MOLECULAR GENETIC AND ENDOSCOPIC STUDIES OF DUODENAL POLYPOSIS

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A thesis submitted to Cardiff University for the degree of Doctor of Medicine

September 2015

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Summary

Duodenal polyposis and cancer have become a key issue for patients with familial adenomatous polyposis (FAP) and *MUTYH*-associated polyposis (MAP). The incidence of duodenal adenomas in MAP appears to be lower than in FAP but the very limited available data suggest a comparable increase in relative risk and lifetime risk of duodenal cancer. The work reported in this thesis addressed gaps in current knowledge in endoscopic and molecular genetic aspects of duodenal polyposis in MAP and FAP.

A prospective study examined the impact of chromoendoscopy on assessment of the duodenum in MAP and FAP. It demonstrated enhanced adenoma detection in both MAP (p=0.01368) and FAP (p=0.002516), but did not affect measurement of adenoma size. In both conditions there was a significant increase in Spigelman stage after chromoendoscopy compared to endoscopy without dye-spray.

A European collaborative project established a cohort of 207 MAP patients who had undergone surveillance upper GI endoscopy. There was a cumulative incidence of 30% of duodenal adenomas, and a 2.3% cumulative incidence of duodenal adenocarcinoma in MAP by 70 years of age. Patients that were Y179C homozygotes had a greater number of duodenal adenomas (and consequently higher Spigelman score) than patients with either two truncating mutations, G396C homozygotes, or G396D / Y179C compound heterozygotes, consistent with a more severe colorectal phenotype previously reported in Y179C homozygotes.

To investigate the somatic mutation rate and patterns of mutations in MAP and FAP duodenal adenomas, exome sequencing, Sanger sequencing and arrayCGH of adenoma tissue and matched blood DNA from patients undergoing upper GI surveillance was performed. This demonstrated a higher load of somatic mutation in MAP than FAP adenomas (p=0.035). With the exception of *APC* and *KRAS* mutations, there were very few somatic mutations in genes that have been found to drive colorectal tumourigenesis, but several other genes including *PLCL1* were found to be recurrently mutated in duodenal adenomas, suggesting that distinct molecular genetic pathways to adenoma development may be operating in the duodenum.

Acknowledgments

Firstly, I would like to thank Dr Sunil Dolwani for the opportunity to undertake this project and for his supervision of the endoscopic studies. I would also like to offer my gratitude to Professor Julian Sampson and Dr Laura Thomas for their excellent support and supervision. In particular, Laura's support and guidance during the last two years has been invaluable. I look forward to continuing working with you.

Sarah-Jane Walton and Professor Sue Clark, and the endoscopists at St Mark's Hospital (Dr Adam Haycock, Dr Noriko Suzuki and Dr Siwan Thomas-Gibson) were extremely helpful in enabling me to obtain vital duodenal polyp samples from their MAP patients. Thank you also to Fiona Lalloo and Jim Hill in Manchester.

I am also grateful to Shelley, Hala, Iris, Helena and Angharad for all their help with the lab work, encouragement and cakes! Sian Jose for guiding me through the array work, and Kevin Ashelford for his assistance with the exome data bioinformatics. Matthew Mort has been very generous in offering advice on the statistical analysis of the endoscopic chapters. A special mention goes to my European co-collaborators who provided data for the MAP study and also to Professor Geraint Williams and Dr Meleri Morgan for reviewing all the histology slides.

I am grateful to all of the patients that participated in the studies described in this thesis. I could not have managed to undertake the endoscopy lists without the help of Suzanne Ventrice, and I really appreciate all the extra effort it took to make sure they ran smoothly. Thank you to Jeff for always listening and for insightful advice, and to my colleagues Liz and Peter for their support.

Finally, I would like to thank my family for all of the love and support they have given me. My wonderful parents Mary and Tony, and brothers Oliver and Alex, who have encouraged me throughout my education and career. Also, the extended Rowe and Hurley families who have always been so supportive. My husband Denis, for all of his hard work (and patience) so that I could concentrate on writing up and for always being so positive, you deserve a medal! This thesis is dedicated to you and to our beautiful daughter Francesca; you are a remarkable little girl and make me laugh every day. I could not be prouder to be your Mum.

List of Abbreviations

A	Adenine
aa	Amino acid
AFAP	Attenuated FAP
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
APC	Argon plasma coagulation
BER	Base excision repair
bp	Base pair
C	Cytosine
cDNA	Complementary DNA
CHRPE	Congenital hypertrophy of the retinal pigment epithelium
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CLE	Confocal laser endomicroscopy
CNV	Copy number variants
CRC	Colorectal cancer
DBE	Double balloon enteroscopy
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
ddNTP	Dideoxynucleotide triphosphate
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
EMR	Endoscopic mucosal resection
EUS	Endoscopic ultrasound
FAP	Familial adenomatous polyposis
FGP	Fundic gland polyp
G	Guanine
GDP	Guanosine diphosphate
GEF	Guanine exchange factor
GI	Gastrointestinal
GTP	Guanosine triphosphate
HGD	High grade dysplasia
HhH	Helix-hairpin-helix
IPAA	Ileal pouch-anal anastomosis

IRA	Ileo-rectal anastomosis
LCR	Locus control regions
LGD	Low grade dysplasia
LOH	Loss of heterozygosity
LS	Lynch Syndrome
MAF	Minor allele frequency
MAP	MUTYH-associated polyposis
MCR	Mutation cluster region
MGD	Moderate grade dysplasia
MLH	MutL homologue
MLPA	Multiplex ligation-dependent probe amplification
MMR	Mismatch repair
mRNA	Messenger RNA
MSH	MutS homologue
MSI	Microsatellite instability
MUTYH	Human MutY homologue (also known as MYH)
NBI	Narrow band imaging
NEIL	Neil like glycosylase
NTHL	Nth endonuclease III-like
NUDT	Nudix (nucleoside diphosphate linked moiety X)-type motif
OGG	8-oxoG glycosylase
ORF	Open reading frame
8-oxoG	7,8-dihydro-8-oxoguanine
PCR	Polymerase chain reaction
PI	Principal investigator
Pol	Polymerase
PPAP	Polymerase proofreading associated polyposis
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Reverse transcriptase
SBE	Single balloon enteroscopy
SDS	Sodium dodecyl sulphate
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variation
T	Thymine
TA	Tubular adenoma
TVA	Tubulovillous adenoma

TAE	Tris-acetate-EDTA
TGF	Transforming growth factor
UTR	Untranslated region
VCE	Videocapsule endoscopy
VA	Villous adenoma

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PUBLICATIONS RELEVENT TO THIS THESIS

Hurley, J.J., Ewing, I., Sampson, J. et al (2014) Gastrointestinal polyposis syndromes for the general gastroenterologist. *Frontline Gastroenterol*. 5: 68-76

Ewing, I., **Hurley, J.J**., Josephides, E. et al (2014) The molecular genetics of colorectal cancer. *Frontline Gastroenterol*. 5:26-30

Short, E., Thomas, L.E., **Hurley J.J**. et al (2015) Inherited predisposition to colorectal cancer: towards a more complete picture. *J Med Genet*. [Epub ahead of print]

Thomas, L.E., **Hurley, J.J**., Jose, S. et al (2015) Genetic mechanisms in duodenal polyposis (submitted)

Hurley, J.J., Thomas, L.E., Walton, S.J et al (2015) The role of chromoendoscopy in duodenal surveillance in MUTYH-associated polyposis (MAP) (in preparation)

Chapter 1

Introduction

1. Introduction and background

1.1 Inherited adenomatous polyposis syndromes

Familial adenomatous polyposis (FAP; OMIM 611731, NM_001127511), *MUTYH*-associated polyposis (MAP; OMIM 604933, NM_012222) and Polymerase proof-reading associated polyposis (PPAP; OMIM 174762, NM_006231 and OMIM 174761, NM_002691) are characterised by the development of colorectal adenomas that over time will progress to colorectal cancer, in contrast to Lynch Syndrome (LS), where multiple colorectal polyps are often not present at the time of cancer diagnosis. FAP and MAP are also defined by the development of extra-colonic manifestations, including duodenal adenomas and cancer, which have now become a leading cause of death as patients undergo prophylactic colectomy at an early stage in their disease. Life expectancy in these patients remains lower than that of the general population (Nugent et al. 1993). In PPAP, families with *POLD1* mutations had endometrial cancer as a feature, and one individual was reported to have two primary brain tumours. To date, there have been no reported occurrences of extra-colonic neoplasms in individuals with *POLE* mutations (Palles et al, 2013). Very recently, Weren et al (2015) applied whole exome sequencing to 51 patients with multiple colorectal adenomas who did not have identified mutations in *APC* or *MUTYH*. They found that 7 members of 3 families had homozygous germline mutations in *NTHL1.* Tumours from affected individuals showed a significant increase in the proportion of C:G>T:A transitions, consistent with the predicted effects of homozygous loss-of-function mutations on *NTHL1* on the accumulation of somatic mutations. Major clinical features of *NTHL1*-associated polyposis include colorectal polyposis, CRC, and endometrial neoplasia but studies of larger patient cohorts are required to fully characterise this disorder.

These polyposis syndromes commonly have overlapping phenotypes, with similar numbers, distribution and histological subtype of polyps within the gastrointestinal (GI) tract, and so a molecular genetic diagnosis is required in order to facilitate planning of surveillance and preventive measures. Identification of a mutation can determine the risk of inheritance (autosomal dominant vs. recessive) and allows predictive testing of at-risk asymptomatic family members.

1.2 Familial Adenomatous Polyposis (FAP) – Clinical Features

FAP is an autosomal dominant disorder, first described in the literature in 1925 by Lockhart-Mummery (Lockhart-Mummery, 1925). It occurs in approximately 1 in 7000-8000 individuals (Cunningham et al. 2010). Multiple (>100) adenomas develop in the colorectum, and there is virtually a 100% life time risk of developing CRC. Traditionally, the clinical diagnosis of FAP is based on the identification of greater than 100 colorectal polyps (Vasen et al, 2008).The polyps usually appear by adolescence or the third decade of life; the average age at diagnosis of CRC being 39 years if left untreated. Although historically, evidence suggests that the risk of developing CRC before the age of 20 years is low (Bussey, 1975), European registries have shown that there is a small proportion (1.3%) of patients that have developed CRC at ages 16-20 years and one case between age 11 and 15 years. No patient in this study had CRC at or before the age of 10 years (Vasen et al. 2008). Prophylactic surveillance of the colorectum by endoscopy in FAP has been shown in FAP to reduce CRC and CRC-associated mortality (Bulow et al. 1995), and in FAP it has been recommended that endoscopic surveillance comprises a 2-yearly flexible sigmoidoscopy starting at age 10-12 years until adenomas are detected (Vasen et al, 2008). Once adenomas are detected, patients should undergo at least biennial colonoscopy until colectomy is planned. The 2 year interval is based on evidence form studies on the natural history of FAP, that have

demonstrated that it takes on average 15-20 years from the first development of adenomas to the development of CRC (Bussey, 1975). However, the presence of symptoms, including rectal bleeding, mucous discharge, loose stools and abdominal or back pain should prompt urgent investigation at any age.

Once florid polyposis develops, the most common form of treatment to prevent the development of CRC is a colectomy, and surgical options include a total colectomy with ileo-rectal anastomosis (IRA) and procto-colectomy with ileal pouch-anal anastomosis (IPAA). There are no guidelines regarding the exact timing of surgery, but the definition of 'florid polyposis' encompasses large numbers of adenomas >5mm in size and adenomas showing high grade dysplasia. Most FAP patients with the classical form of the disease undergo cancer prevention surgery between the age of 15 and 25 years. Factors including patient age, severity of rectal polyps, desire to have children, and possibly the site of the mutation in *APC* may influence the decision on the type of surgery undertaken. Patients with an IRA require regular endoscopic examination of the remaining rectum and removal of any new adenomas. Patients with a pouch should undergo annual endoscopic surveillance of the pouch.

The duodenum is the second most common site for adenoma development in FAP. Duodenal adenomas are found in 30 to 70% of patients, and there is a risk of developing duodenal carcinoma of 5% (Vasen et al, 2008). Periampullary duodenal carcinoma is the commonest cause of cancer-related death in patients that have undergone prophylactic colectomy (Jagelman et al, 1988). There are also several extra-intestinal manifestations which include congenital hypertrophy of the retinal pigmented epithelium (CHRPE), epidermoid cysts, desmoid tumours, thyroid cancer and malignant brain tumours (Table 1.1). Desmoid tumours occur in 10-15% of cases (Gurbuz et al. 1994). They are benign connective tissue tumours that can

lead to life-threatening complications because they can grow to a significant size and encroach onto vital structures. Desmoids frequently arise in the abdominal wall and intra-abdominally in FAP and commonly recur after surgery. They are a cause of significant morbidity and mortality in FAP. Gastric fundic gland polyps and adenomas of the gastric antrum also occur, but no studies have confirmed an increased incidence of gastric cancer in FAP in Europe, however there are a few case reports of FAP-related gastric cancer that originate from Japan and Korea (Park et al, 1992). Gardner's syndrome is now regarded as a phenotypic variant of FAP, and refers to the association of colonic polyps with osteomas of the jaw and epidermoid sebaceous cysts, particularly of the scalp. Turcot syndrome is also the consequence of a germline mutation in *APC* and is the association between multiple colorectal polyps and cerebellar medulloblastoma (Hamilton et al. 1995).

There is a milder variant of the disease, characterised by the development of fewer adenomas and a later onset of colorectal cancer, known as attenuated FAP (AFAP). CRC develops 10-15 years later than in 'classical' FAP and there is a lower burden of extra-colonic features (Knudsen et al. 2003). An international collaborative study proposed the following diagnostic criteria for AFAP: (1) a dominant mode of inheritance and (2) 3-99 colorectal adenomas at age 20 or over. However, only a minority of such patients have mutations in *APC*. Surveillance colonoscopy is recommended every 2 years starting from ages 18-20 years, as localised right-sided colonic adenoma development has been described in this cohort (Knudsen et al, 2010).

Benign lesions	Malignant lesions
CHRPE (70-80%)	Thyroid cancer (2-3%)
Epidermoid cysts (50%)	Brain tumour (<1%)
Osteoma (50-90%)	Hepatoblastoma (~1%)
Desmoid tumour (10-15%)	
Supernumery teeth (11-27%)	
Adrenal gland adenomas (7-13%)	

Table 1.1 *Extra-intestinal features in FAP, excluding duodenal adenomas and carcinoma. Deregulation of the APC gene has been shown to play a role in carcinogenesis of all these tissues (Vasen et al. 2008).*

1.2.1 The *APC* gene

FAP is caused by a germline mutation in one of the adenomatous polyposis coli (*APC*) alleles located within chromosome region 5q21-22 (Bodmer et al. 1987). The chromosomal location was suggested by patients with colorectal polyposis in association with mental retardation, where a deletion in the chromosomal band 5q21 was detected (Herrera et al, 1986). Accurate localisation of the gene to 5q21 resulted from linkage analysis of families with FAP and led to positional cloning (Groden et al 1991). The *APC* gene consists of at least 21 exons (Santoro and Groden, 1997), and the largest of these, exon 15 encodes over three-quarters of the protein. Exons 2 to 15 are coding. Due to splicing, there is a range of alternative transcripts, and the most common transcript consists of 15 exons spliced together to form a transcript 8535 base pairs long (Groden et al, 1991).

1.2.2 The APC protein

APC is a large protein (312kDa) containing multiple functional domains which enable it to interact with numerous other proteins (Figure 1.1) (Fodde et al. 2001). The oligomerisation domain of APC is found at the N-terminus, and contains heptad repeats that allow APC to form homo-dimers (Su et al. 1993). Wild type APC may form dimers with both wild-type and truncated mutant APC proteins. The armadillo region is made of seven repeats and is highly conserved. It binds to the regulatory B56 subunit of protein phosphatase 2A (PP2A), an enzyme that binds axin via its catalytic subunit (Hsu et al 1999). The armadillo region also binds to the APC-stimulated guanine nucleotide exchange factor (ASEF) and KAP3 (Näthke, 2004). The central region of APC contains three 15-amino acid (aa) repeats followed by seven 20-aa repeats, both of which can bind to β-catenin (Rubinfeld 1993; Su et al. 1993). The 15-aa repeats can also bind to α-catenin (Su et al. 1993) and C-terminal binding protein (CtBP) (Hamada and Bienz, 2004) and are retained in the majority of mutant APC proteins. The 20-aa repeats contain a TPXXFSXXXSL motif (Groden et al. 1991) and can bind glycogen synthase kinase 3β (GSK3β) to form binding sites for β-catenin (Rubinfeld et al. 1996). Downregulation of β-catenin is reliant on the presence of at least three of the seven 20AARs in APC (Rubinfield et al. 1997). Scattered between the 20-aa repeats are 3 segments of 31-32 amino acids, each containing the sequence SAMP. SAMP repeats can bind to axin and its homologue conductin (also known as axin2) (Behrens, 1998; Hart et al. 1998). The C-terminal region of APC contains a basic domain (Groden et al.1991) containing arginine and lysine residues that can bind microtubules (Hanson and Miller, 2005). The EB/RP family members EB1 and RP1 also bind to APC through a C-terminal domain (Juwana et al. 1999). The N-terminal and C-terminal regions of APC have been shown to interact, suggesting intramolecular interactions may also occur (Li and Näthke, 2005).

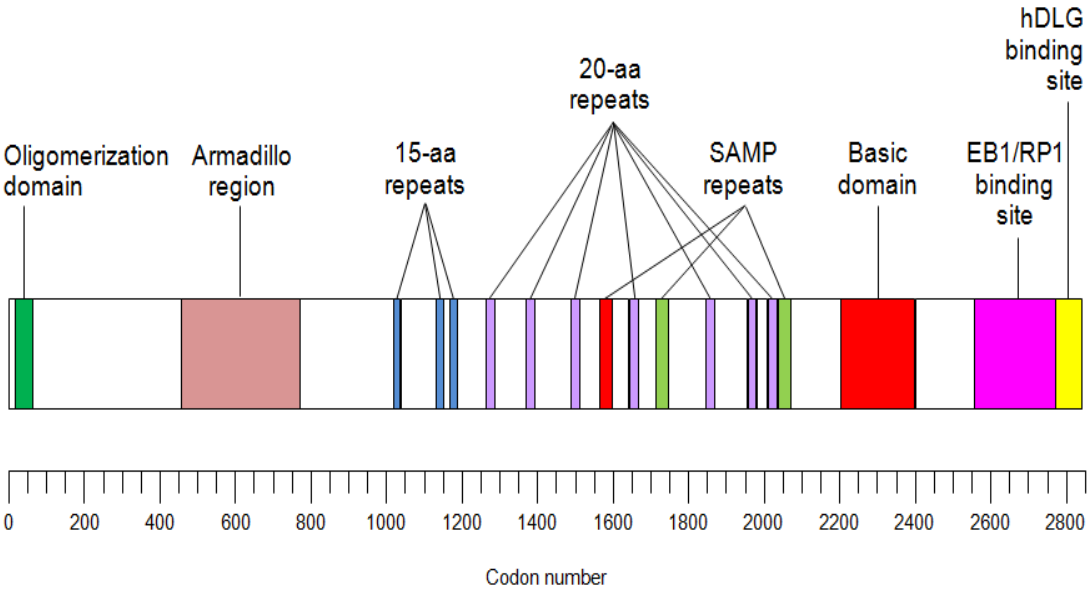


Figure 1.1 *Functional domains of the APC protein*.

1.2.3 Functions of APC

APC is a multifunctional protein involved in a variety of cellular processes including Wnt signalling, cell adhesion, cell migration, mitotic spindle formation, chromosomal segregation and apoptosis (Fodde et al 2001).

1.2.4 Wnt signalling and cancer

The Wnt pathway is crucial in controlling processes such as cell proliferation and differentiation, and the regulation of cell death. The APC protein has tumour suppressor functions and is a key part of the canonical Wnt signalling pathway, thus playing a central role in the development and homeostasis of the intestine and many other tissues. APC functions as part of a destruction complex influenced by Wnt signals (shown in figure 1.2, parts a-c) that targets β-catenin for degradation. The

destruction complex facilitates phosphorylation of both APC and β-catenin by GSK3β. Phosphorylation of APC results in improved β-catenin binding and the ultimate consequence of enhanced APC-mediated GSK3β phosphorylation and degradation of β-catenin. If this is inhibited, abnormal accumulation of intracellular β-catenin occurs (Klaus and Bichmeier, 2008). This allows translocation of β-catenin into the nucleus, where it acts to activate the transcription of Wnt target genes such as *cyclin D1* and *c-myc*, which are involved in cell cycle regulation. Constitutive activation of Wnt signalling results in an enlargement of the GI stem cell population by the inhibition of cell differentiation, or by stimulating de-differentiation (Kielman et al. 2002). This tips the balance between mitosis and cell loss in the colon and leads to tumourigenesis. Other mechanisms can also contribute to the neoplastic transformation of a cell through loss of regulation of Wnt signalling. Activating mutations of β-catenin in colonic tumours without an APC mutation (Sparks et al. 1998) and variants of axin affecting the binding of GSK3β in colon cancer cell lines (Webster et al. 2000) have been reported.

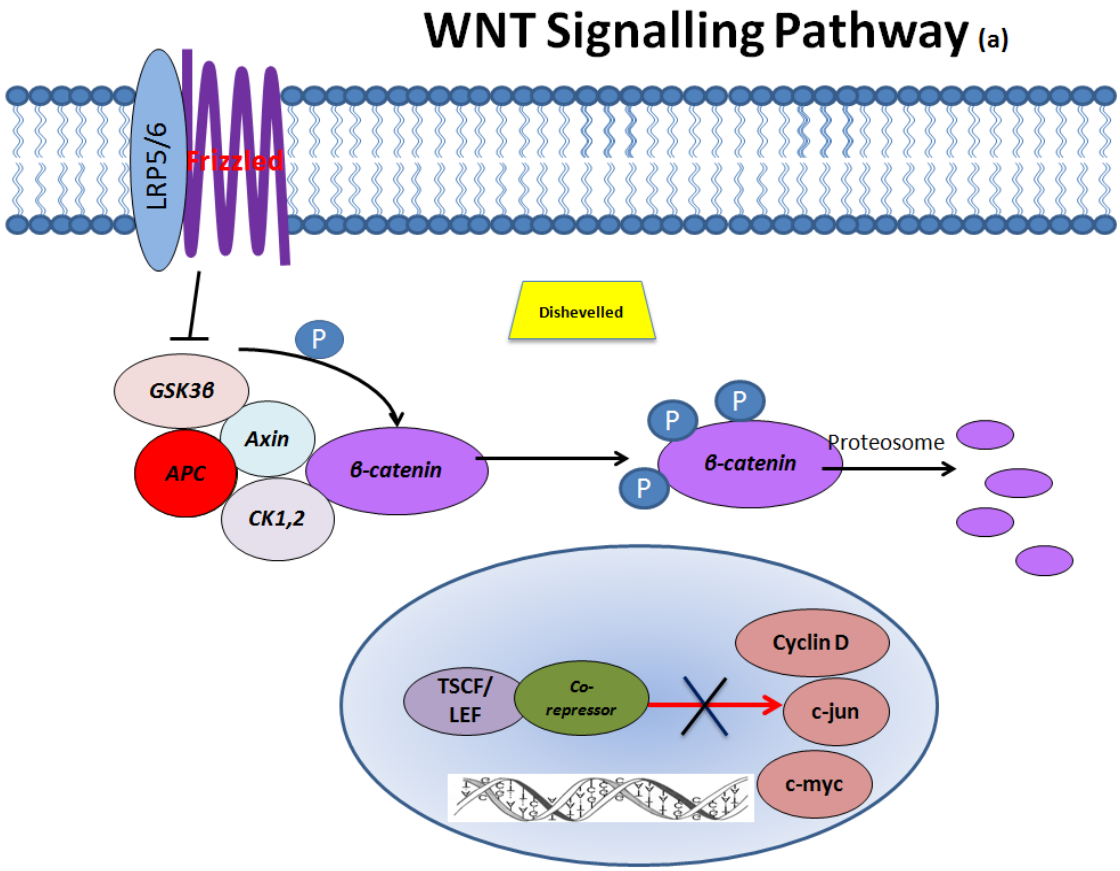


Fig 1.2 - Wnt signalling in colonic epithelial cells.

a) In the absence of Wnt, β-catenin is recruited to the destruction complex (that includes APC, CK1, Axin and GSK3β) and is subsequently proteosomally degraded. Restriction on the amount of intracellular β-catenin enables a transcriptional repressor Groucho and the TSCF/LEF machinery to interact, and represses Wnt target gene transcription.

