani *et al.*, 2000; Martínez *et al.*, 2001). Strategies aimed at reducing the incidence of adenomas, such as screening colonoscopy, have successfully reduced colorectal cancer related mortality confirming that removal of adenomas is important in preventing CRC formation (Thiis-Evensen *et al.*, 1999; Mandel *et al.*, 1999; Scholefield *et al.*, 2002; Atkin *et al.*, 2010; Jørgensen *et al.*, 1993).

The role of adenoma detection in CRC survivors is less well understood. Although, adenomas have been found to predict a higher rate of synchronous and metachronous cancer (Chu *et al.*, 1986; Neugut *et al.*, 1996), the rate of adenoma recurrence in these patients varies substantially between 8 % and 46 % across the literature (Khoury *et al.*, 1996; Patchett *et al.*, 1993; Barlow & Thompson, 1993; McFall *et al.*, 2003; Kawai *et al.*, 2012), depending upon the frequency of surveillance colonoscopy and mode of reporting outcomes. If patients who undergo resection for CRC continue to develop polyps in the remaining bowel, it suggests that they are at increased risk of developing a metachronous cancer. Furthermore, it suggests that the mucosa is preconditioned to develop neoplasia as described by the concept of field cancerisation. When field cancerisation was first described by

Slaughter (SLAUGHTER *et al.*, 1953) , his theory was based on the observation that higher rates of recurrent head and neck squamous cell cancer occurred at the site of previous resection. Although there have been several reports investigating the factors that predispose to polyp recurrence and metachronous colorectal cancer (Kawai *et al.*, 2012; Huang *et al.*, 2015; Gervaz *et al.*, 2005; Chu *et al.*, 2003), few have examined the link between the incidence of polyps and neoplastic risk, in terms of metachronous lesions and local recurrence. Previous reports have found that patients who develop polyps in the remaining bowel after CRC resection actually have an improved disease free survival (Kronborg *et al.*, 1986; Chu *et al.*, 1986). The implications of this are twofold; firstly, polyps may not be an early marker of a field defect but represent areas of mucosa that have already become neoplastic. In other words, polyps are too far along the neoplastic process to represent field change. To clarify this further, studies need to investigate the macroscopically normal mucosa (MNM) around a polyp. If this mucosa contains aberrant cellular processes and signalling, it would suggest that polyps, like cancer, also develop in fields of altered mucosa. Secondly, these studies highlight that detection and removal of polyps is protective against cancer development and this may be the reason for the improved survival. The group of patients who do not develop polyps cannot benefit from endoscopic examination in the same way and may be developing recurrence through alternative CRC pathways such as the serrated polyposis pathway. Alternatively, patients with synchronous polyps are more likely to have DNA mismatch repair and undetected HNPCC which confers a survival benefit (Stigliano *et al.*, 2008). In addition, there are a multitude of other factors that can have an effect on survival and these have not been accounted for in the earlier reports. More recent studies, however, have also demonstrated similar findings (Mattar *et al.*, 2005). Nevertheless, there is a body of literature which links synchronous polyps at time of resection with a higher rate of metachronous polyps and cancers highlighting that polyps could be marker of a field effect.

3.1.2. Aims and objectives

The objective of this study is to investigate if polyps or adenomas are an indicative marker of field cancerisation in CRC. If they represent mucosa at risk of malignant transformation, it would follow that presence of polyps or adenomas is associated with higher incidence of metachronous neoplastic lesions and local recurrence thus, poorer survival of these patients would be expected.

The aim of this study is to investigate the impact of

i) Synchronous adenomas (SA) (adenomas identified at preoperative colonoscopy)
ii) Metachronous adenomas (MA) (adenomas identified on surveillance colonoscopy)

on local recurrence, 5 year overall and disease free survival of patients with CRC.

Patients undergoing colonoscopy where a polyp was found and either not removed or not retrieved were classified as having no adenomas. Only patients in whom the polyp was examined histologically and confirmed to contain adenomatous tissue were included in the SA or MA group. Patients with hyperplastic, metaplastic or inflammatory polyps were not included in the SA or MA groups.

3.2 Materials and methods

University Hospitals of Coventry and Warwickshire (UHCW) NHS Trust is a tertiary referral centre with a catchment population of half a million. Over the last 12 months, 270 patients were diagnosed with CRC, of whom, 167 underwent major surgery. UHCW keeps a prospective database of all patients diagnosed with CRC whom are discussed at the

multidisciplinary team (MDT) meeting. This database was utilised to identify patients with CRC between 2006 and 2012. The following inclusion and exclusion criteria were applied –

Inclusion criteria

- Patients diagnosed with colorectal adenocarcinoma who have undergone surgical resection at UHCW between 2006 and 2012.
- Any mode of presentation so that both screening and symptomatic patients were included.

Exclusion criteria

- Patients with stage IV disease at time of diagnosis or at time of resection
- Patients with cancer confined to an adenoma
- Patients who have not had a surgical resection
- Patients that are known to have genetic predisposition to CRC
- Patients with inflammatory bowel disease

Hospital electronic records were scrutinised to obtain data on patient demographics, operative details, pathological findings, endoscopic observations and survival.

The local Trust policy regarding follow up of CRC patients involves yearly clinic review with routine blood tests including an annual serum carcinoembryogenic antigen (CEA). All patients undergo inspection of their anastomosis within one year of surgery with either a flexible sigmoidoscopy or colonoscopy depending upon site of surgery. A further colonoscopy is performed at 5 years unless there are positive findings in the first investigation. These patients undergo interval colonoscopies as per the polyp surveillance guidelines. A CT scan is performed at 3 years and 5 years after surgery. Patients are usually discharged after 5 years if they remain disease-free.

3.2.1. Data analysis

Synchronous polyps were defined as either polyps found at index colonoscopy or polyps found in the resection specimen by the pathologist. The findings of the pre-operative investigation were combined with the pathological details and the cumulative rate of synchronous polyps (SP) with polyp and adenoma characteristics have been presented. Any polyps that were discovered on subsequent colonoscopy after surgery were classified as metachronous polyps (MPs). Short term (0-24 months) and mid-term outcomes (0-60 months) were calculated based on cumulative colonoscopy findings over this period of time.

Overall survival (OS) was calculated from the date of surgery to the date when the patient was last seen alive in the hospital. Disease free survival (DFS) was calculated between the date of surgery and the date when the patient was last seen without disease recurrence in the oncology or surgical clinic. In patients with local recurrence or metastasis, the date when this was diagnosed on imaging was utilised to calculate disease free survival.

Local recurrence was defined as either soft tissue growth at the anatomical site of previous resection on CT or PET-CT or intraluminal recurrence at the anastomotic site on endoscopy.

Metastasis was defined as the presence of either of the following –

i) Peritoneal metastases

ii) Lymphatic metastases resulting in a nodal mass

iii) Distant organ spread to either liver, lung, bone or other intra-abdominal organ

3.2.2. Statistical data analysis

Differences in clinical variables amongst the different groups were tested with chi-squared test. A multivariate logistic regression model was built to determine which factors affect incidence of metachronous polyps/adenomas and local recurrence in CRC survivors. Differences in survival outcome were tested using the log rank test. A multivariate Cox

regression model was designed to evaluate how different clinical variables affect overall and disease free survival.

3.3 Results

Over the study period, 2101 patients were identified from the MDT database. Patients (n=981) were excluded based on criteria shown in figure 3.1. Hence, 1120 patients were identified for potential inclusion. After further investigation using electronic records, 393 patients were found not to meet the inclusion criteria. Patients who did not have complete colonic examination prior to surgery were excluded, hence, 562 patients were included in the final analysis. The demographic, operative and pathological details of these patients are outlined in table 3.1.

Figure 3.1. Flow chart showing how patients were selected from the MDT database.

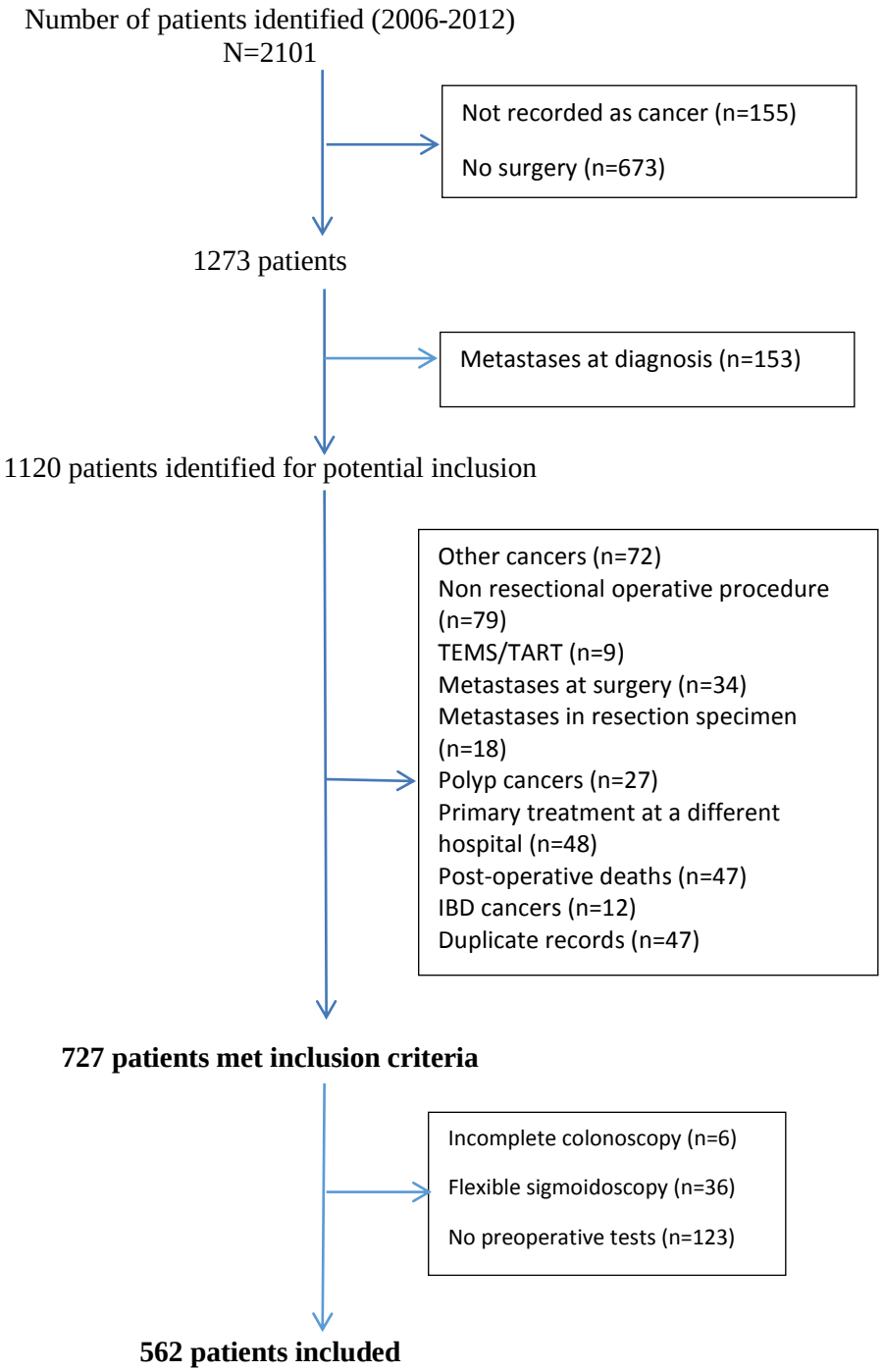


Table 3.1. Clinical, Operative and Pathological details of patients who met the inclusion criteria (n=562).

Clinical details		**Operative details**	
Median age	69 yrs.	**Mode of surgery**	
(IQR)	*(62-77yrs)*	Elective	335
Male: Female	323:239	Emergency	9
(Right sided tumour)	105:131	Scheduled	173
(Left sided tumour)	218:108	Urgent	45
Status of referral		**Operative Procedure**	
Screening	81	Anterior Resection	188
2-week wait	303	Extended Right Hemicolectomy	20
Urgent	202	Left Hemicolectomy	32
Routine	87	Hartmann's Procedure	29
Emergency	36	Right Hemicolectomy	212
Other	19	Sigmoid colectomy	20
		Transverse colectomy	2
		Subtotal colectomy	9
		Panproctocolectomy	2
		Laparotomy	2
		Combined procedure	3
Pathological details			
T-stage		**N-stage**	
pTx	5	pNx	1
pT0	4	pN0	377
pT1	22	pN1	120
pT2	83	pN2	64
pT3	355		
pT4	91	**Median LN yield**	19
		(IQR)	(13-26)
Mucinous		**Extramural vascular invasion**	
Non-mucinous	456	Absent	415
Small focus	17	Present	145
<50 % mucinous	38	Missing	2
>50 % mucinous	49		
Missing	1		

3.3.1. Characteristics of patients with synchronous polyps at presentation

Patients who had undergone complete colonic investigation with either a pre-operative colonoscopy (n=532), CT pneumocolon (n=14) and barium enema (n=16) were included for further analysis (see figure 3.2). Based on these preoperative investigations, 181 patients had polyps. In the remaining 381 patients, there were 66 patients who had polyps found in the resection specimen that were not identified at initial investigation. Thus, 247 patients had synchronous polyps (SP) at time of diagnosis, of which, 197 had adenomas. Male gender and older age was significantly associated with presence of SAs ($p<0.001$ and $p=0.020$ respectively) (see table 3.2. below). There were no significant differences in other clinical and pathological parameters recorded.

Figure 3.2. Flow chart of patients included in analysis. SP-synchronous polyps, SA-synchronous adenoma, MP- metachronous polyp, MA – metachronous adenoma

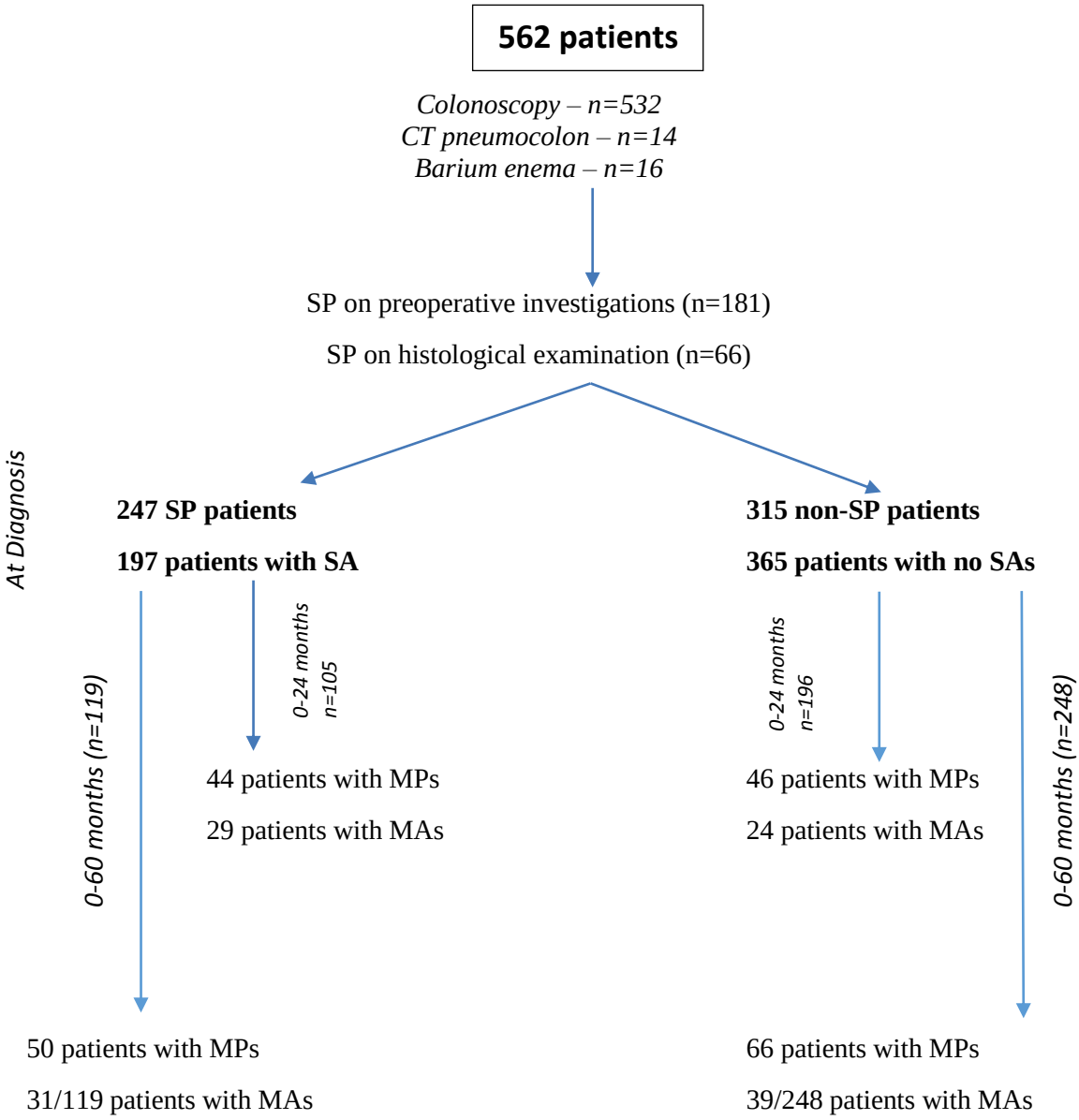


Table 3.2. Clinical, operative and pathological details of SA and non-SA patients.

	SA patients (n=197)	Non-SA patients (n=365)	P value	Odds ratio
Age				
<60 years	29	81	0.020*	0.605
>60 years	168	284		
M:F	139:58	184:181	<0.001***	2.35 (Male)
Right sided tumour	87/197	149/365	0.249	0.872
Mode of operation	118	217		
Elective	0	9	0.148	
Emergency	61	112		
Scheduled	18	27		
Urgent				
T-stage				
pT0	5	5	0.328	
pT1	7	15		
pT2	31	52		
pT3	130	226		
pT4	24	67		
N-stage				
pN0	135	243	0.886	
pN1	40	80		
pN2	22	42		
Mucinous content				
None	166	290	0.279	
Focus	2	15		
<50 %	13	25		
>50%	16	33		
Metachronous polyps				
0-24 months FU	44/105	46/196	0.001***	
0-60 months FU	50/118	66/248	0.002**	
Metachronous adenomas				
0-24 months FU	29/105	24/196	0.001***	2.74
0-60 months FU	31/118	39/248	0.014*	1.91
Local recurrence				
Present	3/193	26/361	0.002**	4.96
Overall survival (months)				
Mean	121	92	0.442	
Disease –free survival (months)				
Mean	79	89	0.530	

3.3.2. Presence of synchronous adenomas indicates future risk of metachronous adenomas

By 24 months, 301 patients underwent colonoscopy, of whom, 53 (18%) were found to have metachronous adenomas (MAs). An additional 83 patients had incomplete examination with a flexible sigmoidoscopy to inspect the anastomosis rather than for investigation of metachronous lesions. The remainder did not undergo any endoscopic examination in this period of time. SA patients were more likely to develop MAs than those without (28 % versus 12 % respectively, Odds ratio 2.74, p=0.001).

In the first 5 years after surgery, 70/366 (19 %) patients who underwent colonic examination had developed MA. SA patients were more likely to develop MA than non-SA patients (26 % versus 16 % respectively, Odds ratio 1.91, p=0.014).

In a multivariate logistic regression model, the only independent predictor of MA by 24 months was SA at presentation (see table 3.3.). In comparison, male gender and right sided tumours were independent predictors of risk of developing MA over 60 months (see table 3.4.). Presence of SA at diagnosis was not an independent predictor of future MA in at longer term follow-up. Gender, site of tumour and SA incidence were collinear which may explain this result. Men were more likely to have SAs (Odds ratio 2.35) and made up a larger proportion of patients with left sided tumours (M: F (right sided tumours): 72:73 versus M: F (left sided tumours): 153:69, p<0.001).

Table 3.3.1 Univariate and multivariate factors that predict the development of MAs in CRC patients 0-24 months after surgery (n=301)

Variable	MA present	Univariate p value	Odds ratio	95 % CI	Multivariate p value
Age	<60 yrs-13/74 >60 yrs-40/227	0.573	0.830	0.399-1.728	0.619
Sex	39/189 M 14/112 F	0.049	0.552	0.272-1.126	0.102
Site of tumour	29/131 R 24/170 L	0.049	0.566	0.294-1.092	0.090
Mucinous content	39/251 none 14/50 present	0.032	2.132	0.995-4.567	0.052
Synchronous adenoma	24/196 none 29/105 present	0.001	2.735	1.494-5.005	0.003**

Table 3.4. Univariate and multivariate factors that predict the development of MAs in CRC patients 0-60 months after surgery (n=366)

Variable	MA present	Univariate p value	Odds ratio	95 % CI	Multivariate p value
Age	<60 yrs-17/94 >60 yrs-53/272	0.448	0.952	0.505-1.794	0.879
Sex	53/224 M 17/142 F	0.004	0.400	0.213-0.752	0.004**
Site of tumour	36/144 R 34/222 L	0.016	0.469	0.266-0.829	0.009**
Mucinous content	56/306 none 14/60 present	0.230	1.266	0.626-2.560	0.512
Synchronous adenoma	39/248 none 31/118 present	0.018	1.910	1.120-3.257	0.104

3.3.3. Local recurrence is not affected by presence of adenomas

On univariate analysis, SA patients were significantly less likely to develop local recurrence than non-SA patients (3/193 (1.5%) versus 26/361 (7.2%), p=0.004). However, with multivariate logistic regression (see table 3.5), the independent predictors of local recurrence included presence of an involved margin after surgery and extramural vascular invasion; the presence of synchronous or metachronous adenomas did not affect the incidence of local recurrence. There were 38 patients with an involved margin, of whom, 9 patients had a local recurrence. If these 9 patients with involved margins were excluded from analysis, SA patients had a significantly lower local recurrence rate compared to non-SA patients (2/188 (1%) versus 18/314 (5 %), p=0.008). In contrast, patients who developed MAs over 5 years were more likely to develop recurrence of their cancer compared to those that did not (3/68 (4.4%) versus 7/279 (2.5%), p=0.309), however, this result did not reach statistical significance.

Table 3.5. Univariate and multivariate factors that predict development of local recurrence at site of previous resection

Variable	Local recurrence	Univariate p value	Odds ratio	95 % CI	Multivariate p value
Age	<60 yrs- 8/110 >60yrs 21/452	0.187	1.878	0.708-4.983	0.205
Sex	14/323 M 15/239 F	0.201	0.977	0.426-2.244	0.957
T-stage	1/121 T0-T2 51/605 T3-T4	0.001	0.252	0.032-1.974	0.189
N-stage	13/378 N0 16/168 N1-N2	0.009	0.762	0.316-1.840	0.546
Mucinous	20/455 none 9/107 present	0.496	0.496	0.193-1.275	0.145
EMVI	13/415 absent 15/128 present	0.001	0.383	0.155-0.944	0.037*
Involved margin	9/38 involved 20/520 not	<0.001	0.146	0.055-0.383	<0.001**
Synchronous adenoma	3/197 present 26/365 absent	0.002	3.366	0.951-11.919	0.060

3.3.4. Overall and disease free survival do not differ with adenomas

After correction for age, sex and pathological T stage, there were no differences observed in overall (OS) or disease free survival (DFS) between SA patients and non-SA patients (Hazards Ratio 0.926, p=0.687 and Hazards ratio 2.367, p=0.460 respectively). Patients with right sided tumours who presented with SAs were likely to survive longer than non-SA patients (mean OS 118 months versus 90 months, p=0.587), however, this was non-significant. In comparison, there was no difference in OS in patients with left sided tumours with and without SAs (91 months versus 93 months, p=0.597). Patients who developed MAs over the 5-year period after surgery had similar OS compared to those that did not (OS 99 months versus 102 months, p=0.504).

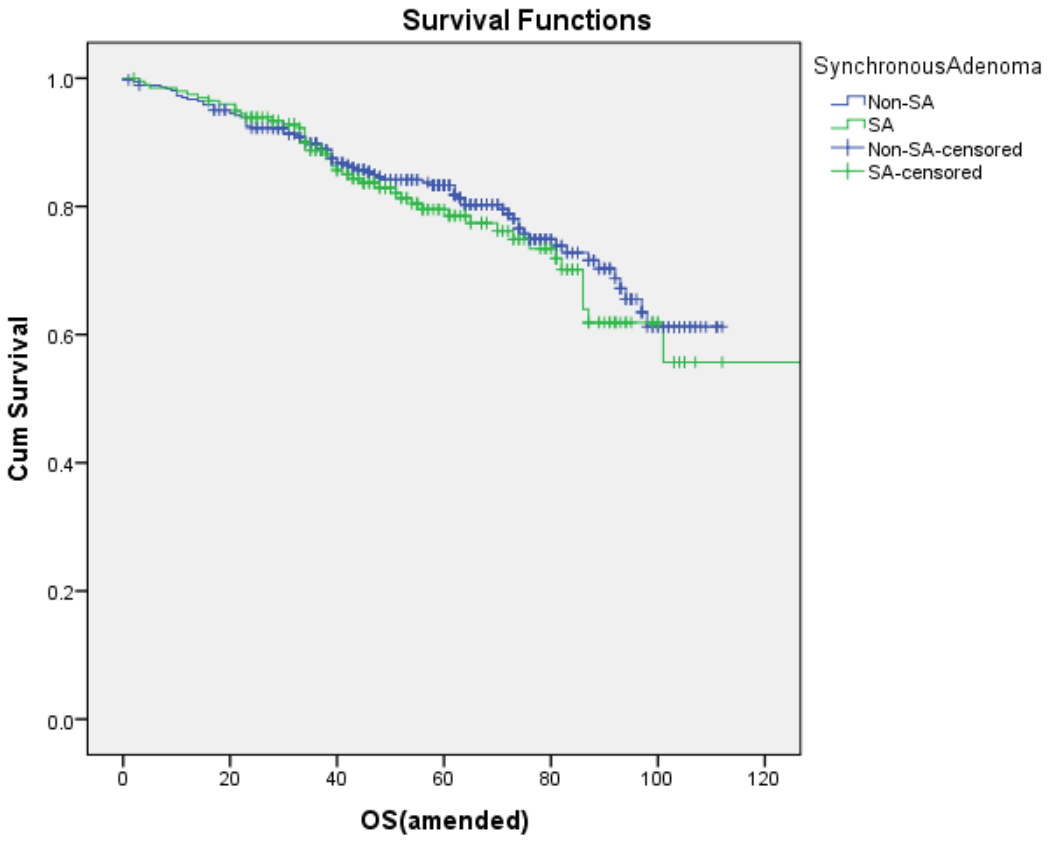


Figure 3.3. Kaplan-Meier survival analysis demonstrating overall survival (in months) in patients with SAs and non-SAs.

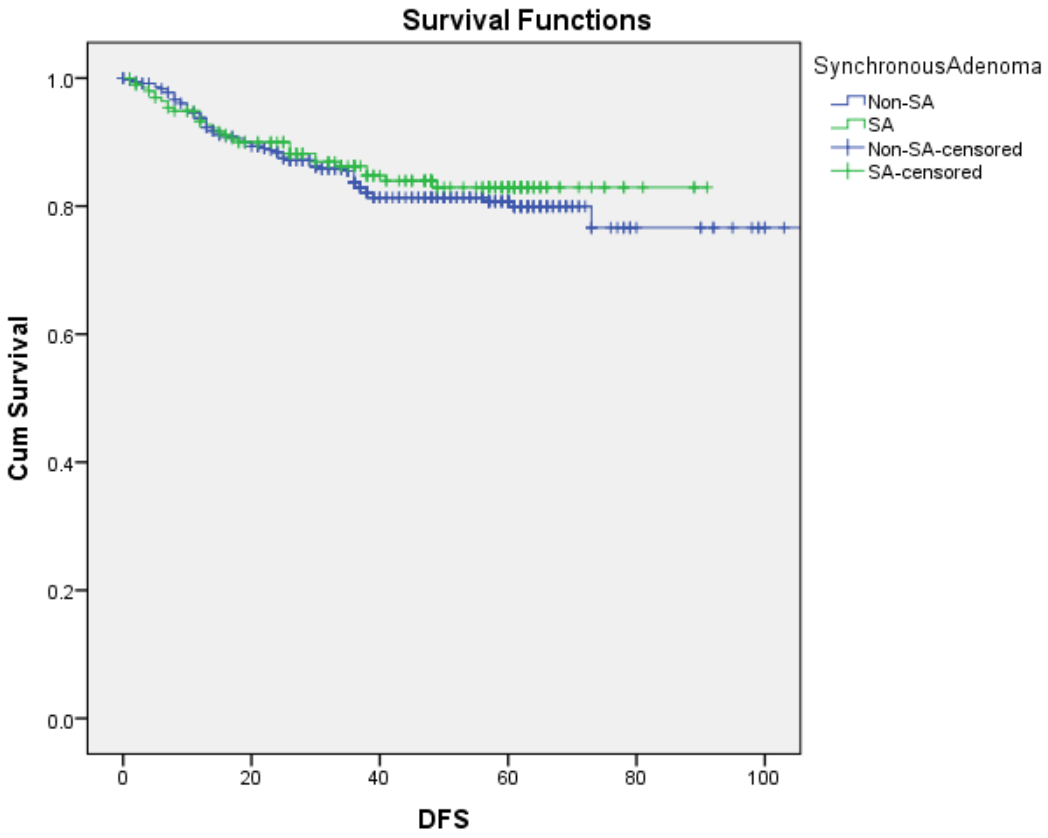


Figure 3.4. Kaplan-Meier survival analysis demonstrating disease free survival (in months) in patients with SAs and non-SAs.

3.4. Discussion

This study has highlighted that SAs are predictive for development of subsequent metachronous lesions in patients who are undergoing surgery for colorectal cancer. However, this does not appear to be related to increased risk of local recurrence nor is there any difference in overall survival or disease free survival in patients who develop adenomas compared to those that do not. This suggests that despite being pre-neoplastic, adenomas are not a reliable marker of field cancerisation. Although they indicate which patients will develop metachronous adenomas, they do not help to identify which patients will develop local recurrence. Based on Slaughter's field cancerisation concept (SLAUGHTER *et al.*, 1953), recurrence of cancer occurs in a field of altered mucosa. If adenomas are a marker of field cancerisation, one would expect that they would be positively associated with the development of local recurrence. In fact, in this study, univariate analysis has demonstrated that patients with synchronous adenomas (SAs) were less likely to develop local recurrence (LR). However, when multivariate analysis was performed, the only independent predictors of local recurrence were an involved margin after surgery or extramural vascular invasion. Patients with SAs were less likely to have an involved margin and this confounded the relationship seen between SAs and LR. In order to counteract this, patients with an involved margin were excluded from the analysis. SA patients were still significantly less likely to develop local recurrence suggesting that other factors besides the presence of an involved margin were responsible for this association. There are two possible explanations for this observation: either adenomas are not a reliable indicative marker of field cancerisation or field cancerisation does not predispose to local recurrence and may be responsible for metachronous neoplastic lesions. Although the differences in survival were non-significant, patients with right sided tumours and SAs had a longer OS. In comparison, patients with left sided tumours did not exhibit such differences. Presence of SAs or subsequent development of MAs did not impact upon OS and DFS in patients with CRC which contradicts previous reports (Kronborg *et al.*, 1986; Chu *et al.*, 1986; Mattar *et al.*, 2005).

The incidence of metachronous adenoma seen in this study (19%) is comparable to other reports which quote rates varying between 8 % and 46 % (Khoury *et al.*, 1996; Barlow & Thompson, 1993; McFall *et al.*, 2003; Kawai *et al.*, 2012). There is considerable variation in the literature because of differences in the surveillance protocols used. Despite the publication of several meta-analyses (Rex *et al.*, 2006; Desch *et al.*, 2005; Jeffery *et al.*, 2007), there is no universally agreed consensus on how CRC patients should be followed up (Scheer & Auer, 2009). Furthermore, there is variation introduced on an individual basis as invasive tests such as colonoscopy are not offered to frail elderly or medically unfit patients. This is reflected in the large proportion of patients that never underwent further endoscopic examination after curative resection in the present study. Despite these shortcomings, this study has highlighted that synchronous adenomas are more likely to develop in men and older patients. Others have reported similar findings with obesity (Scarpa *et al.*, 2014), male gender (Mulder *et al.*, 2011), mucinous cancer (Piñol *et al.*, 2004), proximal tumours (Chen & Sheen-Chen, 2000; Gervaz *et al.*, 2005) and stage II disease (Piñol *et al.*, 2004) being significantly associated with synchronous lesions. Detection of SAs is important as it enables identification of patients who are likely to develop metachronous lesions and therefore helps to inform surveillance schedules. In a recent study addressing factors that are predictive of MAs (Kawai *et al.*, 2015), the only independent predictors identified were age and presence of SAs at diagnosis; male gender was initially found to be predictive, however, on multivariate analysis, there was no strong relationship. Similarly, in the present study, the only predictive marker of MAs at short term follow up (0-24 months), was presence of SAs at diagnosis. Others have also reported similar findings (Rajaratnam & Dennett, 2009; Neugut *et al.*, 1996). Over a longer period of follow up, male gender and right sided tumours were independent predictors of subsequent development of MA. SAs in patients with right sided tumours were related to a higher incidence of MA. For left sided tumours, presence of SAs at diagnosis did not predict future risk of MA. Others have also reported that a proximal location is associated with MA and MC in CRC (Gervaz *et al.*, 2005), however, recent reports have suggested that patients with distal tumours are more likely to develop MAs

(Borda *et al.*, 2012). Previously, the link between SAs and subsequent development of MAs was attributed to underlying undiagnosed HNPCC which is associated with multiple lesions (Dykes *et al.*, 2003; Bae *et al.*, 2012; Nosho *et al.*, 2009; Pedroni *et al.*, 1999). However, there have also been reports to suggest that risk of MAs is not linked to microsatellite instability (MSI) (Ballesté *et al.*, 2007) nor is the incidence of MA in patients with various degrees of MSI different (Kang *et al.*, 2010). Hence, the observed higher incidence of MAs in patients with right sided tumours may reflect other differences in tumour biology compared to distal tumours.

No SA characteristics were found to be predictive of MA patients in this study. A recent report suggested that synchronous tubular adenomas were not predictive of MAs and only advanced SAs (more than 1 cm, villous architecture or high grade dysplasia) were associated with increased risk of finding MAs (Moon *et al.*, 2010). Others have also shown that a single nonadvanced adenoma has a very low risk of forming an advanced adenoma at follow up (McFall *et al.*, 2003). However, in this study, presence of any adenoma was associated with increased risk of developing subsequent adenomas suggesting that it is the presence of adenomas that is important rather than its appearance.

Previous reports have linked presence of SAs with increased incidence of MCs and proposed that they can be utilised to identify patients at higher risk of malignant transformation (Ballesté *et al.*, 2007; Gervaz *et al.*, 2005). However, incidence of metachronous lesions has included both anastomotic recurrence and new cancers in a single category. In this study, no relationship was observed between incidence of local recurrence and SAs after multivariate analysis which contradicts these earlier reports. It appears that incomplete resection was more important in predicting local recurrence than the presence of polyps at a distant site.

There were also no differences in overall and disease free survival between the SA patients and the non-SA patients. As previously alluded to, there may be little survival benefit in removing synchronous and metachronous lesions in the elderly population who undergo CRC resection (McFall *et al.*, 2003). Several large scale studies have failed to demonstrate a survival benefit with removal of polys in CRC survivors. Also, a recent report has proposed that patients with synchronous cancer have worse disease free survival if the primary tumour is microsatellite stable (MSS). The presence of synchronous lesions in patients with MSI did not affect outcome (Malesci *et al.*, 2014). This implies that adenomas could be a marker of neoplastic risk in MSS patients but not in MSI patients. In MSI patients, development of adenomas could be a consequence of widespread epigenetic change in the colon rather than indicating risk of malignant transformation. It is not possible to confirm nor refute such reports based on the findings of the present study as microsatellite stability was not examined in all cases. This is one of the limitations of this study. Furthermore, given the time taken for a polyp to become cancerous (Eide, 1986; Otchy *et al.*, 1996; Morson & Bussey, 1985; Wilson & Lightwood, 2001), a longitudinal study with longer follow up would be required to assess the impact of removing MAs in CRC survivors.

The other limitation of this study is that it is retrospective with some missing data that could not be retrieved. However, this was less than one per cent and given the number of patients included is unlikely to have significantly changed the findings. Those patients undergoing CRC resection included in the study have considerable variation in follow up with a large proportion never undergoing any endoscopic examination. This could have influenced the outcome as patients who underwent colonoscopy and had polyps were more likely to undergo further colonoscopy which would increase the number of MAs detected in this group. However, the proportion of patients undergoing colonoscopy at 24 months is equal in both SA and non-SA groups (around 55 %) and a larger proportion of patients in the non-SA group compared to the SA group underwent colonoscopy by 60 months (42% versus 31%).

Furthermore, it was not the number of MAs that was utilised in the analysis but rather the incidence of MA which is a binary outcome. Thus, it is unlikely that the results could have been influenced by the number of procedures.

3.5 Summary and conclusions

- SA patients were at increased risk of developing MAs after colorectal cancer resection at short term (24 months) and long term (60 months) follow up.
- SA patients were significantly less likely to develop local recurrence, however, the only independent predictive markers of local recurrence were an involved margin after surgery and extramural vascular involvement
- No differences in overall or disease free survival were observed in patients who develop adenomas compared to those that do not, including presence of SAs or MAs.
- Collectively, the data suggest that adenomas are poor markers of field cancerisation and highlight the need for further investigation to identify more reliable biomarkers indicative of neoplastic risk.
- Adenomas are currently used as a surrogate endpoint for CRC chemoprevention trials and due to the length of time it takes to develop adenomas, long periods of follow up are required in these trials to determine therapeutic benefit.
- A biomarker based on the field cancerisation concept could enable earlier diagnosis and would be more effective as a surrogate marker of colonic mucosa at neoplastic risk in chemoprevention trials.

Chapter 4: Characterisation of the gene expression profile of the mucosal field around colorectal cancer and polyps

4.1. Introduction

Advances in molecular medicine have elucidated several different molecular phenotypes of colorectal cancer (CRC) (Jass, 2007; Grady & Carethers, 2008; Sinicrope *et al.*, 2015). CRC is a complex disease characterised by dysregulation of multiple signalling pathways (Sinicrope & Sargent, 2012; Phipps *et al.*, 2015). Epigenetic modification further complicates the disease process but has been recognized as an important mode of silencing the many genes that protect against carcinogenesis (Ogino *et al.*, 2009). Identification of the genetic changes that occur in the tumour tissue has enabled development of targeted molecular therapy (De Roock *et al.*, 2010; Lièvre *et al.*, 2008; Peeters *et al.*, 2013; Benvenuti *et al.*, 2007). However, CRC prognosis remains poor unless patients are diagnosed at an early stage (NCIN). Current screening modalities that are utilised lack specificity and often lead to invasive tests being performed in individuals who do not have cancer (Burch *et al.*, 2007; Logan *et al.*, 2012). Screening tests that are able to identify early molecular changes indicative of CRC are therefore needed to enable more accurate diagnosis and prevent unnecessary morbidity. The field cancerisation concept proposes that there are genetic changes in the macroscopically normal colonic mucosa (MNM) around a cancer (SLAUGHTER *et al.*, 1953). This renders it premalignant but does not produce morphological changes (Luo *et al.*, 2014). Characterisation of these gene expression changes could enable identification of the processes that are dysregulated in the early stages of CRC formation with clear clinical implications.

4.1.1. Gene expression profiling in CRC

Previous studies have investigated the global expression profile of cancerous and non-cancerous tissues comparing tumour tissue with paired normal mucosa (Lin *et al.*, 2002; Croner *et al.*, 2005; Kitahara *et al.*, 2001; Bertucci *et al.*, 2004; Birkenkamp-Demtroder *et al.*, 2005). The assumption being that the paired 'normal' mucosa is in fact not biologically

altered. Based on the field cancerisation concept, many authors have shown that the 'normal' mucosa in cancer patients is biologically altered and this phenomenon is observed at considerable distances from the tumour itself (Shen *et al.*, 2005; Polley *et al.*, 2006; Anti *et al.*, 2001). Previous studies have failed to identify changes that occur early in CRC formation as the comparison is being made between MNM and tumour taken from the same patient. If the MNM is altered in these individuals, only genetic changes that take place later in the carcinogenesis pathway will be identified; the early genetic changes that take place will be found in both the MNM and tumour and therefore will cancel each other out.

Others have adopted a different approach by taking colonic tissue from healthy individuals as a control and have successfully demonstrated genetic dysregulation in a number of cellular processes (Grade *et al.*, 2007; Bianchini *et al.*, 2006; Alon *et al.*, 1999; Notterman *et al.*, 2001). However, it is difficult to ascertain where along the adenoma-carcinoma sequence these abnormalities lie. This has been addressed in studies where the gene expression profile of adenomas has also been characterised (Notterman *et al.*, 2001; Lin *et al.*, 2002; Lechner *et al.*, 2003). In many of these studies, similar genetic changes have been noted in adenomas and tumours compared to healthy colonic mucosa. The level of expression of these genes often lies in between that found in cancer and that in control subjects (Habermann *et al.*, 2007; Buckhaults *et al.*, 2001; Galamb *et al.*, 2006; Wang *et al.*, 2005) lending support to the adenoma-carcinoma sequence. However, it has been difficult to translate the findings from these studies into molecular screening tests. The reason for this is that many adenomas do not progress to CRC and some CRCs arise without a recognizable polyp stage. Additional information regarding the molecular biology of adenomas is required if they are to be utilised for screening. This could come from examination of the MNM as patients with a field defect are at higher risk of malignant transformation therefore instead of identifying polyps, the molecular makeup of the MNM around polyps and cancer should be examined.

This would enable better screening tests to be developed and assist in more accurate risk stratification of patients who have already developed polyps or cancer.

Micro-array technology offers the opportunity to examine gene expression profiles in a candidate-free approach and has the added benefit of being able to understand network pathways through multiple gene analysis. One of the most successful genetic profiling studies in CRC has been the discovery of an 18 gene signature that can be used to identify patients with a poor prognostic CRC who require adjuvant chemotherapy (Salazar *et al.*, 2011). This suggests that gene expression profiling can be readily translated into the clinical arena and may help elucidate how CRC can behave differently in different individuals. Similarly, based on the field cancerisation concept, gene expression profiling could be utilized to identify how the MNM around a cancer or a polyp is biologically altered. This would aid our understanding of CRC pathogenesis and could enable more accurate screening tools and targeted chemo preventative therapy to be developed.

4.1.2. Aims and objectives

The aims of this study were as follows –

i) To investigate field cancerisation in CRC by characterising the key cellular processes and genes that are dysregulated in the MNM around cancer and polyp compared to that found in control subjects

ii) To identify individual genes that may contribute to field cancerisation for further investigation

4.2. Methods

Subjects (n=16) were recruited and mucosal pinch biopsies were taken at time of endoscopy. Biopsies taken from the adjacent macroscopically normal mucosa (MNM) to tumour (n=5) and polyp (n=6) were compared with biopsies of MNM in the caecum or rectum from control subjects (n=5). A two channel micro-array experiment was performed as outlined in section 2.3.

Two different types of statistical analysis referred to as lmFt (LIMMA) and robust regression (RR) were performed to improve the reliability of the genes found to be differentially expressed (Xu *et al.*, 2013) (see section 2.3 for further details). Based on the list of differentially expressed genes (DEGs), two different types of functional analysis were conducted, either using DAVID software (Huang *et al.*, 2009a) or the PANTHER database (Mi *et al.*, 2013) (see figure 2.4).

The cohort contained a patient with a hyperplastic polyp who was included in the initial analysis using the LIMMA method, however, subsequently, was excluded in all other analysis. As the aim of the study was to characterise the gene expression profile of adenomas, it was felt that inclusion of a hyperplastic polyp in the group would be inappropriate and therefore the patient was excluded. The initial analysis had already been conducted at this stage therefore it was not possible to exclude this patient at the time.

The second analysis using PANTHER software focused on approaches to combine both methods therefore the same threshold was set for both LIMMA and RR to prevent any one statistical test identifying a large number of genes and would then be over-represented in the analysis. Genes that were only identified using both statistical analyses, referred to as the

'intersect' region in the Venn diagrams were used to identify genes for further investigation. These genes were ranked based on fold change and a combined rank was calculated by averaging the individual ranks. The genes were then reordered based on the combined rank. A list of 50 up and down regulated genes in each comparison (cancer versus control, polyp versus control and cancer versus polyp) was created. Based on this list, only genes included in at least two of the comparison categories were considered potential candidates for validation.

In comparison, the 'union' DEGs, that is, those that were discovered using either method were subject to functional analysis. A larger number of genes were preferred for this analysis to ensure that all biological terms of relevance were identified. Three separate analyses were performed based on cellular component, molecular function or biological process. The statistical overrepresentation test (Mi *et al.*, 2013) was then performed to identify biological terms that were more 'enriched' in the list of DEGs compared to the background genes found on the microarray chip. There are therefore two different types of comparison that were made in the analysis. Firstly, differences in gene expression between the groups were examined using fold change which is a logarithmic function of the mean difference in raw expression values. Thus, it gives an indication of how gene expression of an individual gene differs across the clinical groups. In contrast, fold enrichment is a value that indicates whether a group of genes belonging to a particular cellular or biological function are over expressed or under expressed in the list of DEGs compared to the background expression that would be expected. In the DAVID analysis, a fold enrichment value of greater than 1.33 was considered significant. In the PANTHER analysis, the statistical overrepresentation test was utilised to calculate fold enrichment and an associated p value. Fold enrichment greater than 2 or a p value <0.05 were considered to be significant. Both fold change and fold enrichment are important parameters to include in the analysis as they explain individual gene differences and global functional differences respectively.

4.3. Results

4.3.1. The global gene expression profile of patients with adenomas and CRC differs

Based on the expression level of all probes tested in the micro-array experiment, there appeared to be a clear distinction between MNM adjacent to a cancer compared to that next to adenoma (see fig 4.1.). It was therefore possible to draw a hypothetical line that can separate both groups based on the global gene expression profile of the samples examined. In comparison, the global gene expression profile of samples taken from control subjects overlaps the other two groups and is not as well demarcated.

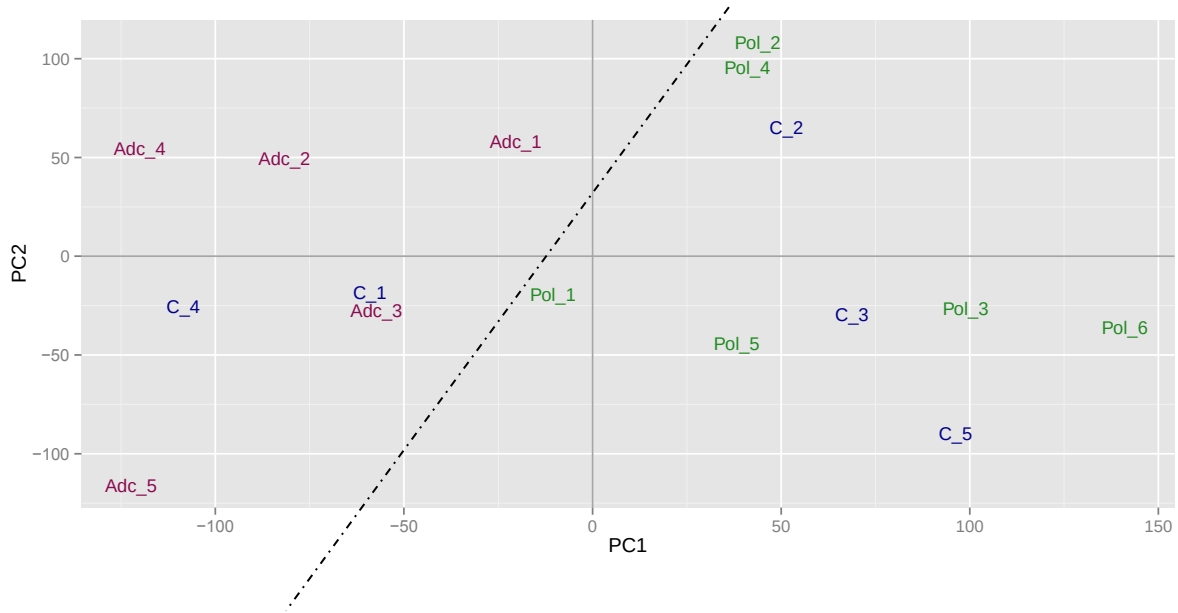


Figure 4.1: Principal components analysis of the global gene expression profile of all 16 patients. A hypothetical line is able to demarcate the patients with adenomas from CRC. C1-5 refers to samples from control subjects, Pol1-6 refers to samples from 6 polyp patients and Adc 1-5 refers to samples from 5 cancer patients

4.3.2. Multiple signalling pathways are dysregulated in the colonic field (DAVID analysis)

LIMMA and RR methods were used to identify DEGs and biological processes that were dysregulated as shown in figure 4.2. The biological processes that were identified were ranked based on the enrichment score and are shown in Appendix 1.

4.3.2.1. Cancer versus control

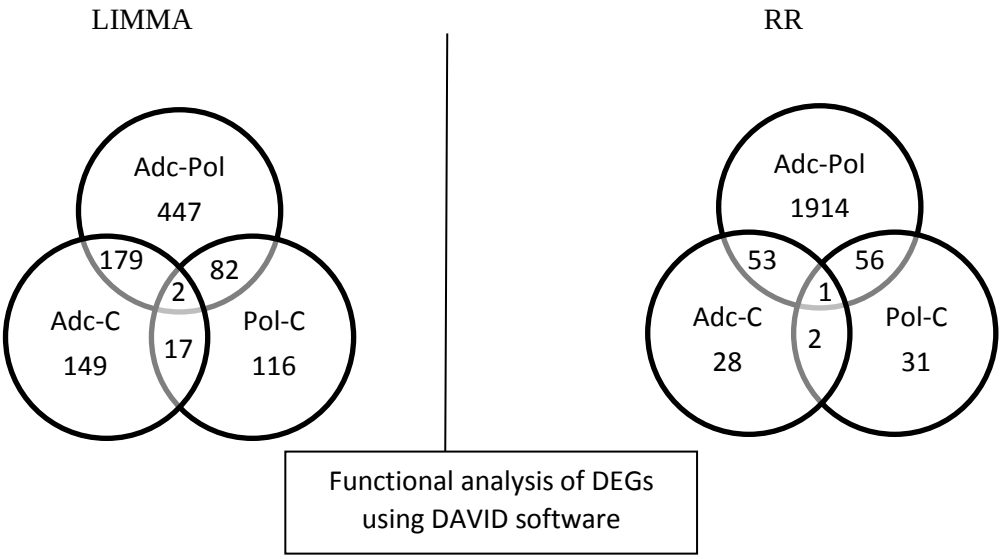
Using the LIMMA method, only two functions were enriched. These included transmembrane signalling and glycosylation; the numbers of genes included in these two categories are shown in Appendix 1. No enriched terms were found using the robust method.

4.3.2.2 Polyp versus control

Using the LIMMA method, there were two terms that were enriched; these included extracellular signalling and response to injury. Despite only 90 probes being differentially expressed with the robust method, 5 terms were enriched: extracellular signalling, coagulation, eye morphogenesis, signalling and cytoskeleton.

4.3.2.3 Polyp versus cancer

The most striking differences were observed between patients with cancer and polyp. Using the LIMMA method, 8 enriched terms were found compared to a staggering 28 enriched terms with the RR method. The details of the enriched terms using both methods are given in Appendix 3. Key processes that are identified included chromatin organization, protein assembly and RNA transport.



LIMMA	Cancer versus control	Polyp versus control	Cancer versus polyp
Upregulated genes	220	151	383
Downregulated genes	127	66	327
Genes for validation	24 UP 25 DOWN	20 UP 21 DOWN	21 UP 22 DOWN
Genes in pathway 1	31	19	11
Genes in pathway 2	25	10	30

RR	Cancer versus control	Polyp versus control	Cancer versus polyp
No of DEGs	84	90	2024
Upregulated genes	18	38	247
Downregulated genes	20	8	188
Genes for validation	16 UP 11 DOWN	20 UP 8 DOWN	22 UP 20 DOWN
Genes in pathway 1	13	30	196
Genes in pathway 2	3	3	190

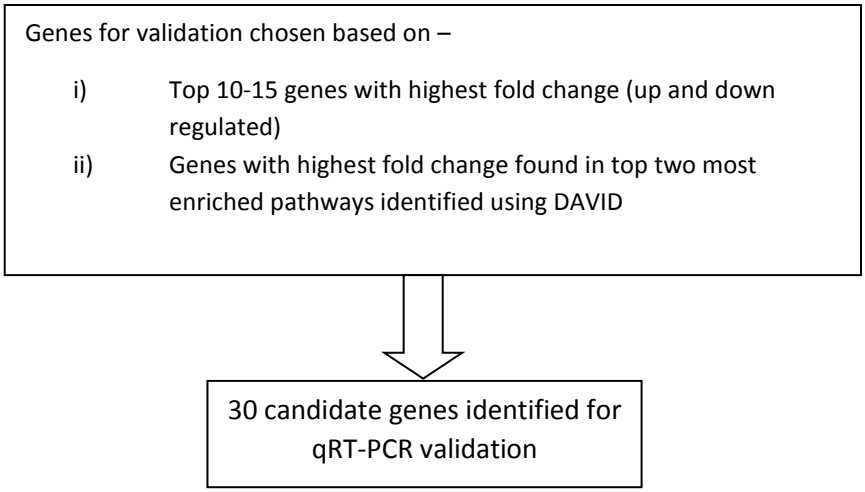


Figure 4.2. DAVID analysis. Number of DEGs identified using the two different statistical methods and subsequent functional analysis with criteria for discrimination of individual genes for further enquiry.

4.3.2.4. Selection of genes for validation and further enquiry

A list of candidate genes for further validation was created based upon the genes recorded under the two most enriched terms of the functional analysis and a list of the top 10-15 genes with the greatest fold change observed in each category. Based on a literature review to explore biological relevance of these genes, 30 potential genes for validation were formulated. These genes are shown in table 4.1. and are involved in a number of different biological processes. From this list, MUC2, MUC5AC and GADD45B were chosen for further validation using quantitative real time polymerase chain reaction (qRT-PCR). The results of this are presented in chapter 5.

Table 4.1: **Candidate genes for further validation** with fold change differences, statistical significance and biological function displayed. A short synopsis of the literature linking the gene to cancer biology and CRC is also given. Numbers in brackets refer to the comparison with 1=cancer versus control, 2=polyp versus control and 3=cancer versus polyp. Italicized values refer to downregulated genes.