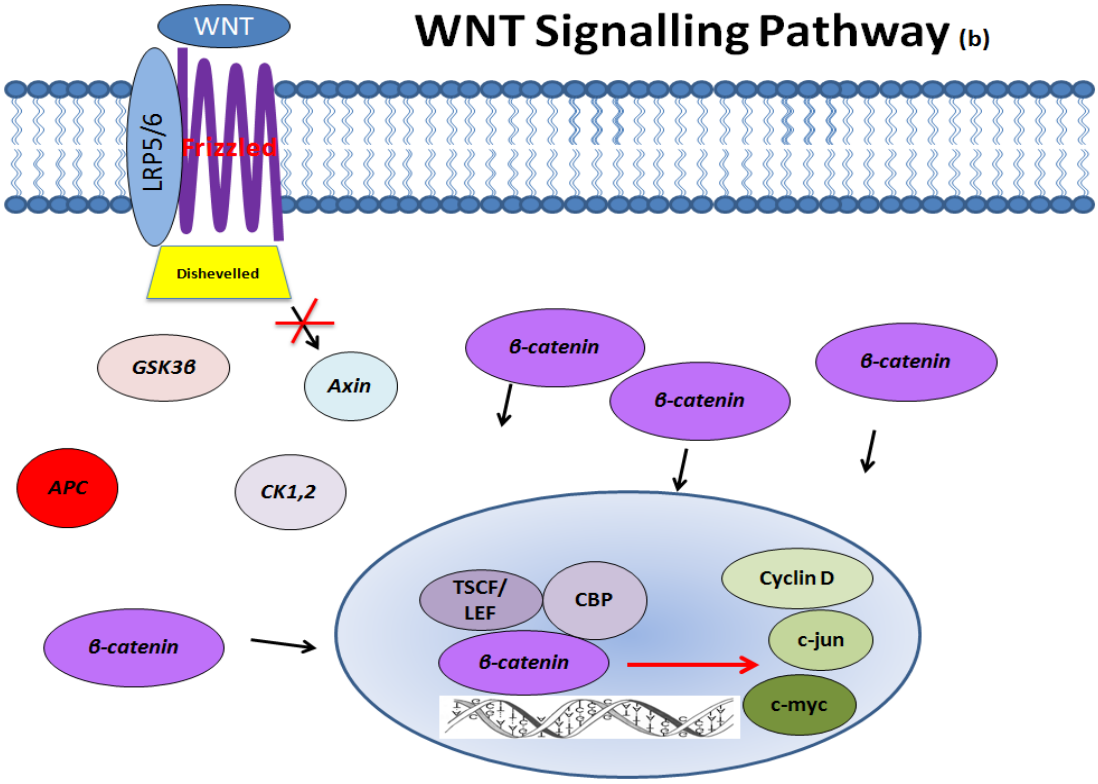


Fig 1.2 - Wnt signalling in colonic epithelial cells.

b) Activation of the Wnt receptor causes dishevelled to bind to and de-phosphorylate axin preventing the formation of the destruction complex. β-catenin accumulates and translocates into the nucleus where it binds with TSCF/LEF transcription factors, activating transcription of Wnt target genes such as c-myc and cyclin D.

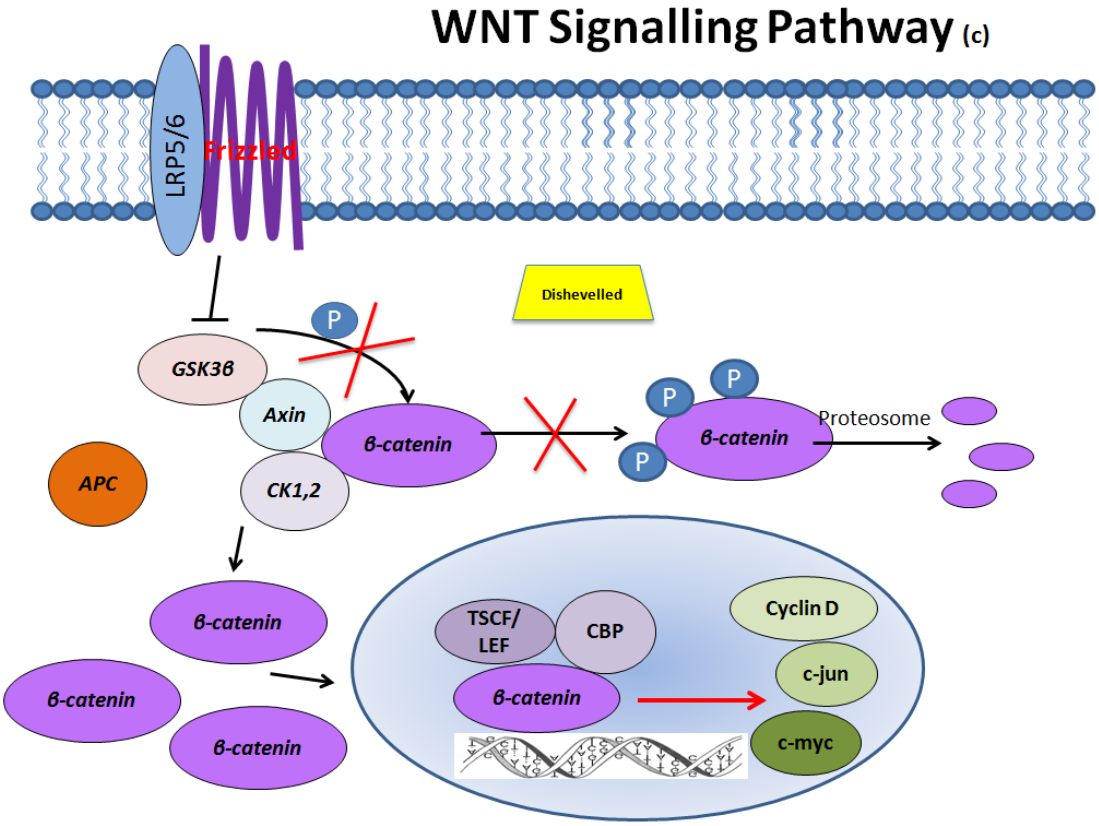


***Fig 1.2 - Wnt signalling in colonic epithelial cells**.*

c) When APC is inactivated in FAP, axin cannot bind to APC. The multiprotein degradation complex is unable to form, and GSK3β-mediated phosphorylation and subsequent β-catenin degradation is inhibited.

1.2.5 Germline *APC* mutations

A large proportion of the germline mutations in FAP patients are seen in exon 15, as would be expected as it accounts for more than 75% of the coding sequence. The majority (~95%) are frameshift or nonsense, resulting in a truncated protein with abnormal function. Frameshift mutations are more frequent than nonsense mutations, and the most common nonsense changes are C>T mutations, which are thought to result from spontaneous deamination of 5-methylcytosine (Laurent-Puig et al. 1998). In 5% of FAP cases, deletions of the whole gene or exons have been reported (Sieber et al. 2002). Aretz et al (2005) identified 14 different large submicroscopic genomic deletions in 26 families of FAP patients where no point mutation had been identified. The size of deletions ranges from single exons to the whole gene, including the promoter region. Larger, cytogenetically detectable interstitial deletions at 5q22 have been reported in FAP patients with a degree of mental retardation and dysmorphism (Aretz et al.2005).

The 1699 germline *APC* mutations recorded (figure 1.3) are spread evenly between codons 200 and 1600, but are rarely seen beyond codon 1600. The most frequent mutational hotspots are at codon 1309, where there is a 5bp (AAAAG) deletion (Beroud and Soussi, 1996) and at codon 1061. Together, these mutations account for a third of reported *APC* germline mutations (Beroud and Soussi, 1996).

The most important functional domains of the *APC* gene from a mutation perspective are the first serine alanine methionine proline (axin binding) repeat at codon 1580 and the first, second and third 20-amino acid repeat (20AARs) which are involved in beta-catenin binding and degradation (Segditsas and Tomlinson, 2006).

AFAP generally results from germline mutations at the 5' or 3' ends of *APC* (codons <163 and 1596-2644) or between codons 329 and 338 in the alternatively spliced region of exon 9 (Galiatsatos and Foulkes, 2006).

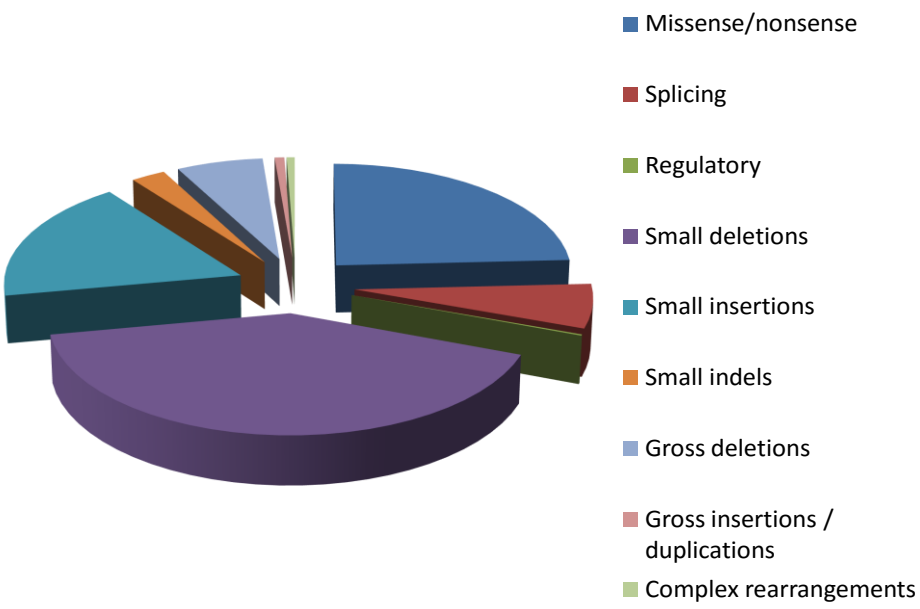


Figure 1.3 Proportions of germline mutation type in APC*. There are 1699 APC variants recorded on the Human Gene Mutation Database (Stenson et al. 2014).*

Whereas truncating germline mutations of the adenomatous polyposis coli (APC) gene give rise to FAP, missense polymorphisms of *APC* may confer a weak risk for colorectal cancer. The missense germline variant I1307K in Ashkenazi Jews is associated with an increased risk of developing multiple adenomas and CRC (Gryfe et al 1999; Frayling et al. 1998). The variant consists of a T→A substitution, which creates a poly (A) tract which is hypermutable and appears to be inherently prone to further mis-pairing and slippage, ultimately resulting in an *APC* allele that has

increased susceptibility to somatic inactivation (Laken et al. 1997; Zauber et al. 2003). A recent meta-analysis demonstrated that compared with those who carried the wild-type I1307K, Ashkenazi Jews who carried the I1307K variant were at a significantly increased risk for colorectal neoplasia, with a pooled odds ratio of 2.17 (Liang et al. 2013). However the tumour risk associated with this variant is controversial and most tumours from I1307K patients have not shown a mutation within the poly (A) tract (Sieber et al. 2003) which may suggest an additional distinct predisposition to CRC. Another missense germline *APC* variant E1317Q (Frayling et al.1998) has been associated with colorectal tumourigenesis, but has also been detected in normal controls. It codes for a mutation in *APC* that occurs in a functionally significant region of the gene and might act through a dominant negative effect on the APC/ β- catenin pathway (see section 1.2.4; Wnt signalling and cancer), resulting in a predisposition to adenoma formation (Frayling et al. 1998). Most studies have lacked the statistical power to confirm the role of E1317Q in CRC risk, and as carriers often lack a personal or family history of colorectal adenoma or carcinoma, this suggests it has a low penetrance (Lamlum et al. 2000).

10-25% of FAP cases have no family history of polyposis, suggesting a new spontaneous mutation has been acquired. Somatic mosaicism for *APC* gene mutations, where the new mutation occurs post-fertilisation and is present in only a subset of cells or tissues may be responsible for a proportion of these sporadic cases. In their cohorts of sporadic FAP cases, Aretz (2006) and Hes (2008) reported that 11% and 21% respectively showed somatic mosaicism.

1.2.6 Somatic *APC* mutations

Molecular genetic evidence has established that inactivation of both copies of the *APC* gene is required for tumour development, suggesting that *APC* follows Knudson's 2 hit model for tumour suppressor genes (Powell et al. 1992). In FAP somatic mutations provide the 'second hit' that is necessary for tumour formation (Knudson, 1996; figure 1.4) and are found in the majority of colorectal adenomas and carcinomas, including in adenomas less than 5mm in size (Powell et al. 1992). Somatic *APC* inactivation may be due to protein truncating mutations or allelic loss that may be manifest as loss of heterozygosity (LOH).

Somatic mutations/LOH are detected in over 75% of sporadic CRCs as well as in tumours from FAP patients (Miyoshi et al, 1992; Rowan et al, 2000). The spectrum and location of somatic mutations in sporadic colorectal tumours is similar to that seen in FAP polyps. It has been suggested that the number of adenomas in the colon in FAP is too great for each one to acquire an individual second hit and that multiple adenomas may arise from one original somatic mutation rather than distinct somatic events (Segditsas and Tomlinson, 2006).

Over 60% of all somatic mutations in *APC* occur within the mutation cluster region (MCR), which accounts for less than 10% of the coding sequence of the gene between amino acid 1281 and 1580 of exon 15 (Cheadle et al. 2002). There are two mutational hotspots for somatic mutations at codons 1309 and 1450 within the MCR (Beroud and Soussi, 1996) seen in both sporadic and FAP tumours, and at codon 1554 in sporadic tumours (Rowan et al. 2000). The vast majority of *APC* mutations result in a truncated protein that lacks all axin binding motifs and a variable number of 20AARs which ultimately act to inactivate the β-catenin down-regulating activity of *APC*.

The locations of the *APC* mutations therefore determine the number of 20-amino-acid beta-catenin binding and degradation repeats that remain in the truncated protein. In the colorectum, most FAP tumours retain one to two 20-AARs, and this is also observed in sporadic CRCs with *APC* mutations. However, in the duodenum, three to four 20-AARs are commonly retained, which is thought to modulate the level of wnt signalling that results from somatic APC mutations (Crabtree et al. 2003; Lamlum et al. 1999).

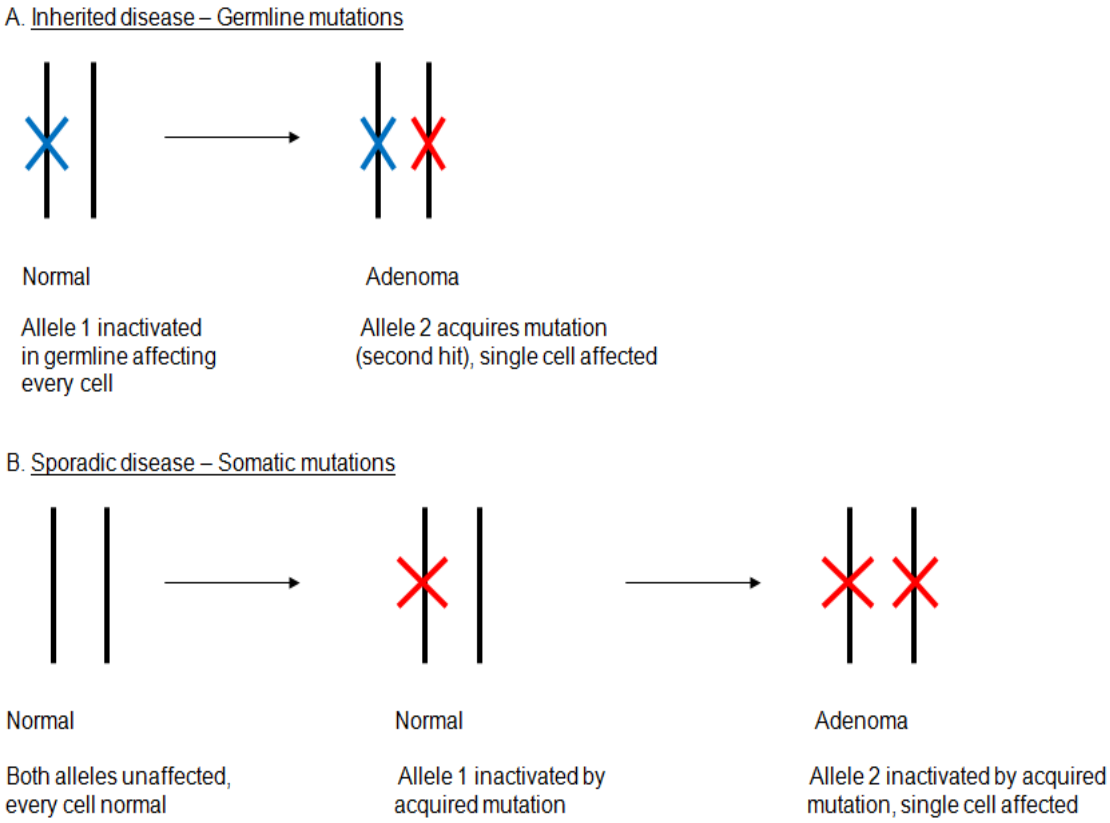


Figure 1.4 *– Knudson's two hit hypothesis for loss of tumour suppressor function in tumourigenesis. In inherited disease, a mutation of one allele is inherited in every cell, and a mutation of the second allele is acquired in tumourigenic cells. In sporadic disease two somatic mutations are required (Knudson, 1996).*

1.2.6.1 Types of mutations

The proportions of somatic frameshifts and point mutations in *APC* are almost equal to that of germline mutations (Beroud and Soussi, 1996). Somatic mutations result in truncation of the *APC* protein either by a nonsense mutation (30%) or by a frameshift mutation (68%) (Crabtree et al. 2003). Less than 15% of all the somatic mutations in *APC* in FAP are due to G:C→T:A transversions (Thierry Soussi database). Most patients have no notable excess of any particular type of somatic mutation (Crabtree et al. 2003; Albuqerque 2002; Miyaki et al. 1994). In their study of 133 FAP colorectal adenomas, Albuquerque et al (2002) reported that 79% of somatic APC mutations found were point mutations, with 21% showing allelic loss. Orbrador-Hevia et al. 2010reported a detectable second-hit in the APC gene in only 25% of FAP adenomas, with LOH being found more frequently than point mutations, however only the MCR region of the gene was analysed.

Single base pair deletions and insertions (indels) account for 33% of all somatic mutations in *APC* (Nagase et al. 1992). Micro insertions and deletions in the *APC* gene are commonly found to cause frameshifts where the correct reading of the frame is disrupted. Frameshift mutations were described in 71% of FAP tumours, with a large proportion of these being 1- to 16 base pair deletions (Miyaki et al. 1995). In non-FAP sporadic colorectal tumours, 59% of *APC* mutations were frameshift. In tumours arising in the stomach or duodenum of FAP patients, there is a similar somatic mutation spectrum to colorectal tumours. In 75 gastro- duodenal polyps from 21 FAP patients, 47 somatic mutations were described; 77% were frameshift mutations, including 11 cases of 2/4 bp deletion and 25 cases of 1-2 bp deletion (Toyooka et al. 1995). AG or AGAG deletion at codons 1462-1465 was detected in 10 tumours, and an A insertion at codons 1554-1556 was detected in 22 cases. However, there is a higher frequency of mutations in codon 1450 in the colorectum (Miyaki et al. 1994).

Base pair substitutions include missense variants, where one amino acid is substituted for that of another, nonsense mutations in which a premature stop codon is introduced, and splice site alterations. In colorectal tumours in FAP, 29% of the somatic mutations identified were nonsense point mutations (Miyaki et al. 1994).

Transitions and transversions which involve the substitution of two-ring purines (A>G) or one ring pyrimidines (C>T) are generally more frequent than transversions that involved changes of purine for pyrimidine bases. Of the point mutations in the colorectal tumours of patients with FAP, 65% of all the point mutations were GC>AT transitions, 22 of the 34 described were found to occur at the CpG sequence (Miyaki et al. 1994). When somatic alterations in the MCR were analysed, only four somatic mutations were detected in 60 adenomas studied and these were all nonsense mutations near to codon 1300. One of them was a C→T transition and the rest were transversions (Obrador-Hevia et al. 2010). In MAP, the majority of adenomas have G:C→T:A transversions (section 1.3.1).

Splicing is a complex process by which introns are removed from pre-messangerRNA (mRNA) and successive exons are joined to produce a mature mRNA molecule. Splicing mutations can generate several outcomes on the mature mRNA, including exon skipping from classical splice site mutations and single nucleotide substitutions within introns, which create de novo, splice sites resulting in cryptic exon inclusion. Single nucleotide substitutions within exons can act to cause the creation of de novo splice sites resulting in the loss of sections of an exon if these new splicing sites are used. Aretz et al (2004) described 917 FAP patients in whom about 5% (23/441) of the identified mutations were localized in intronic splice-site sequences. They demonstrated by complementary DNA (cDNA) analysis that mutations at invariant AG/GT sites led to a reduction of splicing efficiency that

usually results in complete deletion of the corresponding exons. In two cases, mutations generated new splice sites or led to the use of cryptic splice sites, which resulted in frameshifts at transcript level with similar deleterious effects on phenotype as mutations resulting in exon skipping. The use of new splice sites has also been published for mutations at other invariant splice sites in the *APC* gene (Wallis et al., 1999; Charames et al., 2002).

Methylation of CpG dinucleotides is a common transition in mammalian DNA and involved the deamination of methylated cytosine in CpG dinucleotides, most commonly resulting in a C>T transition (Cooper and Krawczak, 1993). Methylation of cytosines in CpG dinucleotides located in the promoter region of tumour suppressor genes can result in transcriptional silencing. The majority of germline and somatic point mutations in the APC gene are C>T transitions occurring at GCA codons (Beroud and Soussi, 1996).

1.2.6.2 Loss of heterozygosity (LOH)

LOH at 5q21 usually occurs by mitotic recombination where there is loss of the wild type allele and reduplication of the mutated allele leading to copy neutral LOH. LOH can also occur by deletion or chromosomal loss due to non-disjunction (Serra et al. 2001). It is seen in both FAP tumours and sporadic CRCs. In sporadic CRC, *APC* LOH may be seen in 30-40% of cases (Rowan et al. 2000). Two studies of FAP reported allelic loss as the second hit in 20% and 22% of tumours (Lamlum et al. 1999; Crabtree et al. 2003).

1.2.6.3 Copy number variations (CNVs)

At least 85% of sporadic CRCs have chromosomal instability (CIN) (Issa, 2008). Aneuploidy is a consequence of chromosomal instability that leads to the gain or loss of whole chromosomes or parts of chromosomes (partial aneuploidy) (Gordon et al. 2012). Copy number variations (CNVs) are common forms of structural variants due to CIN, and are defined as a gain or a loss of copies of DNA segments that are larger the 1kb in length, when compared to a reference genome (Redon et al, 2006). CNVs can affect gene expression and are associated with disease susceptibility, and it alterations in gene dosage can be correlated with changes in expression level (Chaignat et al. 2012, Thomas et al. 2015).

Previous studies on CRCs have reported gains at chromosome 8q, 13 and 20q and losses at chromosome 8p, 17p and 18q (Lassmann et al. 2007; Jones et al. 2005; Alcock et al. 2003; Lipska et al. 2007). Loss of chromosome 18 has frequently been observed in CRC, and this chromosome harbours a number of tumour suppressor genes, for example *SMAD4* (Thiagalingham et al 1996). CNVs affecting tumour suppressor genes, oncogenes, or non-coding RNAs may all contribute to cancer development. For example, loss of an *APC* allele at chromosome 5q21 can lead to its deregulated expression in CRC (Camps et al 2009). Voorham et al (2012) demonstrated copy number changes in 60% of all flat and polypoid colorectal adenomas and specific loss of 5q15.5-q31.1 in adenomas that had a flat morphology that are thought to follow a more aggressive course. Loss of 5q has also been described in CRC cases with metastases (Diep et al. 2006), Cardoso et al (2006) reported that up to 80% and 60% of MAP and FAP colorectal polyps showed aneuploid changes respectively. Both MAP and FAP adenomas were characterised by frequent losses at chromosome 1p, 17, 19 and 22 with gains affecting chromosomes 17 and 13. Their data suggested that a considerable degree of aneuploid change occurs at an early stage of adenoma development. However, this

contrasted with the findings of Lipton et al (2003), where flow cytometry analysis of 13 MAP adenomas was reported to show no detectable aneuploid change.

1.2.6.4 Hypermethylation of the 1A promoter

Hypermethylation of the 1A promoter region of the *APC* gene has been postulated as a possible second hit, acting to prevent expression of the APC protein (Esteller et al. 2000). It has been detected in colorectal carcinomas and adenomas, but not in adjacent normal colonic mucosa, and has also been reported in oesophageal, gastric, hepatic and pancreatic carcinomas in humans (Tsuchiya et al. 2000). However, it also is found in normal gastric mucosa, suggesting that it may be an alternative mechanism of *APC* inactivation in the early stages of colorectal tumour development but a normal event in gastric mucosa. In a further study, hypermethylation of *APC* promoter 1A has been shown to contribute to moderate activation of Wnt signalling (Wnt signalling and cancer; section 1.2.4) in a subset of serrated adenomas, but this was not found in traditional adenomas or CRC, however the numbers of polyps studied were small (Fu et al. 2009).

1.2.7 Interdependence of the first and second hit in *APC*

The precise nature of the somatic *APC* 'second hit' in FAP is highly dependent on the 'first hit' or germline mutation (Lamlum et al, 1999). This pattern is also seen in sporadic tumours with two somatic *APC* hits (Rowan et al, 2000). In FAP, if the germline mutations occur between codon 1194-1392, it has been demonstrated that there is strong selection for allelic loss of *APC* as the 'second hit' in development of a colorectal adenoma. If the germline mutation lies outside of this region, the 'second hit' is most likely to produce a truncating mutation in the mutation cluster region (MCR) (Lamlum, 1999). LOH is strongly associated with germline mutations

between the first and second 20AAR (codons 1285-1379). Germline mutations before codon 1280 are associated with mutations between the second and third 20AAR (codons 1400 and 1495), and germline mutations after codon 1400 are associated with somatic mutations before codon 1280 (Albuquerque et al. 2002; Crabtree et al. 2003). Most colorectal tumours therefore contain *APC* alleles that encode a total of two 20 AARs.

Tumours from FAP patients carrying germline mutations close to codon 1300 show a high frequency of allelic loss and these patients tend to have a more severe form of the disease. Patients carrying germline mutations away from codon 1300 show LOH less frequently (Lamlum et al, 1999).

1.2.8 The 'just-right' hypothesis

The growth advantage gained by different combinations of *APC* mutations varies and is not based simply on loss of function (Rowan et al. 2000, Cheadle et al. 2002). Albuquerque et al (2002) proposed that rather than constitutive activation of wnt signalling (described in section 1.2.4), an optimum level of wnt signalling and a specific lower level activation of β-catenin signalling is favourable for tumourigenesis – the 'just right' hypothesis. The strongest selective advantage is believed to result when the function of *APC* is impaired sufficiently for β-catenin levels to over-activate growth genes, driving cell growth without apoptosis or cell death pathways being initiated. *APC* genotypes retaining one or two of the β-catenin binding repeats (and some residual activity) are most highly selected for. Thus, the mode of inactivation of the wildtype allele of the gene influences the extent of wnt pathway activation and the number and growth of polyps that arise. However, the association between 'first hits' and 'second hits' is not so strong that tumourigenesis arises only if the

genotype is optimum, some variation appears to be tolerated, with the 'loose fit' model more accurately describing this situation (Crabtree et al. 2003).

1.2.9 The 'three-hit' hypothesis

Studies on tumours from patients with attenuated FAP (AFAP) offered evidence for a new mechanism of tumour development. Unlike most tumours from classical FAP patients, AFAP tumours frequently contain two somatic mutations, one of which affects the germline mutant *APC* allele (Spirio et al. 1998; Su, L-K. et al. 2000; Sieber et al. 2006). The highest frequency of these 'third hits' is found in tumours from patients with a germline mutation in the alternatively spliced region of exon 9. The third hit often leaves three 20-aa repeats intact on the germline mutant allele, with either LOH or a proximal somatic mutation of the wild type allele (Sieber et al. 2006). Not all AFAP tumours require three hits in APC which could be a result of modifier alleles affecting levels of functional APC protein and its splicing efficiency, or the presence of a third hit at another locus (Sieber et al, 2006).

The three hit hypothesis has recently been refined, to the 'multiple hit' model. When true LOH occurs at *APC,* it typically involves no copy number change (Sieber et al. 2002). However, in CRC, copy number changes at *APC* are common, and can take the form of a gain or deletion in aneuploid and near –diploid tumours – and it has been postulated that the copy number changes are effectively 'third hits' (Segditsas et al. 2009).

1.2.10 Genotype-phenotype variability in FAP

Genotype/phenotype correlations for colonic disease in FAP have been well recognised and can be useful to guide management decisions. FAP patients do not all develop the same phenotype and this variation is partially accounted for by the position of the germline *APC* mutation. In particular, mutations between codon 1250 and codon 1464 are associated with classical FAP with severe polyposis (Laurent-Puig et al. 1998), and a particularly severe form of disease with early onset of colorectal cancer has been found in patients with a mutation in codon 1309 (Freidl et al. 2001). The reason for the association of a particularly severe form of FAP with germline mutations around codon 1309 is likely related to selection for LOH "second hits" in these tumours that give an optimal level of β-catenin signalling for tumourigenesis. LOH occurs spontaneously at a higher frequency than truncating mutations close to codon 1300 so the frequency of pathogenic mutations in these patients is effectively higher (Lamlum, H. *et al*, 1999; Crabtree, M. *et al*, 2003). FAP patients with germline mutations between the second and third 20AARs have more severe disease than patients with mutations before the first 20AAR (not seen in AFAP), thought to be because patients with mutations before the first 20AAR are constrained to the second hit being between the second and third 20AAR, whereas those with a mutation between the second and third 20AAR can acquire a 'just right' second hit in a substantial part of the gene before the first 20AAR. Will et al (2010) observed the difference within the large bowel in the type of 'second hit' found in FAP polyps. They reported that patients with germline codon 1309 *APC* mutations, leaving 1 x 20AAR, have higher frequencies of LOH as the tumour location becomes more distal. This may partly explain why many FAP patients with codon 1309 have an increased polyp burden in the rectum.

AFAP patients with 5' germline mutations tend to have a more severe phenotype than those with mutations in exon 9 or at the 3' end *of APC* (Soravia, 1998; Sieber

et al. 2006). AFAP mutations often seem to result in production of an APC protein without adequate function, so that the wild type allele also needs to be mutated for tumour initiation to occur. AFAP patients may therefore have a milder phenotype than classical FAP patients because of the requirement for 'three mutational hits' rather than two (Sieber, 2006).

Extra-colonic manifestations are also associated with particular germline *APC* mutations. CHRPE is found in patients with germline mutations between codons 457 and 1444 (Fearnhead et al. 2001) and desmoid tumours are associated with mutations at codons 1445-1580 (Friedl et al. 2001). Individuals appear to be at a higher risk of developing severe duodenal disease if the germline mutation lies between codons 976 and 1067 (Galiatsatos and Foulkes, 2006). Papillary thyroid cancer has been found where germline mutations are located from codon 140 to 1309, and an increased risk of osteomas is associated with mutations after codon 1444 (Galiatsatos and Foulkes, 2006)

1.3 MUTYH-Associated Polyposis (MAP) – Clinical features

The true prevalence of MAP remains unclear, but it is thought to account for ~1% of all CRCs (Fleishman et al, 2004) and the phenotypic characteristics are still being determined. Most information on the MAP phenotype has come from studies of national or regional polyposis registers that are maintained in specialist centres. Patients can develop tens to hundreds of colorectal adenomas and colorectal cancer by the fifth or sixth decade of life, with an average age of diagnosis of CRC of 48 years (Sampson et al, 2003). The lifetime colorectal cancer risk if untreated may approach 100% (Farrington et al, 2005), with a preponderance for the right side of the colon. In a European collaborative study (Neilsen et al. 2009), 33% of the patients had at least another synchronous or metachronous CRC. One third of

individuals with bi-allelic mutations develop colorectal cancer in the absence of multiple adenomas (Croitoru et al. 2004; Tenesa et al. 2006). And only 2 cases of MAP with greater than 500 polyps have been reported (Aretz et al. 2006).The smaller number of colorectal polyps in MAP than in classical FAP is likely to signify the requirement for two rather than just one somatic mutation of *APC* for the commencement of adenoma formation, much as proposed in AFAP. In addition, co-existing hyperplastic polyps have been reported in patients with MAP. One study reported the detection of hyperplastic polyps and / or sessile serrated adenomas in 47% of MAP patients (Bopari et al. 2008). Mutational analysis of these hyperplastic polyps demonstrated G to T transversion mutations suggesting a similar mechanism of tumorigenesis as seen in MAP-associated adenomas. As there are smaller numbers of colorectal adenomas in individuals with MAP than FAP, some patients may be amenable to endoscopic removal of polyps, but if surgery is required, then an IRA is appropriate. If rectal polyposis is severe, then an IPAA is indicated (Vasen et al, 2008).

Extra-colonic features of MAP include duodenal polyps (see section 1.6.2), gastric fundal polyps, sebaceous gland adenomas and epitheliomas. MAP carries an estimated cumulative lifetime risk of duodenal cancer of 4% (Vogt et al. 2009). One case report described a MAP patient with 3 synchronous jejunal carcinomas at age 39 years (de Ferro et al. 2009), and except for 2 carcinoid tumours in the small bowel, no other small bowel carcinomas have been reported. Two patients in a European register series of 270 were found to have carcinoid tumours of the appendix (Jones, 2007). Vogt et al. also reported on the incidence of extra-intestinal manifestations in MAP patients, with 28% having at least 1 extra-intestinal tumour. A total of 110 lesions were recorded, and of those, 44 (40%) were malignant. Compared to the general population, the incidence of extra-intestinal malignancies was almost doubled in MAP patients. The types of cancers described included

breast, endometrial, ovarian, bladder and skin cancer. Benign lesions included sebaceous gland adenoma, epidermoid cysts, lipomas, and benign endometrial and breast tumours. In contrast to FAP, no osteomas or desmoids tumours were observed (Vogt et al, 2009). Key comparisons to FAP are shown in table 1.2.

	FAP	**MAP**
Mode of inheritance	Autosomal dominant	Autosomal recessive
Gene	APC	MUTYH
Primary pathway affected	Wnt signalling	Base excision repair (BER)
Number of colorectal adenomas	>100	10-100
Average age of onset of CRC	39 years	47 years
Incidence of desmoid tumours	10-15%	No reported cases
Duodenal adenomas	70-100%	17%

Table 1.2 - *Key differences in FAP and MAP*

In patients with a mono-allelic mutation in *MUTYH* (*MUTYH* heterozygotes) several studies have tried to estimate the risk of CRC. Farrington et al (2005) showed a statistically significant excess of *MUTYH* heterozygotes among CRC cases aged over 55 years, but this was not corroborated by a meta-analysis of case control studies which found only a non-significant increase in CRC (Balaguer et al, 2007). Analysis of a series of 350 obligate heterozygote parents of MAP patients from the UK, Netherlands and Germany suggests that any increase in CRC risk in *MUTYH* heterozygotes is modest (less than that for the first degree relatives of sporadic CRC patients), with no need for any regular colonoscopic surveillance (Sampson and Jones, 2009). However, Win et al (2014) described an estimated CRC risk of 7.2% for male carriers of monoallelic mutations (95% confidence interval [CI], 4.6%-11.3%) and 5.6% for female carriers of monoallelic mutations (95% CI, 3.6%-8.8%), up to 70 years of age, irrespective of family history. They conclude that the risks of

CRC for carriers of monoallelic mutations in *MUTYH* with a first-degree relative with CRC are sufficiently high to warrant more intensive screening than for the general population.

1.3.1 The *MUTYH* gene

MAP is an autosomal recessive polyposis syndrome caused by inherited mutations in the *MUTYH* gene, the human homolog of the *E. coli* mutY gene. It was first discovered in 2002 by a group in Cardiff, UK, through somatic mutation analysis of the *APC* gene in adenomas and a cancer from patients exhibiting multiple colorectal adenomas with no known germline mutation (Al-Tassan et al, 2002). This analysis revealed an excessive number of G:C→T:A transversions in APC in tumours from a single family. MUTYH has no known involvement in the Wnt pathway. Transversion mutations of G:C→T:A usually occur as a result of oxidative damage to guanine, which leads to production of the highly mutagenic 8-oxo-7,8- dihydro-2'-deoxyguanosine (8-oxo-G). It is the role of DNA glycosylases OGG1 and MUTYH to remove 8-oxo-G and the mispaired adenine respectively, during the repair of oxidative damage by base excision repair pathway (BER; discussed in section 1.3.5). Germline screening revealed bi-allelic mutations in *MUTYH* (Al-Tassan et al, 2002; Jones et al 2002). Subsequent screening of 167 unrelated patients with multiple colorectal adenomas failed to identify any bi-allelic combinations of rare alleles in other BER enzymes such as NEIL1, NEIL2 or NEIL3 (Dalloso et al, 2008). MAP is the first polyposis syndrome with a recessive mode of inheritance. In MAP tumours LOH is rare, because of the predominance of G:C→T:A changes, and mutations between the first and second 20AAR are also rare when compared to sporadic colorectal tumours (Segditsas and Tomlinson, 2006).

1.3.2 The MUTYH protein

MUTYH is a 59kDa protein when encoded by transcript α3 (Slupska et al. 1999; Tsai-Wu et al 2000) which has 41% identity to its *Escherichia coli* (*E.coli*) homologue, MutY (Slupska et al 1996). It contains functional domains, enabling interaction with other proteins and DNA. The study of MutY functional domains and their homology to the MUTYH protein has made possible the identification of several highly conserved motifs in the N-terminal domain of MUTYH. These are the DNA minor groove reading motif, helix-hairpin-helix motif (HhH), pseudo HhH motif, iron-sulphur cluster and adenine recognition motif (Guan et al 1998). Through a C-terminal binding site which contains a QXXLXXFF motif, MUTYH can also interact with proliferating cell nuclear antigen (PCNA) (Parker et al 2001). There are six recognised MUTYH serine phosphorylation sites; three in the N-terminal region (codons 9, 49 and 85) and three within the C-terminal half of the protein at codons 349, 494 and 504 (Parker et al. 2003).

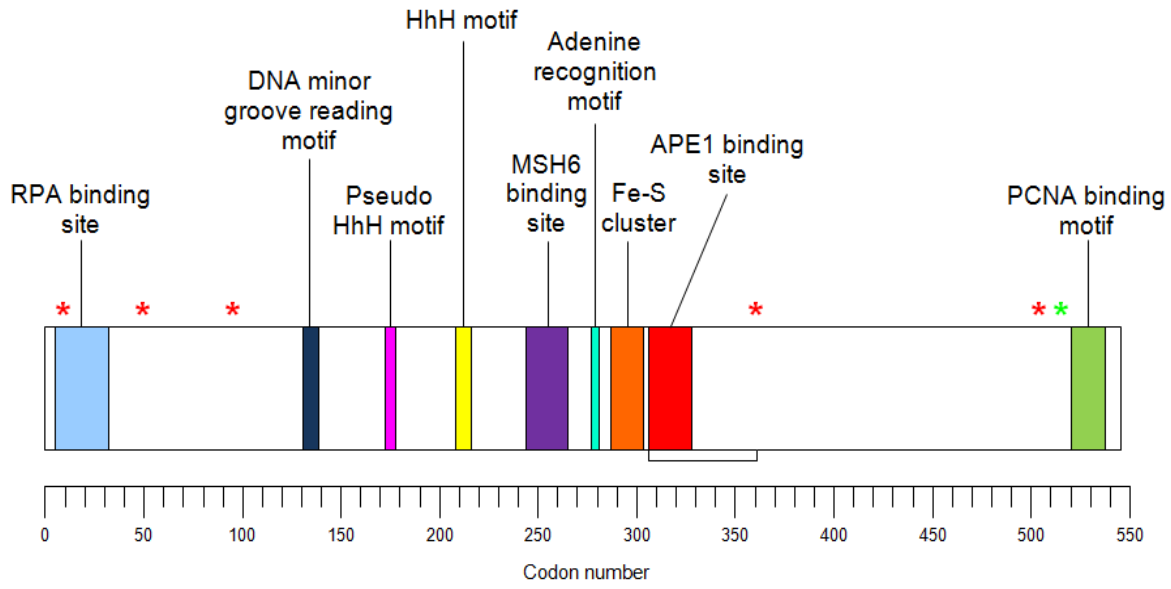


Figure 1.4 *The functional domains of the MUTYH protein* (adapted from Parker et al. 2003)

1.3.3 MUTYH Function

MUTYH is a base excision repair (BER) DNA glycosylase located on chromosome 1p34.1. It consists of 16 exons and encodes a protein 535 amino acids in length. It contributes to the maintenance of genomic stability through its role in the BER of oxidative DNA damage. As a result of oxidative DNA damage, there are approximately 10^4 lesions generated per cell, per day (Ames and Gold, 1991). Key causes of this oxidative damage are reactive oxygen species (ROS), which include hydroxyl radicals, superoxide and hydrogen peroxide, and can be by-products of cellular metabolism or secondary to environmental exposure. Substantial damage can result in the death of a cell, and partial damage can be repaired with no significant adverse outcome to the cell.

1.3.4 *MUTYH* mutations

Cheadle and Sampson (2007) previously reported 30 mutations that are predicted to produce truncated proteins in *MUTYH*, consisting of 11 nonsense, 9 small insertion/deletions and 10 splice site variants. There are currently 102 different germline mutations recorded for *MUTYH* that include 77 missense / nonsense mutations (table 1.3; Stenson et al 2014). To date, the commonest mutations result in *MUTYH* proteins containing amino acid substitutions Tyr165Cy (Y165C) and Gly382Asp (G382D), they accounts for approximately 73% of MAP patients reported in Caucasian populations (Cheadle and Sampson, 2007).

Mutation type	Total number of mutations
Missense / nonsense	77
Splicing	14
Regulatory	0
Small deletions	13
Small insertions	5
Small indels	1
Gross deletions	2
Gross insertions / duplications	1
Complex rearrangements	2
Repeat variations	0

Table 1.3 - Germline Human MUTYH variants recorded in the Human Gene Mutation database *(Stenson et al, 2014)**.** In contrast to APC where truncating mutations (small indels and nonsense) account for the majority of changes, in MUTYH the majority of mutations are missense and there are far lower numbers of gross deletions and insertions. The two genes show a very different mutational spectrum, probably related to their roles in dominant and recessive disorders respectively.*

The mutations Y165C and G382D are the most common mutations in Western populations. Their allelic frequency is estimated at 0.2 and 0.6% in the general population (Jones et al. 2009). The frequencies of other mutations have not been properly evaluated and are therefore likely to be underestimated. The frequency of mono-allelic *MUTYH* mutation carriers is estimated to be at least 1-2% in the general population (Win et al. 2011), and between 1-2 individuals out of 10 000 are thought to have bi-allelic mutations.

A specific somatic *K-RAS* mutation has been identified in some MAP adenomas and is associated with increased dysplasia. The G12C activating mutation is the result of a G:C to T:A transversion (Lipton et al. 2003; Jones et al. 2004).

1.3.5 Genotype-phenotype correlation in MAP

Farrington *et al* (2005) predicted CRC in all G393D homozygotes, but only by the age of 65 years. Biallelic Y176C mutations have been previously suggested to have a greater effect on CRC risk than biallelic G393D mutations, although the difference was not statistically significant (Tenesa et al 2006). Balaguer et al (2007) reported individuals with a Y176C mutation (homozygotes or heterozygotes) were diagnosed with CRC at a younger age and had more adenomas and right-sided CRCs than CRC patients who did not carry this mutant allele. Nielsen et al (2009) reported on the results of a multicenter study, analysing genotype and phenotype data from 257 MAP patients. Data included age at presentation of MAP, polyp count, and the occurrence, location, and age at presentation of CRC. They described patients with a homozygous G396D mutation or compound heterozygous G396D/Y179C mutations who presented later with MAP and had a significantly lower risk of developing CRC than patients with a homozygous Y179C mutation, concluding that the phenotypic effects of Y179C are relatively severe in comparison to those of G396D. The number of truncating *MUTYH* alleles a patient carries was also not found to correlate with disease severity in this study.

Intra-familial variation in phenotype has been reported (Raoof, M. 2007), suggesting that as for other colorectal cancer syndromes, additional genetic or environmental factors are modifying the MAP phenotype (Crabtree et al. 2002).

1.3.6 Base Excision Repair (BER)

The major mechanism that protects the cell against oxidative DNA damage is the multi-step BER pathway (Hazra et al, 2007), however this pathway can also repair damage that results from methylation, deamination and hydroxylation (Hoejimakers, 2009). This process is shown in Figure 1.5.

MUTYH repairs the most frequent and stable form of oxidative damage, 8-oxo-G. When an oxo-G: A mismatch is present in the next round of replication, a G→C to T→A transversion will occur (Shibutani et al, 1991). *MUTYH* recognises this mismatch and excises the undamaged adenine base. DNA polymerases can then restore an oxo-G: C. This can be acted on by another BER glycosylase, OGG1, that will then replace the oxidised guanine with a guanine. Guanine is at particular risk of oxidative damage due to its low oxidative potential (Neeley and Eissigmann, 2006).