Gene	Gene name	Function	Fold change (LIMMA)	P value (LIMMA)	Fold change (RR)	P value (RR)
MUC2	Mucin 2	Goblet cell derived mucin	1.60 (1) 1.58 (3)	0.0007 (1) 0.0007(3)	1.61 (3)	0.001(3)
MUC5AC	Mucin 5, subunit AC	Gastric foveolar mucin	*1.82 (1)*	*0.0005(1)*		
MUC4	Mucin 4	Integral membrane glycoprotein	1.52 (1) 1.74 (3)	0.028 (1) 0.014(3)	1.95 (3)	0.0003(3)
GADD45A	Growth arrest and DNA-damage-inducible protein GADD45 alpha	Stress response	1.73 (3)	0.0001 (3)	1.74(3)	0.0003(3)
GADD45B	Growth arrest and DNA-damage-inducible protein GADD45 beta	Stress response	1.79(1) 1.54 (3)	0.014(1) 0.049 (3)	1.96 (1) 1.82 (3)	<0.0001(1) <0.0001(3)
MT1X	Metallothionien 1X	Transcription co-factor	2.26 (3) *1.83 (2)*	0.005 (3) *0.030 (2)*		
COL3A1	Collagen, type III, alpha 1	Component of type III collagen	*1.66 (3)* 1.88 (2)	*0.009 (3)* 0.002(2)	*1.93 (3)* 2.13(2)	*0.0001(3)* <0.0001(2)
COL5A2	Collagen, type III, alpha 2	Component of fibrillar collagen	*1.51 (3)* 1.70 (2)	*<0.0001(3)* <0.0001(2)		
FGL2	Fibrinogen-like protein 2	Lymphocyte function			2.02 (2)	<0.0001(2)
CTSA	Cathepsin A	Autophagy/platelet activation			*1.74 (2)* 2.27(3)	*<0.001 (2)* <0.0001(3)
CTSG	Cathepsin G	ECM degradation	*2.42 (3)* 1.76(2)	*<0.0004(3)* 0.012 (2)	2.48 (3)	0.0003(3)
IL-17B	Interleukin 17 B	Releases TNFα and IL-1-β	*1.93 (3)*	*0.001 (3)*	2.23 (2) 2.48 (3)	<0.001 (2) <0.001 (3)
MMP1	Matrix metalloproteinase 1	ECM degradation	2.67 (2) *1.87 (3)*	0.003(2) *0.045(3)*		
MMP12	Matrix metalloproteinase 12	ECM degradation and aneurysmal	2.32 (1) 2.08 (2)	0.027(1) 0.042 (2)		

		disease				
FGFBP1	Fibroblast growth factor binding protein 1	Cell proliferation, differentiation migration, growth	2.24 (2) *2.28 (3)*	0.024 (2) *0.022 (3)*		
GREM1	Gremlin 1	BMP antagonist	2.11 (2) *1.61 (3)*	0.027 (2) *0.031 (3)*	3.12 (3)	<0.001 (3)
SFRP1	Secreted frizzled related protein	Modulates Wnt signalling	1.61 (2) *1.83 (3)*	0.022 (2) *0.005 (3)*		
ELANE	Neutrophil elastase	Modifies natural killer cells			1.29 (1)	<0.001 (1)

4.3.3. Multiple differences in cellular component, molecular function and biological processes in the colonic field (PANTHER analysis)

4.3.3.1. Intersect genes

Using both LIMMA and RR identified similar number of genes to be upregulated and downregulated in all three comparison groups (see figure 4.3.). Genes that were found using both statistical tests (intersect genes) were ranked based on the fold change differences. The individual rank values were then combined across the three categories of cancer versus control, polyp versus control and cancer versus polyp. The top 50 genes identified based on the combined rank value were investigated further for their biological relevance in CRC formation (see table 4.2. for further details). Candidate genes (n=5) were identified from this list using a stratified sampling approach where genes are selected at regular intervals along the list.

LIMMA

LIMMA	Cancer versus control	Polyp versus control	Cancer versus polyp
Upregulated genes	220	151	383
Downregulated genes	127	66	327

RR

RR	Cancer versus control	Polyp versus control	Cancer versus polyp
Upregulated genes	204	190	442
Downregulated genes	130	72	380

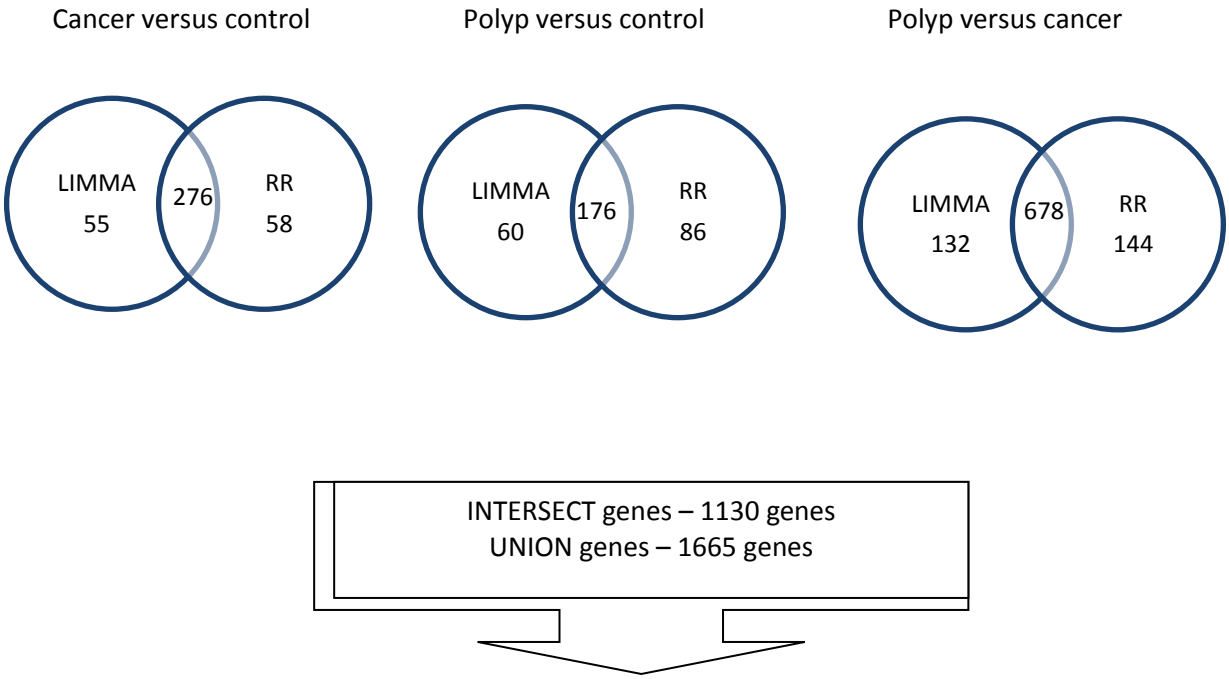


Figure 4.3. PANTHER analysis. Number of DEGs identified using the two different statistical methods. Details of subsequent functional analysis using PANTHER software and analysis of intersect genes to identify candidate genes for further enquiry is also given.

Table 4.2.: **Potential candidate genes for qRT-PCR validation**. Top 20 genes identified based on combined rank displayed with corresponding fold change (FC) differences and statistical significance. Combined rank is given under each gene name. Numbers in brackets refer to the comparison with 1=cancer versus control, 2=polyp versus control and 3=cancer versus polyp. Italicized values refer to downregulated genes.

Gene name *Combined rank*	Gene name	FC (LIMMA)	P value (LIMMA)	FC (RR)	p value (RR)
PSCA ***2***	Prostate Stem Cell Antigen	6.62(1) 7.23(3)	0.0006(1) 0.0004(3)	6.62(1) 7.35(3)	0.0032(1) 0.0022(3)
UGT2B15 ***3***	UDP glucuronosyltransferase 2 family, polypeptide B15	5.11(2) 5.38(3)	0.0145(2) 0.0121(3)	2.66(2) 2.88(3)	0.0230(2) 0.0158(3)
PYY2 ***5***	Peptide YY, 2 (pseudogene)	3.00(1) 2.88(3)	0.0001(1) 0.0002(3)	3.00(1) 2.82(3)	0.0018(1) 0.0030(3)
SLC46A1 ***11***	Solute carrier family 46 (folate transporter) Member 1	2.06 (1) 2.78(3)	0.0027(1) 0.0001(3)	1.98(1) 2.87(3)	0.0065(1) 0.0002(3)
TMCC2 ***13***	Transmembrane and coiled coil domain family 2	*2.39(1)* *2.38(3)*	*0.0036(1)* *0.0039(3)*	*2.35(1)* *2.43(3)*	*0.0074(1)* *0.0062(3)*
JAKMIP3 ***13***	Janus kinase and microtubule interacting protein 3	*2.45(1)* *2.38(3)*	*0.0012(1)* *0.0016(3)*	*2.30(1)* *2.26(3)*	*0.0011(1)* *0.0015(3)*
TFF1 ***14***	Trefoil factor 1	2.37(2) *2.29(3)*	0.0146(2) *0.0182(3)*	2.34(2) *2.26(3)*	0.0296(2) *0.0356(3)*
SLC1A7 ***15***	Solute carrier family 1 (glutamate transporter), membrane 7	2.06(1) 2.40(3)	0.0486(1) 0.0198(3)	2.41(1) 2.55(3)	0.0036(1) 0.0030(3)
SDS ***15***	Serine dehydratase	2.85(1) 2.28(3)	0.0029(1) 0.0143(3)	3.28(1) 2.83 (3)	0.0003(1) 0.0002(3)
FUT2 ***16***	Fucosyltransferase 2	2.05(1) 2.62(3)	0.0489(1) 0.0110(3)	1.78(1) 3.74(3)	0.0023(1) 0.0002(3)
KNG1 ***17***	Kininogen 1	*2.05(1)* *2.30(2)*	*0.0022(1)* *0.0006(2)*	*2.22(1)* *2.50(2)*	*0.0010(1)* *0.0003(2)*
CTSG ***18***	Cathepsin G	1.92(2) *2.64(3)*	0.0054(2) *0.0002(3)*	1.91(2) *2.48(3)*	0.0042(2) *0.0003(3)*
CCL21 ***19***	Chemokine (C-C Motif) Ligand 21	*2.13(1)* *2.27(3)*	*0.00008(1)* *0.0029(3)*	*2.13(1)* *2.25(3)*	*0.0002(1)* *0.0025(3)*
SECTM1	Secreted and	*2.15(2)*	*0.0079(2)*	*2.28(2)*	*0.0070(2)*

20	transmembrane 11	2.29(3)	0.0047(3)	2.42(3)	0.0043(3)
CYP3A4 ***20***	Cytochrome 450, family 3, subfamily A, polypeptide 4	*2.01(1)* *2.26(3)*	*0.0186(1)* *0.0075(3)*	*1.88(1)* *2.11(3)*	*0.0044(1)* *0.0012(3)*
UGT2B11 ***23***	UDP Glucuronosyltransferase 2 family, polypeptide B11	*2.21(2)* 2.16(3)	*0.0150(2)* 0.0178(3)	*2.17(2)* 2.23(3)	*0.0120(2)* 0.0107(3)
HSD11B1 ***24***	Hydroxysteroid (11-Beta) Dehydrogenase 11	1.82(1) 2.44(2)	0.0241(1) 0.0018(2)	1.84(1) 2.27(2)	0.0168(1) 0.0026(2)
S100P ***46***	S100 Calcium Binding Protein P	2.05(2) *1.91(3)*	0.0202(2) *0.0334(3)*	2.15(2) *2.01*(3)	0.0159(2) *0.0254(3)*
CXCL2 ***48***	Chemokine ligand 2/GRO-2	2.06(1) 1.85(3)	0.0107(1) 0.0261(3)	2.00(1) 1.80(3)	0.0203(1) 0.0449(3)
MUC2 ***50***	Mucin 2	1.84(1) 1.98(3)	0.0014(1) 0.0006(3)	1.84(1) 2.00(3)	0.0031(1) 0.0001(3)
GREM1 ***66***	Gremlin 1, DAN family BMP antagonist	1.64(2) *1.71(3)*	0.0326(2) *0.0208(3)*	1.76(2) *1.87(3)*	0.0110(2) *0.0060(3)*

4.3.3.2. PANTHER analysis of UNION genes

In total, 1665 genes were identified for functional analysis when genes identified using either LIMMA or RR were combined. These genes were analysed using PANTHER software to determine which cellular component the gene belongs to and ascertain its biological and molecular function. A number of different biological processes were found to be over or underrepresented in the list of DEGs compared to the number of genes found under that function in the micro-array chip.

4.3.3.3. Cancer versus control

The cellular component analysis showed that the DEGs identified in this category were responsible for intracellular and organelle function (see figure 4.4.). Genes involved in chemokine and cytokine activity were more enriched compared to the background and there was decreased structural molecular and nuclear acid activity (fold enrichment >5, $p<0.001$ and fold enrichment 0.5, $p<0.001$, respectively). In terms of biological processes, genes involved in the immune response, antigen processing and natural killer cell activation were more enriched (see table 4.3). The other terms that were enriched include glycolysis and tricarboxylic acid cycle suggesting that there were changes in metabolic activity (fold enrichment >5, $p<0.001$). Genes that were involved in regulating biological processes and involved in mesoderm/ectoderm development were observed at a lower frequency than the background level (fold enrichment 0.77, $p<0.001$ and fold enrichment 0.38. $p<0.001$ respectively).

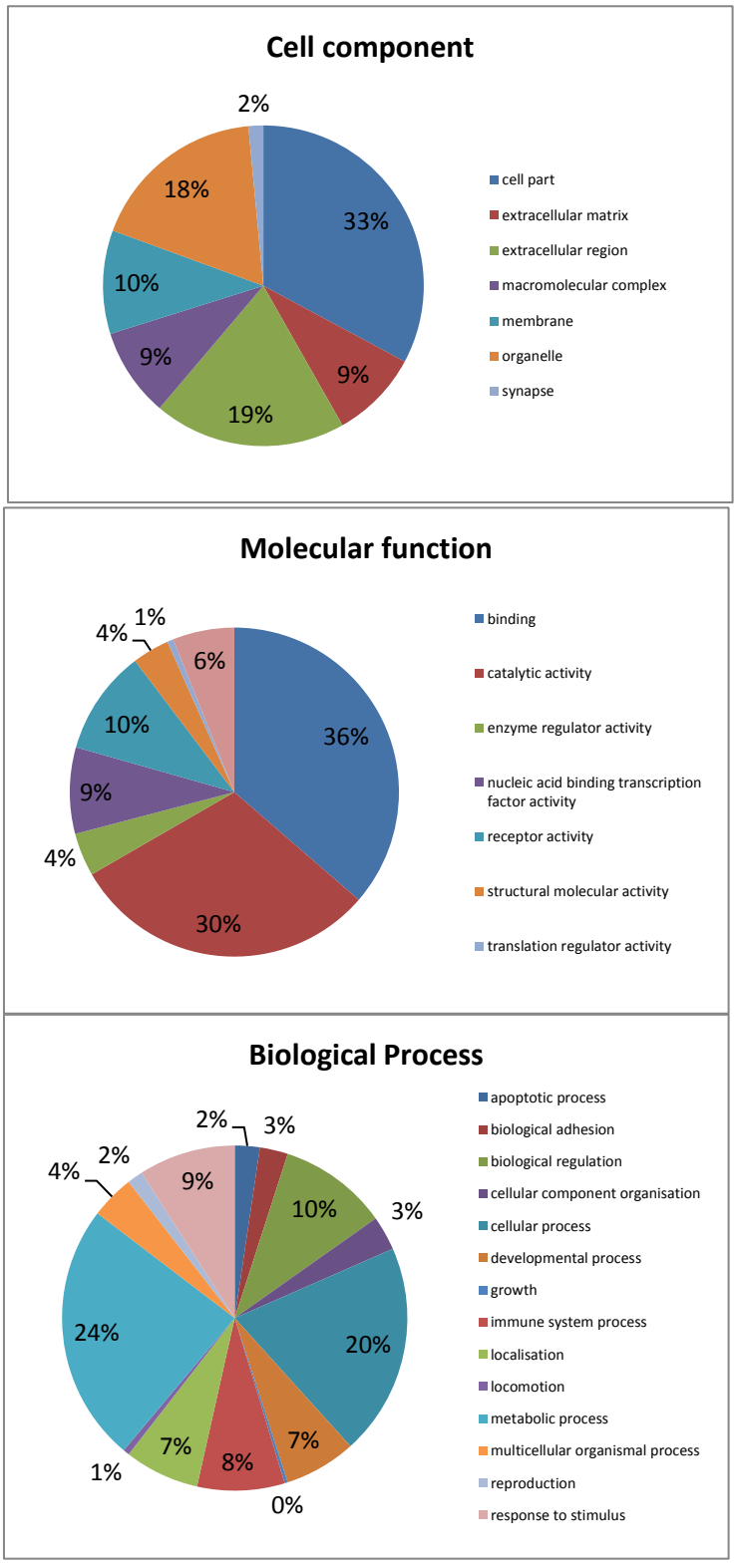


Figure 4.4. PANTHER analysis of DEGs found between cancer and control (n=389) showing which cellular component, molecular function and biological process these genes belong to.

Table 4.3. Statistical overrepresentation test for cancer versus control. Fold enrichment is calculated by taking the ratio of the expected number of genes (based on the genes found under that category in the micro-array chip0 to the actual observed number of genes in that category.

Biological Process	Fold enrichment	P value	No of genes	Genes
Immune system process	1.74	0.003	28	HLA-DQA1, CCL4, IER3, B2M, LILRB1, GADD45B, HLA-DPB1, CCL13, NR4A2, CCL19, LILRB5, PLA2G7, CEBPB, MARCH2, TSPAN32, KLK3, FPR3, CCL21, CCL23, CXCL2, CCL3, CXCL13, NFKBIA, CCL3L1, DDTL, LILRB2, GADD45G
Immune response	2.16	0.008	13	IRF5, CCL4, LILRB1, CCL13, CCL19, LILRB5, CEBPB, KLK3, CCL21, CCL23, CCL3, CCL3L1, LILRB2
Antigen processing and presentation	>5	0.010	3	HLA-DQA1, HLA-DPB1, B2M
Visual perception	2.67	0.017	7	INPP5J, DHRS4, PCDH11X, HSD11B1, LRRC26, RIMS3, TLR3
Behaviour	>5	0.018	2	CXCL2, CXCL13
Natural killer cell activation	3.9	0.020	4	LILRB1, LILRB5, CEBPB, LILRB2
Tricarboxylic acid cycle	>5	0.024	2	LDHA, LDHC
Cell growth	>5	0.034	1	APOE
Glycolysis	> 5	0.041	2	LDHA, LDHC
Mesoderm development	0.38	0.040	3	CEBPB, PCDH11X, NYX
Ectoderm development	0.38	0.045	3	NR4A2, HES5, ABLIM2

4.3.3.4. Polyp versus control

The majority of the DEGs discovered between polyp and control were involved in the cytoskeleton, both actin and microtubule components (fold enrichment 2.87, $p<0.001$). This was again seen with the molecular function analysis which identified the following terms to be more enriched than background: cytoskeletal protein binding, extracellular matrix (ECM) structural constituents and growth factor activity (fold enrichment 3.26, $p<0.001$; 3.92, $p<0.001$ and >5, $p<0.001$ respectively). Similar to the cancer versus control category, genes involved in nucleic acid binding activity were less enriched amongst the list of DEGs (fold enrichment 0.44, $p<0.001$). A large number of biological processes were found to be more enriched including antigen processing, macrophage activation, complement activation, cell-matrix adhesion and cellular process (see table 4.4.). The biological terms that were highlighted could be broadly classified into three categories: cell adhesion, cell morphology and cellular process. Genes that were involved in RNA metabolic process, transcription and regulation of biological processes were less enriched when compared to the number of genes found under these categories in the background.

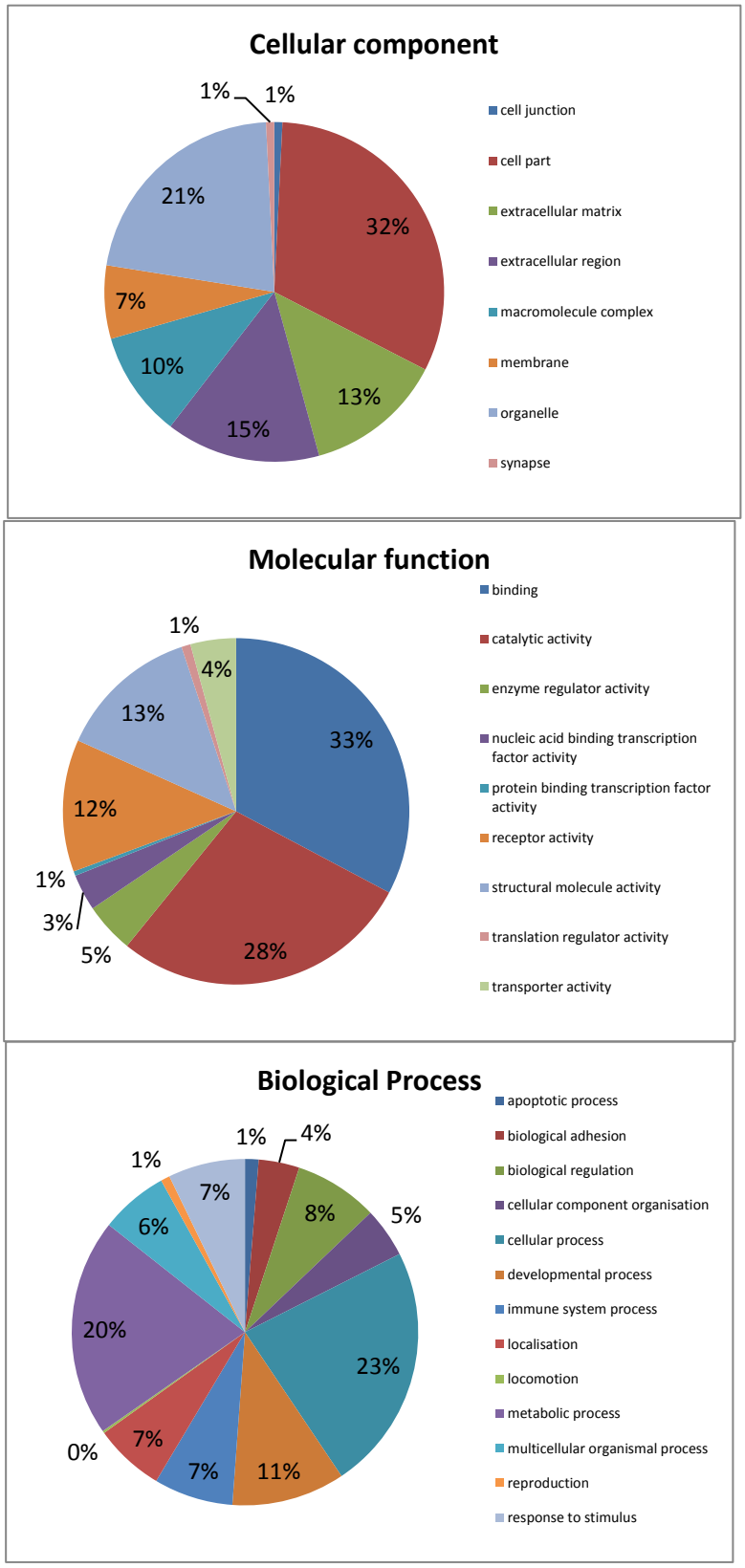


Figure 4.5. PANTHER analysis of DEGs found between polyp and control (n=322) showing which cellular component, molecular function and biological process these

Table 4.4. Statistical overrepresentation test for polyp versus control. Fold enrichment is calculated by taking the ratio of the expected number of genes based on the genes found under that category in the micro-array chip and comparing this to the actual observed number of genes in that category. Only biological processes with fold enrichment <0.5 or >2 have been shown.

Biological Process	Fold enrichment	P value	No of genes	Genes
Anatomical structure morphogenesis	2.29	<0.001	19	COL3A1, GPM6B, COL4A1, CALD1,PLS3,LDB3,TPPP,TPPP3, CCDC102B,ESPNL,AXIN2,PDLIM3,ACTL8,MYOC,SYNAPO2,COL5A2,CFL2,TPM2,ACTA2.
Cellular component morphogenesis	2.42	0.001	16	GPM6B, COL4A1, CALD1, PLS3, LDB3, TPPP, TPPP3, ESPNL, PDLIM3, ACTLB, MYOC, SYNAPO2, COL5A2, CFL2, TPM2, ACTA2
Biological adhesion	2.2	0.002	18	LAMA2, COL4A1, LUM, CLEC4A, LEPREL1, COL14A1, VCAM1, HAPLN1, FGL2, CTSK, TNS1, TIMD4, COL5A2, PCDH18, LRRC66, ADAMTS5, EMCN
Cell adhesion	2.17	0.003	17	LAMA2, COL4A1, LUM, CLEC4A, VCAM1, FGL2, CTSK, TNS1, TIMD4, COL5A2, PCDH18, LRRC66, CFB, ADAMTS5, EMCN, FGF7
Muscle contraction	3.05	0.003	9	PPP1R14A, VCAM1, EDNRA, LDB3, CCDC102B, CCK, PDLIM3, CRYAB, TPM2
Homeostatic process	2.88	0.007	8	COL3A1, COL4A1, ENRA, CCL23, COL5A2, LRRC66, ATP12A, AGR2
Regulation of liquid surface tension	>5	0.008	4	COL3A1, COL4A1, COL5A2, AGR2
Steroid metabolic process	3.09	0.008	7	DHR7SC, CELA3A, CELA3B, CYP3A4, HSD11B1, INSIG1, HSD17B6
Antigen processing and presentation	>5	0.009	4	HLA-DQA1, HLA-DQA2, HLA-DPA1, HLA-F
Monosaccharide metabolic process	3.27	0.011	6	UGTB11, UGTB10, UGTB15, EDRNRA, PCK1, FUT2

Complement activation	>5	0.020	3	C4BPB, CFB, SRPX
Cell-cell adhesion	2.02	0.022	11	LAMA2, COL4A1, LUM, CLEC4A, LEPREL1, FGL2, COL5A2, LRRC66, CFB, ADAMTS5
Macrophage activation	2.64	0.028	6	COL3A1, CLEC4A, S100A14, DMBT1, GPB3, PLEKHS1
Blood coagulation	2.62	0.029	6	CLEC4A, COL14A1, PROCR, C4BPB, CFB, SRPX
Cell-matrix adhesion	3.19	0.038	4	LAMA2, COL14A1, FGL2, ADAMTS5
Cellular defence response	2.21	0.042	7	HLA-DQA1, CLEC4A, BTN3A2, PLEKHS1, HLA-DPA1, STAT6, HLA-DQA2
Nucleobase containing compound metabolic process	0.5	<0.001	23	LUM, RUNXT1, ABCA8, S100A14, COL14A1, RGS5, HOXB9, LDB3, APOBEC1, ISL1, HIST1H4A, AXIN2, PDLIM3, BHLEH22, S100P, PRRX1, TSEN2, MAOB, BMP3, APOBEC3B, STAT6
RNA metabolic process	0.43	0.0001	13	LUM, RUNTX1, HOXB9, LDB3, APOBEC1, ISL1, PDLIM3, BHLHE22, PRRX1, TSEN2, BMP3, APOBEC3B, STAT6
Transcription	0.4	<0.001	10	LUM, CBFA2T1, HOXB9, LDB3, ISL1, PDLIM3, BHLHE22, PRRX1, BMP3, STAT6
Ion transport	0.21	0.003	2	KCTD12, ATP12A
Cation transport	0.26	0.017	2	KCTD12, ATP12A
Organelle organisation	0.38	0.043	3	HIST1H4A, MAOB

4.3.3.5. Polyp versus cancer

In comparison to the changes found between cancer/polyp and control, most of the DEGs in this category were associated with membrane structures or the extracellular matrix. Similarly, genes that were involved in cell signalling (acetyltransferase activity), protein degradation (cysteine type peptidase activity, racemase and epimerase activity) and microtubule binding were more enriched (fold enrichment 3.01, $p<0.001$: 2.66, $P<0.001$ and 3.62, $p<0.001$, respectively). Similar to the previous analyses, genes involved in transcription and nucleic acid binding were less enriched compared to the background. The biological process analysis revealed enrichment of genes involved in metabolism (tricarboxylic acid cycle, monosaccharide metabolic process and glycolysis) and reduced enrichment of genes required for regulation of RNA transcription (RNA metabolic process, transcription, mRNA process, nitrogen compound metabolic process and biological regulation) (see table 4.5).