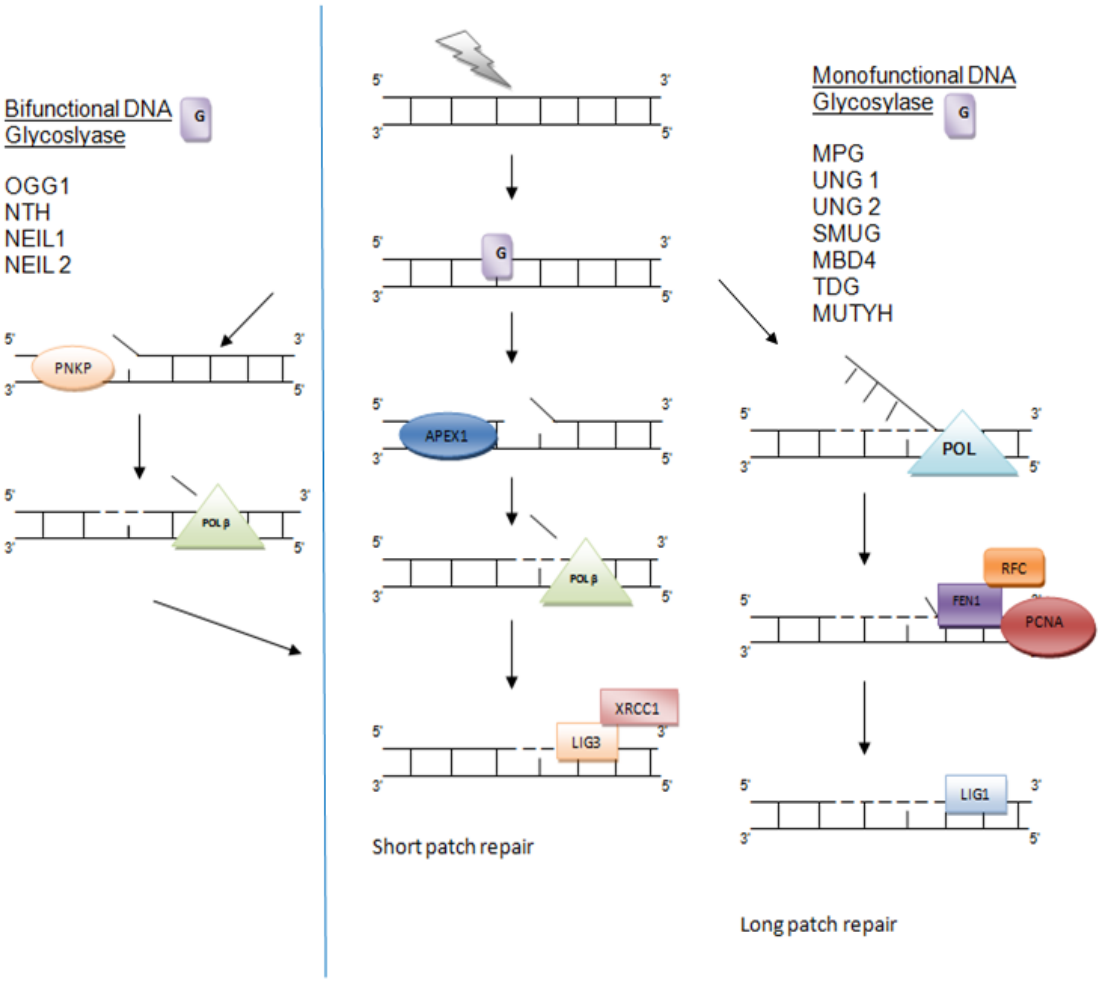


Figure 1.5- Base excision repair*. Following single base DNA damage, DNA glycosylases recognise and initiate repair by removing the damaged base. If monofunctional, such as MUTYH, it removes the base by hydrolysis of the N-glycosidic bond, 'flipping' it out of the helix. This results in an apurinic/apyruic (AP) site which is incised to form a single strand break (SSB) by AP endonuclease (APEX1). AP sites can also occur spontaneously as a result of hydrolysis (Dianov et al, 2003). A 5'deoxyribose 5'-phosphate residue (dRP) and a normal 3'hydroxyl (3'OH) group is left. If the DNA glycosylase is bifunctional, it acts to first remove the bases and then scores open the phosphodiester DNA backbone using its own DNA lyase activity. Depending on the glycosylase, and the group at the 3' end of the break, either APEX1 or polynucleotide kinase 3' phosphatise (PNKP) process the*

DNA in preparation for DNA synthesis. The pathway then follows either a long patch or short patch repair (Wilson and Bohr, 2007). Short patch repair is the most common in mammals. In short patch repair, DNA polymerase β (POLB) repairs the damaged base and removes the overhanging dRP (Matsumoto and Kim, 1995). The remaining break is sealed by an XRCC1 (X-ray repair cross complementing 1)/DNA ligase III (LIG3) complex. In long patch repair, POLB or polymerase Ɛ (POLE) elongate 2-12 nucleotides from the 3'incision site, this creates a flap (Dianov et al. 2003). A repair patch is synthesised from the damaged cell, aided by proliferating cell nuclear antigen (PCNA) and replication factor C (RFC), and the flap is then excised by flap endonuclease 1 (FEN1). The strand is then ligated by DNA ligase (LIG1).

1.4 The adenoma to carcinoma sequence

Bi-allelic mutations in APC appear to be sufficient for initiation of a tumour but the progression to malignancy requires multiple mutations in distinct genes. A model for the stages of tumourigenesis has been proposed, with the genetic events usually occurring over many years (Figure 1.6) (Vogelstein and Kinzler, 2004). Each stage results from mutations in genes involved in cell cycle control and key signalling pathways. Although different genes within the same pathway can be mutated in different colorectal cancers, only one gene in a particular pathway is usually mutated in any one CRC (Vogelstein and Kinzler, 2004). The type of genomic instability present in the adenoma or carcinoma influences the gene mutated in a specific pathway.

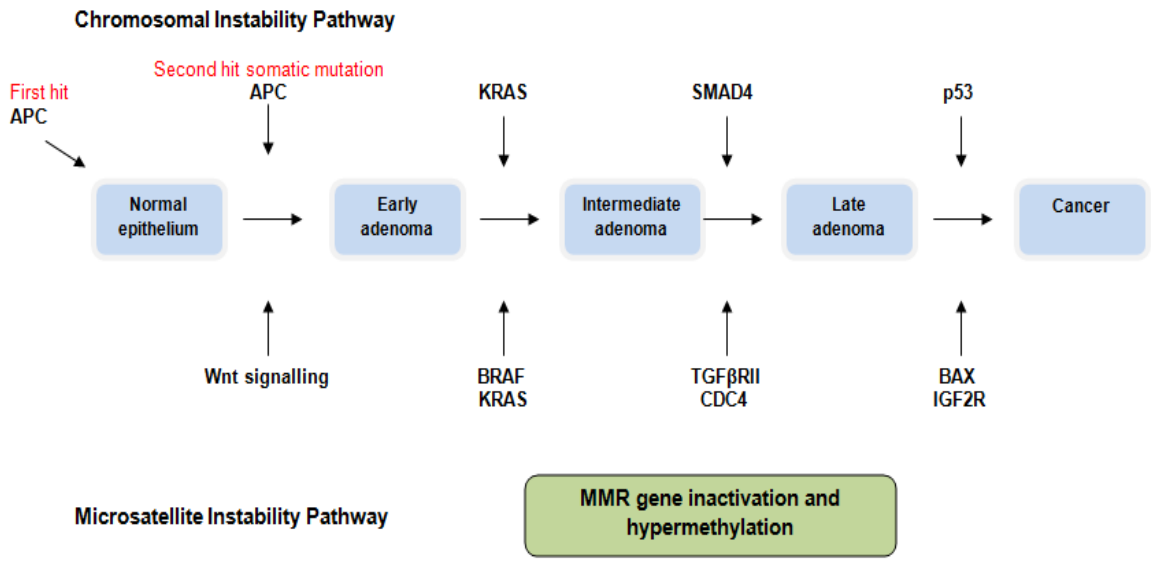


Figure 1.6 - The adenoma-carcinoma sequence pathway*. This can occur via two main routes; the chromosomal instability pathway and the microsatellite instability pathway. The first step of tumorigenesis is the formation of an adenoma.*

1.4.1 Genomic instability

Genomic instability is occurs in most cancers (Grady 2004). Major mechanisms of genomic instability in colorectal tumours include chromosomal instability (CIN) and microsatellite instability (MSI). CIN results in structural aberrations or changes in chromosome copy number whereas MSI causes genetic alterations through defective DNA mismatch repair (MMR) proteins (Söreide et al. 2006).

The alterations in CIN result from mitotic recombination or aberrant segregation of chromosomes at mitosis and lead to the aneuploidy seen in these cancers (Kinzler and Vogelstein, 1996). Allelic loss (loss of heterozygosity, LOH) at 17p, 18q and 5q often occurs and these chromosomal regions harbour the loci of *p53*, *SMAD4* and *APC* respectively (Terdiman, 2000). Deletion of 1p and 8p is also frequently seen,

but the underlying genes involved are yet to be defined. *KRAS, CTNNB1* and *PIK3CA* oncogenes are often mutated in CIN tumours (Santini et al. 2008; Sparks et al. 1998 and Samuels et al. 2004). Several studies have confirmed that CIN takes place at an early stage of tumourigenesis in both sporadic CRC (Shih et al 2001) and inherited CRC (Cardoso et al. 2006).

MSI is the next most common form of genomic instability. Microsatellites are sequences consisting of 1-5 base pairs, repeated throughout all DNA (Wheeler et al. 2000). During DNA replication, the microsatellites can mutate, requiring repair by MMR enzymes. Mutations of the genes encoding the MMR enzymes result in the accumulation of microsatellite mutations, particularly frameshift mutations (Söreide et al. 2006). A small proportion of CRCs display genomic instability that does not involve CIN or MSI (Goel et al. 2003), but show hypermethylation of promoters containing CpG islands, known as the CpG island methylator phenotype (CIMP). This instability promotes tumorigenesis by transcriptionally silencing genes that prevent tumour formation (Toyota, 1999)

1.4.2 Tumour suppressor genes

The outcome of mutations in tumour suppressor genes is an absence or inactive form of the resulting protein. Mutations that cause a truncated protein or lead to nonsense mediated decay (NMD), missense mutations that target residues essential for the proteins function, insertions, deletions or gene silencing by promoter hypermethylation (Vogelstein and Kinzler, 2004) lead to this loss of function. *APC* somatic mutations resulting in loss of function are the most common tumour suppressor gene defects observed in sporadic CRC (Groden et al. 1993). *TP53* is critical in the development of a number of cancers and is mutated in 50% of CRC cases (Hollstein et al. 1991). An inactivating mutation of *TP53* results in loss of

regulation of the cell cycle arrest and cell death. Other tumour suppressor genes involved in CRC tumourigenesis include *SMAD4* (Woodford-Richens et al, 2001), *TGFβRII* (Markowitz et al 1995), *PTEN* (Nassif et al. 2004) and *BAX* (Rampino et al. 1997). In general, both alleles of a tumour suppressor gene need to be mutated to have a tumourigenic effect.

1.4.3 Oncogenes

An oncogene is a gene that becomes constitutively active when mutated or active when the wild type gene would not be. Only one allele of an oncogene needs to be somatically mutated for it to have a tumourigenic effect. Activation can be caused by chromosomal translocations (such as CIN), gene amplifications or point mutations within a specific part of the gene that consequently affects the activity of the protein (Vogelstein and Kinzler 2004). *KRAS* is an example of a commonly mutated gene in colorectal tumourigenesis, and is found to be mutated in 40-50% of CRCs. The *KRAS* gene encodes a protein essential to signal transduction through a number of intracellular pathways and couples growth factors to the mitogen-activated protein kinase (MAPK) cascade which results in expression of genes involved in cell proliferation. The KRAS protein is a membrane-associated GTP-coupled protein that is activated by the binding of GTP enabling it to stimulate the MAPK cascade. Mutation of the KRAS gene causes persistence of the active GTP protein and continued cell division signalling results, stimulating cell proliferation (Grady and Markowitz, 2002). Other oncogenes involved in CRC tumourigenesis include *CTNNB1* (Morin et al. 1997), *BRAF* (Davies et al 2002) and *PIK3CA* (Samuels et al 2004).

1.5 The duodenum – anatomy and function

The duodenum is the first section of the small intestine, preceding the jejunum and ileum. Its position within the gastrointestinal tract is shown in figure 1.7. It is the shortest part of the small intestine measuring 25-38cm in length, often described as a C-shape or horseshoe shape, and is almost entirely retroperitoneal. Anatomically it is divided into four parts; the first part or duodenal bulb (5cm); the second or descending part (10cm); the third part (7.5cm) and the fourth part (2.5cm) that continues as the jejunum.

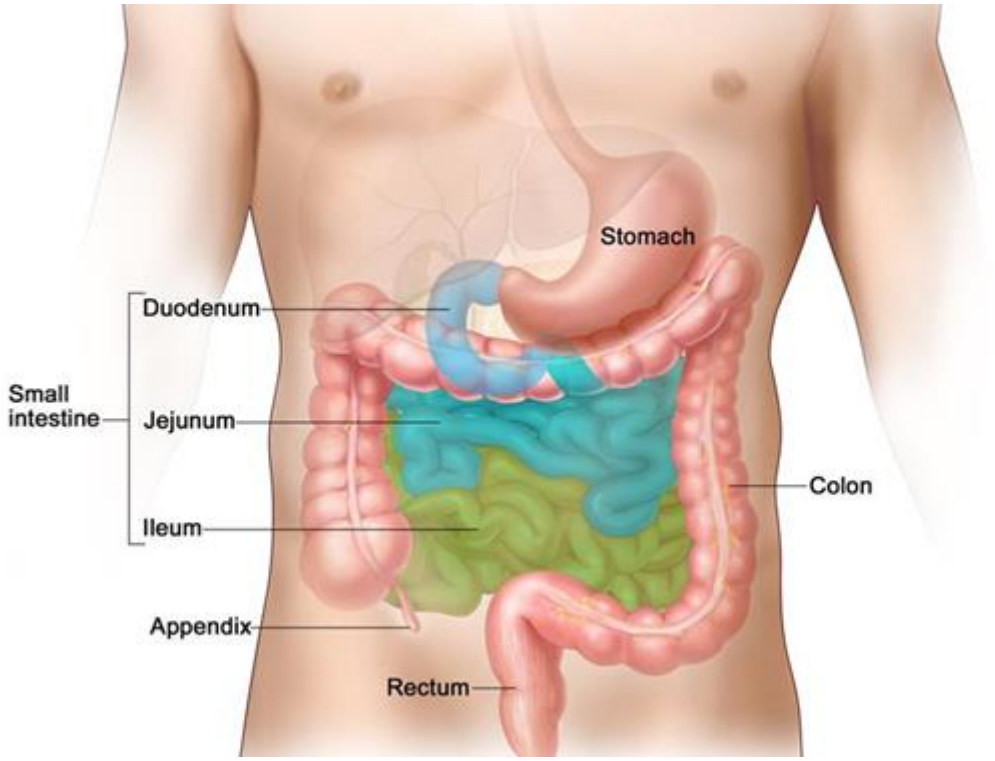


Figure 1.7 - *Schematic diagram of the gastrointestinal tract, highlighting the duodenum. www.cancer.gov*

In the second part of the duodenum the ampulla of Vater, also known as the hepatopancreatic ampulla, is formed by the union of the pancreatic duct and the common bile duct. The ampulla is specifically located at the major duodenal

papilla. The ampulla of Vater is located halfway along the second part of the duodenum and marks the anatomical transition from foregut to midgut, and hence the point where the coeliac trunk stops supplying the gut and the superior mesenteric artery takes over.

The small intestine has the same wall structure as the colon, consisting of mucosal, submucosal, muscularis and serosal layers. The first 5cm of mucosa within the duodenum is smooth, but in the second part of duodenum the mucosa which comprises a simple columnar epithelium and a lamina propria, forms finger like projections into the lumen known as villi. Deep cavities, the crypts of Lieberkuhn are found between the villi. The crypts and villi increase in size in the second and third parts of the duodenum, and increase the surface area available for the absorption of nutrients. The duodenum is largely responsible for the breakdown of food within the small intestine using enzymes, and also regulates the rate of emptying of the stomach via hormonal pathways. Secretin and cholecystokinin are released from cells within the duodenum in response to fatty and acidic stimuli from the contents of the stomach. In turn, this triggers the liver and gallbladder to release bicarbonate and digestive enzymes such as trypsin, lipase and amylase into the duodenum as required. In addition, Brunner's glands are found exclusively in the duodenum, acting to neutralise gastric acids by secreting mucous and bicarbonate.

1.5.1 Sporadic duodenal adenocarcinoma

Despite the fact that the small intestine comprises 75% of the gastrointestinal tract, small bowel cancer is rare, accounting for less than 5% of gastrointestinal cancers (Neugut et al. 1998). The duodenum is the most frequently involved segment, with 55-82% of small bowel cancer cases (Bilimoria et al. 2009). The average age onset

is in the mid 60s, with an overall incidence of between 0.01 and 0.04% (Sexe et al. 1996; Adedeji et al. 1995).

Genetic predisposition aside, other predisposing conditions associated with small bowel cancer include Crohn's disease, usually in an inflamed segment of small bowel, which may not include the duodenum, and coeliac disease which is thought to arise from immunological disruption and damage to epithelial cells that can induce premalignant change.

Studies on the pathogenesis of small bowel carcinoma are limited by the small numbers of cases, but alcohol consumption and smoking have been shown to be positively associated with an increased risk (Chow et al. 1993). Because of the marked differences in small bowel and colorectal adenocarcinoma incidence, it has been proposed that this may result from different exposure to carcinogens. Possible explanations include a shorter contact time in the small bowel between cells and dietary carcinogens, due to a shorter transit time; epithelial cells in the small bowel have a wider range of enzymes, including benzopyrene hydroxylase, which may protect cells against carcinogens (Delaunoit et al. 2005); and a higher density of gut microbiotia in the colon, where the microbiotia produce xenobiotic transformation during which bile salts are deconjugated and dehydroxylated to form desoxycholic acid which may act as a tumour promoter (Schottenfeld et al. 2009).

1.5.2 Clinical features of duodenal disease in FAP and MAP

Although duodenal adenomas are found in 30-70% of FAP patients, the cumulative lifetime risk of developing duodenal adenomas is almost 100% (Nugent et al. 1993). Data so far suggests involvement of the upper gastrointestinal (GI) tract in MAP is not as common as in FAP. However historical data on upper GI adenomas in MAP

may be an underestimation, as upper GI endoscopy was not previously routinely advised in patients with between 10 and 100 colorectal polyps. A multicentre European study reporting on the extra-intestinal manifestation in a large cohort of MAP patients found duodenal polyps in 26 of 150 (17%), however only 16 were confirmed histologically as adenomas (Vogt et al. 2009). Therefore the confirmed duodenal adenoma frequency was actually 11% in this study. Hyperplastic duodenal polyps were observed in 1 patient, and in the other 9 patients the polyp type was unknown.

The estimated lifetime risk of developing duodenal or periampullary cancer in FAP is approximately 4.5%, and between 100 and 330 times that for the general population (Offerhaus et al. 1992). The average age of diagnosis is 52 years, earlier than that of sporadic cases. In MAP, despite the lower frequency of duodenal adenomas than in FAP, the increase in relative risk and the lifetime risk of duodenal cancer (~4%) appear similar (Vogt et al. 2009). The natural history of duodenal polyposis in MAP is less well defined than that in FAP.

In FAP, adenomas can be found throughout the duodenum, but the most commonly affected sites are the second and third parts and the peri-ampullary region. Polyp distribution often mirrors mucosal exposure to bile, suggesting a role for bile in duodenal adenoma development (Spigelman et al. 1989). An excess of DNA adducts, described as chemical modifications of DNA implicated in the initiation of carcinogenesis, has been shown in duodenal biopsies from patients with FAP (Spigelman et al. 1991), supporting a role for bile in the pathogenesis of adenomas.

FAP duodenal adenomas can be small and flat or lobulated (figure 1.9), but in more advanced cases mucosal carpeting can occur when larger plaques of abnormal tissue coalesce (figure 1.10). Microscopically, the adenomas are similar to colonic

adenomas, and histologically resemble sporadic adenomas (Dominizo et al. 1990). There is little data published on the morphology of duodenal polyps in MAP, however MAP adenomas in the colon are small, tubular and tubulovillous adenomas, usually with low grade dysplasia and MAP cancers are similar in histological appearance, stage and grade to sporadic colorectal cancers but often with a lymphocytic infiltrate similar to Lynch syndrome cancers (Nielsen et al. 2009)

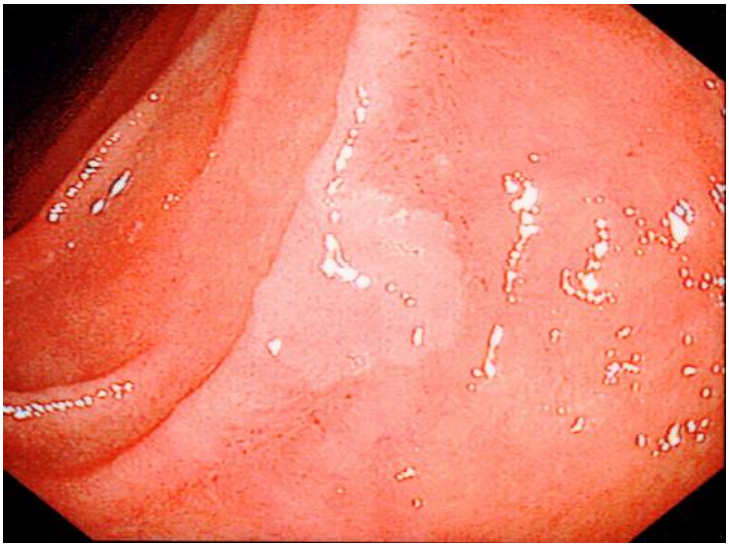


Figure 1.9 – Small flat adenoma in the duodenum of a FAP patient

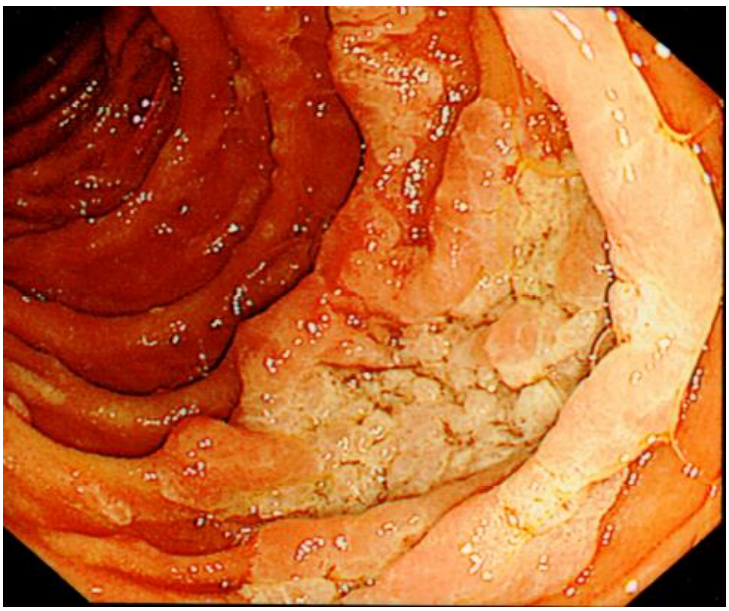


Figure 1.10 – 25mm plaque-like adenoma in the duodenum of a FAP patient

1.6 Surveillance of the upper gastrointestinal tract

A system for rating the grade of severity of duodenal adenomatosis was developed by Spigelman et al (1989), using the results of data from an upper GI screening programme in FAP which aimed to identify patients that appeared to be at higher risk of duodenal cancer and improve outcomes. It remains the most commonly used method of risk-stratification in FAP patients. Points are accumulated for size, number, histology and severity of dysplasia of polyps (table 1.4). According to the updated Vienna classification of gastrointestinal epithelial neoplasia (low grade dysplasia or high grade dysplasia) (Schlemper et al. 2000), the Spigelman score has been modified by attributing one point for low grade and three points for high grade dysplasia; removing moderate grade dysplasia as a variable within the overall point scoring system.

	1 POINT	**2 POINTS**	**3 POINTS**
No of polyps	1-4	5-20	>20
Polyp size (mm)	1-4	5-10	>10
Histology	Tubular	Tubulovillous	Villous
Dysplasia	Mild		Severe

Stage 0 = 0 points; stage I = 1-4 points; stage II = 5-6 points; stage III = 7-8 points; stage IV = 9-12 points

Table 1.4 – *Modified Spigelman classification of duodenal polyposis in FAP*

An individual's Spigelman stage is of importance because of the risk of disease progression and ultimately cancer development (discussed in chapters 2 and 3). It is also important because of the potential benefit from more intensive surveillance and early treatment. The aim of surveillance of the upper GI tract is ultimately to prevent the development of duodenal cancer. The recommended surveillance interval between upper GI endoscopic examinations therefore depends on the severity of duodenal disease and is based on our current understanding of growth rates and

outcomes. Groves et al (2002) proposed an algorithm for the management of duodenal polyposis in FAP, which has been recommended by the Mallorca group in their 2008 guidelines (Vasen et al. 2008; table 1.5). The American College of Gastroenterology recommend shorter surveillance intervals, and suggest surveillance should be repeated after 4 years in stage 0 disease; 2-3 years in stage I disease; 1-3 years in stage II and 6-12 months where there is stage III polyposis (Syngal et al. 2015). Both the Mallorca group and the American College of Gastroenterology recommend that surveillance should begin between the ages of 25 and 30 years. For MAP, as there are no prospective studies on the development of duodenal adenomas, the Mallorca group advises upper GI endoscopy also should be initiated between the ages of 25 and 30 years, and that screening intervals should depend on disease severity, following the same protocol as based on Spigelman stage for FAP.

SPIGELMAN SCORE	SURVEILLANCE INTERVAL (YEARS)
0/I	5
II	3
III	1-2
IV	Consider surgery

Table 1.5 -*Recommended upper GI endoscopic surveillance intervals in relation to Spigelman classification (Vasen et al. 2008)*

1.7 Management of Duodenal Disease

There is no consensus about how to treat patients with duodenal polyposis in FAP or in MAP. Treatment options include continued endoscopic surveillance, endoscopic therapy, surgery and pharmacologic treatment. Management is generally individualised to each patient and where disease is more advanced often

involves a multidisciplinary team, including a surgeon, endoscopist, pathologist and a radiologist. The morbidity and potential mortality associated with interventions such as endoscopic treatment or surgery must be weighed against the risk of developing duodenal adenocarcinoma. Recent studies have suggested that duodenal adenomatosis in FAP progresses slowly and sequentially through stages defined by increasing size and dysplasia towards cancer (Serrano et al. 2014), highlighting the importance of identifying and treating patients with higher stage disease in a more frequent and intensive manner. Progression of duodenal adenomatosis and risk of cancer will be discussed in detail in chapter three.

1.7.1 Lifelong endoscopic surveillance

In patients with only a few small adenomas (Spigelman stages I and II), studies have shown that the risk of developing duodenal cancer is very low (Groves et al. 2002; Lepisto et al. 2009; Bulow et al. 2011) and is outweighed by the potential risks of endoscopic or surgical intervention. In selected patients, lifelong surveillance might be indicated rather than attempts at adenoma eradication.

1.7.2 Endoscopic therapy

The rationale for endoscopic treatment of adenomas in the duodenum applies to patients with multiple larger adenomas (Spigelman stages III and IV), as their risk of duodenal cancer is higher. Data published only pertains to FAP, no studies have reported on endoscopic therapy of MAP duodenal adenomas. Endoscopic treatments available include endoscopic mucosal resection (EMR), snare excision polypectomy, thermal ablation, argon plasma coagulation (APC) and photodynamic therapy (PDT). Ablative therapies such as APC and PDT can be used to remove small or flat lesions, for polyps greater than 20mm, and for extensive ‘carpeting’

lesions either as for debulking or for treatment of recurrent adenomatous tissue. APC is also used to treat the edge of a resection after EMR as studies have shown that routine use of APC can minimise the risk of recurrent adenoma formation in the colon (Zlantanic et al. 1999). Results of the use of PDT in eradication of neoplastic lesions in the upper GI tract have not been promising (Regula et al 1995; Mlkvy et al. 1995). APC has a good technical success rate in achieving endoscopic ablation, however recurrent or persisting adenoma was reported in 12 of 16 patients (75%) in one recent study (Jaganmohan et al. 2012), with progression from tubular adenoma to tubulovillous adenoma observed in 4 patients despite ablation with APC.

Duodenal adenomas are often flat and non-polypoid lesions, which can make traditional snare polypectomy difficult. Many centres now use EMR, where a submucosal injection of saline, indigo-carmine and adrenaline solution provides a cushion under the polyp, which facilitates removal and reduces the risks of bleeding and perforation (Groves et al. 2002). However, there is an increased risk of haemorrhage following EMR in the upper GI tract as compared to EMR during colonoscopy. Much of the available literature suggests that endoscopic treatment is usually associated with a significant risk of complications and rarely guarantees a completely adenoma-free duodenum. The duodenal wall is significantly thinner than the colon, with a mean thickness of 1.6mm in healthy subjects (Nyland et al. 2012) and so the perforation rate is much higher; 1:30-1:50 in experienced hands (Bjorn Rembacken, personal communication). Other challenges include limited space within the duodenum, reducing the manoeuvrability of the endoscope and the curved shape of the duodenum, which can make access to the lesion difficult and maintenance of a stable endoscope position very difficult (Basford et al. 2014). The location of the polyp, whether near the ampulla or in the more distal duodenum, can also affect recurrence rates and complication rates. Even in experienced hands the complications associated with endoscopic papillectomy are high compared to other

endoscopic procedures and include pancreatitis, cholangitis and papillary stenosis. Han et al (2006) reported a morbidity rate of 23% and mortality rate of 0.4% when evaluating cases series of endoscopic resection of ampullary adenomas. Serrano et al (2014) reported that amongst 104 endoscopic polypectomies in patients with advanced duodenal polyposis in FAP, the complication rate was 20%, with 11 cases of bleeding, 9 cases of pancreatitis and 1 death due to severe pancreatitis. All patients in this study developed recurrence after polypectomy and ablative techniques. Other studies have reported that the recurrence rate of adenoma development after endoscopic therapy was high (50%-100%), with a 17% complication rate (including perforation, haemorrhage and pancreatitis) (Apel et al. 2005; Brosens et al. 2005). Norton and colleagues (2002) reported slightly lower recurrence rates after one year of follow up, but the series also contained patients without FAP. A recent series by Moussata et al (2014) evaluated endoscopic treatment in 35 patients with stage IV polyposis, but only where 'the adenomas appeared accessible by an endoscopic approach according to expert advice'. They reported a rate of 90% of "down- staging" to a lower Spigelman stage in addition to a high success rate of endoscopic ampullectomy, (95%) with local recurrence in 28%. However, a limitation of this study was the lack of a standardised endoscopic treatment approach and uncertainty if there was a significant effect on the overall outcomes. Ma et al (2014) also observed a recurrence rate of 58% after endoscopic ampullectomy in FAP, but there was no correlation between recurrence and resection margin, and of the 3 patients that went on to have surgery, this was not for recurrent adenomas; 2 cases were for high grade dysplasia and one was patient choice.

However, sampling error in biopsy taking and polyp removal in high Spigelman stages remains a concern. In the St Mark's and Middlesex series, 5 patients of 16 (31%) undergoing prophylactic pancreaticoduodenectomy for apparently stage IV

polyposis were subsequently found to have ampullary carcinoma that had not been detected on biopsy (Gallagher et al. 2004).

Lepisto et al (2009) demonstrated a reduction in Spigelman stage in half of patients 1-3 months after endoscopic excision (papillectomies and snare excision), which was statistically significant, the majority of patients being stage II or III initially. Thus, it may be advantageous to try to achieve local disease control in those with stage II-III disease, with the aim of delaying or avoiding radical surgery and its significant complication rates. Recently published data from St Mark's Hospital Polyposis registry (Balmforth et al. 2011) followed up 41 patients with stage IV duodenal polyposis that were down-staged to either stage III, II or I disease either by endoscopic or pharmacological therapy. They found that all patients demonstrated an increased rate of disease progression back to severe disease in comparison to reported rates of primary disease progression. This suggests that once a patient has been classified as having stage IV disease, they should forever be managed as high-risk and that the current surveillance protocol should be amended accordingly.

1.7.3 Surgical management

The surgical options for management of duodenal disease in FAP include local surgical treatment, pancreas-sparing duodenectomy and (pylorus sparing) pancreaticoduodenectomy (Whipple's procedure). There is often difficultly in deciding which surgical option is appropriate due to absence of randomised controlled trials. There is no literature available on the upper GI surgical management of MAP polyposis. Early surgical referral for patients with large duodenal adenomas, bulky ampullary disease or severe dysplasia on biopsy seems appropriate in view of reports of carcinoma detected in resection specimens in a significant number of patients undergoing supposedly prophylactic surgery

(Gallagher et al. 2006). Two patients (6%) with benign duodenal adenomatosis pre-operatively were found to have cancer following surgical resection in one series (Van Hueman et al. 2011). Local surgical treatment includes duodenotomy with polypectomy and/or ampullectomy, but results have been disappointing. Penna et al (1998) reported recurrent disease in all patients after 6-36 months in patients undergoing duodenotomy with polypectomy, with progression to stage IV polyposis after a mean of 53 months. Brosens et al (2005) reviewed the results from studies of local surgical treatment and reported a high recurrence rate after local surgery in FAP patients with severe polyposis. In the most recent study by Lepisto et al (2009), Spigelman stages had decreased in 5 patients (29.4%), increased in 2 (11.8%) and remained unchanged in 6 (35.3%) patients at the first post-operative endoscopy performed 6 months to 2 years after duodenotomy. The change in mean Spigelman stage was statistically significant. Pancreaticoduodenectomy was subsequently performed on 6 (35.3%) of patients. Despite these results, duodenotomy may have a role in patients who have one or two worrying or dominant lesions where there is an otherwise minimally involved duodenum, especially if patients are young and where the aim is to delay major surgery.

Severe polyposis (stage IV), failed endoscopic or local surgical treatment and carcinoma development are all indications for more radical surgery – either a Whipple's procedure or pancreas-sparing duodenectomy. Published data confirms low recurrence rates of polyposis within the proximal small bowel with these procedures. However, there is significant morbidity and mortality associated with a Whipple's procedure, with a mortality rate of 5% in expert centres. Immediate complications include pancreatic anastomotic failure, sepsis and bleeding as well as more chronic problems such as pancreatic endocrine insufficiency, where insulin therapy is required in 3-6%, and exocrine insufficiency seen in 30-60% of patients (Vasen et al. 1997). Although preserving the pancreatic head in a pancreas-sparing

duodenectomy may result in limited pancreatic exocrine and endocrine insufficiency, the majority of malignancies occur in the periampullary region, and may not be obvious when undertaking duodenoscopy (Gallagher et al. 2004). Of the surgical outcomes reviewed by Brosens et al (2005) there was no significant difference in recurrence rates between the two operations. Farnell et al (2000) noted that in 25 FAP and sporadic patients, there were no recurrences following either surgical method but that morbidity was higher after pancreas-sparing duodenectomy. A Dutch group have more recently reported that there was a 78% (14 of 18 patients) rate of newly formed adenomas in the neo-duodenum after duodenectomy, occurring after a mean of 46 months (Alderlieste et al. 2013). Advanced adenomas recurred in 7 patients, with 2 patients requiring further surgery. The specific choice of procedure appears to be related to local expertise and polyp location, but caution must be taken when deciding on the surgical options as a prophylactic procedure.

Other considerations include preservation of the pylorus, which decreases biliary reflux into the stomach - a risk factor for gastric polyp formation in FAP (Spigelman et al. 1991b) and the need for continued monitoring of the remaining proximal small bowel. The Mallorca Group have recommended that the Roux-en-Y be constructed in such a way that endoscopic follow up is possible as there is a risk of jejunal polyposis or jejunal carcinoma following duodenal surgery.

Prophylactic radical surgery should be reserved for well informed and carefully chosen patients, in expert regional or national centres with experience of these complex procedures, and in patients where the burden of disease is weighed against the operative risks. However, some argue that the poor prognosis of duodenal cancer may justify an aggressive prophylactic surgical approach in advanced but ‘benign’ duodenal disease.

1.7.4 Pharmacological treatment

In early duodenal disease, the risks of surgery far outweigh the risk of malignant progression. There are also those patients who are unsuitable for surgery even if they have advanced disease, due to other co-morbid conditions, and those lesions which cannot be removed endoscopically for technical reasons. Consequently, pharmacological treatment options have become a focus of ongoing research.

In 1983, sulindac (a non-selective cyclo-oxygenase inhibitor) was shown to be effective in FAP (Waddell et al. 1983), reducing the number of colorectal adenomas by greater than 50%, as well as in the retained rectal segment after colectomy. However, it did not prevent initial adenoma development in FAP (Giardiello et al. 2002). Further studies have since confirmed that non-steroidal anti-inflammatory drugs (NSAIDs) are beneficial in both sporadic and FAP-associated colorectal adenomas due to their anti-proliferative effects. Table 1.6 provides a brief summary of their mechanisms of action.

COX DEPENDENT MECHANISMS	**COX INDEPENDENT MECHANISMS**
• Inhibition of COX-1 & COX-2 enzymes • Block conversion of arachidonic acid to prostaglandins • Inhibition of prostaglandin synthesis • Disruption of cellular functions involving prostaglandins such as angiogenesis and cell proliferation. • COX-2 inhibition may induce apoptosis, via inhibition of PGE2 NB: COX-1 expressed in most tissues, COX-2 expressed in response to growth factors, lipopolysaccharide, cytokines, mitogens and tumour promoters.	• High doses of NSAIDs induce apoptosis in COX-1 or COX-2 deficient cells • Prostaglandins do not rescue cells from apoptosis • Apoptosis induced via membrane bound and mitochondrial pathway Other targets for NSAIDS: • Beta-catenin • Proteins of the Bcl-2 family • TGF-beta signalling • Peroxisome proliferator activated receptor (PPAR) family

Table 1.6 - *Proposed mechanisms of action of NSAIDs in chemoprevention*

However, these results have not been reproduced in the duodenum. Nugent et al (1993b) found no statistically significant difference in patients on 400mg sulindac daily after 6 months, although they did reveal a statistically significant effect on duodenal polyps less than 2mm. The authors reported that sulindac treatment actively reduced mucosal proliferation in both the duodenum and rectum. Cell proliferation is a well validated marker of tumour formation in the gastrointestinal tract, and an increase in the cell proliferation rate has been shown to equate with a rise in the rate of tumour formation in the colon in both animal and human studies. However the fall in mucosal proliferation was associated with significant polyp regression in the rectum, but not the duodenum. Another study of 8 patients with residual small periampullary polyps treated with 300mg sulindac daily for at least 10 months, found that no patient showed regression of adenomas (Richards et al. 1997). In addition, side effects meant that sulindac was discontinued in 3 patients.

Celecoxib, a selective cyclo-oxygenase-2 inhibitor (COX-2) has been shown to reduce the number of colorectal adenomas by 25% (Steinbach et al. 2000) and is the only agent to have been shown to be of benefit in duodenal adenomas in FAP. At a dose of 400mg twice daily, celecoxib was found to have a statistically significant effect on duodenal polyp number, but not total polyp area, with a 14% reduction in the number of polyps after 16 months compared to placebo (Phillips et al. 2002). Although COX-2 inhibitors are reported to have fewer gastrointestinal related side effects than non-selective NSAIDs, there are significant concerns about the reported cardiovascular side effects. Meta-analysis has shown rofecoxib to have an increased risk of stroke or myocardial infarction (Wallace et al. 2001) but showed that celecoxib at a dose of 200mg per day was not associated with a higher risk of cardiovascular disease. At present, data is being collected to determine the long term side effects of higher dose celecoxib use in FAP. The Mallorca group guidelines conclude that the use of celecoxib might be justifiable for patients with

severe duodenal polyposis (Spigelman stage III-IV), because the endoscopic and surgical treatment in such cases are associated with significant complications, and that COX-2 inhibitors should only be considered in those patients with no cardiovascular risk factors until more data is available.

Other agents that have been used in small trials include H2 receptor anatagonists (rantidine) (Wallace et al. 2001) and calcium with calciferol (Seow-Choen et al. 1996). None of these trials found a significant reduction in the duodenal polyp number over the study period. A recently published randomised, placebo controlled trial described the use of an enteric coated formulation of eicosapentaenoic acid (EPA), as the free fatty acid EPA-FFA in patients with a retained rectum after ileorectal anastomosis in FAP (West et al. 2010). EPA is an omega-3 polyunsaturated fatty acid which has been shown to have anticolorectal cancer activity in vitro and in pre-clinical models, although the precise mechanism of its antineoplastic action remains unclear. Polyp number, size and overall polyp burden all decreased significantly after treatment with EPA-FFA 2g daily compared with placebo over a 6 month period. This exciting development should now prompt future studies of this agent in duodenal adenomatosis. Oestrogen receptors (ERs) have been suggested as having a pivotal role in preventing malignant transformation of colon epithelial cells in humans (Weyant et al. 2001). Thus vegetable rich diets may prevent CRC due to their high content of phytoestrogens (Bjork et al. 2001). Phytoestrogens include a variety of vegetable derived compounds with oestrogen-like chemical structure, and a recent study evaluating the effect of a patented blend of phytoestrogens and indigestible and insoluble fibres (Eviendep, CM&D Pharma Limtied, UK) has been shown to reduce the number of duodenal polyps in FAP patients with IPAA (Calabrese et al. 2013). The polyp number in the duodenum reduced by 33% and size was reduced by 51%, however this was a small study totalling 11 patients.

1.8 Aims

The current study had threefold aims:

a) To investigate the impact of chromoendoscopy on the characterisation of duodenal adenomatosis in patients with MAP and compare this to patients with FAP to establish effects on Spigelman staging and clinical management.

b) To initiate a long term prospective study of duodenal disease in MAP and describe disease characteristics via a cross sectional study using initial data

c) To characterise the somatic mutational landscape in duodenal adenomatosis in MAP and FAP using genomic approaches

Chapter 2

The Role of Chromoendoscopy in the Surveillance of the Duodenum of Patients with MAP and FAP

2.1 Introduction

The Spigelman scoring system for risk stratification of duodenal polyps in FAP was developed to allow an estimation of the risk of developing duodenal carcinoma. The Mallorca group advocate the same surveillance programme for MAP in the upper GI tract (2008). In FAP approximately 80% of patients have stage I-III disease and 10-20% have stage IV disease (Vasen et al. 2008), and the risk of developing cancer in Spigelman stages III-IV is reported to be between 7-36% despite an overall cancer risk in all patients of 5%. However, recent data suggests this risk may be even higher with one recent study reporting a lifetime risk of duodenal carcinoma of 18% (Bulow et al. 2011). Studies have reported that advanced duodenal cancers have been shown to develop in 4 of 11 patients with duodenal polyps that were evaluated at stage III or less, and these patients were not diagnosed with cancer at surveillance but rather when they became symptomatic (Groves et al. 2002; Bjork et al. 2001; Nugent et al. 1995). In their 10 year prospective study Groves et al. (2002) estimated the cancer risk at 36% in patients with stage IV disease; 4 of 11 patients with Spigelman stage IV disease at initial examination went on to develop duodenal cancer. However, the progression of duodenal polyps in terms of size number and histology was slow, suggesting the transformation from adenoma to carcinomas may take longer than 15 to 20 years. Bulow et al (2004) reported a cumulative incidence of cancer of 4.5% at 57 years of age, and observed that this cancer risk was higher in patients with a Spigelman score of IV at the first endoscopy. Given this evidence, accurately identifying patients at an increased risk of harbouring or developing duodenal cancer is the principal goal. Because many patients with FAP develop duodenal polyps, yet the majority of patients do not develop invasive cancer, the clinical management of the duodenum in patients with FAP remains problematic. There is little published data on the natural history of duodenal adenomatosis and no long term studies of upper GI surveillance in MAP.

Endoscopic surveillance of the GI tract is recommended to be carried out with a forward-viewing and a side-viewing (ERCP) endoscope in order to obtain maximum views of the ampulla, stomach, duodenal bulb and second part of the duodenum; then into the third and fourth parts of the duodenum to the duodeno-jejunal junction (see figure 1.7). Conventional gastroscopes often do not adequately observe the third part of the duodenum and beyond. Saurin et al (2004) observed a progression in Spigelman score during follow up in 50% of patients, with high grade dysplasia developing in 32% of patients, a higher rate of progression that has been reported previously. A possible explanation for this may be the methodology of duodenal examination, which was carried out under general anaesthesia, allowing for a precise and comfortable examination of all parts of the duodenum. However there is no evidence from any randomised controlled trials to support this explanation. Upper GI endoscopy examines the duodenum, but does not inspect any of the small bowel beyond the ligament of Treitz. The clinical significance of small intestinal polyposis, in particular jejunal adenomas, in FAP is unclear. There have been several studies published that suggest the occurrence of small adenomas with low grade dysplasia in the proximal jejunum, with prevalence rates varying from 25-87%, but these largely are observed in patients with advanced stages of duodenal polyposis (Burke et al. 2005; Iaquinto et al. 2008; Monkemuller et al 2007). Adenomas in the jejunum and ileum are not known to occur in patients without duodenal adenomas.

Invasive endoscopic methods of surveying the whole of the small bowel include single balloon enteroscopy (SBE) and double balloon enteroscopy (DBE). Both techniques have been shown to be safe and effective; providing good quality visualisation of the proximal jejunum and detection of adenomas (Gunther al al. 2010; Yamada et al. 2014; Alderlieste et al. 2013). Videocapsule endoscopy (VCE) is a minimally invasive technique where a small capsule (25-30mm in length)

containing a video camera, white light–emitting diode illumination sources, a power source, and a radio telemetry transmitter, is swallowed by the patient in order to record thousands of high quality images as the capsule passes through the small bowel. Alderliste et al (2013) found that counting and estimating the size of polyps, identifying the junction between the duodenum and the jejunum when compared with enteroscopy was poor, with other studies reporting that the accuracy of VCE for detection of polyps within the duodenum itself as inadequate (Wong et al. 2006). Matsumoto et al. (2005) reported that DBE was superior to VCE for the detection of diminutive polyps in FAP. In contrast, Yamada et al (2014) reported a prevalence of 40% of small intestinal polyps in their study of VCE in FAP, and showed that endoscopy and VCE detected different polyps in the duodenum. This led to a conclusion that VCE may actually detect additional duodenal polyps, but the numbers included in the study were small. Current data available suggests that although the finding of small adenomas distal to the duodenum is common, progression to advanced adenomas or carcinoma is rare, and that routine small bowel surveillance is not indicated in patients with FAP, even in those patients with advanced duodenal polyposis. In particular, the role of enteroscopy may be reserved for patients with symptoms that warrant investigation of the distal small bowel or in the context of pre-operative assessment to avoid reconstruction with a segment of jejunum that has a high number of adenomas.

In the colon there is an overall polyp miss rate of 22% using white light endoscopy for sporadic polyps (van Rijn et al. 2006). Polyp detection rate in the colon is influenced by a variety of factors such as a family or personal history of CRC and polyps, and endoscopic factors such as colonoscopist technique, withdrawal time and the quality of bowel preparation. The detection or miss rate of lesions within the upper GI tract has not been as widely studied, and rarely in the duodenum. Moreover, many of the factors associated with miss rates in the colon are not

applicable to upper GI endoscopic examination. In the majority of patients undergoing upper GI endoscopy for a variety of presenting symptoms, no duodenal lesions are found (Kiesslich et al. 2003). Since the widespread adoption of the Spigelman score, there have been many developments that have led to significantly improved resolution in endoscopic images. Endoscopic techniques to optimise polyp detection rate have been sought to improve the overall miss rate of lesions during endoscopy.