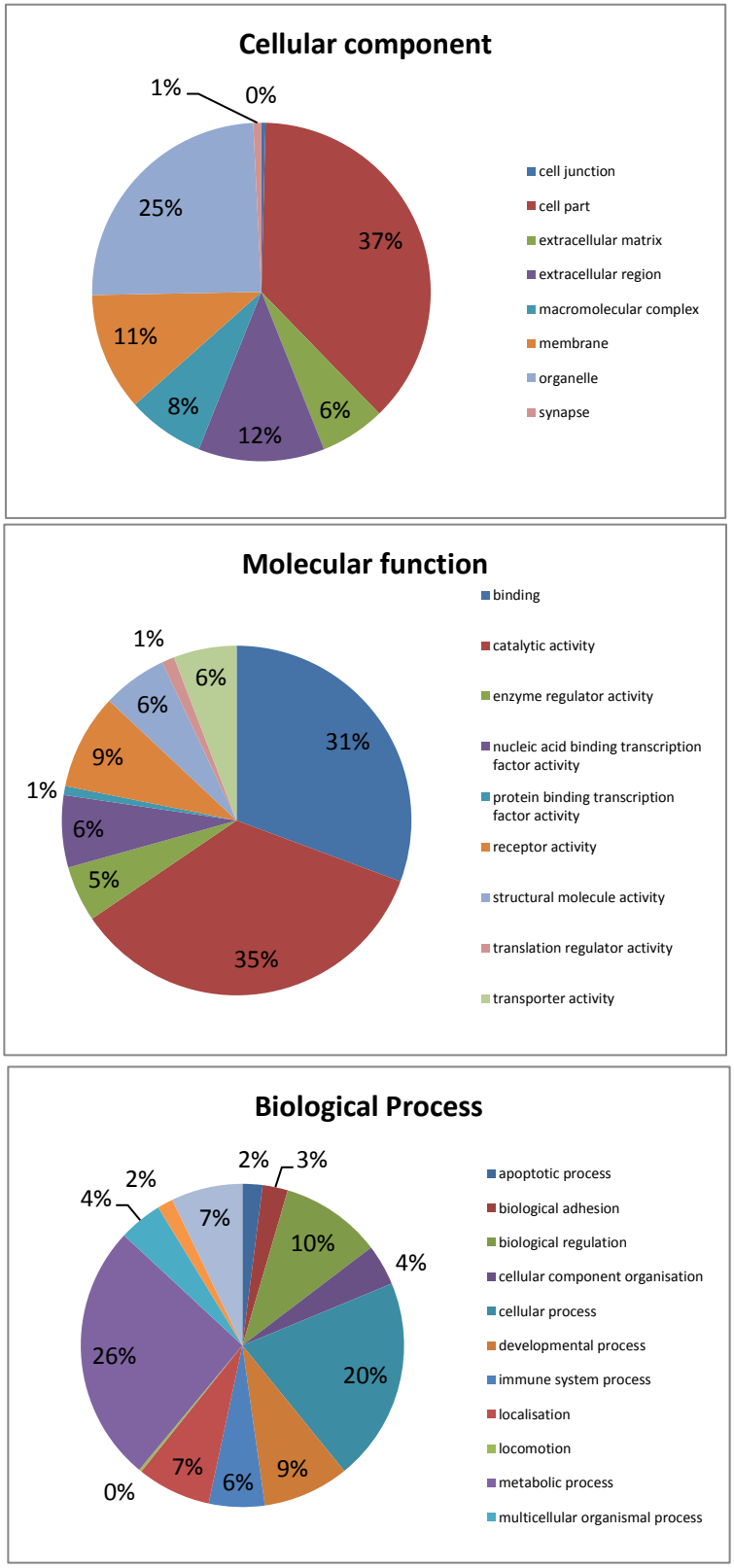


Figure 4.6. PANTHER analysis of DEGs found between polyp and cancer (n=954) showing which cellular component, molecular function and biological process these genes belong to.

Table 4.5. Statistical overrepresentation test for polyp versus cancer. Fold enrichment is calculated by taking the ratio of the expected number of genes based on the genes found under that category in the micro-array chip and comparing this to the actual observed number of genes in that category. Biological processes with fold enrichment ratio >2 or <0.66 have been shown.

Biological Process	Fold enrichment	P value	No of genes	Genes
Chromatin organisation	2.50	<0.001	25	LIN6,HIST1H3A, BRD3, SMC2, NAP1L, SMC4,HIST1H4A,HIST1H2BC, HIST1H2AG, HIST1H2AB, CARM1, H1FO, HIST2HAA3, HIST1H1B, HIST1H2AC, HIST1H1D, TNRC18
Tricarboxylic acid cycle	>5	0.001	5	LDHB, ACO2, ME1, LDHA, LDHC
Monosaccharide metabolic process	2.17	0.015	11	LDHB, UGTB11, PPARG, PFKL, LDHA, UGTB15, LDHC, FUT2, PMM2, INPPL1.
Glycolysis	4	0.019	4	LDHA, PFKL, LDHB, LDHC
mRNA processing	0.3	<0.001	3	UPF1, PPP1CB, APOBEC3B
Nitrogen compound metabolic process	0.66	0.009	26	BMP5, CDC42EP1, APOA1BP, MLXIP, SPDEF, RGS5, PARP1, FOXA2, THNSL2, CYB5R2, AXIN2, HIST1H2BC, CARM1, DHX34, PPARGC1B, TSEN2, CYC5, MAGOHB, GPT, ATP5D, POLR2E, DAB2IP, ABCA7, KIF13B, TNRC18, FOXP4
RNA splicing	<0.2	0.038	1	TSEN2

4.3.3.6. Several cellular processes are dysregulated in the colonic field

The findings from the study can be summarized as shown in figure 4.7. The mucosal field around a polyp contains cells that have altered cytoskeleton and dysregulated growth factor activity. Some of the genes highlighted by the functional analysis were involved in epithelial –mesenchymal transition (EMT) suggesting that cell structure and interaction with surrounding stromal cells may become modified early on in the carcinogenesis pathway. Subsequently, cells acquire the ability to survive in poor nutrient conditions and evade the host immune response as seen by the genes that were dysregulated between polyp and cancer. In all three categories, there was less enrichment of genes that are involved in regulation of RNA replication and transcription suggesting that the cells in the mucosal field progressively accumulate disordered nuclear division.

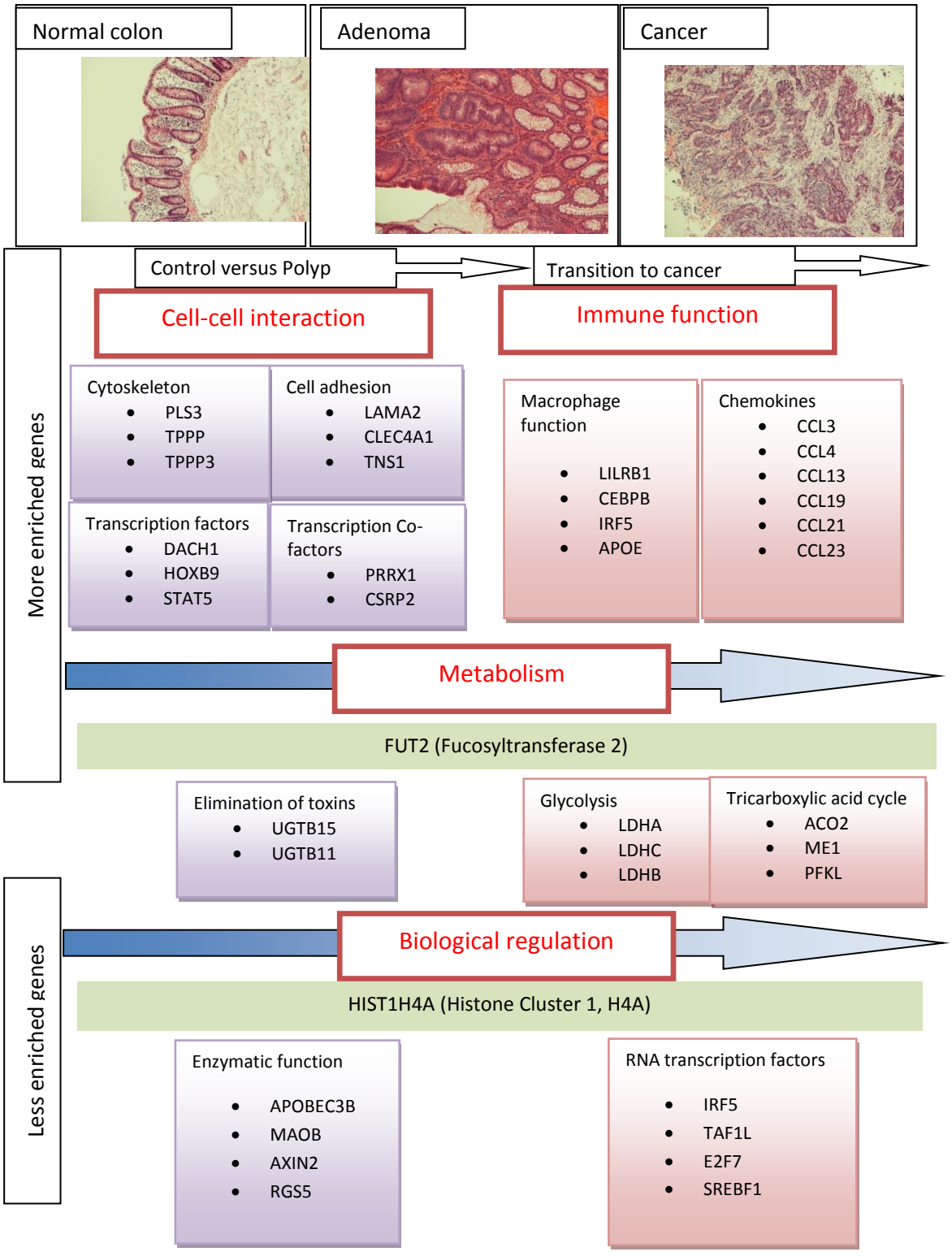


Figure 4.7. **Proposed cellular alterations in the colonic field** during progression along the adenoma-carcinoma sequence based upon genes identified in the PANTHER analysis.

4.3.3.6.1. Immune response

Several of the genes that were identified under the immune response category were related to macrophage function included LILRB1, CEPB, IRF5 and APOE. LILRB1 is a leukocyte immunoglobulin receptor which is found on immune cells where it binds to MHC class I molecules on antigen presenting cells. CEBPB is a transcription factor that binds to certain DNA regulatory elements on other acute phase and cytokine genes. IRF5 acts as a molecular switch determining whether macrophages promote or inhibit inflammation.

The cytokines that were found to be more enriched in cancer versus control included CCL3, CCL4, CCL13, CCL19, CCL21 and CCL23. CCL19 and CCL21 have homeostatic function, being constitutively expressed in certain tissue and attracting leukocytes as part of normal physiological function. In comparison, CCL3 and CCL4 are pro-inflammatory and are only expressed under pathological conditions.

4.3.3.6.2. Metabolism

Enrichment of genes involved in cellular metabolism was found in all three groups, however, only two terms were enriched in the polyp versus control comparison: steroid metabolic process and monosaccharide metabolic process. UDP glucuronosyltransferase 2 family, polypeptide B15 (UGTB15) and UDP glucuronosyltransferase 2 family, polypeptide B11(UGTB11) were found in both polyp versus control and polyp versus cancer. These genes encode enzymes that are important for the conjugation and subsequent elimination of potentially toxic xenobiotics and endogenous compounds.

In the cancer versus control and cancer versus polyp comparison, three common processes were enriched which included glycolysis, tricarboxylic acid cycle (Hoy *et al.*) and lipid

metabolic process. In terms of glycolysis and TCA, lactate dehydrogenase A (LDHA) and C (LDHC) were found in both of these categories whilst lactate dehydrogenase B (LDHB), Aconitase 2 (ACO2), Malic enzyme (ME1) and Phosphofructokinase, Liver (PFKL) were only found in the cancer versus polyp comparison.

Lipid metabolic process was the other biological term that was found to be enriched in the analysis comparing cancer with control or polyp. However, only 8/50 genes found in the cancer versus control category were also found in the cancer versus polyp category.

Fucosyltransferase 2 (FUT2) was the only gene that was identified in all three comparisons. This gene encodes a protein that is a Golgi stack membrane protein, involved in the creation of a precursor of the H antigen in body fluids and intestinal mucosa. The H antigen is an oligosaccharide which acts as a carbon source and attachment site for intestinal bacteria.

4.3.3.6.3. Cell-cell interaction

Most of the genes that were identified under the polyp versus control category contribute to cytoskeleton function, microtubule assembly, cell adhesion and cell proliferation. Key genes identified included PLS3, TPPP, TPPP3, LAMA2, CLEC4A1, TNS1, HOXB9, STAT6, PRRX1 and FGF7. PLS3 encodes an actin-bundling protein that inhibits cofilin mediated depolymerisation of actin fibres. LAMA2 encodes the alpha 2 chain of laminin 2 and 4. Laminin is an extracellular protein that is a component of the basement membrane and interacts with other ECM constituents to mediate migration and attachment of cells. STAT6, HOXB9 and PRRX1 are involved in transcription.

4.3.3.6.4. RNA transcription

In all three categories, the biological processes where genes were less enriched compared to the background population included those involved in biological regulation or RNA transcription suggesting dysregulation of homeostasis and production of proteins. Only one gene which fell under these terms was found in all three categories; HIST1H4A (Histone Cluster 1, H4A) which encodes a member of the Histone 4 (H4) family. Histone proteins form an octamer around which DNA is wrapped. Despite the similarity in the terms that were enriched, only 11/27 and 2/27 of the genes found in the control versus cancer comparison were also found in the cancer versus polyp and polyp versus control categories respectively. This suggests that different genes with variant cellular and biological functions are dysregulated along the adenoma-carcinoma sequence. In the polyp versus control category, most of the genes identified had enzymatic activity such as hydrolase activity (APOBEC3B), oxidoreductase activity (MAOB), GTPase activity (AXIN2, RGS5). In comparison, most of the genes identified in the cancer versus polyp category were transcription factors or cofactors suggesting that altered transcription occurs later in the cancer process.

4.4 Discussion

This study offers unique opportunity to examine the cellular processes that are altered in the MNM and identify genes that may play a role in field cancerisation. Furthermore, it is possible to cluster the patients that have polyps and cancers from controls based upon the gene expression profile of MNM suggesting a possible role of field cancerisation in screening and early diagnosis.

Micro-array analysis is an efficient way to measure gene expression and samples the genome in an unbiased fashion enabling a candidate-free approach to identification of putative biomarkers. In CRC, most studies have been conducted comparing normal mucosa with cancer tissue to determine the molecular pathways that are involved in carcinogenesis (Alon *et al.*, 1999; Notterman *et al.*, 2001; Birkenkamp-Demtroder *et al.*, 2005; Grade *et al.*, 2007; Croner *et al.*, 2005). Whilst these studies have been successful in clustering normal versus tumour samples and to a lesser extent, different progression stages (Frederiksen *et al.*, 2003; Groene *et al.*, 2006; Croner *et al.*, 2008), MSI status (Kruhøffer *et al.*, 2005; Banerjea *et al.*, 2004) and anatomical location (Bertucci *et al.*, 2004; Komuro *et al.*, 2005; Birkenkamp-Demtroder *et al.*, 2005), there have been few previous reports where the MNM in cancer patients has been profiled. Although only a small fraction (approx. 2 %) of the genes used on a microarray platform have been found to be differentially expressed between cancer and control tissues (Cardoso *et al.*, 2007), a number of common processes have been identified across studies. Genes that are dysregulated between cancer and control are usually involved in cell adhesion, cell communication, cell cycle regulation, cellular and nucleic acid metabolism and the cellular response to external stimuli which includes the immune and stress response (Cardoso *et al.*, 2007). However, there are multiple genes that have been highlighted in these studies which have no proven role in CRC and require further investigation (Nannini *et al.*, 2009).

Across the DEGS identified, the gene expression profile of adjacent MNM in cancer and polyp patients varied the most. Amongst many of the DEGs found, there was an inverse relationship between gene expression in cancer compared to polyp suggesting that either the genes involved have a dual function similar to that found with TGF β (Derynck *et al.*, 2001; Wakefield & Roberts, 2002) or that there are dynamic gene expression changes along the adenoma-carcinoma pathway. In this setting, genes that play an integral role in polyp formation may reach a threshold after which upregulation of the gene is of no further benefit to the cell and consequently it is turned off.

A number of cellular processes were found to be dysregulated in the colonic field around CRC and adenomas including the immune response, metabolism, epithelial-mesenchymal transition (EMT) and cellular proliferation.

4.4.1. Immune response

Several of the genes highlighted under immune system response play a role in macrophage function. In some studies, macrophages have been shown to enhance tumour growth and progression. They are usually attracted by hypoxic and necrotic tumour cells and release pro-inflammatory compounds such as TNF-alpha which activate NF-KB. NF-KB enters the nucleus and switches on anti-apoptotic genes and genes that increase cell proliferation or inflammation. Tumour growth is further augmented by the macrophages with release of pro-angiogenic factors such as VEGF, GM-CSF and IL-1/IL-6 (Jedinak *et al.*, 2010). However a more recent study (Ong *et al.*, 2012) has challenged this view and shown that tumour associated macrophages in CRC exert a tumour suppressive effect and are pro-inflammatory. In another study, it was possible to blunt the innate pro-tumoral effects of macrophages by forcing IFN-alpha expression tumour infiltrating macrophages and convert the tumour

microenvironment towards more effective dendritic cell activation and immune effector T cell cytotoxicity (Escobar *et al.*, 2014).

The enrichment of genes involved in macrophage function was coupled by enriched genes having chemokine/cytokine activity which reflects the requirement to attract immune/inflammatory cells. The role of the immune system in carcinogenesis is supported by the observation that there is an increased incidence of CRC amongst immunocompromised individuals (Sint Nicolaas *et al.*, 2010). Furthermore, an increase in the inflammatory/immune component of a tumour has been associated with reduced risk of recurrence and improved survival (Pagès *et al.*, 2005; Nosho *et al.*, 2010; deLeeuw *et al.*, 2012). This suggests that the immune system may be important in counteracting cancer growth and if dysregulated, could contribute to invasion and the formation of metastasis. This is of further interest as pharmacological manipulation of this immune response could aid the host in fighting against cancer and can contribute to chemotherapy induced cell death.

4.4.2. Metabolic process

Several of the genes that were dysregulated between cancer and control tissue played a role in mitochondrial function and Tricarboxylic acid cycle (Hoy *et al.*). Several TCA genes have been found to be mutated in hereditary and sporadic cancer (Gaude & Frezza, 2014). Under normal physiological conditions, anaerobic respiration only occurs when there is insufficient oxygen available. However, in cancer cells, there is increased LDHA activity which enables cells to convert pyruvate into lactate even when there is sufficient oxygen. This has been termed the Warburg effect (WARBURG, 1956) and explains how the energy demands of rapidly dividing cells can be met without relying upon glucose delivery (Vander Heiden *et al.*, 2009). Increased lactate levels have been correlated with poor disease free survival and

increased incidence of metastases in a number of different cancers (Schwickert *et al.*, 1995; Walenta *et al.*, 1997) including rectal cancer (Walenta *et al.*, 2003).

Dysregulation of the TCA also leads to production of higher reactive oxygen species which contributes to cancer formation. ME1 is found in the cytoplasm (in contrast to ME2 which is mitochondrial) and catalyses the oxidate decarboxylation of malate into pyruvate. Malate metabolism has been shown to be compartmentalized (Moreadith & Lehninger, 1984). Malate generated in the mitochondrion proceeds through the TCA whereas cytosolic malate is converted to pyruvate and then citrate, potentially fuelling fatty acid and cholesterol biosynthesis, aiding tumour growth. ME1 and ME2 expression have been shown to be regulated by p53 (Jiang *et al.*, 2013) which is one of the key genes to be dysregulated in CRC suggesting a potential role of ME1 in colorectal carcinogenesis.

4.4.3 Epithelial-mesenchymal transition (EMT)

Similar to previous reports (Lin *et al.*, 2002), this study demonstrated that there are changes in gene expression of genes encoding components of the cytoskeleton and extracellular matrix in patients with polyps compared to controls. These changes could contribute to epithelial mesenchymal transition (EMT) whereby epithelial cells become spindle shaped, motile and develop the ability to invade the basement membrane. Contrary to previous studies which have linked EMT with more aggressive, metastatic tumours (Tam & Weinberg, 2013) or proposed that it facilitates drug resistance against chemotherapy agents (Kim *et al.*, 2015), this study did not find similar enriched terms in the other two comparisons. The findings could suggest that EMT may be taking place earlier in the colorectal cancer pathway than previously thought. Alternatively, changes in cell morphology and adhesion could occur at the polyp stage with the complete process of EMT only taking place once key genes, such as TGF β are activated. A recent study (Yokobori *et*

al., 2013) identified elevated levels of PLS3 in circulating CRC cells and demonstrated that it was independently associated with prognosis. The authors postulated that it plays a role in driving EMT and therefore cancer cells with high expression have greater malignant potential through acquiring a mesenchymal phenotype. A subsequent study (Sugimachi *et al.*, 2014) has shown that PLS3 induces EMT via activation of the TGFβ pathway and confers the ability to invade. Similarly, TPPP and TPPP3 also bind tubulin and have microtubule bundling activity and help maintain the integrity of the microtubule network.

Several of the other genes that were identified were involved in cell adhesion thus alterations in these genes could also contribute to the process of EMT.

4.4.4. Cell proliferation

The other term that was found only to be enriched between polyp and control was 'cellular process'. Multiple different genes were included under this term including transcription factors, transcription co factors and supportive growth factors such as FGF7. STAT6 is induced by IL4 and upon phosphorylation, translocates to the nucleus where it induces the expression of the anti-apoptotic proteins BCL2L1/BCL-X(L). In one study, CRC cell lines where STAT6/IL4 are upregulated displayed increased resistance to apoptosis and increased metastatic potential (Li *et al.*, 2008). The authors went onto show that xenograft tumours of STAT6 active HT29 cells displayed a predisposition to TH2 cytokines (IL-4 and IL5) and were more pro-apoptotic and pro-metastatic (Li *et al.*, 2012). HOXB9 encodes a protein with a homeobox DNA-binding domain which acts as a sequence specific transcription factor which is involved in cell proliferation and differentiation. HOXB9 expression has been shown to correlate with poor survival and higher incidence of metastasis in CRC patients (Huang *et al.*, 2014). DACH1 is a chromatin associated protein that interacts with other DNA-binding transcription factors to regulated gene expression and determine cell fate. Expression of this gene has been shown to be lost in some forms of cancer including breast,

endometrial and prostate cancer (Nan *et al.*, 2009; Wu *et al.*, 2006; Wu *et al.*, 2007a; Yamada *et al.*, 2008). In a recent study, DACH1 was found to be switched off by promoter region hypermethylation in CRC and subsequent reduction in DACH1 expression correlated with late tumour stage, poor differentiation grade and increased incidence of lymph node metastasis (Yan *et al.*, 2013). Loss of DACH1 expression lead to TGF-β induced EMT which contributed to increased cell growth, motility and invasiveness (Wang, 2015).

4.4.5. Field cancerisation

Most of the previous studies that have investigated field cancerisation in CRC have shown differences in cellular proliferation and apoptosis along the colon, at distant sites to the primary tumour (Anti *et al.*, 2001; Badvie *et al.*, 2006; Anti *et al.*, 1993). More recently, investigators have demonstrated that there are methylation changes in the colonic field around a CRC which mirror the epigenetic changes found in the tumour itself (Worthley *et al.*, 2010; Belshaw *et al.*, 2008). Others have shown that there is reduced protein expression of important DNA repair proteins up to 10 cm away from the tumour in the macroscopically normal mucosa around a cancer (Facista *et al.*, 2012). The differences in immune response, cellular metabolism and cell-cell interaction that have been reported in this study have previously not been investigated in the context of field cancerisation. Thus, a candidate free approach was successful at identifying novel cellular processes that are dysregulated in the colonic field. Although some of these genes have been implicated in CRC formation previously, their role in being a marker of the field defect or driving biological alteration in the colonic field has not been evaluated. Further validation of the genes identified could help to elucidate if they can act as CRC biomarkers and help to risk stratify individuals based on the field cancerisation concept.

4.4.6. Limitations

There are some limitations to this study that require further consideration. Although the number of patients investigated may appear small, it is comparable to earlier microarray exploratory studies designed to investigate global differences in gene expression (Kitahara *et al.*, 2001; Zou *et al.*, 2002; Dai *et al.*, 2012). The other limitation is that no molecular subtyping of the CRC was performed. Previous studies investigating field cancerisation have highlighted that gene expression changes in the MNM parallel those found in the tumour itself suggesting that they may predate or contribute to the malignant process (Worthley *et al.*, 2010; Facista *et al.*, 2012). This also implies that there may be differences in the various molecular subtypes of CRC with field cancerisation being observed in only specific types. However, there is insufficient evidence in the current literature to determine which molecular subtypes of CRC are more important when investigating field cancerisation. Thus, it was felt that it would be more important to ascertain which cellular processes are dysregulated across several molecular subtypes of CRC rather than focusing on any individual subtype.

One of the shortcomings of microarray technology has recently been highlighted in a meta-analysis which found little overlap in the differentially expressed genes reported in different studies. Amongst the genes that were reported by multiple studies, there were inconsistencies in the direction in which the genes were changed. This may relate to differences in sample collection and processing (with some using laser capture microdissection), use of different array technology (cDNA versus oligonucleotide array) and discrepancies in analysis techniques or definitions of what constitutes differential expression (Cardoso *et al.*, 2007). It highlights that individual genes that are identified by these studies require validation using different experimental techniques to support the micro-array findings.

4.5. Summary and conclusions

- Multiple cellular processes were altered in the MNM around cancer and adenomas supporting the field cancerisation concept
- Several of the genes identified were related to the extracellular matrix, cytoskeletal components, cell adhesion and epithelial-mesenchymal transition.
- The transition to invasive malignancy was accompanied by alterations in immune function and metabolism in the MNM around cancer.
- Across all three groups, genes that regulate RNA transcription were under-represented suggesting inactivation of these genes which could contribute to the genomic instability observed in tumour tissue itself.
- It is unclear whether these biological processes occur prior to or after the development of cancer which requires further investigation
- It is important to both validate and investigate the differences in global gene expression observed in this study as this will facilitate identification of biomarkers that can not only aid early diagnosis but could be utilised to risk stratify individuals prior to the development of any histological abnormality.

Chapter 5: Differences in gene expression in the colonic field around colorectal cancer and polyps

5.1. Introduction, aims and objectives

5.1.1 Introduction

The concept of field cancerisation, first described by Slaughter (SLAUGHTER *et al.*, 1953), has been applied to several different cancers (Wong *et al.*, 2001; Kim *et al.*, 2006; Grepmeier *et al.*, 2005; Kakizoe, 2006; Kitago *et al.*, 2004; Jonason *et al.*, 1996) including colorectal cancer (CRC). Over the last three decades, there has been a gradual increase in the number of reports describing alterations in the macroscopically normal mucosa that could potentially hold the clue to the process that underlies field cancerisation (Shen *et al.*, 2005; Polley *et al.*, 2006; Badvie *et al.*, 2006; Bernstein *et al.*, 2013). There have been a few attempts to translate these findings into the clinical arena enabling early CRC diagnosis based upon biological changes detected at a distant site to the tumour (Gomes *et al.*, 2009). Despite these advances, the biological processes that underlie field cancerisation remain poorly understood. In the previous chapter (chapter 4), a candidate free approach with microarray technology was utilised to identify novel cellular processes and genes that were dysregulated in the macroscopically normal mucosa (MNM) around CRC and colorectal adenomas. A number of genes were identified based on the two different analytical methods utilised (see chapter 4) including MUC2, MUC5AC, GADD45B, PSCA, FUT2, CXCL2, S100P and SLC46A1 (see table 5.1.). These genes were chosen based on statistical significance and biological relevance as discussed in chapter 4.

Table 5.1. Micro-array genes. Genes identified for further investigation based on the microarray study findings. Information given regarding the family of genes to which the gene belongs, its biological function and role in cancer biology.