Chromoendoscopy (dye spray) was first used in the stomach (Yamakawa et al. 1966) and Tada et al (1977) later described its use in the colon. There are three principal roles of chromoendoscopy: (1) to improve detection of lesions during screening or surveillance procedures, (2) to assess and distinguish non-neoplastic from neoplastic lesions and (3) to demarcate and define margins of lesions in order to delineate the extent of lesion and thus aid decision making as to endoscopic resectability.

Methylene blue and indigo carmine are the two dyes commonly used in the detection of lesions (Shim 1999). Methylene blue is actively absorbed into the intestinal epithelial cells, staining them blue and is classed as an absorptive (vital) stain. Abnormal mucosa is highlighted where there is absence of or altered uptake of dye. There have been concerns raised regarding this due to its potential to cause DNA damage to cells in vitro and in vivo, and the resulting carcinogenic effect (Davis et al. 2007). However, no published clinical evidence supports an increased malignancy risk associated with its use (Denis-Ribeiro M et al. 2008). Indigo carmine is a contrast (reactive) stain which combines a blue plant dye (indigo) and a red colouring (carmine) (Fennerty, 1994). This dye is not absorbed by the epithelial cells, but instead collects between the cells in the pits and grooves of the mucosal surface (Canto, 1999).

For lesion detection in the upper GI tract, the segment of mucosa to be examined must be clear of any pools of fluid or food residue. The dye is most commonly applied through a plastic spray catheter that is passed through the accessory channel of the gastroscope, until the tip is just seen in the lumen. A continuous stream of dye is injected by the endoscopy assistant through the catheter, during spiral withdrawal of the determined segment that is to be examined by the endoscopist (Wong Kee Song et al. 2007). An even coating of the mucosal surface is achieved by collapsing the lumen by aspiration of the carbon dioxide or air used to inflate the bowel or stomach, and then re-insufflated with any excess pools of dye aspirated to enable careful and methodical evaluation of the mucosal surface.

Within the colon, there are many studies that have examined the impact of pan-chromoendoscopy on polyp detection rates. Hurlstone et al (2004) reported significantly more adenomas identified in the dye spray (66%) versus control (33%) group in a randomised trial of 260 patients using 0.5% indigo carmine dye. They also demonstrated a higher number of flat and diminutive polyps in the chromoendoscopy group, especially in the right colon ($p< 0.05$). A Cochrane review of chromoendoscopy excluding patients with polyposis syndromes and inflammatory bowel disease concluded that chromoendoscopy identifies more patients with at least one adenoma and significantly more patients with three or more adenomas (Brown et al. 2007).

In FAP, a single study of thirteen patients compared white light endoscopy with narrow band imaging (NBI) and autofluorescence imaging (AFI) (both are types of push button technology that are able to mimic the role of dye based chromoendoscopy), and chromoendoscopy with indigo carmine dye within the colon. NBI highlights mucosal surface structures and superficial microcapillaries, which can indicate neoplastic change. A significantly greater number of lesions were identified

using chromoendoscopy than any of the other techniques ($p<0.05$) and this was particularly marked in flat polyps and those with a depressed element (Matsumoto et al. 2009). In patients with Lynch syndrome (LS), the findings are similar in the colon. The largest study to date of 109 patients confirmed significantly more adenomas and hyperplastic polyps were identified in the chromoendoscopy group compared to white light colonoscopy and NBI (Huneburg et al. 2009). This appears to be of particular significance when detecting small flat adenomas, as reported by Hurlestone et al (2005) and Lecomte et al (2005) in LS individuals.

In the duodenum, Kiesslich et al (2003) showed significantly more duodenal lesions were found with use of chromoendoscopy in consecutive patients undergoing gastroscopy; patients with familial polyposis syndromes were excluded. They also reported that significantly more targeted biopsies were possible after chromoendoscopy. Most of these lesions were gastric metaplasia within the duodenal bulb, and did not translate into any change in the endoscopic diagnosis or in the overall management strategy of the patients.

Potential applications to chromoendoscopy within the upper GI tract in FAP appear promising; the diagnostic yield of standard surveillance upper GI endoscopy was demonstrated to be improved by dye spraying alone in a small study of 10 patients with FAP undergoing upper GI endoscopic surveillance (Picasso et al. 2007). A more recent study of 43 patients has also demonstrated chromoendoscopy increases the detection of duodenal adenomas in FAP, but did not induce a significant change in Spigelman stage (Dekker et al. 2009).

The effect of chromoendoscopy on the yield of duodenal adenoma detection in MAP has not been previously investigated. This study aims to evaluate the use of dye-spray with indigo carmine in the duodenum to improve the identification of small

polyps that may be overlooked in MAP during standard white light endoscopic examination. It also aims to determine the impact on the Spigelman stage, and to compare this to individuals with FAP.

2.2 Methods

The research was approved by the South-East Wales research ethics committee in January 2010, reference number: 10-MRE09-43. Cases for the study were recruited prospectively. Patients with confirmed FAP or MAP on genetic testing were recruited from gastroenterology and genetics clinics at the University Hospital Llandough, Cardiff and the University Hospital of Wales Institute of Medical Genetics, Cardiff. The Institute of Medical Genetics upholds a register of patients in Wales with polyposis syndromes. In addition, patients with MAP were recruited from St Marks Hospital, Harrow and St Mary's Hospital, Manchester. There were no healthy volunteer controls recruited for the study. Between August 2011 and December 2013, 51 consecutive patients who were genetically verified with MAP and FAP and scheduled for surveillance endoscopy of the upper GI tract were invited to participate in the study. The endoscopies were performed in two academic centres in the United Kingdom (University Hospital Llandough in Penarth and St Mark's Hospital in London). All participants completed a consent form prior to participation (appendix 1 of supplementary electronic data).

At each centre, experienced endoscopists performed all endoscopies for this study (SD, AH, NS and S T-G). All gastrodudoenoscopies were performed using high resolution forward viewing video endoscopes (GIF-Q260, GIF-H260, GIF XQ260; Olympus Medical Systems) and a side viewing videoendoscope if the ampulla was not adequately visualised with the forward viewing videoendoscope. Procedures were performed under general anaesthetic or conscious sedation using standard

doses of fentanyl and midazolam in line with British Society of Gastroenterology guidance (Teague 2003) depending on patient preference and the presence of co-morbid conditions. Antispasmodic medication (Buscopan) was given during endoscopy at the discretion of the endoscopist.

At introduction, the forward viewing endoscope was advanced until the duodenojejunal junction was reached. During withdrawal, the different parts of the duodenum (D2, D3 and duodenal bulb) were evaluated and the number and sizes of polyps recorded on a standardised proforma before staining (figure 2.1). Polyp size was estimated using Radial Jaw 3 biopsy forceps (Boston Scientific, Natick, USA), with a closed diameter of 2.2mm and an open diameter of 8mm. The endoscopist then sprayed a 0.3% solution of indigo carmine (3mls of indigo carmine and 7mls of water for injection) from D4 proximally to the duodenal bulb onto the duodenal mucosa, distributed in a homogenous fashion by a spraying catheter, passed through the endoscope channel. The residual dye was then suctioned away. After adequate coating of the duodenum with the dye solution, I independently recorded the size and number of polyps (being unaware of the first part of the examination) (figure 2.2). Biopsy samples were not taken until after staining and counting had taken place. Samples were taken from all lesions with high-grade morphology and lesions greater than 1cm. If the number of polyps was small, all lesions were sampled, but if there were numerous polyps at least three of the largest adenomas were biopsied in addition to the criteria above. To further aid accurate staging of duodenal disease, the patients were then examined using a side viewing videoendoscope to assess the ampulla if the forward view was not adequate. If it was adenomatous, a biopsy was taken. All procedures were conducted using the same structural and colour enhancement settings in each centre.

Figure 2.1 *Duodenum of FAP patient pre-chromoendoscopy*

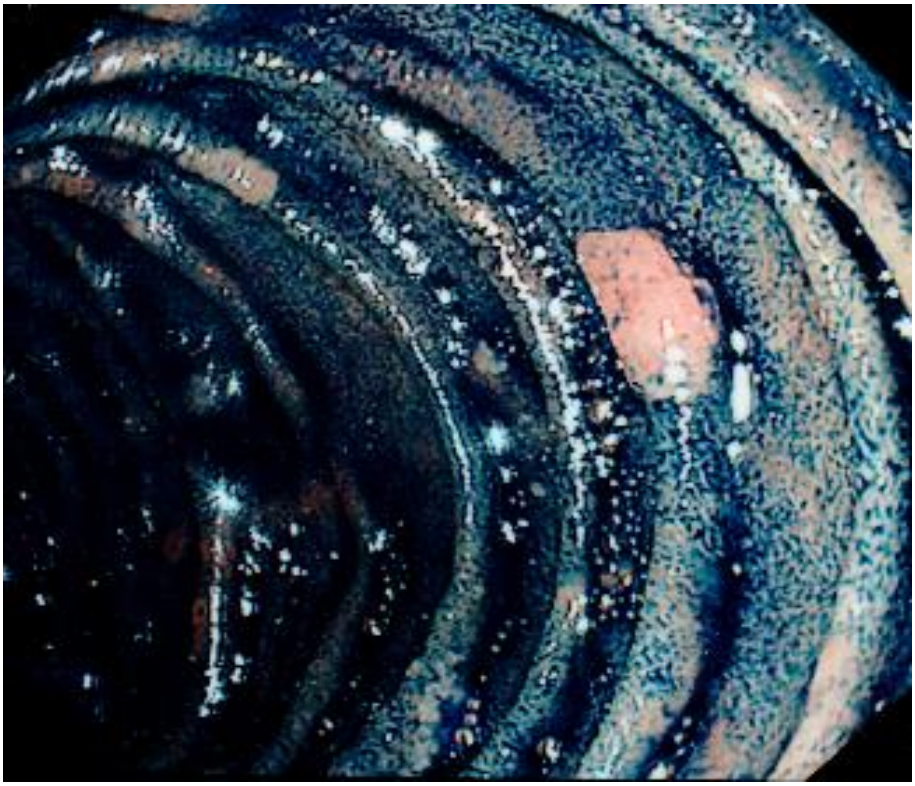


Figure 2.2 *Duodenum of the same patient with FAP post-chromoendoscopy*

The biopsies were evaluated by expert gastrointestinal pathologists at both centres. For all patients Spigelman point totals and stage before and after the application of chromoendoscopy were assessed. The following criteria were used: number of adenomas, largest size, the most advanced histology and most advanced grade of dysplasia. Only histologically confirmed adenomas were included in the analysis.

2.2.1 Statistical analysis

Statistical analysis for the study was performed using R (version 3.0.2) software. Statistical significance for the frequency of duodenal adenomas was calculated using the Wilcoxon signed-rank test and Mann-Whitney U test and a one-sided sign test was used for comparing the Spigelman stages. A *P* value of less than 0.05 was considered statistically significant. The study was powered for 92% at a 5% significance level (1 degree of freedom using a chi squared test to compare the 2 conditions). Statistical data are expressed as medians.

2.3 Results

Between August 2011 and December 2013, 51 patients (19 FAP and 32 MAP) underwent gastrodudoenoscopies (table 2.1). Of the FAP patients, 8 were female and 11 male; median age 41 years (32-69). There were 17 female patients with MAP and 15 male patients with MAP; median age 54 years (25-81). No patient was on any pharmacological treatments for their duodenal disease. There were no complications relating to endoscopic examination (bleeding or perforation) and no complications relating to general anaesthesia or sedation were observed.

Case	FAP or MAP	Age	Sex	No. of polyps white light	Additional no. of polyps after staining	No. of polyp >1cm	Stage before	Stage after
1	MAP	43	F	0	0	0	0	0
2	MAP	55	M	0	0	0	0	0
3	MAP	45	M	0	0	0	0	0
4	MAP	59	F	0	1	0	0	I
5	MAP	56	M	0	0	0	0	0
6	MAP	68	F	0	0	0	0	0
7	MAP	71	M	0	0	0	0	0
8	MAP	81	F	0	0	0	0	0
9	MAP	69	F	0	0	0	0	0
10	MAP	74	M	0	0	0	0	0
11	MAP	46	F	0	0	0	0	0
12	MAP	60	M	6	9	2	III	III
13	MAP	46	F	0	1	0	0	II
14	MAP	25	M	0	0	0	0	0
15	MAP	62	F	1	2	0	I	II
16	MAP	25	F	0	0	0	0	0
17	MAP	75	M	0	0	0	0	0
18	MAP	61	F	1	0	0	II	II
19	MAP	54	F	1	1	0	II	II
20	MAP	48	M	1	2	1	II	II
21	MAP	49	F	1	1	1	II	II
22	MAP	40	M	0	0	0	0	0
23	MAP	54	F	0	3	0	0	II
24	MAP	29	F	0	0	0	0	0
25	MAP	60	M	0	0	0	0	0
26	MAP	57	M	0	0	0	0	0
27	MAP	49	F	0	0	0	0	0
28	MAP	42	M	0	4	0	0	II
29	MAP	49	F	0	0	0	0	0
30	MAP	54	M	0	0	0	0	0
31	MAP	48	F	0	0	0	0	0
32	MAP	52	M	0	0	0	0	0
33	FAP	32	F	0	0	0	0	0
34	FAP	52	M	0	0	0	0	0
35	FAP	43	M	7	10	0	III	III
36	FAP	37	M	24	32	5	IV	IV
37	FAP	44	M	1	0	0	I	I
38	FAP	38	F	18	19	0	II	III
39	FAP	69	M	6	15	0	II	II
40	FAP	36	M	3	3	0	II	II
41	FAP	44	F	0	0	0	0	0
42	FAP	38	M	0	0	0	0	0
43	FAP	41	F	46	64	3	IV	IV
44	FAP	39	F	14	65	2	III	III
45	FAP	32	F	0	8	0	0	II
46	FAP	39	M	10	51	4	III	IV
47	FAP	46	F	4	12	0	I	II
48	FAP	31	M	0	0	0	0	0
49	FAP	46	M	0	0	0	0	0
50	FAP	42	M	10	4	0	IV	IV
51	FAP	49	F	7	19	0	II	III

Table 2.1 *Patient characteristics, polyp numbers and Spigelman scores pre- and post- chromoendoscopy*

The difference in the ages of the patients in the FAP group compared to the MAP group was statistically significant (*P*=0.001). There was no difference in the calculated overall Spigelman stage pre or post dye spraying for any patient when comparing the traditional with the modified Spigelman score.

2.3.1 Number of adenomas

In MAP patients before staining, the median number of adenomas detected per patient was 0 (total 10, range 0-6). After staining, the median number did not change (total 34, range 0-15), however the increase in detected adenomas was significant (table 2.2). Additional duodenal adenomas were detected in 9 (28%) of procedures. The median number of additional adenomas detected was two per patient (total 22, range 1-9).

Gastroduodenoscopy findings	Pre-staining	Post-staining	*P* value
Median number of duodenal adenomas (total)	0 (10)	0 (33)	0.01368*
Median largest size (range)	5mm (2-50mm)	6mm (2-50mm)	0.3711
Median Spigelman stage (range)	0 (0-III)	0 (0-III)	0.03125*

Table 2.2 - *Duodenal adenoma characteristic in patients with MAP (n = 32). * indicates statistically significant result (P < 0.05). Despite no change in the median Spigelman scores, the Mann-Whiney and Wilcoxon tests are rank sum tests and not median tests. It is possible for groups to have different rank sums and yet have equal or nearly equal medians.*

The median number of adenomas in the FAP cohort, per patient, was 4 (total 150, range 0-46). After staining the median number of adenomas that was detected was 14 per patient (total 442, range 0-100), (p=0.002) (table 2.3). Additional adenomas after chromoendoscopy were detected in 13 patients (63% of FAP cases).

Gastroduodenoscopy findings	**Pre-staining**	**Post-staining**	***P* value**
Median number of duodenal adenomas (total)	4 (150)	14 (442)	0.002516*
Median largest size (range)	6mm (2-23mm)	6mm (2-30mm)	0.1814
Median Spigelman stage (range)	II (0-IV)	II (0-IV)	0.03125*

Table 2.3 - *Duodenal adenoma characteristic in patients with FAP (n = 19). * indicates statistically significant with (P < 0.05).*

The number of duodenal adenomas observed post-staining was significantly higher in FAP than MAP (*P* = 0.0002452; Mann–Whitney U test).The post-staining Spigelman stage is significantly higher in FAP versus MAP (*P* = 0.0009646; Mann–Whitney U test).

2.3.2 Size of adenomas

The median largest adenoma size was 5mm compared with 6mm after staining in MAP, and the size of the largest adenomas (15mm, 15mm, 25mm and 50mm) did not change after dye-spraying with indigo-carmine.

In FAP there was also no statistically significant increase in the median adenoma size after staining. However, whilst the largest adenoma did increase by 23mm to 30mm after staining this did not affect the individual patient's Spigelman points total or stage (IV). No significant statistical difference was observed between the post-staining size of largest duodenal adenomas observed in MAP versus FAP ($P \approx 1$; Mann–Whitney U test). There was no significant statistical difference in the overall numbers of adenomas greater than 1cm in patients with MAP versus FAP pre or post staining ($P \approx 1$; Mann–Whitney U test).

2.3.3 Histology of adenomas

In all of the MAP patients, the worst histological diagnosis was tubular adenoma, and the most advanced grade of dysplasia was low grade (when reviewed by a GI pathologist, 3 patients had moderate grade dysplasia). In FAP there were tubular adenomas in 8 (42%) patients, tubulovillous adenomas in 4 (21%) patients and villous adenoma in 1 (5%) patient. Four of the FAP patients had moderate dysplasia on second review by a GI pathologist, and there was no high grade dysplasia detected.

2.3.4 Endoscopic technique

Using the side-viewing endoscope did not detect any additional ampullary adenomas compared with the forward viewing endoscope, and only one patient had an ampullary adenoma detected in this study. Biopsies confirmed a tubular adenoma with low grade dysplasia. One patient was observed to have four further polyps detected by side-viewing endoscopy after dye-spraying and counting.

2.3.5 Spigelman staging

Prior to dye spraying, the Spigelman classification was stage 0 in 27 patients (84%) stage I in 2 patients (6.25%), stage II in 2 patients (6.25%) and stage III in 1 patient (3.5%) with MAP (table 2.4). Staining resulted in an increased Spigelman point total in 9/32 individuals (28%), with a corresponding upgrade in Spigelman stage in 6 patients (18%) (from 0→I, n=1; from 0→II, n= 3; from I→II, n=2; $p<0.05$). In FAP patients staining resulted in an increased Spigelman point total in 13/19 individuals (68%), with a corresponding upgrade in Spigelman stage in 5 patients (26%) (from 0→I, n=1; from I→II, n= 1; from I→IIII, n=2; from III→IV, n=1; $p<0.05$).

	Present series MAP (n=32)		**Present series FAP (n=19)**		**Previous case series (Dekker E et al, 2009 N=43)**	
	Before staining	**After staining**	**Before staining**	**After staining**	**Before staining**	**After staining**
Spigelman stage						
0	27 (84%)	23(72%)	7 (37%)	6 (32%)	3 (7%)	2 (4%)
I	2 (6.25%)	1 (3.5%)	2 (10%)	1 (5%)	2 (4%)	2 (4%)
II	2 (6.25%)	7 (22%)	4 (21%)	4 (21%)	11 (26%)	10 (23%)
III	1 (3.5%)	1 (3.5%)	3 (16%)	4 (21%)	14 (33%)	15 (36%)
IV	0	0	3 (16%)	4 (21%)	13 (30%)	14 (33%)

Table 2.4 - *Spectrum of Spigelman stages in FAP and MAP patients before and after staining, compared with a previous case series of FAP patients*

The spectrum of Spigelman stages expressed as percentages of the total scores after staining for the present study and for patients with FAP (n=43) (Dekker et al. 2009) and a case series of 35 FAP patients undergoing duodenal surveillance endoscopy using indigo carmine dye spray (Saurin et al. 2004) is shown in figure 2.1. The mean age of the patients in the Saurin study was 37 years (SD +/- 10.2).

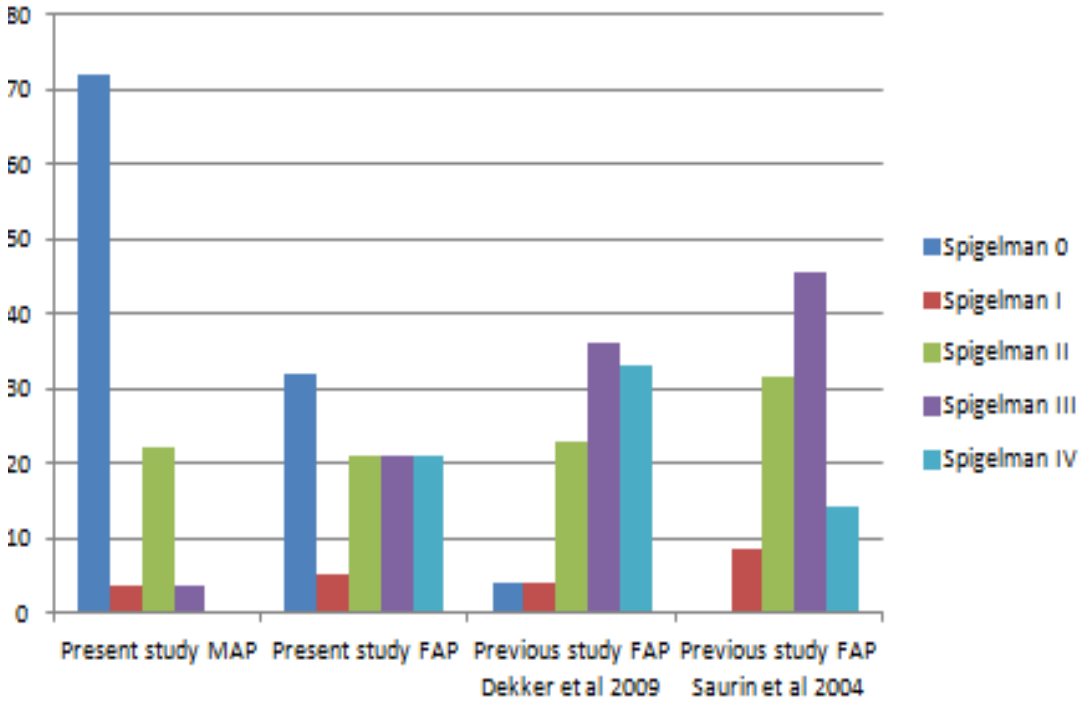


Figure 2.1 - *Comparison of Spigelman stages (in percentages) in MAP and FAP compared to two previous studies of FAP where dye-spraying the duodenum was used to assess duodenal polyposis.*

2.4 Discussion

This novel study describes the largest prospective series of MAP patients in which duodenal adenomas are described and the additional value of chromoendoscopy for the evaluation of duodenal adenomas is assessed. It demonstrates that there is a significant increase in the numbers of duodenal adenomas identified following indigo carmine dye spraying in both MAP and FAP, and that this resulted in a significant increase in Spigelman stage in both conditions. This study has also shown a higher proportion of duodenal adenomas in MAP patients (28%) compared to previously reported data from a large multicentre European study where duodenal adenomas were found in 17% of patients (Vogt et al. 2009).

2.4.1 Number of adenomas

This study has shown that the number of duodenal adenomas detected was increased in both MAP and FAP patient cohorts following chromoendoscopy with indigo-carmine dye spray.