Gene	General description	Specific description	Role in cancer
MUC2 Mucin 2 **MUC5AC** Mucin 5, subunit AC	Family of proteins (MUC1-MUC21) containing tandem repeat structures with high proportion of prolines, threonines and serines. (Timpte *et al.*, 1988; Gendler *et al.*, 1987)	Secreted mucins (MUC2, MUC5AC and MUC6) produce mucous gel - physical barrier. MUC2-colorectal MUC5AC-bronchial	Implicated in lung, gallbladder and GI tract cancer (López-Ferrer *et al.*, 2001; Yu *et al.*, 2005; Briggs *et al.*, 2009). Conflicting reports in tumour vs control.
GADD45B Growth arrest DNA damage inducible 45, subunit B	Small, conserved proteins which act as stress sensors and modulate the cellular response to genotoxic or physiological stress (Liebermann & Hoffman, 2002). Interacts with other proteins involved in the stress response including PCNA, p21, Cdc2/Cyclin B1, MEKK4 and p38 kinase	GADD45B acts in co-ordination with other factors to inhibit cell growth and promote apoptosis	Implicated in human hepatocellular carcinoma and pituitary gonadotrope tumours (Michaelis *et al.*, 2011; Ou *et al.*, 2010; Qiu *et al.*, 2003). Role in CRC unclear.
CXCL-2 Growth related oncogene β (GRO- β)	CXC family - group of small proteins that modulate leukocyte migration to sites of inflammation (Charo & Ransohoff, 2006) and in tumours, induce angiogenesis and modify host immune response (Balkwill, 2012; Koizumi *et al.*, 2007)	CXCL-2 is an ELR ('glu-leu-arg') positive chemokine - pro-angiogenic, able to attract & activate neutrophils & stimulates endothelial cell migration (Vandercappellen *et al.*, 2008; Strieter *et al.*, 2006).	Possible role in CRC though reports are conflicting (Friederichs *et al.*, 2005). Possible role as a serum biomarker of CRC (Zheng *et al.*, 2015)
SLC46A1 solute carrier family 46 (folate transporter), member 1	Proton coupled folate transporter (PCFT), involved in intestinal absorption of dietary folates (Hou & Matherly, 2014; Zhao & Goldman, 2007)	More active in the stomach and tumour tissue as requires a more acidic environment	Linked to longer disease free survival in CRC (Odin *et al.*, 2015)
S100P S100 calcium binding protein P	95 amino acid residue protein, member of EF-hand superfamily of calcium binding proteins which mediate calcium dependent signalling regulating cellular processes such as cell cycle, growth, differentiation and metabolism (Donato, 2001)(Donato 2001)	Interacts with (RAGE) to stimulate Erk and (NF)-κB activity leading to cell growth and migration (Tóthová & Gibadulinová, 2013). Targets E2/EP4 receptor signalling pathway leading to cell invasion and motility (Chandramouli *et al.*, 2010)	Implicated in several cancers including cancer of the pancreas (Dowen *et al.*, 2005), breast (Guerreiro Da Silva *et al.*, 2000), prostate (Wang *et al.*, 2007), lung (Rehbein *et al.*, 2008) and colon (Birkenkamp-Demtroder *et al.*, 2005).
FUT2 Fucosyltransferase 2	Secretor type of α(1,2) fucosyltransferase-regulates expression of H antigen (Wang *et al.*, 1994a; Wang *et al.*, 1994b; Masutani & Kimura, 1995) (precursor of essential A and B antigens)	Determine microbial flora in colon thus provides link between microbiota and CRC	Glycosylation pattern of A/B antigens changes during malignant transformation (Hakomori, 1989).
PSCA Prostate stem cell antigen	Member of the LY-6 family of surface proteins		High in prostate, bladder, endometrium, kidney, lung & pancreas cancer (Liu *et al.*, 2010; Cao *et al.*, 2005; Elsamman *et al.*, 2006).

Early reports addressing the changes in the MNM adjacent to CRC (termed the transitional mucosa) identified an increase in the number of goblet cells (Dawson & Filipe, 1976; Riddell & Levin, 1977) with changes in sulfation of mucin that was present (Nieuw Amerongen *et al.*, 1998; Filipe & Branfoot, 1974). Therefore, it seems plausible that there could be changes in mucin content in the adjacent MNM around cancer or polyp that could play a role in field cancerisation. Several recent reports have highlighted defective DNA repair in the MNM around CRC up to 10 cm from the tumour which could underlie field cancerisation (Shen *et al.*, 2005; Facista *et al.*, 2012). Similarly, GADD45B which is usually released in response to cellular stress, also helps to maintain genomic integrity by arresting cell growth and promoting apoptosis. It has recently been identified in a 12 gene marker panel utilised for risk stratification of stage II CRC patients (O'Connell *et al.*, 2010; Gray *et al.*, 2011). This implies that it is a marker of risk of recurrence which could occur through field cancerisation whereby individuals with a field defect are at higher risk of metachronous lesions. Therefore, GADD45B could be an indicative marker of field cancerisation.

The other five genes that were selected for further investigation have previously not been linked to field cancerisation, though a few of them have been implicated in CRC formation. CXCL2 interacts with its receptor, CXCR2 to activate neutrophils and endothelial cells (Strieter *et al.*, 1995; Addison *et al.*, 2000). There have been a few studies which have linked expression of chemokines to increased metastatic potential and poor clinical outcome in CRC (Kim *et al.*, 2005; Schimanski *et al.*, 2005; Günther *et al.*, 2005; Kawada *et al.*, 2007; Kollmar *et al.*, 2006; Yoshitake *et al.*, 2008). However, studies in which CXCL2 expression in CRC has been examined have yielded conflicting results. Some have proposed that it is increased in CRC (Doll *et al.*, 2010), others have failed to demonstrate a difference in expression levels (Cuenca *et al.*, 1992; Wen *et al.*, 2006) whilst there have been a few reports to imply that it is actually anti-angiogenic (Cao *et al.*, 1995). Further investigation is necessary to elucidate the precise role of this chemokine in CRC.

SLC46A1 has been linked to improved disease free survival in CRC (Odin *et al.*, 2015) whereas S100P has been associated with increased tumourigenic activity and metastatic potential in CRC suggesting that it may have a role in promoting carcinogenesis (Jiang *et al.*, 2011; Wang *et al.*, 2012; Dong *et al.*, 2014). In comparison, despite being linked to susceptibility to infections such as Norovirus (Marionneau *et al.*, 2005; Carlsson *et al.*, 2009) and HIV progression (Kindberg *et al.*, 2006), the role of FUT2 in CRC is poorly understood. In the foetus, ABH antigens are expressed equally along the length of the colon (Dabelsteen *et al.*, 1988; Szulman, 1987). At birth, ABH antigens are no longer expressed in the distal colon which creates a proximal-distal gradient. Several studies have linked differences in expression of H antigens to tumour progression in the distal colon and rectum (Wiley *et al.*, 1981; Yuan *et al.*, 1985; Schoentag *et al.*, 1987). However, there have also been reports suggesting that cell surface fucosylation does not affect development of colon tumours in mice (Domino *et al.*, 2007)(Domino et al, 2007) questioning its role in carcinogenesis.

PSCA had the highest fold change in both types of microarray analysis and was therefore selected. It is usually overexpressed in prostate cancer, particularly with the more aggressive form, (Gu *et al.*, 2000). Reduced levels of expression have been found in bladder, gastric and oesophageal cancer (Bahrenberg *et al.*, 2000). There have only been a few reports investigating the PSCA gene and colorectal cancer which have not yielded any conclusive results.

Although these genes were identified with the previous microarray study, it is generally accepted that validation of microarray findings is necessary. This is usually achieved by examining gene expression in a separate cohort of patients to the original set or utilising different experimental methods.

5.1.2 Aims and objectives

The aim of this study was to

i) Measure the gene expression level of MUC2, MUC5AC, GADD45B, PSCA, SLC46A1, CXCL2, S100P and FUT2 in colorectal tumour tissue, adjacent MNM, at the resection margin and in control subjects to elucidate if these genes have any role in field cancerisation

ii) Validate the findings from the micro-array study (discussed in chapter 4) by measuring mRNA expression of these genes and comparing it to levels identified using micro-array

iii) Determine serum concentration of CXCL2 in control subjects and patients with cancer to determine if it could be used as a serum biomarker

5.2. Materials and Methods

5.2.1 Setting and participants

Ethical approval was sought as detailed in Chapter 2. In brief, after gaining informed consent from participants, mucosal tissue samples were taken from the tumour, adjacent to the tumour and at the most distant resection margin of the colectomy specimen from individuals undergoing surgery. These patients were age and sex matched to control subjects who were recruited at the time of colonoscopy. Mucosal pinch biopsies were taken from the caecal pole and rectum in these individuals. If a polyp was identified during the procedure, a biopsy adjacent to the polyp was also taken. The samples taken from these patients were used to examine field cancerisation around polyps. Only samples proven to be adenomatous on histology were included in this cohort. Right sided polyps and cancers (proximal to mid-transverse) were matched with caecal samples from control subjects and left sided polyps and cancers were matched to rectal biopsies from control subjects. Patients with polyps also had additional biopsies taken from the caecum or rectum that were tested concurrently. No mucosal tissue was taken from the polyp itself as this is routinely analysed to exclude a malignant component. Thus, the results that are presented under the term 'polyp' are actually the gene expression levels of tissue found adjacent to the polyp.

In total, 225 samples were included in the analysis from 30 cancer patients, 29 control patients and 23 patients with polyps. Due to the presence of synchronous lesions in some of these patients, 36 cancers and 32 polyps were assessed in total. Demographic data for the cancer and polyp patients are shown in table 5.2. In a subset of patients, serum CXCL2 levels were measured as outlined in chapter 2.

Table 5.2. Clinical and pathological details of patients included in the analysis

	Control Subjects (n=29)	Polyp patients (n=23)	Cancer patients (n=30)
Median Age (IQR)	69 (60-76)	71 (64-78)	66 (57-73)
Male: Female	19:10	15:8	21:9
Mean BMI (+/- S.D.)	25.9 (3.9)	26.3 (5.2)	26.1 (5.5)
Smokers/Ex-smokers (%)	6 (21%)	10 (43%)	9 (30%)
Diabetics (%)	4 (14%)	7 (30%)	3 (10 %)
Aspirin use (%)	3 (10%)	7 (30%)	3 (10%)
Statin use (%)	7 (24%)	5 (22%)	10 (33%)
Cancers (n=36)			
Operation		**T staging**	
Right Hemicolectomy	11	T1-T2	8
		T3-T4	28
Extended R **hemicolectomy**	5	**N staging**	
		N0	19
Anterior Resection	9	N1-N2	17
Sigmoid colectomy	1	**Grade of tumour**	
Left Hemicolectomy	2	Well/Mod	21
Other	3	Poor	15
Polyps (n=32)			
Type of polyp		**Size of polyp**	
Sessile	24	< 10 mm	14
Pedunculated	7	10-20 mm	10
Villous	1	>20 mm	8
Histology of polyp		**Grade of dysplasia**	
Tubular adenoma	18	Low	29
Tubulovillous adenoma	7	High	1
Serrated adenoma	6	Hyperplastic	2
Other	1		

5.2.2. Experimental methods

The samples were stored in RNA later, kept at 4 ^{o}C overnight and subsequently stored at -20 ^{o}C for future processing. mRNA was extracted using Qiagen RNeasy tissue mini-extraction kit and cDNA synthesis was performed with 250 ng cDNA as previously described (see chapter 2 for further details). Quantitative real time PCR (qRT-PCR) was performed with Taqman gene expression assays. The comparative $2^{-\Delta\Delta}C_T$ method was used to calculate mRNA expression level of the genes compared to expression levels of 18S which was the endogenous control. All values were $\log_{10}$ transformed prior to statistical analysis to normalise the data. Changes in gene expression of MUC2, MUC5AC, GADD45B, SLC46A1, FUT2, S100P and CXCL-2 were measured in all samples. To preserve the small quantities of samples available, PSCA was measured only in a subset of the patients as it was found to be expressed at very low levels, particularly in normal colonic tissue. Serum CXCL2 levels were measured using the MIP2 Human Simple Step Elisa Kit (Abcam, UK) as outlined in chapter 2.

5.2.3. Statistical analysis

Statistical analysis was performed using SPSS version 21 (SPSS Inc, Chicago, Illinois). Normally-distributed variables were described as mean and standard deviation and non-normally distributed variables as median and interquartile range. Categorical variables were described as numbers and percentages. The test of significance to determine a difference between groups was assessed using chi-squared with categorical variables, t test or Mann-Whitney U test for ordinal variables. A p value of <0.05 was considered significant. Multivariate linear regression analysis was used to identify patient and pathological factors that affected expression levels of the genes examined.

The detailed analytical methods of the two types of analysis that were performed of the microarray data has been described in chapter 2. Genes that were chosen for validation in

analysis 1 were based upon statistical significance and biological plausibility with scientific reports linking the gene to field cancerisation. In the second analysis, genes were chosen based on their statistical significance across different comparisons between cancer, adenoma and control subjects. As alluded to previously, there was very little existing literature that linked these genes to field cancerisation.

5.3. Results

5.3.1. Mucin expression is altered in CRC but GADD45B is no different

MUC2 was significantly downregulated (0.4 fold) in tumour tissue compared to control subjects ($^{\Delta\Delta}C_T$ (tumour) 11.41 versus $^{\Delta\Delta}C_T$ (controls) 10.04, p=0.003). However, expression levels in the adjacent MNM and at the resection margin were no different to control subjects ($^{\Delta\Delta}C_T$ (adjacent MNM) 9.87 and $^{\Delta\Delta}C_T$ (margin) 10.09 versus $^{\Delta\Delta}C_T$ (controls) 10.04, p=0.467 and p=0.826 respectively).

In comparison, MUC5AC was significantly upregulated (12 fold) in tumour tissue compared to control subjects ($^{\Delta\Delta}C_T$ (tumour) 13.45 versus $^{\Delta\Delta}C_T$ (controls) 17.12, p<0.0001). Again, the MUC5AC expression in the adjacent MNM and at the resection margin was similar to that found in control subjects ($^{\Delta\Delta}C_T$ (adjacent MNM) 17.83 and $^{\Delta\Delta}C_T$ (margin) 17.25 versus $^{\Delta\Delta}C_T$ (controls) 17.12, p=0.408 and p=0.889 respectively).

In control subjects, there was a significant increase in expression of MUC2 and MUC5AC along the colon with levels in the left colon being significantly higher than the right colon (p<0.001 for both). MUC2 was significantly down regulated in the tumour tissue for left sided tumours only (p=0.017) and MUC5AC was significantly upregulated for right sided tumours only (p<0.001).

No significant differences in gene expression of GADD45B were found between the four groups examined ($^{\Delta\Delta}C_T$ (tumour) 15.65 versus $^{\Delta\Delta}C_T$ (controls) 15.96, p=0.201).

Figure 5.1.1. MUC2 expression levels in control subjects compared to tumour samples, MNM adjacent to tumour and at the resection margin from the colectomy specimen.

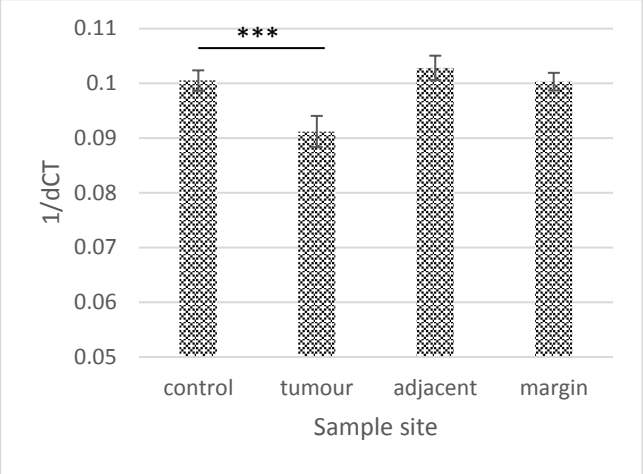


Figure 5.1.2 MUC5AC expression levels in control subjects compared to tumour samples, MNM adjacent to tumour and at the resection margin from the colectomy specimen.

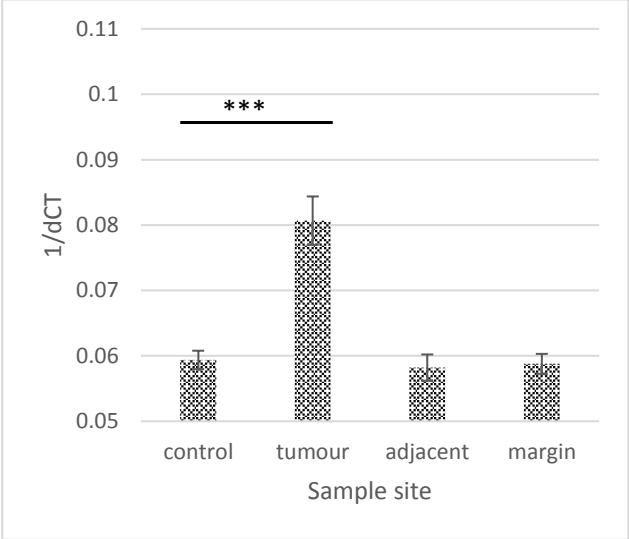
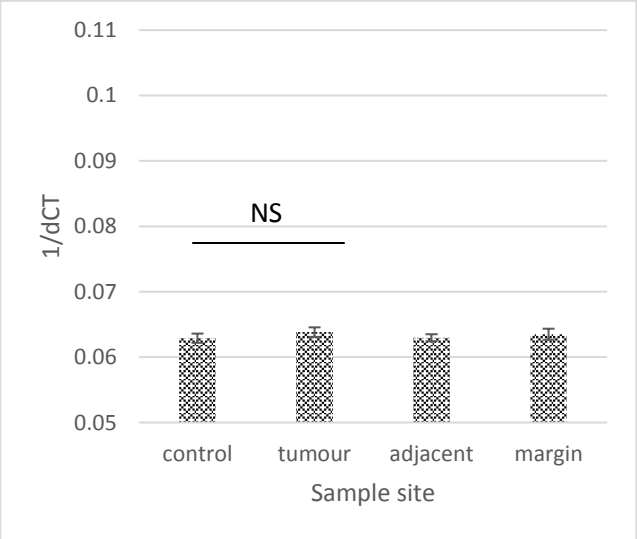


Figure 5.1.3 GADD45B expression levels in control subjects compared to tumour samples, MNM adjacent to tumour and at the resection margin from the colectomy specimen.



Based on paired analysis of samples taken from the colectomy specimen, MUC2 was significantly downregulated and MUC5AC was significantly upregulated in the tumour compared to the resection margin ($p=0.004$ and $p<0.0001$ respectively). No differences were found between the adjacent MNM and resection margin.

Across all three genes, there were no differences in mRNA expression in the adjacent MNM around polyps compared to control subjects (MUC2 $p=0.650$, MUC5AC $p=0.553$ and GADD45B $p=0.511$). However, if the MNM adjacent to polyp was compared to that around cancers, MUC5AC was found to be significantly upregulated ($^{\Delta\Delta}C_T$ (adjacent polyps) 15.73 versus $^{\Delta\Delta}C_T$ (cancer) 17.83, $p=0.017$).

Fig 5.1.4. MUC2 expression levels in control subjects compared to right colonic, left colonic and adjacent MNM taken in patients with polyps.

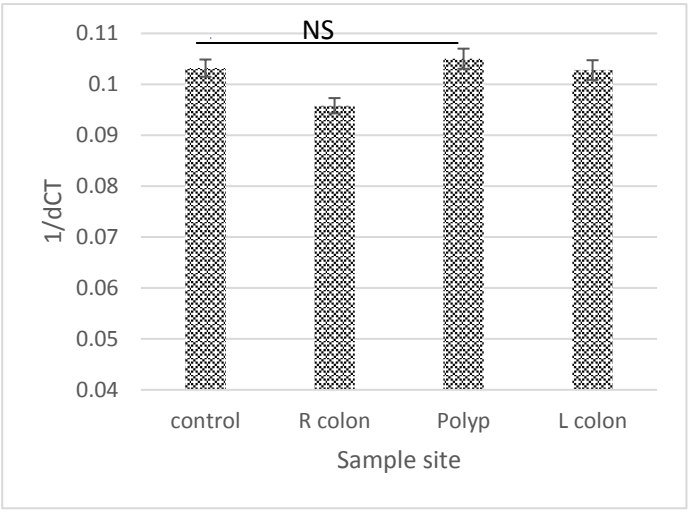


Fig 5.1.5. MUC5AC expression levels in control subjects compared to right colonic, left colonic and adjacent MNM taken in patients with polyps.

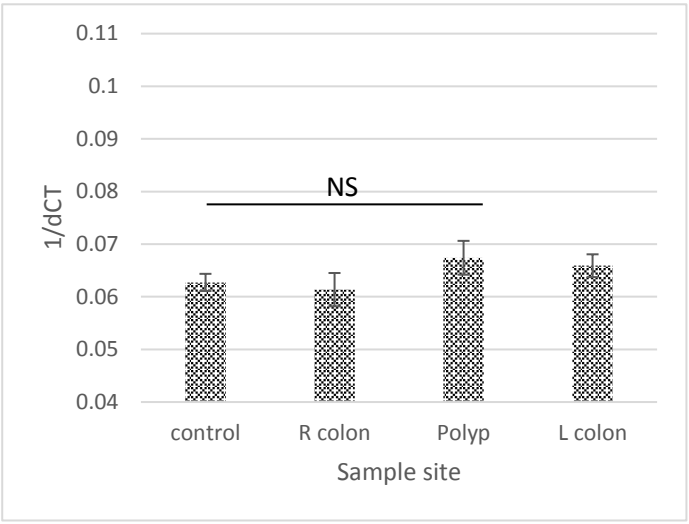
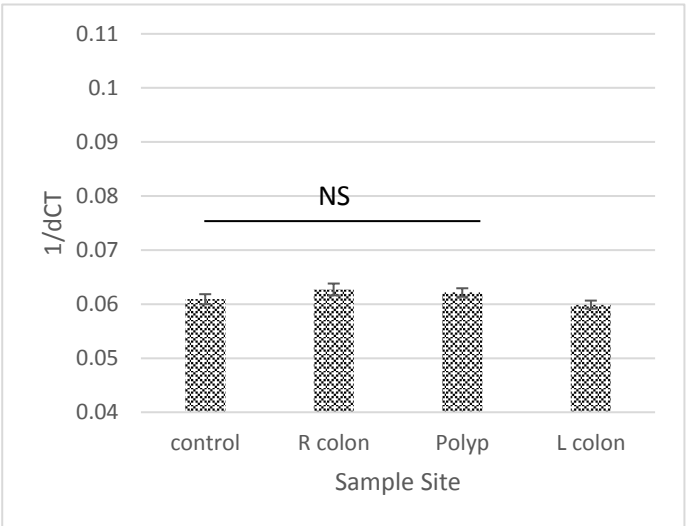


Fig 5.1.6._GADD45B expression levels in control subjects compared to right colonic, left colonic and adjacent MNM taken in patients with polyps.



5.3.2. CXCL2, S100P and FUT2 are dysregulated in CRC

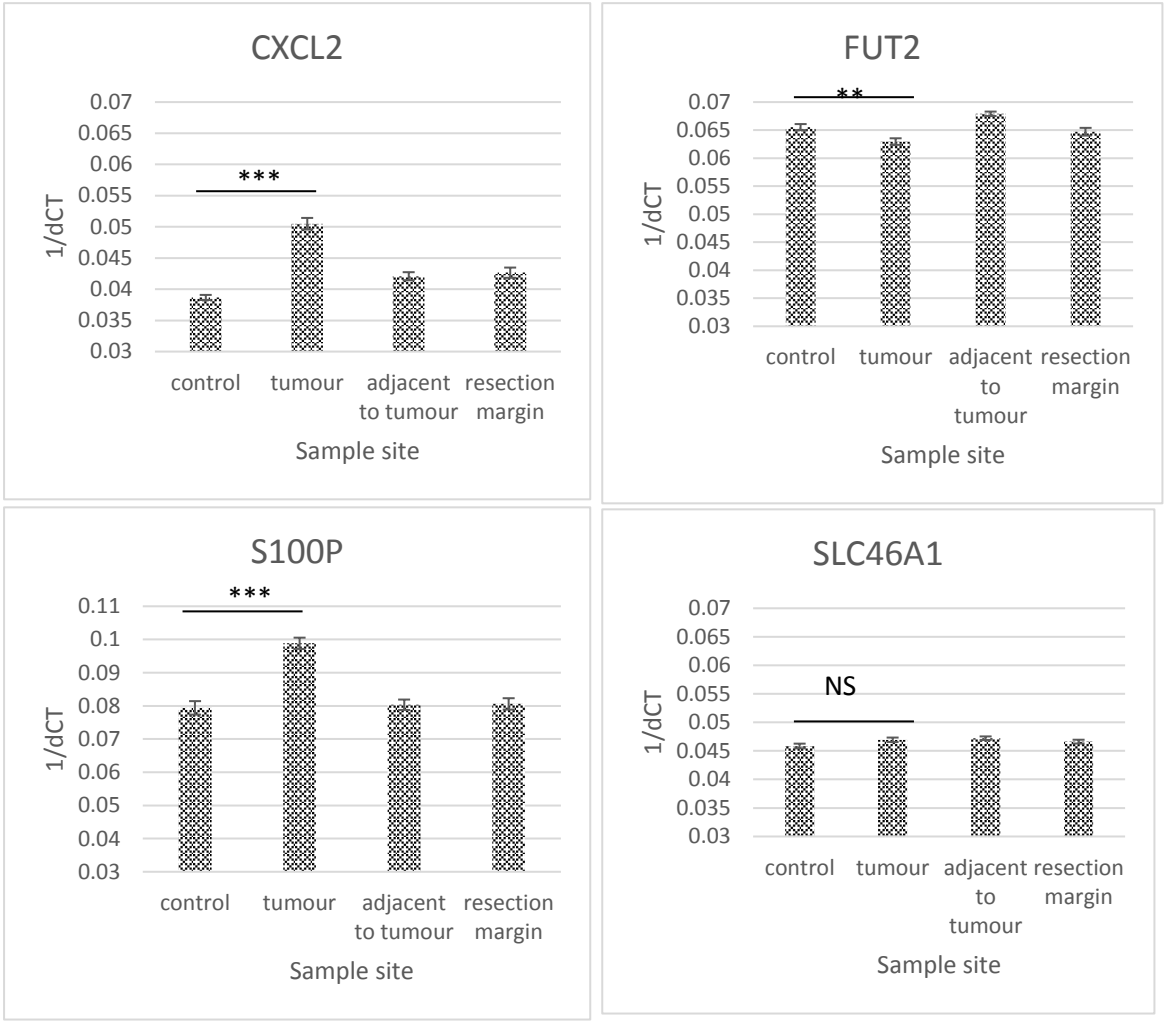
PSCA was expressed at very low levels in colonic tissues (CT > 39 in 60 % samples tested). Hence, only a small proportion of the cohort were included in the analysis. PSCA was expressed in 10/12 tumours and there was no significant difference in expression levels in control subjects compared to the cancer patients (p=0.121).

The expression levels of the other 4 genes are given in figure 5.3.1.6. Both CXCL2 and S100P were significantly upregulated in tumour tissue compared to control subjects (CXCL2: 60-fold difference, p<0.0001; S100P: 6-fold difference, p<0.001), FUT2 was significantly downregulated (1.6-fold difference, p=0.002) and no differences were found with SLC46A1 (p=0.115).

CXCL2 was significantly upregulated in the adjacent MNM and resection margin mucosa of cancer patients compared to controls (4-fold difference and 5-fold difference respectively, p<0.001 for both). SLC46 A1 was only upregulated in the adjacent mucosa and not at the resection margin compared to control subjects. There were no differences in gene expression of S100P and FUT2 in the adjacent mucosa or at the resection margin.

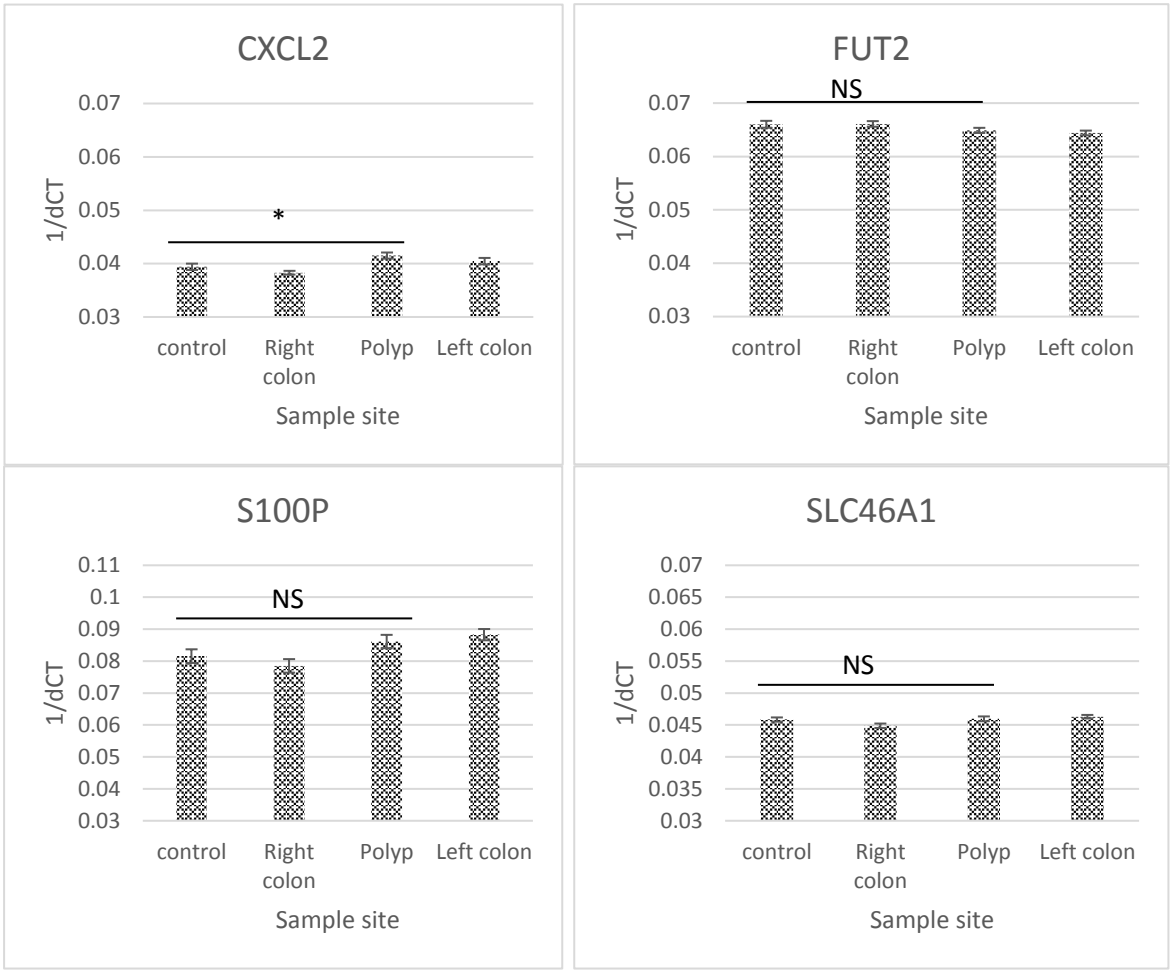
Using paired t tests, gene expression levels were also compared at the three sites of sampling undertaken in patients with CRC. CXCL2 and S100P were significantly upregulated in the tumour compared to the resection margin (p<0.0001 and p=0.009 respectively) and FUT2 was significantly downregulated (P<0.0001). No differences were found with SLC46A1 (p=0.666). The gene expression level of all four genes in the adjacent MNM was no different to that at the resection margin.

Fig 5.2.1. Expression levels of CXCL2, FUT2, S100P and SLC46A1 in control subjects compared to patients with cancer (samples taken from the tumour, adjacent MNM and at the resection margin).



With regards to patients who had polyps (see figure 5.3.1.7), CXCL2 was the only gene that was found to be significantly upregulated (2.4 fold) in the mucosa adjacent to a polyp compared to MNM taken from control subjects ($^{\Delta\Delta}C_T$ (adjacent to polyp) 24.23 versus $^{\Delta\Delta}C_T$ (controls) 25.52, p=0.013). The level of CXCL2 gene expression in paired samples taken at distant points in the colon of patients with polyps did not differ from the level recorded in MNM of control subjects. No differences in gene expression were recorded for FUT2, S100P and SLC46A1 amongst the polyp patients.

Fig 5.2.2. Expression levels of CXCL2, FUT2, S100P and SLC46A1 in control subjects compared to patients with polyps (samples taken from adjacent MNM around polyps (labelled polyp), right colonic and left colonic biopsies).



5.3.3. There is a gradient in gene expression along the colon comparing caecum to rectum

However, paired analysis using samples taken at caecum and rectum from patients with polyps revealed that CXCL2, S100P and SLC46A1 were significantly upregulated adjacent to the polyp compared to expression levels in the caecum ($p<0.001$, $p<0.001$ and $p=0.03$ respectively) whereas FUT2 was significantly downregulated ($p=0.003$). No differences in gene expression were seen if expressions levels adjacent to the polyp were compared to rectal samples taken from control subjects ($p=0.124$, $p=0.295$. $p=0.342$ and $p=0.352$ respectively). S100P expression was significantly higher in the adjacent MNM of left sided polyps compared to right sided polyps ($p<0.001$) and decreased with increasing number of polyps ($p=0.006$). FUT2 expression in the adjacent MNM of polyps correlated with number of polyps in the colon ($p=0.006$, $r=0.422$), size ($p=0.034$, $r=-0.375$) and morphology of the polyps ($p=0.035$, $r=-0.373$).

If expression levels of the genes are compared in the caecum and rectum in controls subjects, there are significant differences along the colon for all four genes (CXCL2 $p<0.001$, FUT2 $p=0.003$, S100P $p<0.001$ and SLC46A1 $p=0.01$). Due to this gradient in gene expression, if right sided tumours are compared to caecal samples from control subjects, all four genes are significantly different in the tumour compared to control subjects, including SLC46A1 ($p=0.006$). If left sided tumours are compared to rectal samples from control subjects, SLC46A1 is no longer upregulated in tumours ($p=0.894$). Despite this gradient in gene expression, there are no differences in gene expression of right sided tumours versus left sided tumours for all genes except FUT2. FUT2 is significantly upregulated in right sided tumours compared to left sided tumours ($p=0.02$).

5.3.4. Serum CXCL2 levels do not differ between the three groups

Serum CXCL2 concentration was measured in 25 patients with cancer, 20 patients with polyps and 29 control subjects. There was no difference in serum CXCL2 concentration in patients with cancer compared to control subjects (mean concentration 401.7 +/- 46.8 pg/mL versus 376.0 +/- 54.6 pg/mL, p=0.73). Similarly, no statistical difference was noted between patients with polyps and control subjects (mean concentration 402.5 +/- 70.0 pg/mL versus 376.0 +/- 54.6 pg/mL, p=0.77).

5.4 Discussion

This study has identified significant differences in CXCL2 gene expression in the MNM around a cancer and adenoma highlighting its potential role in field cancerisation in CRC. In agreement with previous reports, it has also demonstrated differential gene expression of mucin in tumour tissue.

Based on the qRT-PCR experiments, CXCL2 was significantly upregulated in the adjacent MNM of both cancer and polyp patients, with slightly lower levels being observed in the polyp patients. Previous studies have shown that CXCL2 gene expression increases in colonic tumour tissue and adenomas (Doll *et al.*, 2010; McLean *et al.*, 2011), however, this is the first study to demonstrate a difference in gene expression in the adjacent MNM of both pre-neoplastic and neoplastic colorectal lesions suggesting a potential role in field cancerisation. Inflammatory activity in CRC tumour tissue is a known predictor of prognosis (Galon *et al.*, 2006) and *in vitro* studies have shown that colonic tumour cells both secrete cytokines and possess the receptors required to respond to them directly (Schimanski *et al.*, 2005; Sturm *et al.*, 2005; Zipin-Roitman *et al.*, 2007; Li *et al.*, 2004). However, the point at which an inflammatory environment promotes tumour formation is not known. The finding that CXCL2 gene expression is increased in adenomatous lesions and this is accompanied by increased numbers of macrophages, neutrophils and T cells in the stroma (McLean *et al.*, 2011) suggests that inflammatory changes are important early on in carcinogenesis. The findings of this study, however, suggest that these changes may even predate the formation of adenomas. CXCL2 has been shown to have both angiogenic and proliferative activity (Wang *et al.*, 2006a; Bruyère *et al.*, 2011), hence, it is conceivable that changes in its levels could contribute to the early processes that are necessary for malignant transformation. However, there have been reports where no differences in gene expression of CXCL2 have been found when comparing tumour tissue to control tissue (Cuenca *et al.*, 1992; Li *et al.*, 2004; Wen *et al.*, 2006) leading authors to propose that it may be the balance between pro-

angiogenic and anti-angiogenic cytokines that contributes to tumour formation rather than levels of a single cytokine (Strieter *et al.*, 1999). Nevertheless, this study has demonstrated that CXCL2 gene expression at a distant site from the tumour could be utilised to identify patients with cancer. However, these differences in expression were not detected in serum samples taken from the three groups. This contradicts the findings of a recent study which demonstrated higher serum CXCL2 concentration in cancer patients compared to control subjects (Zheng *et al.*, 2015) highlighting the potential role of CXCL2 as a serum biomarker of CRC. Although this study demonstrated a statistical difference between cancer patients and control subjects, there was wide variation in the serum CXCL2 concentration, particularly in the cancer patients. This is similar to the findings of the present study where wide variation was observed in all three groups. There are many factors that can contribute to raised serum CXCL2 concentration (Bauer *et al.*, 2006; Tsai *et al.*, 2014) which may explain the high levels detected in control subjects. Despite the recent report of its potential role as a serum biomarker, given the wide variation observed across individuals, it would be difficult to determine an appropriate cut-off for it to be used as a screening or diagnostic test.

In agreement with several previous reports (Blank *et al.*, 1994; Ajioka *et al.*, 1996; Weiss *et al.*, 1996; Perez *et al.*, 2008; Sylvester *et al.*, 2001; Börger *et al.*, 2007), MUC2 was found to be downregulated in tumour tissue and MUC5AC was upregulated (Losi *et al.*, 2004; Walsh *et al.*, 2013; Arai *et al.*, 2007; Park *et al.*, 2006). *In vitro* studies have shown that loss of MUC2 results in increased cell proliferation, reduced apoptosis and increased cell migration (Velcich *et al.*, 2002). Similarly, MUC2 knockout leads to a 65 % increased incidence of tumours of the small intestine, colon and rectum within 1 year of birth in murine models (Velcich *et al.*, 2002). This study therefore suggests that MUC2 functions as a tumour suppressor, hence, inactivation promotes tumour growth. However, several studies have also shown that MUC2 and MUC5AC are more likely to be expressed in mucinous cancers with microsatellite instability (MSI) (Bu *et al.*, 2010; Biemer-Hüttmann *et al.*, 2000), BRAF

mutation and cancers that are poorly differentiated, proximal or have a high lymphocytic response (Walsh *et al.*, 2013). Generally it is accepted that MUC2 is usually downregulated in CRC (Aziz MA *et al.,* 2014) except in a proportion of mucinous cancers where it can be upregulated (Walsh *et al.,* 2013). In the present study, MUC2 and MUC5AC gene expression were not related to tumour characteristics such as differentiation grade, mucinous histology or lymphocytic response. However, MUC2 was only downregulated in left sided tumours rather than right sided tumours suggesting preserved function with more proximal tumours as suggested in the literature. In contrast, MUC5AC gene expression was significantly upregulated in proximal tumours only and there were no differences in gene expression in left sided tumours compared to control subjects. A recent study which evaluated MUC2 and MUC5AC expression using immunohistochemistry also showed that MUC5AC expression was associated with proximal tumours and mismatch repair deficiency (Imai *et al.*, 2013). This supports the hypothesis that these mucins are more likely to be expressed in proximal tumours which tend to develop along the MSI pathway rather than the conventional CIN pathway. As the differences in gene expression of MUC2 and MUC5AC were only observed at the tumour site and not in the adjacent MNM, these mucins are unlikely to play a role in field cancerisation. On the other hand, in this study, the mucosa adjacent to polyps demonstrated higher MUC5AC expression compared to adjacent MNM around cancers. Previous reports where MUC5AC protein expression has been examined along the colon have shown that there is variable staining of normal goblet cells in the MNM in close proximity to the tumour but no staining of these cells at more distant sites from the tumour (Walsh *et al.*, 2013). This raises the possibility that MUC5AC may be indicative of an early field defect particularly around pre-neoplastic polyps. However, in an earlier study which was designed to evaluate the role of MUC5AC in field cancerisation around polyps, MUC5AC was not detected in any of the samples taken adjacent to polyps which led the authors to conclude that MUC5AC gene expression does not represent a 'field change' around polyps (Longman *et al.*, 2000).

Similar to previous reports, this study also demonstrated that S100P is upregulated in colorectal tumour tissue compared to control tissue (Birkenkamp-Demtroder *et al.*, 2005; Fuentes *et al.*, 2007; Parkkila *et al.*, 2008; Wang *et al.*, 2012). S100P has been found to be overexpressed in several other cancers suggesting that it may play a more generic role in cancer formation by contributing to cell proliferation and migration (Dowen *et al.*, 2005; Guerreiro Da Silva *et al.*, 2000; Wang *et al.*, 2007; Rehbein *et al.*, 2008). *In vitro* studies have shown that S100P induces anchorage independence of tumour cells (Dowen *et al.*, 2005), increases proliferation (Fuentes *et al.*, 2007), promotes migration, invasion and decreases chemosensitivity to conventional agents such as 5-fluoruracil (Dong *et al.*, 2014). Conversely, blocking its action results in reduced cell growth, migration and invasion *in vitro* and smaller tumours with fewer liver metastases *in vivo*. Interestingly, in the present study S100P gene expression levels in the adjacent MNM around cancer were significantly lower than that in adjacent MNM around adenomatous lesions. As the majority of the downstream targets of S100P are cytoskeletal regulators, changes in cell anchorage and migration in the adjacent MNM may be more important in the field around adenoma formation compared to a neoplastic lesion as suggested by the findings of the microarray study described in chapter 4.

In this study, FUT2 was upregulated in tumour tissue, however, no differences were found in the adjacent MNM around polyp or cancer compared to control subjects. Previous reports have shown altered gene expression of fucosyltransferases in colorectal cancer compared to control subjects (Petretti *et al.*, 2000). Aberrant fucosylation can lead to expression of altered antigens on the cell surface which results in dysregulation of key cellular processes and altered cell adhesion. Bacteria in the GI tract often utilise host cell surface molecules, hence, genetic variation in the FUT2 gene has been implicated in susceptibility to several bacterial infections such as Norovirus (Marionneau *et al.*, 2005; Carlsson *et al.*, 2009) and Helicobacter Pylori (Ikehara *et al.*, 2001). Loss of the FUT2 gene has also been linked to

increased incidence of Crohn's disease suggesting that it plays a role in the host-microbial interface (McGovern *et al.*, 2010). Given the link between colonic microflora and CRC, it is conceivable that disordered fucosylation could contribute to the malignant process. Furthermore, in the present study, FUT2 gene expression was significantly different in right sided lesions compared to left sided lesions. This may explain the differences observed in expression of H antigens along the colon and in neoplastic tissues that have been reported in a previous study (Fujitani *et al.*, 2000).

There are some limitations to the present study that require consideration. Firstly, there has been no attempt to subgroup the patients with cancer according to MSI status. Previous reports on field cancerisation in CRC have shown that the most consistent changes at the molecular level reported at distant sites along the colon include changes in methylation and DNA repair (Shen *et al.*, 2005; Worthley *et al.*, 2010; Facista *et al.*, 2012). This suggests that cancers that develop along the conventional pathway with chromosomal instability are potentially less likely to harbour a field defect. However, there is no conclusive evidence in the literature to support this hypothesis. Given that the aim of the study was to identify potential genes that could contribute to field cancerisation, it was felt that excluding patients based on MSI status would mean that field cancerisation in conventional CRCs would not be explored which form the majority of patients.

Secondly, this study has highlighted discrepancies in levels of expression measured using micro-array technology compared to qRT-PCR demonstrating some of the limitations of micro-array. The fact that no differences in gene expression were observed for the gene that was found with the highest fold change on micro-array, PSCA, suggests that validation of micro-array is necessary. Although there is general agreement amongst researchers that micro-array data needs validation (Chuaqui *et al.*, 2002; Firestein & Pisetsky, 2002; Benes

& Muckenthaler, 2003), few attempts have been made to describe the precise methods and selection criteria for testing of individual genes (Brazma *et al.*, 2001; Miron *et al.*, 2006). These protocols are poorly adhered to resulting in considerable variability of reported outcomes across the literature (Cardoso *et al.*, 2007) leading some authors to propose that the focus of enquiry should be based on biological pathways rather than individual genes (Chen *et al.*, 2013b). Others have focussed on selecting genes with the largest effect, either determined by fold change value or statistical significance (Chuaqui *et al.*, 2002; Irizarry *et al.*, 2005; Larkin *et al.*, 2005). However, this may not be the most optimal approach as highlighted in a study which demonstrated that a random stratified sampling approach is more effective at identifying genes for validation with qRT-PCR (Miron *et al.*, 2006). Furthermore, wide ranging correlations between microarray and qRT-PCR data have been described in the literature (Beckman *et al.*, 2004; Etienne *et al.*, 2004; Larkin *et al.*, 2005; Dallas *et al.*, 2005), particularly with genes that are expressed at low levels in the tissue being examined (Czechowski *et al.*, 2004). Other reasons for poor correlation include very high expression levels, at the limits of detection (Etienne *et al.*, 2004) or alternative transcripts of the gene being detected by the two methods (Dallas *et al.*, 2005). Despite these shortcomings, the present study was successful at identifying CXCL2 as a biomarker indicative of field cancerisation in colorectal cancer. As there have been no previous reports linking CXCL2 to field cancerisation, these findings highlight the importance of adopting a candidate free approach to evaluate global gene expression profiles in an exploratory study of this kind.

5.5. Summary and conclusions

- CXCL2 is upregulated in the adjacent MNM around both CRC and adenomas compared to MNM in control subjects therefore it could be an indicative marker of field defect

- MUC2 is downregulated and MUC5AC is upregulated in the tumour tissue compared to MNM in control subjects
- There are no differences in expression of MUC2 or MUC5AC in the adjacent MNM or resection margin of colectomy specimens compared to MNM from control subjects
- The findings highlight the importance of the inflammatory micro-environment in supporting cell growth, invasion and metastasis, possibly at an earlier stage than previously reported.

Chapter 6: The role of fibroblast growth factors in field cancerisation in colorectal cancer

6.1. Introduction, aims and objectives

6.1. Introduction

There is emerging evidence to support the concept that the tumour microenvironment plays a fundamental role in supporting tumour growth facilitating both invasion and metastasis (O'Toole *et al.*, 2014; Chen *et al.*, 2015). Previous reports have demonstrated that there are alterations in cell proliferation and apoptosis in the macroscopically normal colonic field around colorectal cancer supporting the field cancerisation concept (Anti *et al.*, 2001; Badvie *et al.*, 2006; Hanna-Morris *et al.*, 2009). However, few have investigated how the stromal compartment changes in the macroscopically normal mucosa (MNM) around colorectal cancer (CRC) and adenomas (Despotović *et al.*, 2014). The microarray study described in chapter 4 (page 144) identified fibroblast growth factor 7 (FGF7) as being dysregulated in the MNM adjacent to polyps compared to MNM taken from control subjects. FGF7 is also known as keratinocyte growth factor and is a mitogen that is produced by cells of mesenchymal origin (Finch *et al.*, 1989; Rubin *et al.*, 1989). It acts upon a particular subtype of fibroblast growth factor receptor (FGFR) called FGFR2b which is usually expressed by epithelial cells (Miki *et al.*, 1991). There are 20 alternative splicing variants of FGFR2 that have been identified (Katoh, 2008). The major splicing event occurs in the carboxyl terminal half of the third Ig-like domain (D3) which results in two variants of receptor (Eswarakumar *et al.*, 2005) –

i) FGFR2IIIb which binds FGF 1, 3,7,10 and 22

ii) FGFR2IIIc which binds FGF 1,2,4,6,9,17,18.

The distribution of FGF7 and its receptor supports the proposal that it acts as a paracrine signal and plays a role in epithelial-mesenchymal transition (EMT) (Finch & Rubin, 2004). The downstream signalling pathways that are activated upon FGF7 binding to its receptor involve mitogen-activated protein kinases (MAPK) including ERK-1 and ERK-2 (Katoh & Nakagama, 2014).

FGF7 plays a role in epithelial repair in response to a toxic insult (Chen *et al.*, 2004a; Farrell *et al.*, 1998; Ulich *et al.*, 1997; Yi *et al.*, 1998) by stimulating cell proliferation, migration, differentiation, DNA repair and induction of enzymes that are involved in eradication of reactive oxygen species (Finch & Rubin, 2004). FGF7 has been shown to be a mitogen that increases epithelial cell proliferation in many different organs and therefore may augment tumour cell growth (Housley *et al.*, 1994; Nguyen *et al.*, 1996; Ulich *et al.*, 1994). It may also protect malignant cells from the toxic effects of chemotherapy by enhancing cell proliferation and increasing cell survival thus contribute to drug resistance.

In CRC, conflicting results have been observed amongst studies measuring expression levels of FGF7 and FGFR2 with some investigators proposing that expression is higher in CRC (Watanabe *et al.*, 2000) and others finding it to be no different (Otte *et al.*, 2000). Some investigators have shown that expression of FGFR2IIIb confers a well differentiated phenotype of CRC (Yoshino *et al.*, 2005; Otte *et al.*, 2000). Stronger FGFR2 expression was detected in the invasive front of CRC cells compared to the surface or central area of cancerous cells (Matsuda *et al.*, 2011). If FGFR2 expression was blocked in CRC cell lines, there was a reduction in cell migration, invasion and tumour growth highlighting the potential role of FGF7 in augmenting CRC growth.

Several other fibroblast growth factors (FGFs) have also been implicated in CRC (Brooks *et al.*, 2012), most notably, fibroblast growth factor 1 (FGF1) (Dirix *et al.*, 1996; Wang *et al.*, 2010). All four fibroblast growth factor receptors have been linked to cancer formation (Sato *et al.*, 2009; Jayson *et al.*, 1999; Spinola *et al.*, 2005; Jang *et al.*, 2001). However, recent reports have proposed that fibroblast growth factor 19 (FGF19) may also play a role in CRC formation through interaction with the Wnt signalling cascade (Pai *et al.*, 2008; Desnoyers *et al.*, 2008; Katoh, 2006; Liu *et al.*, 2013b). FGF19 belongs to the endocrine family of fibroblast growth factors (Kurosu & Kuro-O, 2009) and regulates bile acid (Holt *et al.*,

2003), glucose (Kir *et al.*, 2011; Potthoff *et al.*, 2011), lipid (Tomlinson *et al.*, 2002) and carbohydrate metabolism (Potthoff *et al.*, 2012). FGF15/19 interacts with FGFR complexed with a membrane bound protein called β-klotho (Goetz *et al.*, 2007; Lin *et al.*, 2007; Tomiyama *et al.*, 2010). If β-klotho is present, FGF19 can activate FGFR1c-3c and FGFR4 (Kurosu *et al.*, 2007), however, most of its biological activities are mediated through FGFR4 as FGFR4 knockout mice show altered bile acid metabolism (Yu *et al.*, 2000). Although FGFR4 plays a role in hepatocyte proliferation and suppression of bile acid synthesis, FGF19 is able to exert its effects on glucose and lipid metabolism independent of FGFR4 (Wu *et al.*, 2011) suggesting that there are multiple pathways through which FGF19 can act independent of the FGFR4 receptor. Several possible roles of FGF19 in driving CRC formation have been proposed in the literature (Nicholes *et al.*, 2002; Sawey *et al.*, 2011; Huang *et al.*, 2009b). Several previous proteomic studies have identified FGFR4 as a potential predictive serum biomarker of CRC (Babel *et al.*, 2009; Barderas *et al.*, 2012). More recently it has emerged that FGFR4 activity drives epithelial-mesenchymal transition in CRC (Peláez-García *et al.*, 2013; Liu *et al.*, 2013b). However, its role in field cancerisation in CRC has previously not been investigated.

6.1.2 Aims and objectives

The aims of the study were as follows -

i) To determine if gene expression levels of FGF7/FGF19 and its receptors FGFR2/FGFR4 differ between MNM adjacent to tumour, MNM adjacent to polyps and MNM taken from control subjects.

ii) To ascertain if these changes in gene expression translate into discernible differences in serum concentration

iii) To investigate which downstream pathways are affected by dysregulation of FGF signalling

6.2 Materials and Methods

6.2.1. Setting and participants

This study was approved by the Coventry and Warwick Local Research Ethics Committee (ref MREC ref no 09/H1211/38) and University Hospital Coventry & Warwickshire Research & Development division. Informed consent was gained from all participants.

Mucosal tissue samples, serum, whole blood and plasma were collected from 117 participants as outlined in section 2.2. There were 37 CRC patients, 23 patients with adenomas and 57 control subjects. The clinical and pathological details of these patients are given in table 6.1.

Table 6.1: **Clinical and pathological details of all participants in the study**. Due to the presence of synchronous lesions, 39 cancers and 32 adenomas were included in the analysis.

	Control Subjects (n=57)	Polyp patients (n=23)	Cancer patients (n=37)
Median Age (IQR)	69 (60-76)	71 (64-78)	66 (57-73)
Male: Female	31:26	15:8	20:17
Mean BMI (+/- S.D.)	26.8 (6.6)	26.3 (5.2)	26.2 (6.7)
Smokers/Ex-smokers (%)	17 (30%)	10 (43%)	10 (27%)
Diabetics (%)	8 (15%)	7 (30%)	2 (5 %)
Aspirin use (%)	11 (19%)	7 (30%)	2 (6%)
Statin use (%)	9 (16%)	5 (22%)	10 (28%)
Cancers (n=39)			
Operation		**T staging**	
Right Hemicolectomy	10	T1-T2	11
Extended R hemicolectomy	4	T3-T4	28
Anterior Resection	17	**N staging**	
Sigmoid colectomy	3	N0	22
En-bloc resection	1	N1-N2	17
Other	2	**Grade of tumour**	
		Well/Mod	26
		Poor	12
Polyps (n=32)			
Type of polyp		**Size of polyp**	
Sessile	24	< 10 mm	14
Pedunculated	7	10-20 mm	10
Villous	1	>20 mm	8
Histology of polyp		**Grade of dysplasia**	
Tubular adenoma	18	Low	29
Tubulovillous adenoma	7	High	1
Serrated adenoma	6	Hyperplastic	2
Other	1		

6.2.2. Experimental methods

Three different types of analysis were performed. Serum concentrations of proteins were measured using standard ELISA kits (see chapter 2) in 77 participants for FGF19 and 36 participants for FGF7. As FGF19 is an endocrine FGF and is secreted into the bloodstream, the aim of the study was to determine how it differs in CRC and whether it could be used to identify patients with CRC. In comparison, FGF7 is not an endocrine FGF, thus, the aim of the study was to ascertain if there were sufficient protein levels in the bloodstream for it to be detected. If the results from this initial investigation were positive, then, further samples would be analysed to determine if it could also be utilised as a diagnostic biomarker.

In a subset of patients, quantitative real time polymerase chain reaction (qRT-PCR) was used to measure changes in gene expression of FGF7/FGF19 and its receptors, FGFR2/FGFR4 respectively.

The first downstream target along the FGF7 signalling cascade is fibroblast receptor substrate 2α (FRS2α) which is phosphorylated upon binding of FGF7 to its receptor FGFR2. The other downstream targets of FRS2α involve Erk 1/2 and Akt, both of which are phosphorylated upon activation (Eswarakumar *et al.*, 2005). The protein expression levels of FRS2α, phospho-FRS2α, Erk 1/2, phospho-Erk 1/2, Akt and phospho-Akt were measured using Western blot analysis. Please see chapter 2 for detailed information regarding the methodology used.

In total, 199 samples were analysed from 17 patients with CRC, 23 patients with polyps and 34 control subjects. The control subjects were age and sex matched to the CRC patients with a 2:1 ratio. mRNA and protein expression were evaluated in the tumour tissue, MNM adjacent to tumour and MNM at the resection margin. In patients with adenomas, caecal and rectal samples were compared to MNM adjacent to the adenoma. There were no samples of the actual adenoma available for analysis as they were utilised for routine histological

analysis to exclude a malignant component. Hence, the results that are presented under the term ‘adenoma’ actually represent the expression levels detected in the MNM adjacent to the adenoma and not the adenoma itself. Right sided cancers or polyps were matched with caecal samples taken from control subjects and left sided lesions were matched with rectal samples from control subjects.

6.2.3. Statistical analysis

All qRT-PCR data was $\log_{10}$ transformed prior to statistical analysis with SPSS version 21.0. Mean expression levels between control subjects and cancer/polyp patients were compared using the unpaired t test. The paired t test was utilised to determine differences in gene or protein expression between samples taken at different sites from cancer patients. Multivariate linear regression was used to ascertain how different variables relate to serum FGF19 concentration. Statistical significance was based on $p<0.05$.

6.3. Results

6.3.1. FGF7 is upregulated in the colonic field

FGF7 was significantly upregulated in the tumour tissue itself ($^{\Delta\Delta}C_T$ 21.4), its adjacent MNM ($^{\Delta\Delta}C_T$ 20.9) and the resection margin ($^{\Delta\Delta}C_T$ 21.1) in patients with cancer compared with control subjects ($^{\Delta\Delta}C_T$ 22.6) (see figure 6.3.1). In comparison, there was significant down regulation of its receptor, FGFR2 in the tumour tissue itself ($^{\Delta\Delta}C_T$ 21.2 in tumour versus 19.4 in controls, p <0.001). However, FGFR2 gene expression in the adjacent MNM ($^{\Delta\Delta}C_T$ 19.6) and resection margin ($^{\Delta\Delta}C_T$ 19.4) of CRC patients was no different from that found in control subjects.