Two previous studies have assessed adenoma number whilst investigating the role of chromoendoscopy in the duodenum in FAP. Picasso et al (2007) studied 10 patients undergoing upper GI surveillance and found a statistically significant increase in the number of duodenal polyps following chromoendoscopy ($p=0.03$), revealing additional polyps in eight of the ten patients. Unlike this current study, the overall change in Spigelman stage was not assessed, so the implications for future surveillance unknown. Dekker et al (2009) studied 43 patients with FAP and showed that significantly more duodenal adenomas were detected after the application of indigo carmine but this did not result in a significant change in Spigelman stage or result in any major additional clinical consequences. Interestingly, of the 43 patients only 26 had an APC mutation that had previously been identified; the other 17

patients all had undergone colectomy because of >100 histologically confirmed colorectal adenomas.

One limitation of the Dekker study was that the endoscopist was not blinded to the number of adenomas prior to staining, which may have biased the results. The current study was designed to blind the second 'counting' endoscopist but there remains the possibility of a systematic bias due to counting differences between the two endoscopists. Both previous studies are in agreement with our findings in FAP, but the increased adenoma detection in MAP has never been studied and is a novel finding.

2.4.2 Size of adenomas

This study has shown no significant effect of chromoendoscopy on the sizing of adenomas in either MAP or FAP; however the study was not powered to detect any apparent differences in size. Dekker et al (2009) reported that the largest adenomas detected at chromoendoscopy were significantly larger than before staining. This was considered to be due to better visualisation of the adenoma borders that extended further than could originally be seen. Interestingly, Picasso et al (2007) reported that the size of the polyps actually were smaller after dye spraying, with a median size of 4mm pre-chromoendoscopy compared to 3mm post-chromoendoscopy ($p=0.03$), also explained by more precise demarcation from normal tissue. However, more lesions may look larger, but smaller lesions detected would result in the maximum diameter to increase but the median size overall to be smaller. This current study of chromoendoscopy also compares to NBI in showing no additional effect on size of chromoendoscopy in the FAP duodenum (Lopez-Ceron et al. 2013). Generally, in studies of the outcomes of surgical intervention in FAP, it is the patients Spigelman stage that has been reported rather than the sizes

of lesions harbouring cancer (Saurin et al. 2004; Van Heuman et al. 2011; Bulow et al. 2011; Serrano et al. 2014). In their prospective 10 year study, Groves et al (2002) reported on six patients that progressed from duodenal adenoma to carcinoma. Four patients had initial Spigelman stage IV, with one patient stage III and one stage II disease. The maximum size of the largest polyp ranged from 2mm (in the stage II patient) to between 20mm and 40mm in the other five patients with stage III and IV disease and none had HGD on biopsy. Saurin et al (2004) found high grade dysplasia in all but one of adenomas that were greater than 1cm, and Lopez-Ceron et al (2013) found that when using NBI for detection of duodenal adenomas, the only trait that was significantly associated with advanced histology was an estimated polyp size of greater than 1cm, with a three-fold increased risk. Although high Spigelman stages do not necessarily imply advanced histological lesions, Saurin et al (2004) reported an original Spigelman score equal to or greater than 7 or 8 was predictive of HGD development. However, the Spigelman score does not take into account very large adenoma sizes, using 1cm as the cut-off in risk stratifying patients, with polyps 20mm or above in size conferring no additional points. A modification to the classification to place more emphasis on adenoma size might improve the predictive value of the scoring system.

HGD can be found in lesions less than 1cm, and size may be only one predictor of high risk adenomas. The focus of adenoma detection may need to be altered to distinguish particular lesions at high risk from their endoscopic appearance. One small study of 14 patients showed potential for NBI to identify ampullary lesions with advanced dysplasia (Uchiyama et al. 2006), but this was not confirmed in a subsequent larger study (Lopez-Ceron et al. 2013).

2.4.3 Histology of adenomas

One observation of this current study is that chromoendoscopy did not detect the presence of any endoscopic features of advanced histology, such as a loss of surface pit pattern. The current study used the updated Vienna classification and thus reported only on low grade or high grade dysplasia, but a specialist GI histopathologist also reviewed all the slides for moderate grade dysplasia. The Spigelman scores were re-calculated for any patient found to have moderate grade dysplasia, however this had no effect on any patient's overall Spigelman stage. Given the lack data on the natural history of duodenal disease MAP it may not be prudent to await the endoscopic appearance of high grade dysplasia in these patients.

A previous study of confocal laser endomicroscopy (CLE) - a technology that provides 1000x magnification imaging - failed to show superiority over magnifying NBI on the correct predication of histological grade of ampullary and non-ampullary adenomas in FAP but it was found to be better than HRE (Pittayanon et al. 2013). However, they were not able to predict HGD in these lesions. Use of endoscopic ultrasound (EUS) has been shown to upstage patients to advanced adenomas of the ampulla by providing an improved assessment of polyp dimensions including identifying growth into the papilla (Gluck et al. 2015). The treatment course was altered in 36% of patients by performing EUS, the effect of which was an increase in lesion size where EUS augmented previous endoscopic size estimate to more than 10mm in 12 of 28 patients, including 2 cases that were defined as microadenomas. Biopsy specimens were not taken at EUS, and there could be no assessment of surface morphology of the lesions. Further evaluation of advanced surveillance techniques and their optimum use, perhaps in combination, in duodenal disease in FAP and MAP is required.

2.4.4 Endoscopic technique

Dekker et al (2009) attributed the minor change in Spigelman score in their study to the use of high resolution endoscopy. They concluded that considerably improved endoscopic visualisation of duodenal adenomatosis with high resolution endoscopy leaves little room for further improvement of clinical consequence. However, a major limitation of the Dekker study was that the endoscopist was not blinded to the number of adenomas prior to staining, unlike the current study. A previous study has suggested that in patients that have duodenal disease progression, both time lapse and technical improvements were determinant factors (Mathus-Vliegen et al. 2011). In a mixed-model analysis, time-lapse, change in image resolution to high resolution technology and dysplasia ranking contributed consistently to an increased Spigelman score and stage. This suggests visibility and histology may result in an over-estimation of the clinical significance of duodenal polyposis by using the Spigelman system. However, other studies appear to demonstrate a lack of correlation between the findings seen at endoscopy and pathology results from biopsy specimens, and between progressive structural changes within the polyps and the pathology findings (Moozar et al. 2002) . This led the authors to argue that the overall aim of identifying patients who are at high risk of developing duodenal and ampullary cancer remains problematic. The importance of the polyp number, polyp size and even histology may need to be revised within the risk stratification model, but as yet no such alternative scoring system has been proposed. Further research is required to prospectively validate the Spigelman classification in the risk stratification of duodenal polyposis.

In a large previous case series where high resolution endoscopy was not used in the upper GI surveillance of FAP patients (Bulow et al. 2004) there was a large percentage of patients with Spigelman stage 0 (34%) and a much smaller percentage of patients with stage IV disease (7%). This could be due to the fact that

high resolution endoscopy leads to an improved adenoma detection rate. The present study found that with use of high resolution endoscopy, for the FAP cohort 31% had Spigelman stage 0 disease which is more in keeping with studies that did not use this technology. This may be explained by the small numbers in the FAP cohort (n=19). Compared to the study by Dekker et al (2009), this current study has a larger proportion of Spigelman stage 0 disease, but although the distributions of Spigelman stages II-IV are not mirrored, both studies have the majority of their cohort observed to be stage II -IV. When compared to the only other published series where indigo-carmine dye spraying was used in duodenal surveillance (Saurin et al. 2004) interestingly no patients were reported to have with Spigelman stage 0 disease. When this group of 35 patients were followed up over a median of 47 months, 42.8% of patients were found to have Spigelman stage IV disease. It must be borne in mind, however that 7 of these patients reported by Saurin et al. (2004) were referred to the study centre with severe duodenal polyposis, which may have introduced bias to the overall data.

A recent pilot study (Pittayanon et al. 2013) demonstrated that magnifying NBI was able to distinguish between adenomas and non-adenomas better than high definition endoscopy in all 29 duodenal lesions studied in 14 FAP patients (P=0.003). Lopez-Ceron et al. (2013) are the only group that have evaluated the effect of NBI (following examination with high resolution endoscopy) on the detection rates of duodenal adenomas in patients with FAP. In contrast to this current study they found that in their study group of 37 patients, there was no clinically relevant upgrade in the Spigelman classification using NBI. They also concluded that there was no improvement in the detection of gastric polyps using NBI. More duodenal adenomas were detected in 16 examinations (35.6%), leading to an upgrade in the score in 5 patients but only an increase in Spigelman stage in two patients (2.2%) which was not statistically significant. The Spigelman stage in one patient increased

from II to III because of a higher score for number and size of polyps, and in the other patient the stage increased from III to IV solely due to increased polyp number.

This current study supports the use of chromoendoscopy over NBI in the improvement of adenoma detection in duodenal polyposis, but further studies are needed to confirm this.

The technique of chromoendoscopy itself is time-consuming and requires training. For example, for surveillance colonoscopy in inflammatory bowel disease, the British Society of Gastroenterology guidelines (2010) recommend a 3 point time allocation instead of a 2 point allocation for colonoscopy in order to provide enough time for the technique of dye-spraying (pan-colonic chromoendoscopy) and for careful inspection of the colonic mucosa. This would impact waiting times, and patients may prefer a prolonged procedure to be undertaken under general anaesthesia rather than in the endoscopy department, with resulting need for theatre time, staff and anaesthetic support. In addition, the upstaging of patients Spigelman scores would also have resource implications, as patients would require more frequent surveillance endoscopies.

2.4.5 Spigelman Staging

This study had an expected finding of a higher number of duodenal adenomas observed post-staining in FAP compared to MAP, with a post-staining Spigelman stage significantly higher in FAP. In MAP, the significant change in Spigelman score following dye spraying is of clinical importance because of the impact of more intensive duodenal surveillance programmes. Four MAP patients in this study who were Spigelman stage 0 pre-chromoendoscopy became stage I or II after dye

spraying, accounting for 44% of MAP patients that had adenomas detected in this study. Those MAP patients who have developed duodenal cancer have done so on a background of minimal duodenal polyps (Nielsen et al. 2009), in contrast to those who have developed duodenal cancer in FAP, suggesting that the acquisition of different mutations or a greater mutational load in MAP polyps. The MAP-associated DNA repair defect may drive a more rapid progression to cancer. Thus improved adenoma detection rates seen in MAP following chromoendoscopy has the potential to be clinically useful.

The ages of patients with MAP in this study were significantly higher than that of the FAP patients. The numbers of adenomas were higher in the FAP group pre and post staining, but interestingly no significant statistical difference was observed between the post-staining size of largest duodenal adenomas observed in MAP versus FAP and there was no significant statistical difference in the overall numbers of adenomas greater than 1cm in patients with MAP versus FAP. In FAP, it is time since diagnosis, age and Spigelman stage at initial endoscopy that have been found to be determining features of the severity of duodenal polyposis (Saurin et al. 2002; Saurin et al 2004; Bulow et al. 2004; Vasen et al. 2008). These variables may not apply for MAP. As MAP was formally characterised in 2003, most patients in this study were relatively recently diagnosed compared to FAP. Our study suggests that the effect of increasing age on the number of polyps is not the same for MAP as it is for FAP. Lepisto et al. (2009) described a risk of severe dysplasia or cancer of 5.7% at 40 years, 15.2% at 50 years and 23.2% at 60 years, with a cumulative incidence of duodenal cancer of 34% at 75 years in their FAP cohort, which was not reflected in our small group of MAP patients. The natural history of duodenal polyposis in MAP is a topic that requires further study.

In this present study, in patients with FAP there was no change in Spigelman stage IV disease after dye-spraying, suggesting where there are greater than 20 small adenomas seen with white light endoscopy, detecting more adenomas is of limited benefit and that other factors may play a role in the malignant progression in duodenal adenomatosis. Whether detection of multiple small polyps ultimately influences the natural history of carcinoma development in FAP or MAP has yet to be determined.

2.4.6 Study limitations

A limitation of this study is the inability to determine the additional value of chromoendoscopy in the assessment of ampullary adenomas; because examination of this area with the side viewing endoscope was done after dye spaying had taken place. In addition, we were unable to systematically assess the utility of side-viewing endoscopy itself on the number of polyps in the peri-ampullary region, although data has shown that in a previous case series, ampullary adenomas were seen in 66% of cases with a side viewing endoscope (Burke et al. 1999). This study did not detect a large number of ampullary adenomas (only two patients had ampullary adenomas), which also may have caused bias as the majority of malignancies occur in the periampullary region. Also, no lesions with HGD were detected and our series may not be representative of the FAP and MAP population. In their prospective five nation study of the long term natural history duodenal adenomas in FAP, Bulow et al (2004) reported that 12% of adenomas were diagnosed only histologically, where no visible polyps were seen. This study did not incorporate the taking of routine biopsies of the background duodenal mucosa to exclude dysplasia and relied on the visualisation of precisely demarcated adenomatous tissue as morphological polyps. Picasso et al (2007) did take random

biopsies in their study, of mucosal folds in the second and upper third part of the duodenum, including the papilla, but found no additional adenomatous tissue. Dekker et al (2009) did not include the taking of random background biopsies in their protocol. In their study of the role of HRE and NBI in the evaluation of duodenal polyps Lopez-Ceron et al (2013) did not take any random biopsies. Of note, although not part of our study protocol, in all of the MAP patients included from the Cardiff cohort without visible polyps, background duodenal mucosal biopsies were taken. No patient had dysplasia reported on histology.

2.4.7 Conclusions

This study demonstrates that chromoendoscopy of the duodenum enhances adenoma detection in both MAP and FAP. In both conditions there was a significant increase in Spigelman stage after chromoendoscopy and therefore clinical consequence to the patient in terms of follow up according to current management guidelines. As screen-detected duodenal cancers have been shown to have a much better prognosis than symptomatic cancers in FAP - 8 years after a screen detected cancer versus 0.8 years (Bulow et al. 2011), there is a strong argument for regular endoscopic surveillance. However, there are shortcomings in applying the same surveillance programme to MAP as in FAP because of a paucity of knowledge of the risk of malignant progression in MAP duodenal adenomas, with a need for further studies to focus on this question.

Chapter 3

The clinical spectrum of duodenal adenomatosis in MAP: A European cross-sectional study

3.1 Introduction

Although there have been no randomised controlled trials of surveillance of the upper GI tract versus no surveillance in FAP, screening of the duodenum may lead to the identification of patients with advanced but asymptomatic disease and therefore to treatment that may lead to the reduction of duodenal cancer related mortality (Vasen et al. 2008). Bulow et al (2011) recently showed a considerable improvement in prognosis of selected patients in a 20 year prospective follow up of patients undergoing a surveillance programme based on Spigelman classification combined with prophylactic surgery for advanced but benign disease. Duodenal polyposis is seen less frequently in MAP than FAP, occurring up to 17% of patients in the largest study to date (Vogt et al. 2009; table 3.1) and there is no current evidence to support adoption of the same surveillance approach. Indeed reports of a small number of MAP patients that have developed cancer suggest that may have done so with low Spigelman scores that would have resulted in an inappropriately extended surveillance interval when following recommendations for FAP (Nielsen et al, 2005; Nielsen et al, 2006; Stomorken et al, 2006; Croitoru et al. 2007; Beucher et al 2008).

Study	Number of patients with duodenal adenomas (%)
Sieber et al. (2003)	2 of 24 (8.3%)
Nielsen et al. (2005)	4 of 16 (25%)
Lejeune et al (2006)	3 of 39 (7.7%)
Bouguen et al (2007)	1 of 56 (1.8%)
Vogt et al (2009)	26 of 150 (17%)
Walton et al (2014)	13 of 34 (38%)

Table 3.1 – *Published studies reporting duodenal adenomas in MAP*

The use of a staging system has aided in a better understanding of the natural history of duodenal polyposis in FAP and the risk of duodenal cancer. Retrospective studies had shown a low frequency of progression with time (Burke et al. 1999; Matsumoto et al. 2000), but more recent studies have shown high rates of progression (table 3.2), and the development of HGD in 32% of patients (Saurin et al. 2004). As discussed in section 2.1, prospective studies of duodenal surveillance (Groves et al. 2002; Bulow et al. 2004, 2011; Saurin et al. 2004) have shown a cumulative risk of the most advanced stage (IV) of duodenal polyposis to be between 35 and 52%, and this risk worsens substantially with increasing age. Progression to stage IV disease appears to carry a one in three risk of subsequent duodenal cancer (Groves et al. 2002). By comparison, the natural history of duodenal polyposis in MAP remains unclear.

A better understanding of the natural history and development of duodenal adenomas in MAP may result in the surveillance strategy for upper GI disease being improved and rendered more clinically effective. A long-term prospective collaborative study was therefore established to collect data on the results of duodenal surveillance in MAP to better describe the endoscopic, morphological and histological features of duodenal adenomas in patients with MAP and to determine the effects intervention including endoscopic or surgical treatment.

Characteristic	Saurin et al (2004)	Bjork et al (2001)	Groves et al (2002)	Nugent et al (1994)	Burke et al (1999)	Matsumoto et al (2000)	Lepisto et al (2009)	Bulow et al (2011)	Serrano et al (2014)
Study type	P	R	P	P	R	R	R	P	P
No of subjects	35	180	99	70	114	18	129	304	218
Mean age (years)	37	-	42	42	-	-	41	38	-
Male sex, %	57.1	-	55.2	55.7	-	38.8	49		49
Mean follow up (months)	47.9	72	-	40	51	196	102	168	-
Stage progression, % of patients	40.0	-	16.6	14.3	-	-	34	44	32.5
Stage IV polyposis, %									
Initial examination	14	7.8	9.6	14.3	-	-	1.5	4.9	8
Final examination	35	-	14.0	17.1	-	-	12.4	-	30
Number of Invasive carcinomas	0	5	6	3	1	0	5	20	5

Table 3.2 - *Studies of progression of duodenal polyposis in FAP. P=prospective, R=retrospective study design*.

3.2 Methods

A collaborative project was established between the Institute of Medical Genetics (IMG) Cardiff, UK; The Centre of Human and Clinical Genetics, Leiden, the Netherlands; St Marks Polyposis Registry in London, UK and two centres in France; Lyon and Paris, resulting in a cohort of 207 MAP patients with identified biallelic *MUTYH* mutations. These patients were identified from prospectively maintained polyposis registry databases, and data on upper GI surveillance procedures was retrospectively collected using medical notes, endoscopy reports and histopathology reports. Only those patients that had undergone a previous surveillance upper GI endoscopy as part of their routine clinical management were included in the study. The study was approved in the UK by the South-East Wales research ethics committee in January 2012, reference number: 11-WA/0208. Informed consent was obtained according to protocols approved by the appropriate national and / or local ethics committees. A copy of the data sheet used for information collection can be found in appendix 2 in the electronic supplementary information.

3.2.1 Genotype-phenotype analysis

To examine potential genotype-phenotype correlations of duodenal disease in MAP, *MUTYH* genotypes were classified as described in previous studies (Nielsen et al. 2009, Vogt et al. 2009). Genotypes were classified into six groups: (1) homozygotes for a non-truncating mutation, (2) compound heterozygotes for a non truncating and a truncating mutation, (3) homozygotes for a truncating mutation, (4) G396D homozygotes, (5) G396 / Y179C compound heterozygotes, and (6) Y179C homozygotes. Separate analyses for other biallelic combinations were not done, due to small numbers of corresponding MAP patients.

3.2.2 Statistical analysis

Values of median and range are provided for non-normally distributed data. Categorical data are presented with absolute numbers and percentages. The change in mean Spigelman class after treatment was analysed by the Wilcoxon signed rank test, and the cumulative incidence of adenomas by age was calculated.

To compare the number of polyps between the different genotypes, a one way ANOVA with Tukey post hoc testing was performed. A *P* value of less than 0.05 was considered statistically significant. Statistical analysis for the study was performed using R (version 3.0.2)

3.3 Results

The study included 207 patients from five countries (77 from the UK, 63 from The Netherlands 52 from France and 15 from Spain) with a median follow up of 5 years (range 0.5 to 25) years. The sex distribution of patients was 112 males and 95 females.

Of the 206 patients with complete clinical data, 100 had been diagnosed with colorectal cancer (48.5%) prior to the commencement of upper gastrointestinal surveillance. The presence of other malignancies is shown in table 3.3.

Data on all patients and their surveillance endoscopies can be found in appendix 3 of the electronic supplementary information.

Cancer type	Number of patients
Endometrial cancer	3
Breast cancer	2
Prostate cancer	2
Lymphoma	1
Jejunal cancer	1
Duodenal neuroendocrine tumour	1

Table 3.3 *- Retrospective identification of other malignant manifestations in 207 MAP cases*

At the index endoscopy the median age was 51 years (range 21-81 years), and the Spigelman stage distribution was: stage 0, *n*=169 (82%); stage I, *n*=25 (12%); stage II, n=6 (3%); stage IV, *n*=0; duodenal adenocarcinoma, *n*=1 (0.5%). The incidence of adenomas at first endoscopy was 17% (table 3.4), median age 50.5 years (range 21-70 years). Seventy-seven (37%) only had one endoscopy. In the total study period, 63 patients (30%) were found to have histologically confirmed duodenal adenomas, and the median age at which duodenal adenomas occurred was 51 years (range 21-70 years). Random biopsies showed adenomatous tissue in one patient without visible polyps at endoscopy.

Age range	Number of patients	Percentage overall	Percentage of those with adenomas
21-25	1	0.5%	3%
26-30	0	0	0
31-35	1	0.5%	3%
36-40	4	2%	11%
41-45	2	1%	6%
46-50	9	4.5%	25%
51-55	8	4%	22%
56-60	4	2%	11%
61-65	4	2%	11%
65-70	3	1.5%	8%
71-75	0	0	0
75-80	0	0	0

Table 3.4 *– Ages of those with duodenal adenomas at first endoscopy*

Of those that went on to have further surveillance endoscopy (*n*=130), the Spigelman stage distribution at last endoscopy was: stage 0, *n*=88; stage I, *n*=13; stage II, n=12; stage III, *n*= 11; stage IV, *n*=4; duodenal adenocarcinoma, *n*=2. Among those followed up that did not have evidence of adenomatosis at the beginning of the study the cumulative incidence of adenoma development was 21.5%. Forty patients out of 130 that underwent repeat surveillance endoscopy had adenomas at the end of the study, a cumulative incidence of 30% by age 70 years, and the cumulative incidence of Spigelman stage IV disease was 3%. The progression of duodenal adenomatosis is shown in figure 3.1. The median age at last endoscopy was 56 years (range 29-84 years).