With paired analysis of samples taken from CRC patients, FGF7 and FGFR2 were both downregulated in the tumour tissue compared to the resection margin (p=0.036 for FGF7 and p=0.013 for FGFR2).

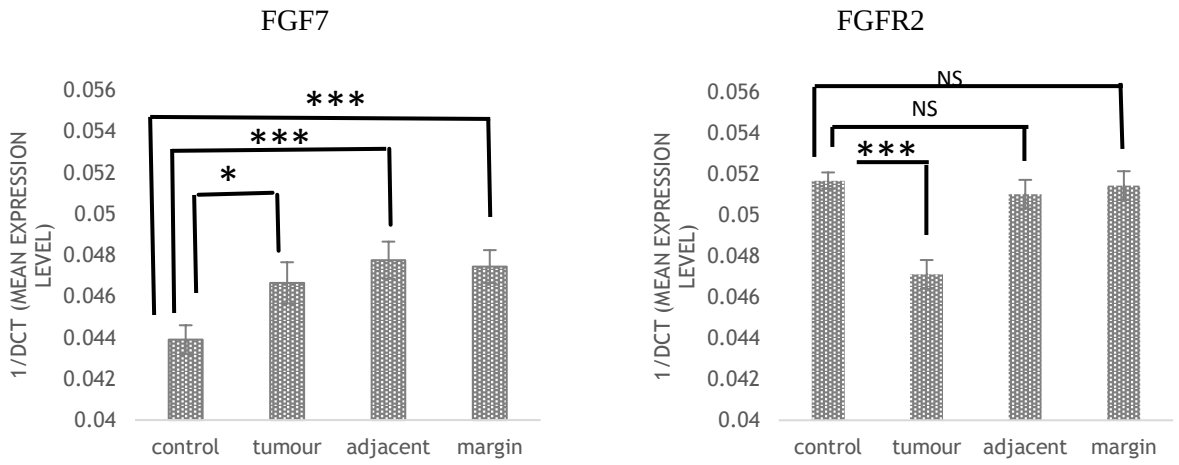


Figure 6.1: **Mean gene expression level of FGF7 and FGFR2** in caecal or rectal samples taken from control subjects compared to samples taken from tumour tissue, adjacent MNM and resection margin of colectomy specimen from CRC patients. Statistical analysis using unpaired t test - P value < 0.05=*, <0.01= ** and <0.001= *** and NS=non-significant.

6.3.2. The FGF7-FGFR2 signalling axis is dysregulated in tumour tissue

FGF7 gene expression was negatively correlated with FGFR2 gene expression in samples taken from control subjects. However, this relationship was lost in tumour tissue and no correlation was detected between expression levels of the genes.

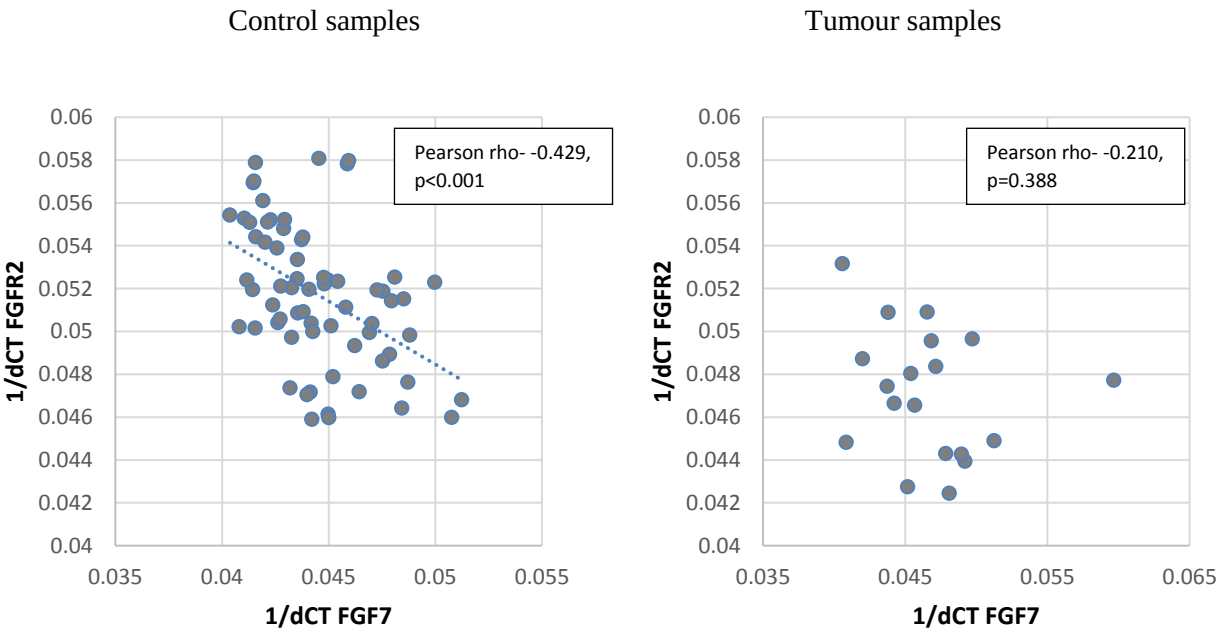


Figure 6.2: **FGF7 and FGFR2 correlation**. Mean gene expression level of FGF7 and FGFR2 plotted against each other for control samples (n=71) and tumour samples (n=19). Pearson correlation coefficient and p value are displayed.

6.3.3. FGF7 and FGFR2 gene expression are no different in patients with polyps

FGF7 gene expression in the right colon was no different to the left colon, both in control subjects and in patients with polyps. However, there was a significant difference in FGFR2 gene expression between right and left colon ($^{\Delta\Delta}C_T$ 16.2 in right colon versus $^{\Delta\Delta}C_T$ 17.4 in left colon, p=0.009).

There was no difference in gene expression of FGF7 adjacent to the polyp ($^{\Delta\Delta}C_T$ 21.0) compared to gene expression from samples taken from control subjects ($^{\Delta\Delta}C_T$ 21.4) (p=0.386) (see figure 6.3.). Similarly, there was no difference in FGFR2 gene expression between the two groups (p=0.216).

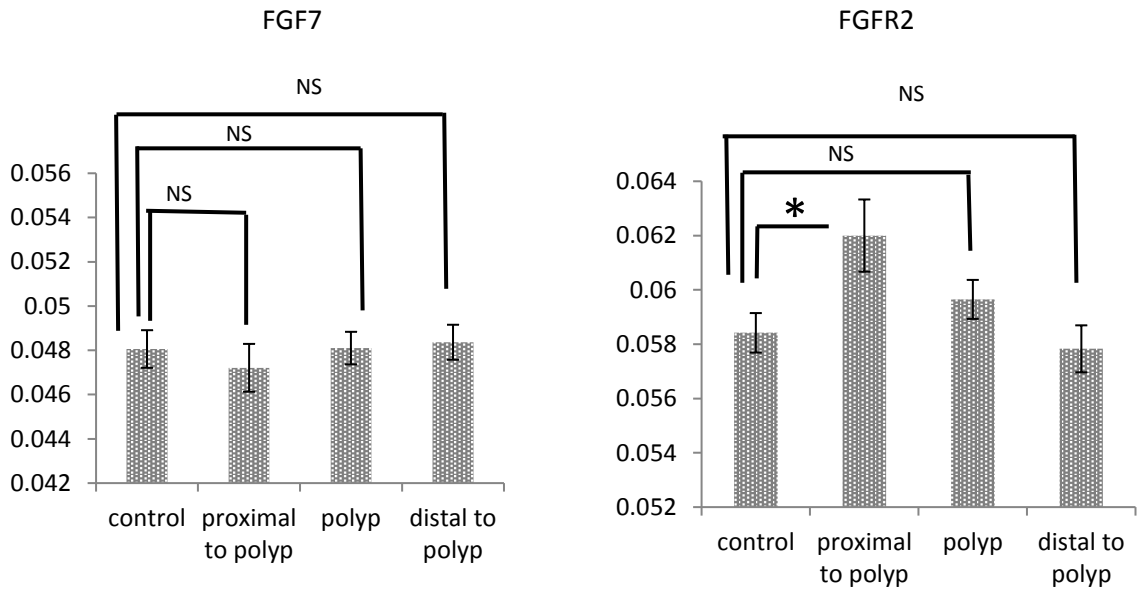


Figure 6.3: **FGF7 and FGFR2 gene expression in patients with polyps**. Mean gene expression level of FGF7 and FGFR2 in biopsies taken proximal, adjacent and distal to polyp compared to matched samples taken from control subjects. Statistical analysis using unpaired t test - P value < 0.05=*, <0.01= ** and <0.001= *** and NS=non-significant.

6.3.4. The FGF7-FGFR2 signalling axis is dampened in CRC tissue

Protein expression of both FRS2 and phospho-FRS2 was reduced in the tumour tissue compared to the mucosa at the margin suggesting that the FGF7 signalling cascade was dampened in the tumour tissue itself. This was accompanied by a reduction in Akt signalling but no changes were observed in Erk 1/2 protein expression.

There was no significant difference in protein expression between the adjacent mucosa and the resection margin.

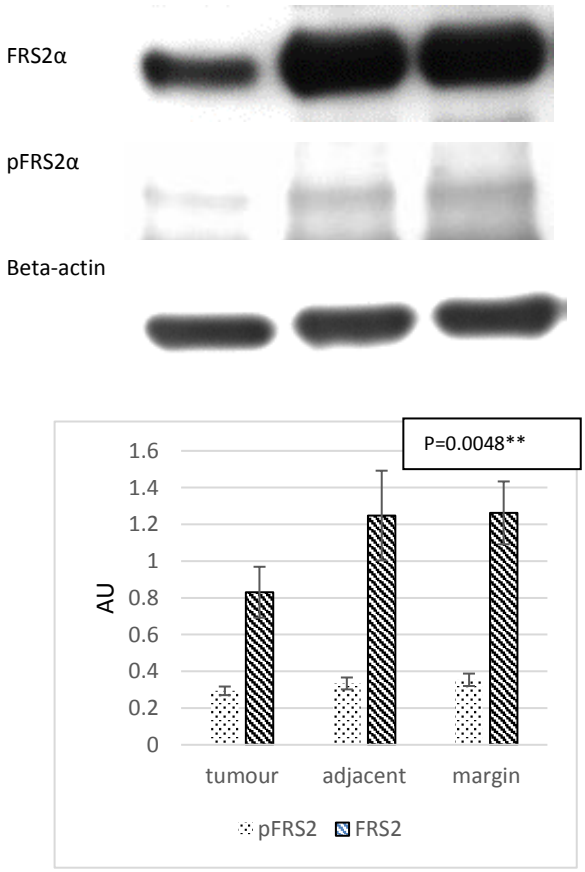


Figure 6.4: **Protein expression of FRS2α and pFRS2α** measured using Western blot analysis (n=6). Paired t test used to determine statistical difference between expression at tumour site and adjacent to tumour compared to expression at resection margin. The ratio of pFRS2/FRS2 comparing tumour and resection margin is displayed.

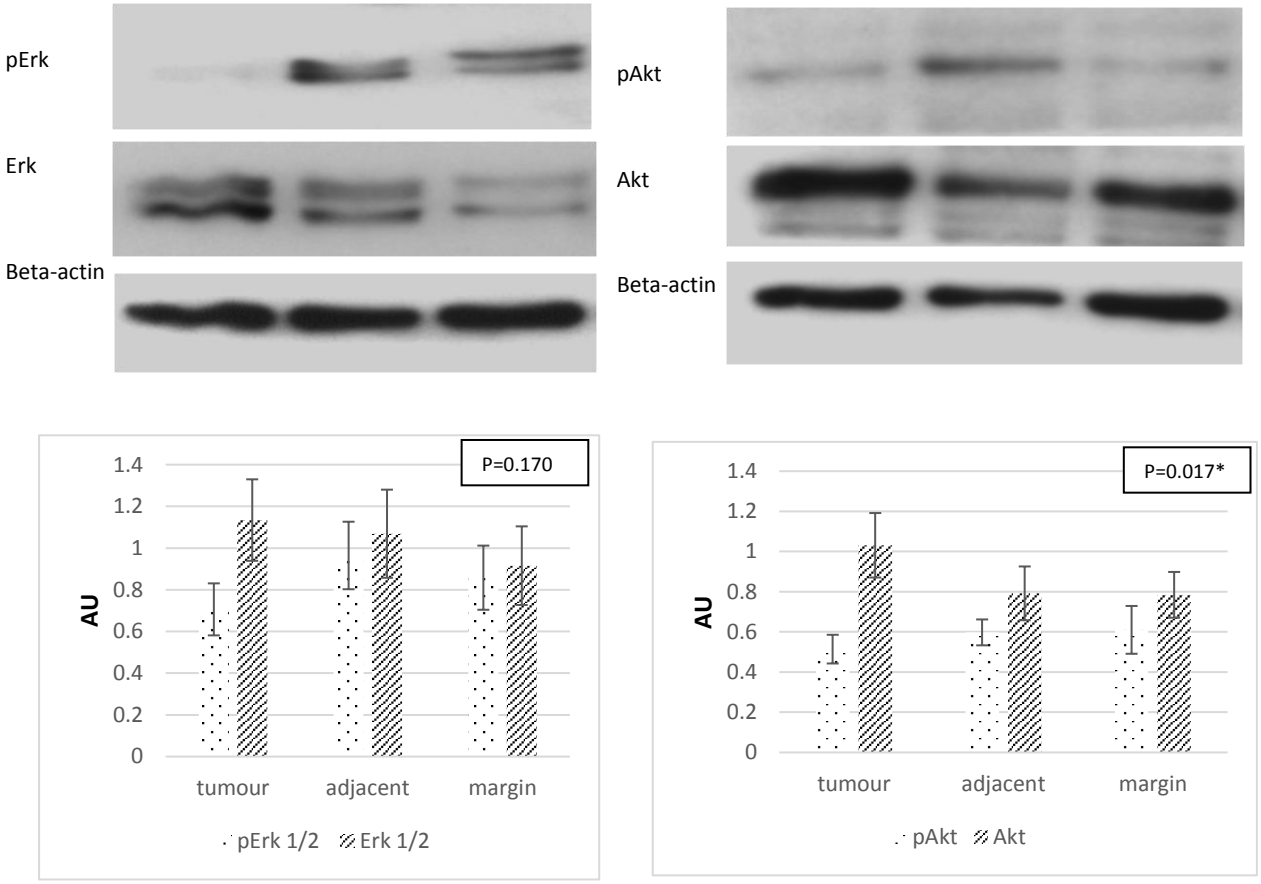


Figure 6.5: **Protein expression of phospho-Erk (pErk), Erk, phospho-Akt (pAkt) and Akt** measured using Western blot analysis (n=6). Paired t test used to determine statistical difference between expression at tumour site and adjacent to tumour compared to expression at resection margin. The ratio of the phosphorylated form to unphosphorylated form was used in the analysis. The p value comparing expression at the tumour site compared to the resection margin is shown.

6.3.5. FGF7 serum concentration does not differ between cancer and control

No significant differences in FGF7 serum concentration were found between patients with CRC compared to control subjects (mean concentration 33.16 (+/- 5.67) pg/mL versus 33.81 (+/- 5.43) pg/mL respectively, p= 0.72).

6.3.6. FGF19 is expressed at low levels in colonic tissue

Based on the results obtained with qRT-PCR, FGF19 was only expressed in 9/17 tumours at a very low level (CT 30-38 cycles). Analysis of samples taken from control subjects did not give reliable values for expression level across triplicates. Therefore, tissue samples taken from control subjects were not analysed further for FGF19 gene expression due to limited biopsy material available. However, FGFR4 was detected across all samples that were examined. There were no differences in gene expression of FGFR4 in cancer tissue itself ($^{\Delta\Delta}C_T$ 20.3), its adjacent MNM ($^{\Delta\Delta}C_T$ 20.6) and the tissue at the resection margin ($^{\Delta\Delta}C_T$ 20.5) compared to the expression levels in healthy colonic mucosa ($^{\Delta\Delta}C_T$ 20.8) (p=0.175 for cancer versus control). When patients with cancer were divided into two groups based on those whose tumours expressed FGF19 (denoted FGF19 positive) and those that did not (denoted FGF19 negative), there was no significant difference in expression of the receptor, FGFR4 (p=0.222).

6.3.7. FGF19 serum levels are lower in CRC patients with right sided tumours

Amongst the 77 patients whose serum FGF19 concentration was measured, there was no significant difference between cancer patients and healthy controls (mean concentration 171.67 +/- 121.21 pg/mL versus 202.28+/- 113.46 pg/mL respectively, p=0.256). The only clinical parameter with which there was an association with serum FGF19 concentration was smoking (see table 6.3.7). There were 14 patients with right sided tumours and 25 patients with left sided tumours. The mean serum FGF19 concentration was significantly lower in patients with right sided tumours compared to left sided tumours (124.64+/-62.25 pg/mL versus 198.0+/-138.38 pg/mL respectively, p=0.029).

There was no correlation between serum FGF19 concentration and gene expression of FGFR4 in the colonic tissue (Pearson's correlation coefficient, r=0.053, p=0.841). However,

if the patients with synchronous cancers are excluded as they appear to have one tumour which expresses FGF19 and another that does not, serum FGF19 levels are lower in patients whose tumours express FGF19 compared to those whose tumours do not (p=0.041).

Table 6.2: **Serum FGF19 concentration and correlation with clinical factors**. Relationship between clinical variables and serum FGF19 concentration determined using Pearson correlation in patients with cancer (n=37) and control subjects (n=57)

Clinical variable	Pearson's correlation coefficient	p value
Age	-0.172	0.134
Sex	-0.034	0.767
BMI	0.040	0.730
Smoker	0.304	0.007*
Fasting sample	-0.155	0.178
T stage	-0.214	0.191
N stage	-0.262	0.107
Grade of tumour	-0.068	0.683

6.4. Discussion

Using a novel sampling strategy, this study has shown that FGF7 is significantly upregulated along the colon in patients with CRC and could potentially act as a biomarker of field cancerisation. This is accompanied by disruption of the FGF7-FGFR2 signalling axis in the tumour tissue itself with reduced protein expression of some of its downstream targets. This suggests that loss of FGF7 activity may contribute to malignant transformation of the colonic mucosa. However, contrary to previous reports, there were no differences in gene expression of FGF19 or its receptor, FGFR4.

FGF7 signalling was found to be disrupted in CRC patients with uncoupling of the negative reciprocal relationship seen between a ligand and its receptor. Under normal physiological conditions, when FGFRs are activated they undergo endocytosis which reduces the number of receptors found in the membrane. This negative feedback loop is important in regulation of FGFR activity and has recently emerged as a potential source of disruption in growth factor activity and may contribute to cancer (Haugsten *et al.*, 2005; Mosesson *et al.*, 2008; Haglund *et al.*, 2007; Abella & Park, 2009). The authors of a recent study found that Src and its phosphorylation target, Eps8 facilitate clathrin mediated endocytosis of FGFRs (Auciello *et al.*, 2013). FGFs increased the number of clathrin coated pits and events of clathrin mediated endocytosis suggesting that an increase in FGF7 expression would be expected to lead to downregulation of its receptor. Depletion of Eps8 inhibited FGFR trafficking with FGFR being retained in peripheral early endosomes and led to a reduction in burst of Erk 1/2 activation suggesting that Eps8 is important for both FGFR trafficking and signalling. The uncoupling of FGF7 and FGFR2 gene expression found in this study could be explained by alterations in FGFR trafficking.

Previous studies have shown that FGF7 plays a role in the epithelial response to toxic injury. IL-7 is produced by intraepithelial lymphocytes in response to a toxic insult which acts upon the stromal cells to induce FGF7 release which drives epithelial cell proliferation. FGF7 treatment of intestinal cells led to increased expression of epithelial cell derived IL-7 in vitro and in a mouse model (Cai *et al.,* 2012). In a subsequent study, they showed that FGF7 also increased Lovo cell proliferation and increased intestinal wet weight, villus height, crypt depth and crypt cell proliferation in mice. With blockade of two further downstream targets of FGF7, IRF-1 and IRF-2 there was a reciprocal reduction in IL-7 expression suggesting a feedback loop where IL-7 induces epithelial cell growth through FGF7 (Cai *et al.*, 2013).

The reduction in protein expression of the downstream targets of FGF7 that was observed in this study could be explained by postulating that FGF7 protects against toxic injury and regulates cell differentiation. Loss of the FGF7 signal would thus disable the epithelium from responding appropriately to toxic/ inflammatory injury and could contribute to uncontrolled growth and tumour formation. A previous study found higher FGFR2 expression in well differentiated tumours and those with shallow wall invasion (Yoshino *et al.*, 2005) compared to poorly differentiated tumours suggesting that the FGFR2 signal is lost during malignant transformation into a more aggressive phenotype. Loss of FGFR2b expression has also been found in other tumours including cancer of the prostate, bladder and salivary gland (Diez de Medina *et al.*, 1997; Naimi *et al.,* 2002; Amann *et al.*, 2010; Zhang *et al.,* 2001). Mutations where there is inactivation of the FGF7-FGFR2 axis have also been reported in melanomas (Gartside *et al.,* 2009). Thus, it is plausible that in certain tumours, FGF7 protects against malignant transformation by contributing to cell differentiation and a less aggressive phenotype.

In a further study, FGF7 was found to be a strong mitogen only for normal epithelial cells and not tumour cells (Otte *et al.*, 2000). Similarly, another study reported that recombinant FGF7 was only able to stimulate growth of primary, immortalised keratinocytes and not malignant head and neck squamous cancer cells (Hille *et al.*, 2010). A neutralising antibody against FGF7 only abolished cell proliferation of the keratinocytes and again, had no effect on the malignant cells. FGFR2b was only expressed in the keratinocytes and not the malignant cells. Thus, they postulated that FGF7 acts in a paracrine manner in normal cells, however, it is unable to stimulate cell growth in malignant cells. Similar findings were observed in the present study whereby there was loss of FGFR2 expression in the tumour cells despite high expression of FGF7. This was accompanied by a reduction in Akt protein expression and not Erk 1/2 expression. In a study using pancreatic duct cells, FGF7 stimulated proliferation through MEK-Erk 1/2 pathway and cell differentiation through the Akt pathway (Uzan *et al.*, 2009). Similarly, the Akt activity has been shown to be important in driving cell differentiation in embryonal carcinoma cells (Chen *et al.*, 2013a). Hence, loss of Akt expression found in the tumour tissue in the present study could suggest that the ability of FGF7 to regulate cell differentiation via Akt has been lost whilst its ability to stimulate cell proliferation via Erk may have been retained. This may seem to contradict previous reports which have associated Akt activation with more aggressive forms of cancer (Agarwal *et al.*, 2013) highlighting that Akt actually supports cell proliferation and tumour growth. This dual role of Akt can be explained by different effects depending upon the subcellular location of PI3K, its upstream activator. In sub confluent proliferating cells, the p85 regulatory subunit of PI3K was distributed in the cytoplasm whereas in confluent cells, it was found at cell-cell interfaces (Laprise *et al.*, 2002). E-cadherin mediated cell-cell contact led to increased Akt activity and suppressed Erk activity. If PI3K was blocked, AKT activity was abolished and a sustained activation of Erk was observed in confluent differentiating cells but not in undifferentiated proliferating cells (Laprise *et al.*, 2004). The authors proposed that PI3K-Akt activity supports proliferation in sub confluent dividing cells and once confluence is reached, it drives cell differentiation. Thus, PI3K-Akt plays an

important role in mediating the transition between proliferation and differentiation. It is therefore plausible that loss of its activity could render the cell in a permanent state of proliferation and prevent its ability to undergo differentiation contributing to malignant transformation.

There have been reports of increased expression of FGFR2 in CRC that contradicts our findings. In one study, stronger FGFR2 expression was observed in the invasive front of CRC cells compared to the surface/central area of cancerous cells (Matsuda *et al.*, 2011). Reduced expression of FGFR2 in CRC cells was met by a reduction in cell migration, invasion and tumour growth in vitro and in vivo. FGFR2 may also play a role in tumour angiogenesis by increasing VEGF-A production (Narita *et al.,* 2009). Thus, it remains unclear whether FGF7 acts to regulate angiogenesis and in so doing augment tumour growth or restore regulated cell growth following inflammatory injury.

Under normal physiological conditions, FGF7 has also been implicated in epithelial-mesenchymal transition therefore defective cell signalling through this pathway could lead to altered cell-cell adhesion (Kudo *et al.*, 2007). Using a FGF7 transfected CRC cell line, the authors demonstrated a higher degree of adhesion to ECMs (type IV collagen, fibronectin), enhanced ERK1/2 phosphorylation and increased focal adhesion kinase (FAK) expression. Therefore, FGFR2 could play a role in preventing malignant seeding by enabling CRC cells to adhere to underlying collagen. This may explain its association with less aggressive, well differentiated tumours that has previously been observed. However, in this study, there was no correlation between the histological differentiation grade of the tumour and FGF7 gene expression thus suggesting that the role of FGFR2 in preventing metastasis may be less likely.

Previous reports have demonstrated that FGF19 is overexpressed in human colon cancer tissue and in colon cancer cell lines (Desnoyers *et al.*, 2008). When FGF19 activity is blocked with a neutralising antibody, the mouse tumour xenografts created using these cells are significantly smaller than those using wild type cells suggesting that FGF19 augments tumour growth. However, FGF19 was only expressed in 6/10 tumours tested suggesting that in some tumours, it did not drive carcinogenesis. This is similar to the present study which found that FGF19 was expressed in 9/17 tumours. As normal colonic tissue samples did not express FGF19, detection of FGF19 in the tumour tissue itself suggests that it was upregulated and therefore may contribute to malignant transformation. Similarly, no differences in FGFR4 gene expression in the tumour, adjacent MNM or resection margin were detected. This contradicts previous reports which have observed that FGFR4 is upregulated in CRC (Heinzle *et al.*, 2012) and many other cancers including breast (Jaakkola *et al.*, 1993), prostate (Sahadevan *et al.*, 2007), gastric, rhabdomyosarcoma (Taylor *et al.*, 2009) and pituitary adenocarcinoma (Ezzat *et al.*, 2002). However, FGFR4 was only upregulated in 20/71 cancers tested in the former study suggesting that in many of the colorectal tumours examined or investigated, FGFR4 was not upregulated. Similarly, in this study, FGFR4 was also only upregulated in a proportion of the tumours examined suggesting that it may not be a very good candidate for a clinical biomarker. This was further confirmed with no differences being detected in serum FGF19 between cancer patients and control subjects. Therefore, based on the findings of the present study, FGF19 does not appear to be a candidate for a clinical CRC biomarker.

There are some limitations to this study that require further consideration. The specific isoform of the FGFR2 receptor with which FGF7 interacts was not measured in the qRT-PCR experiments. Recent evidence has emerged to support the concept that there may be a class switch from FGFR2IIIb isoform to FGFR2IIIc isoform in CRC which confers a more malignant phenotype and contributes to the neoplastic process (Matsuda *et al.*, 2012). This

highlights that it may not be the total expression level of the FGFR2 gene that is important but the relative expression levels of its different isoforms. Despite this limitation, however, there were significant differences observed in gene expression of FGFR2 supporting its role in CRC.

6.5. Summary and conclusions

- Using a novel sampling strategy in a well characterised cohort of patients, FGF7 was found to be upregulated throughout the resection specimen in cancer patients compared to healthy controls.
- Dysregulation of the FGF7/FGFR2 signalling cascade was only observed in tumour tissue and not in the adjacent MNM suggesting that either decreased FGF7 activity contributes to malignant growth or the transformed cells found in tumours contain mutations that inactivate this pathway.
- Reduced Akt signalling downstream of FGF7 suggests loss of cell differentiation with preserved Erk signalling which is usually responsible for cell proliferation
- FGF7 could play a role in field cancerisation and help to elucidate in whom risk of neoplasia is greatest.
- Pharmacotherapy aimed at restoring the FGF7 signal could be utilised in cancer patients to restore regulation of cell differentiation and antagonise malignant growth.

Section 7: Discussion

7.1. Introduction

Colorectal cancer (CRC) remains a source of considerable health burden for most communities worldwide. Despite substantial advances in our understanding of the molecular biology that underpins CRC formation, patients are still diagnosed at a late stage with wide heterogeneity in response to available chemotherapeutic agents (Ferlay *et al.*, 2013; Coleman *et al.*, 2011). The field cancerisation concept (SLAUGHTER *et al.*, 1953) offers an alternative approach to solving some of the clinical challenges that remain in the modern treatment of CRC. Improvements in our knowledge about the changes that occur in the colonic field prior to development of histological abnormalities may aid earlier diagnosis of CRC and could pave the way to unravelling some of the complexities that underlie this disease. Better risk stratification of both screened and symptomatic patients can help to develop more accurate screening and diagnostic tests. Assessment of risk in this manner may also aid design of surveillance schedules for those already deemed at high risk, that is, those with a genetic predisposition or CRC survivors. Furthermore, changes in the colonic field could serve as a surrogate endpoint for chemoprevention trials where CRC risk is modulated by various pharmacological interventions. With such wide application, it is important that the field cancerisation concept in CRC is investigated further.

7.2. Field cancerisation in CRC – parallels between IBD and sporadic CRC

Field effects have readily been accepted in CRC associated with inflammatory bowel disease (IBD-CRC) (Leedham *et al.*, 2009; Risques *et al.*, 2011; Katsurano *et al.*, 2012), the reason being that inflammation is believed to be the primary cause for the increased predisposition to malignant transformation. In IBD-CRC, the presence of DALMs (dysplasia associated lesion or mass) has been associated with malignant risk supporting this hypothesis (Bernstein *et al.*, 1994; Thomas *et al.*, 2007). Studies using animal models corroborate this observation by demonstrating molecular changes in the mucosa at distant sites to tumour formation (Katsurano *et al.*, 2012). In humans, mutations in KRAS, CDKN2A (p16) and

TP53 were observed in non-tumour, non-dysplastic and dysplastic epithelium of patients with Crohn's disease (Galandiuk *et al.*, 2012). However, field cancerisation in sporadic CRC is generally not as well accepted. The difference highlighted by investigators is that sporadic CRCs occur in otherwise biologically normal colon. As there is no histological abnormality in the mucosa, the assumption is made that this mucosa is 'healthy' and not biologically altered. However, in a similar manner to IBD, pre-neoplastic lesions such as aberrant crypt foci and adenomas are detected at multiple sites along the colon in patients who go onto develop CRC (Morson, 1974; Bird, 1987). The findings presented in the first chapter of this thesis demonstrated that there is a higher risk of developing metachronous lesions in the residual bowel after cancer surgery if synchronous adenomas are present at time of presentation. This implies that the colonic mucosa at sites distant to the tumour is also preconditioned to tumour formation and this predilection does not disappear after the primary tumour is removed. This has two implications; firstly, it supports the concept of field cancerisation in CRC and secondly, it suggests that field changes in the colonic mucosa are not a consequence of the presence of tumour itself and persist after curative resection implying that they predate tumour development. Earlier antagonists of field cancerisation had proposed that it was the presence of tumour which was inducing biological changes in the surrounding mucosa (Kuniyasu *et al.*, 2000). This contention is not supported by the findings in the present study. It also suggests that a proportion of CRC patients may not exhibit a field defect along their colon or that polyps are poor markers of field cancerisation and different molecular changes should be sought.

7.3. Existing scientific evidence to support field cancerisation in CRC

There have been several reports that have demonstrated cellular, genetic and epigenetic differences in the normal colonic mucosa at distant sites to the tumour supporting the field cancerisation concept in CRC. Earlier studies that were conducted provided evidence that the colonic field distant to the tumour exhibited differences in cell structure with altered

karyometry (Bibbo *et al.*, 1990; Verhest *et al.*, 1990; Alberts *et al.*, 2007; Cherkezyan *et al.*, 2014), changes in crypt morphology (Dawson & Filipe, 1976; Riddell & Levin, 1977) and altered cell kinetics (Terpstra *et al.*, 1987; Anti *et al.*, 2001; Badvie *et al.*, 2006; Hanna-Morris *et al.*, 2009). Subsequent studies have sought to clarify how the genetic and protein milieu of the cells in the colonic field around cancer differ from those found at distant sites (Chen *et al.*, 2004b; Hao *et al.*, 2005; Polley *et al.*, 2006). Most of the genetic alterations proposed have been in DNA repair and methylation (Facista *et al.*, 2012; Nguyen *et al.*, 2010; Shen *et al.*, 2005; Ramírez *et al.*, 2008). A recent study measured transcript expression and chromosomal changes along the colon (Hawthorn *et al.*, 2014). The authors showed that although there were chromosomal abnormalities in the colonic field, these were much smaller in number than those observed in the tumour and were not consistently seen at distant sites from the tumour. There was variation in the abnormalities detected at 3,5 and 10 cm from the tumour in the same individual. The authors proposed that the findings supported field cancerisation and could be explained by the mutator phenotype model for cancer whereby cells within a tumour are heterogeneous and do not always share the same mutations. Others have shown that that epigenetic makeup of cells in the colonic field mirrors the epigenetic changes that have occurred in the tumour itself (Worthley *et al.*, 2010) suggesting that the tumour may have arisen within a field of biologically altered cells.

7.4. A candidate free approach to identify novel genes

The aim of this thesis was to investigate the concept of field cancerisation in CRC to determine which cellular processes and genes are dysregulated in the colonic field around cancer and adenomas. Although there have been reports identifying different epigenetic and genetic changes that could play a role in field cancerisation, there have been no previous studies which have taken a candidate free approach. It was felt that this would be the most appropriate way to identify novel genes or signalling pathways that had not previously been described. It would also enable a more general interrogation of the cellular machinery that

becomes altered in field cancerisation. Microarray technology was utilised to identify differences in the global genomic expression profile of macroscopically normal mucosa (MNM) taken from patients with cancer or polyps compared with control subjects. This identified a number of different biological processes that were dysregulated including immune response, metabolism and epithelial-mesenchymal transition. Interestingly, genes that would be conventionally considered to contribute to tumourigenesis by regulating cell proliferation or apoptosis were not identified. Amongst the genes that were further validated, CXCL2 and FGF7 were both found to be significantly upregulated in the adjacent MNM to cancer compared to the MNM taken from control subjects. In contrast to previous studies which have shown that these field changes are limited to the immediate vicinity of the tumour (Shen *et al.*, 2005; Facista *et al.*, 2012), both these genes were found to be significantly upregulated at the resection margin compared to control subjects. This highlights that field changes can occur at considerable distances along the colon from the primary tumour indicating a wide field of biological change. With the use of protein expression studies, FGF7 was upregulated along the colon, its receptor, FGFR2 was locally downregulated at only the tumour site. This implied that disruption of this signalling axis could contribute to malignant transformation and in response, FGF7 could be upregulated along the colon. Protein expression studies confirmed down regulation of downstream targets, pFRS2α and pAkt only in the tumour and not at the resection margin site nor in the adjacent MNM. Based on these findings, FGF7 could play a role in contributing to CRC formation. Moreover, its widespread upregulation only in the colonic mucosa of cancer patients supports a potential role in field cancerisation.

7.5. Implications of the findings of this thesis

The findings of this thesis contribute to the existing body of scientific literature that proposes field cancerisation in CRC. It highlights that there are multiple biological processes that are dysregulated in the colonic field around a cancer or polyp. Although no field effect

around a polyp could be demonstrated with FGF7, the difference in CXCL2 expression found adjacent to polyp was intermediate to that observed in the MNM adjacent to the tumour and MNM in control subjects. This implies that there may be a field of altered mucosa around polyps, however, the differences in expression are small suggesting that it is closer to the mucosa in control subjects than the MNM found in cancer patients. Previous studies which have investigated field cancerisation around polyps have also yielded conflicting conclusions. In one study, an upward shift in the crypts' replicative compartment of rectal mucosa in patients with adenomas heralded a higher risk of adenoma recurrence (Anti *et al.*, 1993). However, in a subsequent study (Anti *et al.*, 2001), no global changes in cell proliferation and apoptosis were observed in individuals with adenomas compared to those without. However, more recent studies where optical spectroscopy and light scattering technology have been used to evaluate changes in microvasculature and cell ultrastructure have highlighted important biological changes in the colonic field of patients with polyps. In a study with 222 subjects, an endoscopically compatible polarisation gated spectroscopic probe was used to measure changes in microvasculature around polyps. A gradient in the measure of blood supply was observed up to 30 cm from the polyp suggesting that there was a local increase around the polyp to support adenoma growth (Roy *et al.*, 2008). In a subsequent study, however, this measure of microvasculature was found in be increased by 50 % in the rectal mucosa of patients with adenomas compared to those without irrespective of the adenoma site (Gomes *et al.*, 2009). The authors proposed that rectal measurement of microvasculature could be utilised to screen for patients with advanced adenomas without having to perform complete colonic investigation. Findings from this thesis, however, would suggest that the changes that take place in the field around polyps (as measured by CXCL2) are a local phenomenon and are not found at distant sites along the colon. In contrast, patients with CRC show evidence of upregulated FGF7 and CXCL2 at distant sites. Any clinical application based on these findings could enable discrimination between cancer and polyp patients using the gene expression of either FGF7 or CXCL2 taken from the distant rectal mucosa.

Studies utilising light scattering technology have shown that there are ultrastructural changes in the cells around polyps. The maximal difference between subjects with and without adenoma was observed at 100 μm which correlates with changes in epithelial cells or the extracellular matrix of the lamina propria (Radosevich *et al.*, 2011; Radosevich *et al.*, 2012).. In the fourth chapter of this thesis, a large quantity of the genes identified to be differentially expressed in the adjacent MNM around polyps were involved in the extracellular matrix and lamina propria supporting the findings of these earlier reports.

The main biological processes that were identified to be dysregulated in the colonic field around CRC were concerned with supporting epithelial cell growth rather than being responsible for driving it. Similarly, FGF7 facilitates epithelial proliferation and CXCL2 attracts neutrophils which are pro-tumorigenic and angiogenic. Both of these genes represent changes in stromal elements that interact with epithelial cells. Hence, changes in the stroma maybe required earlier on in the carcinogenesis process than previously thought. Changes may occur in the stroma in preparation for malignant growth with mutations arising in conventional oncogenes and tumour suppressor genes later on. At present, most investigators propose that changes in vasculature and metabolism occur after tumour formation has been initiated to support increased cell proliferation (Hanahan & Folkman, 1996). Based on the findings from this thesis, however, these supportive growth signals may actually be important much earlier along the cancer pathway as they were detected in the colonic field which harbours no evidence of tumour formation macroscopically.

7.6. Limitations

The findings from this thesis demonstrate that field cancerisation exists around CRC and can be identified using FGF7 and CXCL2 at considerable distances from the tumour along the resection specimen. However, there are some limitations that require further consideration.

Firstly, no causal relationship between the observed changes in gene expression and subsequent tumour formation has been investigated. Previous studies utilising animal models have shown that there are changes in the histologically normal mucosa that predates the development of aberrant crypt foci (ACF) (Roy *et al.*, 2004). Changes in light scattering parameters of MNM were found 2 weeks prior to the development of ACF with changes correlating both spatially and temporally with sites of subsequent development of ACF. Investigation to prove a temporal, causal relationship in humans with sporadic CRC is difficult to achieve, however, it has been described in a study on IBD-CRC. Mutations in KRAS, TP53 and CDKN2A were observed in the histologically normal mucosa of patients with Crohn's disease up to 4 years before development of cancer suggesting that they predated the formation of malignancy (Galandiuk *et al.*, 2012).

Secondly, the focus has been on genomic profiling, however, it is important to realise that post translational modification of proteins will play a significant role in governing which signalling cascade is active in the cell. The studies on FGF7-FGFR2 interaction with protein analysis were therefore helpful in elucidating how this signalling axis is disrupted in CRC. Further investigation of CXCL2 would require similar evaluation with protein analysis.

The other aspect of gene modulation that should be considered is plasticity whereby a single genotype can result in different phenotypes depending upon the local environment (Nieto, 2013). This is particularly important in cancer where the cells acquire the ability to survive in hostile conditions and travel to distant organs, adopting a different phenotype to normal cells.

7.7. Future directions

The findings in this thesis have provided the foundation for future work into field cancerisation in CRC by highlighting two potential signalling pathways that could play a role in contributing to a field defect. FGF7 shows promise as a marker of field cancerisation

whose function appears to be important for regulation of cell differentiation. Thus, loss of its signal activity results in malignant transformation. CXCL2 is very significantly upregulated in tumour tissue but also upregulated in the adjacent MNM around both cancer and polyp. This implies that its expression could become altered much earlier on in the carcinogenesis process and thus, it may translate into an earlier biomarker of cancer.

Whilst this thesis has been effective at highlighting the biological changes that occur in the colonic field and provides a novel way of interrogating genes implicated in CRC carcinogenesis, it does not elucidate the clinical implications of these findings. A prospective observational clinical study in which the tissue expression levels of these genes are correlated with clinical findings would help to characterise the clinical importance of a field defect. The mRNA expression level determined using a rectal biopsy could be utilised to cluster individuals with and without CRC. With longer follow up, the expression levels could be correlated with future neoplastic risk in terms of development of adenomas or cancer thereby elucidating if those with a field defect are at higher risk of recurrence. A further study assessing field changes before and after surgery may help to discriminate if these field changes are dependent upon tumour presence and could help identify its role as a prognostic indicator. The relationship between colonic field changes and presence of polyps is particularly interesting in this regard as polyps, at present, are the only means of risk stratification of CRC risk in symptomatic individuals at present.

7.8. Final conclusion

Since the molecular characterisation of the adenoma-carcinoma sequence, there have been considerable advances in our understanding of how CRC forms. However, there has been little translation into effective diagnostic and therapeutic modalities. Researchers need to address the cancer process in a different manner if we are to be successful at halting the process. Field cancerisation offers promise of providing clues into the origins of CRC by

providing a means of investigating what happened before the onset of carcinogenesis. Future investigation will not only need to demonstrate that field changes exist but provide ways in which to link these changes in a causative manner to the cancer process. Several studies have successfully shown that CRC is a heterogeneous cancer with several different pathways and different phenotypes. Instead of addressing the very many different types of CRC, a unified approach where the cancer is interrogated as a single disease may yield more positive results for development of screening tests. The cellular and structural changes that underpin cancer formation in several organs are similar in nature, hence, field cancerisation offers such a united approach. Based on the field cancerisation concept, it would be possible to discriminate between cancer patients and control subjects using distal rectal biopsies obtained through a limited endoscopic examination. Such biopsies could be utilised to risk stratify individuals with CRC or familial risk to inform surveillance schedules. If these genes form the targets for pharmacological therapy, field cancerisation offers the opportunity to not only identify high risk individuals but potentially enable modification of their neoplastic risk prior to the development of any histological abnormality. If used in this clinical setting, it would be possible to achieve prevention of CRC formation before any macroscopic changes occur in the mucosa.

This thesis has demonstrated alterations in several biological processes in the colonic field around CRC which support tumour formation providing evidence for the field cancerisation concept in CRC. Growth signals (FGF7) and a pro-inflammatory environment (CXCL2) appear to contribute to field cancerisation rather than alteration in known oncogenes and tumour suppressors highlighting the importance of the stromal compartment in supporting tumour growth. Future studies will need to establish a mechanistic relationship with in vitro cell culture models and investigate the clinical implications of this field defect in longitudinal observational cohort studies.

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Appendix I: DAVID analysis

Table showing pathways that were found to be enriched using DAVID software.

Cancer versus control

LIMMA			Robust Regression		
Function	**Enrichment ratio**	**Number of genes**	**Function**	**Enrichment ratio**	**Number of genes**
Transmembrane signalling	2.05	29	No enriched functions		
Glycosylation	1.34	23			

Polyp versus control

LIMMA			Robust Regression		
Function	**Enrichment ratio**	**Number of genes**	**Function**	**Enrichment ratio**	**Number of genes**
Extracellular signalling	4.27	16	Extracellular signalling	3.95	26
Glycosylation	1.88	7	Coagulation	2.46	3
			Eye morphogenesis	1.68	4
			Signalling	1.47	6
			Cytoskeleton	1.38	5

Cancer versus polyp

LIMMA			Robust Regression		
Function	**Enrichment ratio**	**Number of genes**	**Function**	**Enrichment ratio**	**Number of genes**
Response to DNA damage	2.71	8	Nucleoplasm	6.35	184
Cell cycle	2.65	23	DNA packaging	6.06	65
DNA packaging	2.41	17	Protein assembly	4.58	57
Metal ion binding	2.20	3	Cell cycle	4.34	90
Regulation of cell cycle	1.76	16	Histone assembly	3.93	18
DNA repair	1.51	16	RNA processing	3.60	68
Cytoskeleton	1.43	31	Cytoskeleton	3.51	217
Lysosome	1.40	8	Microtubule	3.09	29
GTP binding	1.53	36	Chromosome	2.64	19
Protein maintenance	1.49	10	Mitochondria	2.62	102
Cell cycle regulation	1.46	39	Nucleotide	2.60	169
ATP dependent activity	1.46	31	Protein localisation	2.58	86
Tissue homeostasis	1.40	12	Monosaccharide metabolic process	2.53	29
Helicase activity	1.36	6	DNA repair	2.33	58
Response to nutrients	1.34	22	Nuclear transport	2.21	15
			Heat shock protein	2.06	16
			DNA damage	2.05	16
			Biological regulation	1.70	30
			Transcription	1.66	48
			Microtubule cytoskeleton	1.64	67

Appendix II: Patient information sheet

University Hospitals Coventry and Warwickshire NHS Trust

Department of Gastroenterology

University Hospital

Clifford Bridge Road

Walsgrave

Coventry

CV2 2DX

Tel: 024 7696 6087(sec)

Fax 024 7696 6090

www.uhcw.nhs.uk

Version 6 (RPA)

Dec 2014

A study to look at how bowel fermentation and diet affect other diseases

PATIENT INFORMATION SHEET

1. **Invitation**

You are being invited to take part in a research study. Before you decide if you want to take part, you may wish to consider two things: firstly, why the research is being done and secondly what you would have to do. Please take time to read the information below and talk it over with someone else if you want to. If anything is not clear or you would like to know more, please ask.

Please also note that some aspects of this information sheet may not be relevant to yourself. It would largely depend on the specific condition in which you are consulting your specialist. The reason is that this is a large study that covers several different areas of medicine.

Thank you for reading this.

2. What is the reason for the study?

Bowel problems are common and diet is known to be important in certain diseases of the large bowel (colon). Within the bowel there are large number of bacteria which help in the process of fermentation. Changes in diet may result in changes in fermentation which is thought to be contributory not only to certain bowel diseases but other metabolic diseases such as diabetes and obesity and even kidney, bladder, joint and heart disease. We hope that this study will give us a better understanding of the relationship between diet and bowel fermentation and its effects on other diseases. Food is digested by bacteria within our bowels and produces certain gasses (fermentation). These gasses are unique to certain diseases and we detect these using specialised instruments (electronic nose). It is hoped that this study will lead to early diagnosis and avoid invasive tests in the future.

3. Why have I been chosen?

Due to the specific nature of your illness, which is the subject of study in this research, your consultant has invited you to participate in this research.

4. Do I have to take part?

It is for you to decide whether you wish to take part. If you do decide to take part, you will be given this information sheet to keep. You will be asked to sign a consent form. Even if you decide to take part you can withdraw at any time. If you decide to withdraw, you do not need to tell us why and it will not change your treatment in any way.

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

If you agree to take part, we will invite you to sign a consent form to allow us to take samples of tissue. Depending on your medical condition we will collect certain tissue sample types e.g. urine, blood, breath or bowel tissue. However, not all may be relevant to yourself. We also seek permission to look at your medical records. Information sought from your medical records will be your medical history and medication.

For those attending Endoscopy

To obtain bowel tissue, an Endoscopic procedure is required (sigmoidoscopy/colonoscopy). This may already be a pre-requisite as part of your routine clinical investigation. At the time of examination, the doctor doing the test will take pieces of tissue for examination under a microscope (this is known as taking a biopsy). An extra 3 to 4 small pieces of tissue will be taken (each being the size of a grain of rice). Taking these extra biopsies

will not affect your care or add any significant risk. Your flexible sigmoidoscopy/colonoscopy will be the **same** whether or not you take part in the study. The doctor will discuss the results of your test either during or after the examination.

After the examination, a doctor who is part of the research team, will take a sample of blood and urine as well as a breath sample. We will also ask that you complete a food and lifestyle questionnaire which should take about 20 min. This can often be completed when you attend for your clinic visit or for an endoscopic examination. If this is not possible, the form can be completed at home and posted back to us. Recordings of your height, weight, hip and waist circumference will also be made (if not already done on your clinic visit).
After this, you may go home with follow up, if necessary, as planned by your consultant. We will also provide you with a stool pot to provide a stool specimen. A member of the research team will contact you 7 days after your test to arrange a suitable time and place (of your convenience) to collect the stool specimen. If you attend follow-up clinic, we may ask you again for further samples (e.g. urine, stool or blood). You will not be required to make an additional visit to the hospital for this purpose. Below is a chart of what will happen.

Blue text indicates what is additional as a result of this study.

If you agree to participate,
Sign CONSENT form

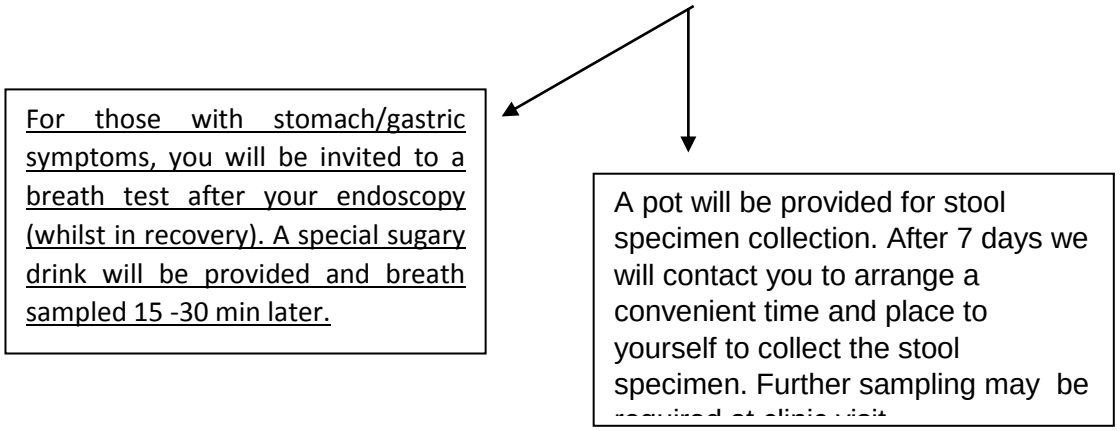
↓

Clinic visit – sampling of blood, urine, breath & completion of questionnaire as well as height/weight measurements

↓

Endoscopy – biopsy tissue sampling (depending on clinical condition).

[PN: If you attend directly for your endoscopic examination then we will collect the above samples and take measurements during this visit. You will not be required to attend a separate visit for this purpose to minimise inconvenience to yourself]



6. What do I have to do?

You need do nothing until you attend for your test. At this point, if you agree to participate, one of the doctors from the research team will discuss this with you and obtain your consent. If you have any questions beforehand, please see no 14.

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

a) Bleeding very rarely occurs following biopsies and the risk of bleeding is small. (In one study [1], there was one case of bleeding noted after 5000 consecutive biopsies i.e. 0.02% risk). Our own experience in a previous study had no immediate complications following 4500 consecutive biopsies in 500 individuals (Arasaradnam et al PhD Thesis 2007; University of Newcastle)
b) Taking a blood sample can be a little uncomfortable and occasionally for some there is a little bruising.
c) The additional biopsies required will add only 3-5 minutes to the procedure.
d) It is hoped that all necessary information can be obtained during the clinic or endoscopic visit and if not this will add no more than 10-15 min to your overall visit.

8. What happens to the samples taken?

Samples of stool will be analysed for changes in fermentation within the bowel and analysis of type of bacteria. The blood, urine and breath will be analysed in a similar manner to look for similarities in pattern compared with stool. The blood and urine samples give us more information about your diet. Samples from bowel tissue will be used to analyse gene, cell and protein changes and compared with the fermentation changes. Specifically, these gene changes include molecular changes such as 'methylation', 'acetylation' and markers of 'mitochondrial' damage. We will then try to link these changes with your diet from the information you gave us in the questionnaire. We value your anonymity hence all samples will be anonymised which means that in the event of any research related findings that come to light from this research study, the results cannot be traced to yourself. However significant clinical findings which relate directly to your care will be fed back to your overseeing consultant and to your GP.
We will also request permission to store some of the bowel tissue, blood urine and breath samples indefinitely. This is because in the future, newer techniques may become available which will allow us to perform more up to date testing. As mentioned above, these samples will be anonymised and therefore cannot be traced back to you.

9. What are the possible benefits of taking part?

We hope that the information we get from this study will help us understand more about how diet affects certain genes and proteins in the normal bowel. In the future this may help us understand more about how certain diseases develop and perhaps even how to better prevent this through alteration in diet.

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

All the information we have about you from this study is strictly confidential. This information will be kept securely while the study is taking place. Only the research doctors will be able to see it. Any information about you that leaves the hospital as part of a research report will have your name, address and any other personal information removed so that you cannot be identified from it. Your GP and your hospital consultant will be told that you are taking part in the study if you wish.

11. What if something goes wrong?

In the unlikely event that something goes wrong, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service (NHS) complaints mechanisms will be available to you.

12. Who is organising and funding the research?

This study has been organised and will be done at UHCW in conjunction with the University of Warwick. The chief investigator is Dr R P Arasaradnam. None of the researchers will be paid in person nor will they receive any financial gain for doing this study. Sponsorship in the form of a Research Grants have been obtained from local charities, research networks and collaborative bids (commercial and non-commercial).

13. Who has reviewed the study?

The Warwickshire Research Ethics Committee has reviewed and approved the study

14. Contact for Further Information or Complaints

Dr R P Arasaradnam

Assoc. Prof of Gastroenterology,

Department of Gastroenterology,

University Hospital Coventry & Warwickshire,

Clifford Bridge Rd,

Coventry CV2 2DX

Tel: 02476 966087

E mail: ramesh.arasaradnam@uhcw.nhs.uk or Nicola.O'Connell@uhcw.nhs.uk

Thank you for taking part in the above study.

A copy of the information sheet and signed consent form is available for your records.

Reference:

1. *Macrae FA, Tan KG, Williams CB. Towards safer colonoscopy: a report on the complications of diagnostic or therapeutic colonoscopies. Gut 1983 May;24(5):376-83*

Appendix III: Consent form

University Hospitals Coventry and Warwickshire NHS Trust
Department of Gastroenterology
University Hospital
Clifford Bridge Road
Walsgrave
Coventry
CV2 2DX

RPA
Version 5 August 2015

Consent for removal and storage of Human Tissue, Blood, Urine, Faeces and breath for Research

Thank you for reading the information about our research project. If you would like to take part, please read and sign this form.

Study number:
Patient identification number for this study:

Food and fermentation using metagenomics in health and disease (FaMIsHED study)

Title of project:……

Dr R P Arasaradnam, Dr M Pharaoh, Dr G Williams, Prof. C Nwokolo, Prof. K D Bardhan and Prof S Kumar

Name of researcher (s):……

02476 966087 or e mail: ramesh.arasaradnam@uhcw.nhs.uk

Contact details for research team:……..

Please initial boxes

1. I have read the attached information sheet on this project, dated…December 2014… (Version 6) and have been given a copy to keep if I wish. I have been able to ask questions about the project and I understand why the research is being done and any risks involved. ☐

2. I agree to give samples of tissue including bowel, blood, urine, faeces and breath for this research project. I understand how the samples will be collected, that giving samples for this research is voluntary and that I am free to withdraw my approval up to the point of anonymisation, for the use of the sample at any time without giving a reason and without my medical treatment or legal rights being affected. I am aware that the samples given will be analysed for gaseous products, gene and protein changes. I understand that the samples given for research purposes will be anonymised and therefore cannot be traced back to myself. Clinical findings will be fed back to my consultant and GP.

☐

3. I give permission for someone from the research team to review my medical records. I understand the information will be kept confidential. I do/do not wish for the research team to inform my GP of my participation in this study.

☐

4. I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test.

☐

RPA
Version 5
August 2015

.....	.....	.....
Name of patient (BLOCK CAPITALS)	Date	Signature
.....	.....	.....
Name of person taking consent (if different from researcher)	Date	Signature
.....	.....	.....
Name of researcher	Date	Signature

Thank you for agreeing to participate in this research