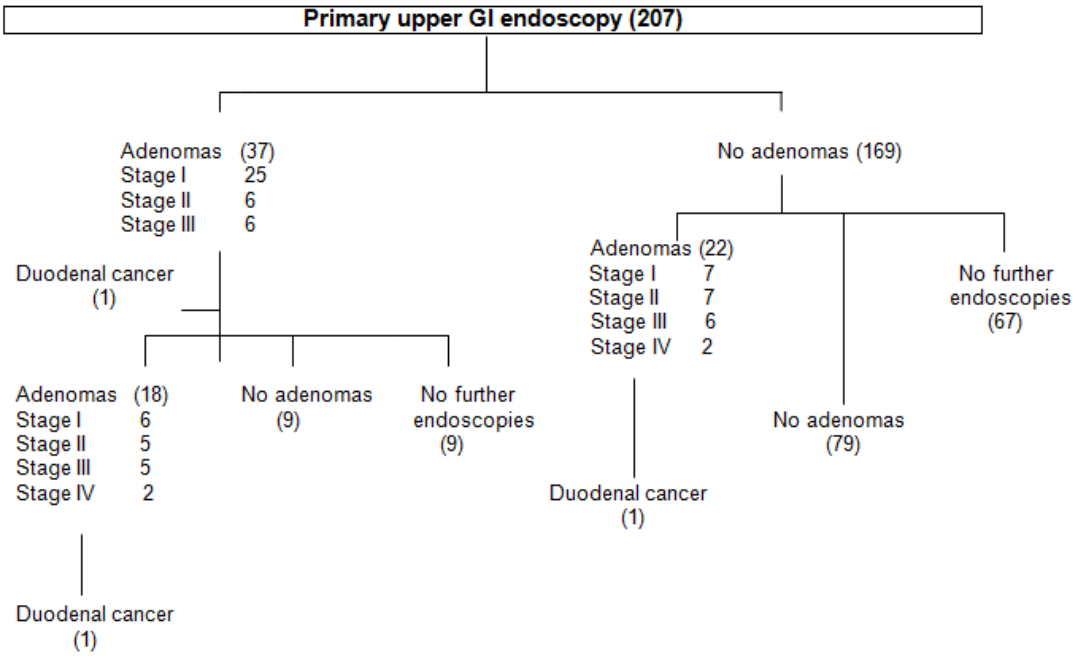
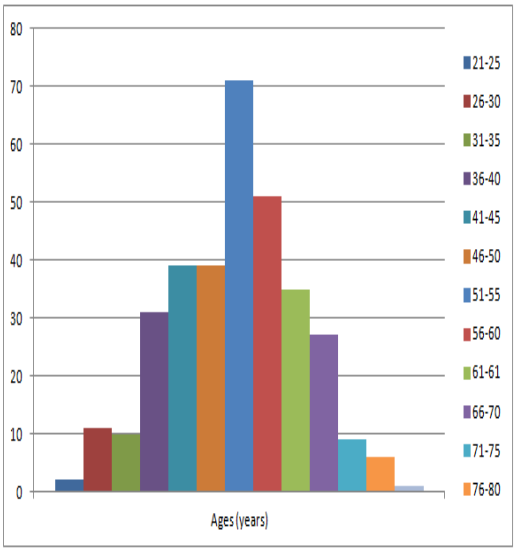


Figure 3.1 *– Progression of duodenal adenomatosis in 207 patients with MAP from primary endoscopy to most recent endoscopy in the study period.*

Of 169 patients that had no adenomas at first endoscopy, 22 (13%) went on to develop adenomas identified at a later endoscopy, 79 (47%) had a normal endoscopy and 67 (40%) had no further endoscopies. The median age of onset for those that developed adenomas was 54 years (range 41- 73 years). A total of 532 surveillance upper GI endoscopies were undertaken during the study period. The median number of endoscopies undertaken in this study was 2 (range 1-13). The distribution of age and Spigelman scores at endoscopy is illustrated in figures 3.2.

A



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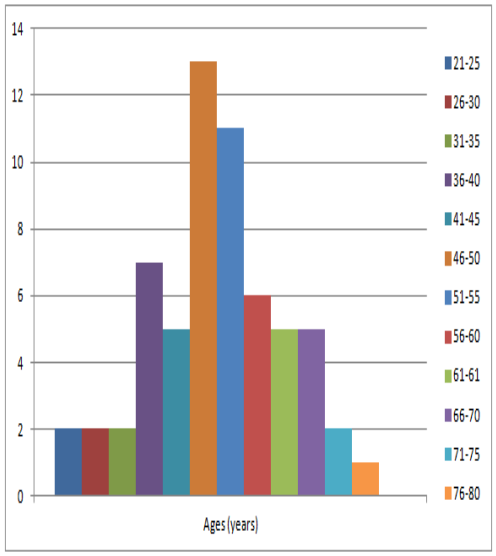

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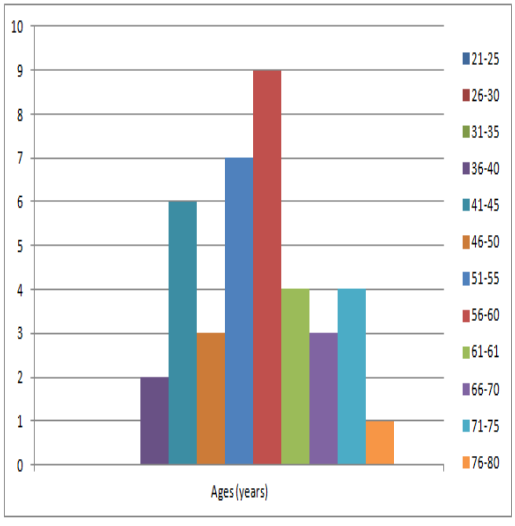

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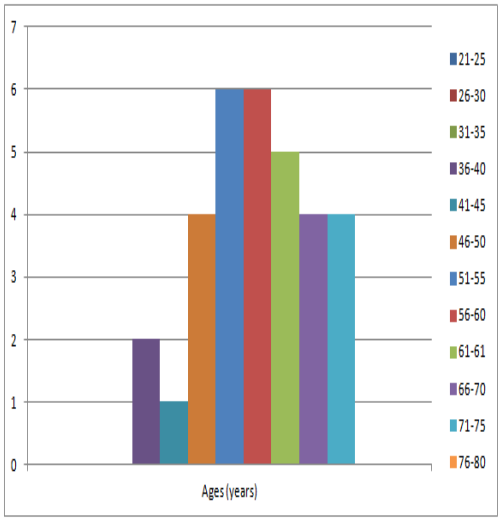

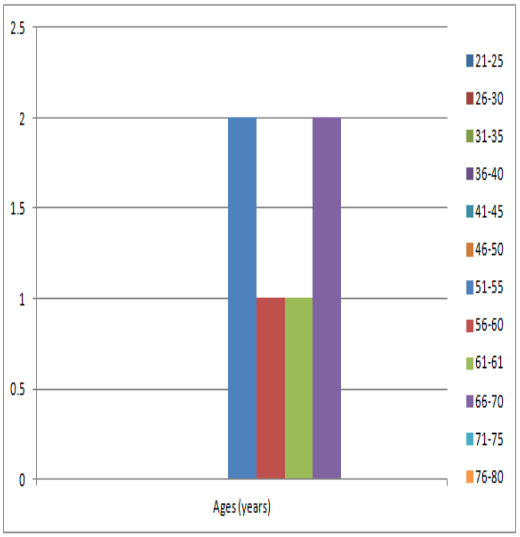

E

Figure 3.2 *- Age distribution of all surveillance endoscopies by Spigelman stage. A= Spigelman stage 0, B= Spigelman stage I, C= Spigelman stage II, D- Spigelman stage III and E= Spigelman stage IV*

The cumulative incidence of adenomatosis at age 50 years was 9%; at age 60 years, 22% and by age 70 years, 30% in this study. Of those patients that underwent repeat endoscopies, 24% of patients progressed to a higher Spigelman score. Two of 22 patients with stage 0 disease at first OGD progressed to stage IV, and 2 of 37 patients with stage I, II or II disease at OGD progressed to stage IV. There was a statistically significant difference (p= 0.041) in the time of adenoma progression when comparing those that were Spigelman stage 0 to those that were observed to have adenomas at first endoscopy. Among those developing stage IV disease, the median time from first endoscopy to stage IV disease was 6.5 years. Nine patients (24%) that had adenomas at first endoscopy were found to have Spigelman stage 0 at follow up, this subgroup of patients had not undergone endoscopic therapy, but diagnostic biopsy only. Details of stage progression are shown in table 3.5.

Details of stage progression	Duration observed (years)
0 → I	4
0 → I	3
0 → I	3
0 → I	5
0 → I	7
0 → I	12
0 → 0 →0 → 0 →0→ I	6
0 → I → II	13
0 → I → II → II	11
0 → 0→ 0 →0 → II	14
0 → 0 →0 →0→ II →II	17
0 → II	1
0 → II	3
0 → II	18
0 → II→ III	5
0 → III	3
0 → III	3
0 → III	8
0 → 0 → III	3
0 → 0 → III	10
0 → IV	3
0 → IV	7
I → II	1
I → II	1
I → II	4
I → II	8
I → III	1
I → III	1
I → III	2
III → III → III → III → IV	6
III → III → III → IV→ IV	8

Table 3.5 - *Progression of Spigelman stages*

In total, 22 patients underwent therapeutic endoscopic treatment. Thirteen patients underwent endoscopic mucosal resection of adenomas greater than 1cm, two underwent argon plasma coagulation (APC) destruction of adenomas, two patients underwent a combination of EMR and APC destruction, three patients underwent conventional biopsy removal of small polyps in combination with EMR, and one patient underwent biopsy removal of all small polyps <5mm. Two of the patients were noted to have high grade dysplasia in the biopsy specimen; one had a recurrence of high grade dysplasia after endoscopic treatment, the other patient developed high grade dysplasia on a background of Spigelman stage III disease

treated by EMR. This patient had been on sulindac therapy for over 10 years prior to the development of high grade dysplasia.

After endoscopic therapy, the Spigelman stage decreased in 17 patients (77%), remained unchanged in 3 patients (14%) and worsened in one patient (4.5%). Follow up endoscopy has yet to be carried out in one patient. At the latest endoscopy, the Spigelman distribution in these 17 patients was: stage 0, 7 patients; stage I, 4 patients; stage II, 3 patients and stage III, 3 patients. The change in the mean Spigelman class after endoscopic therapy was statistically significant (p = 0.0003).

Five patients (13.5%) had an adenoma with high grade dysplasia at first endoscopy, and 5 (9%) of patients with low grade dysplasia or no polyps at previous endoscopy went on to develop an adenoma with high grade dysplasia.

Three patients (1.5%) had a duodenal adenocarcinoma at a median age of 64 years (range 46 – 66 years). One was diagnosed at index endoscopy. The other two cases were asymptomatic interval cancers (‘screen detected’). One patient had a normal endoscopy (Spigelman 0) 11 years prior to diagnosis of cancer, the other patient developed cancer 1 year after Spigelman stage III adenomatosis. The cumulative incidence of developing duodenal adenocarcinoma in this study was 2.3%. The proportion of patients with adenomas that developed duodenal cancer in this study was 4.5%.

The median number of adenomas per patient across the study period was 2.5 (range 1-32 adenomas). Four patients (7%) of those with adenomas had an adenoma size of greater than 2cm (2cm, 2.5cm, 3cm and 3cm) and 67% of patients were found to have between 1-4 adenomas in total. Nine polyps involved the

ampulla, and of these 7 were <1cm in size. The majority of polyps were in the second part of the duodenum and spared the ampulla, one patient had polyps detected in the jejunum. Three of the four patients with Spigelman stage IV disease had 1 adenoma, 1 adenoma and 2 adenomas respectively all 20mm or greater in size and all with histological confirmation of high grade dysplasia. The other patient had 20 adenomas, the largest being 10mm, and all low grade dysplasia with the most advanced type being a tubulovillous adenoma.

Of those patients that had details of mutational status information available, it was possible to classify the genotypes into 6 groups (figure 3.3): 1) homozygotes for a non truncating mutation, *n*= 56; (2) compound heterozygotes for a non truncating and a truncating lesion, *n*=12; (3) homozygotes for a truncating mutation, *n*=37; (4) G396D homozygotes, *n*=30; (5) G396 / Y179C compound heterozygotes, *n*=28 and (6) Y179C homozygotes, *n*= 25.

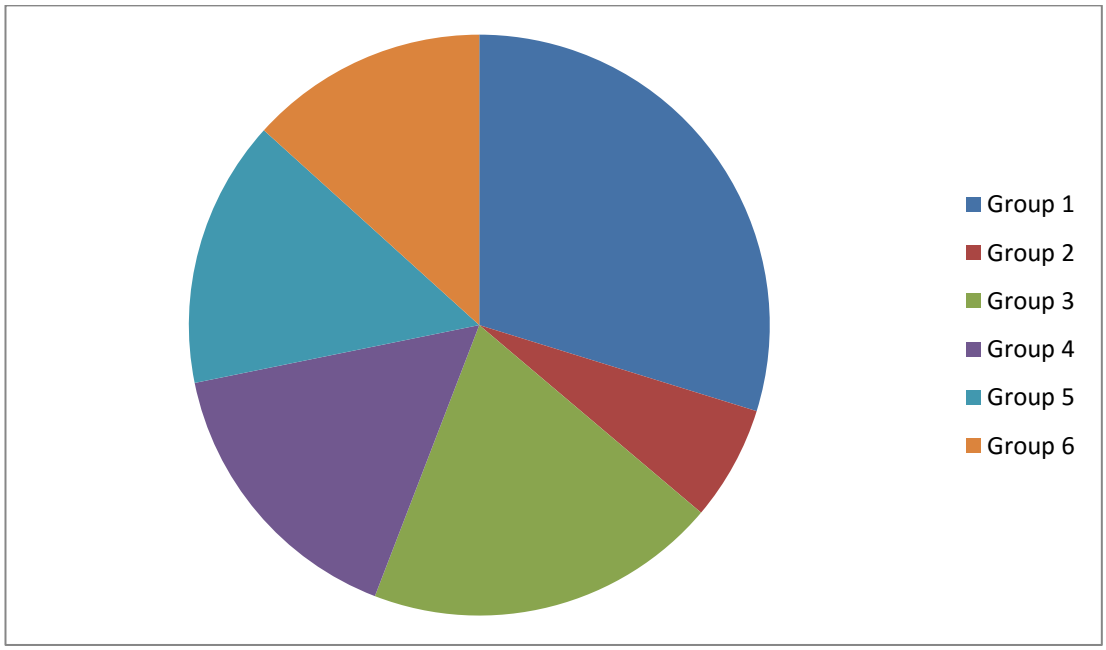

Figure 3.3 *– Proportions of different genotype groups for MAP*

Table 3.6 presents this data in relation to adenoma numbers and Spigelman stages.

Genotype	n	Median age at last endoscopy (range)	Total number of adenomas observed	Median number of adenomas per patient (range)	Median Spigelman score (range)
No truncating mutation	56	54.5 years (29-79)	94	0 (0-19)	0 (0-III)
One truncating mutation	12	53 years (46-79)	8	0 (0-3)	0 (0-II)
Two truncating mutations	37	51.5 years (27-74)	32	0 (0-8)	0 (0-III)
G396D / G396D	30	55 years (37-75)	2	0 (0-2)	0 (0-I)
G396D / Y179C	28	59 years (37-79)	21	0 (0-8)	0 (0-III)
Y179C / Y179C	25	52 years (29-75)	92	1 (0-19)	0 (0-III)

Table 3.6 - *Adenoma numbers and Spigelman stages in patients undergoing upper GI surveillance endoscopy by genotype*.

The mean polyp numbers differ significantly across some of the different genotype groups (*P*=0.00062; One-way ANOVA). There was a significant difference in the number polyps in the cohort with the Y179C homozygote genotype in comparison to those patients with two truncating mutations (*P* = 0.0076983), Y179C homozygotes and G396C homozygotes (*P* = 0.0003410) and Y179C homozygotes and G396D / Y179C heterozygotes (*P* = 0.0097043 groups appear to be significantly different (One-way ANOVA with Tukey Post Hoc Test; significance level = 0.05). There was no statistically significant difference in the ages of patients in each group.

3.4 Discussion

This study is the largest reported cohort study of duodenal adenomatosis in patients with MAP, focusing on adenoma occurrence, progression of adenoma development and the morphological features of duodenal polyps.

3.4.1 Duodenal adenoma incidence

In keeping with previously reported data, we found that 17% of patients had adenomas at their first endoscopy. The largest published study of MAP patients to date (*n*=150) also reported that 17% of patients had duodenal adenomas, but no follow up data was reported (Vogt et al. 2009). This current study adds to the understanding of the natural history of duodenal adenomatosis in MAP and found that the majority of patients with MAP did not develop duodenal adenomatosis with a cumulative incidence of 30% of duodenal adenomas by a median age of 70 (range 21-80 years). This is in contrast to FAP patients where the lifetime incidence of adenomas in the duodenum is up to 95% (Nugent et al. 1993). Our study also found that only 3% of MAP patients developed the most advanced stage (Spigelman IV) by age 70 years which again contrasts with studies in FAP that have shown a risk of 10-15-fold greater. However, it should be noted that of the 207 patients that had an index endoscopy, 28% had not undergone a further upper GI endoscopy during the study period which may have affected led to an underestimate of the occurrence of stage IV disease. The majority of these patients had not undergone repeat surveillance as part of the management of low Spigelman score findings, where the surveillance interval is 5 years between endoscopies and it is reasonable to assume that these patients are at low risk of developing further adenomas. Only 4 patients were lost to follow up in this study.

The median age at first endoscopy in the study was 51 years, and this is likely to reflect the timing of MAP diagnosis. The median age of adenoma observation was found to be 51 years, and the cumulative incidence of adenomatosis at age 50 years was 9%; at age 60 years, 22% and by age 70 years, 29% all of which are much lower than in FAP (Bulow et al 2011).

3.4.2 Adenoma Progression

Walton et al. (2014) reported slow progression of duodenal adenomas in MAP, with only one patient of 34 progressing to another Spigelman stage over 5 years. However this was a small study and might not accurately represent the true progression of duodenal adenomas. In comparison, we found that 24% of patients progressed to a higher Spigelman stage over the study period. Interestingly, 13.5% of patients had high grade dysplasia at first endoscopy, but this did not correlate to a high initial Spigelman score, as no patient had Spigelman stage IV disease. Recent studies of FAP have shown a higher percentage of overall stage progression of between 32 and 44% (Saurin et al. 2004; Lepisto et al. 2009; Bulow et al. 2011 and Serrano et al. 2014) but what is striking in our study is the very low rate of progression to stage IV disease. Spigelman stages do progress in MAP, and appear to do so with time and patient age. This appears to be a slow a process in MAP as it is in FAP, occurring over many years. However, as described by Serrano et al (2014) in a study of 218 FAP patients, they can progress from stage 0 to stages I, II, II or IV with no observation of change sequentially through stages. We found that the increase in Spigelman score in MAP appears to reflect mainly changes in histological grade rather than adenoma numbers suggesting that in MAP, the component parts of the total Spigelman score and their individual points weighting may require modification from the weightings used in FAP. In addition, in this study we found that once a patient had duodenal polyps, the rate of adenoma progression

was shown to be more rapid (p= 0.041) than in those that had no adenomas at first endoscopy. In the colorectum, after the primary diagnosis of polyposis a substantial proportion of patients were shown to develop CRC within a decade (Niewenhuis et al. 2012), an observation that suggests that there may be an accelerated pathway to cancer that could also occur in the duodenum.

3.4.3 Number of adenomas

The median number of adenomas per patient across the study period was 2.5, 67% of patients were found to have between 1-4 adenomas in total, confirming that MAP patients appear to have a lower polyp burden compared to FAP. In FAP, it has been reported that in more advanced cases mucosal carpeting can occur when larger plaques of abnormal tissue coalesce (Sarre et al. 1987; Iida et al. 1989), and this study has also shown the presence of large adenomas in MAP where four patients (7% of those with adenomas) had a single duodenal adenoma greater than 2cm in size. As seen in FAP (Seow-Choen et al. 1992), most lesions were located in the second part of the duodenum with relative sparing of the duodenal cap, but in contrast we did not observe a clustering of adenomas around the ampullary region. However, it was not known if participating centres routinely undertook surveillance with a side viewing endoscope to specific ally assess the ampullary region. Two of the four patients with Spigelman stage IV disease had a single adenoma greater than 20mm in size, with one patient found to have 2 adenomas >20mm, all with histological confirmation of high grade dysplasia, again demonstrating the disparity with the common findings within a ‘classical’ FAP stage IV duodenum.

3.4.4 Effect of endoscopic therapy

In their series of 129 patients with FAP, Lepisto et al. (2009) demonstrated a Spigelman stage decrease following endoscopic therapy of 50%. This current study also demonstrated a high rate of down-staging of 77% in MAP patients, which was statistically significant. In keeping with previous reports (discussed in section 1.7.2), it is important to mention that the patients with larger adenomas treated with APC alone or in combination with endoscopic resection had a 100% rate of adenoma recurrence in this study. Although no patient that underwent endoscopic down-staging was found to develop duodenal cancer in this study, larger prospective studies are needed to definitively evaluate the role of endoscopic therapy in the longer-term and the impact on outcome. In addition, in this study a quarter of patients that had adenomas at first endoscopy were found to be Spigelman 0 at follow up endoscopy. The biopsying of small adenomas 3-4mm or less may have a coincidental therapeutic role, and it is interesting that there was no recurrence of these diminutive adenomas but this could be interpreted as observer bias, as even small changes in the number and size of polyps may change the Spigelman stage (Bulow et al. 2011).

The one patient that had received pharmacological treatment with sulindac developed high grade dysplasia despite long-term use, which supports the findings in FAP that the effect on adenomas is much less in the duodenum than the colorectum (Nugent et al. 1993). However, the reported fall in mucosal proliferation as a marker for the rate of tumour formation observed in that study may have prevented carcinoma development in this patient.

3.4.5 Duodenal adenocarcinoma

Three patients (1.5%) had a duodenal adenocarcinoma at a median age of 64 years in this study, with one was diagnosed at index endoscopy. The other two cases were asymptomatic interval cancers ('screen detected'). The cumulative incidence of developing duodenal adenocarcinoma in this study was 2.3%, and compares to a recent study by Serrano et al (2014) that reported 5 duodenal cancers in a cohort of 218 FAP patients. They concluded that this low rate compared to other studies was a result of effective surveillance and management of duodenal adenomas. However there was a 4.5% proportion of duodenal cancer in this current study in those patients that had duodenal adenomas detected at previous screening which has a greater implication for surveillance. The low rate of duodenal adenocarcinoma in MAP in our study may be attributable to endoscopic intervention. However, some study centres referred patients to different specialist centres for management of complex disease (e.g. six patients from the Netherlands) and the extent to which these other interventions may have prevented cancer is unknown. Only two patients underwent surgery for non-cancer polyposis, and minimisation of cancer overall in this study may be attributed to endoscopic intervention.

Adenoma development (thus carcinoma development) in MAP patients likely develop at a later age than FAP due to the requirement of two somatic *APC* mutations in addition to the bi-allelic *MUTYH* germline mutation (Niuewenhuis et al. 2012), and so as the median age of patients in our study was 51 years at index endoscopy, longer term follow up may demonstrate detection of additional duodenal cancers.

In FAP, no patients with stage 0 or I disease at index endoscopy were reported to have developed a duodenal malignancy (Groves et al. 2002; Bulow et al. 2004; Saurin et al 2004; Bulow et al. 2011), which is in contrast to our findings in MAP

where one patient had a normal endoscopy (Spigelman 0) 11 years prior to diagnosis of cancer. However, this endoscopy took place before the advent of high resolution endoscopy, which is known to increase adenoma detection in FAP (Mathus-Vliegen et al. 2011), and so the possibility of 'missed' adenomas may have occurred. The other patient developed cancer 1 year after Spigelman stage III (6 points) adenomatosis where the patient had less than 10 adenomas, maximum size 4mm with tubulovillous histology and low grade dysplasia. This supports previous reports of duodenal adenocarcinoma occurring in the context of few adenomas in MAP (Nielsen et al. 2006). High initial Spigelman scores (>7) in FAP have been shown to be a risk factor for HGD and carcinoma development but Speigelman grading may be less appropriate for stratifying risk in MAP. However, in this current study, there were too few HGD lesions and cancers detected to make an estimation of hazard ratios.

3.4.6 Duodenal genotype-phenotype correlation

Previous studies have investigated the influence of the site of mutation on the severity of duodenal polyposis in FAP in order to help inform appropriate surveillance and / or treatment decisions. Saurin et al. (2002) reported that a mutation within codon 279-1309 at the centre of the *APC* gene was associated with development of severe duodenal adenomatosis, but this was not confirmed by a larger study (Bulow et al. 2011). There is currently insufficient data to justify treatment decisions based on a patient's mutational status. In MAP, a large multi-centre genotype-phenotype study (Nielsen et al. 2009) found that Y179C homozygotes presented earlier and had a significantly increased colorectal cancer hazard risk than G396D homozygotes and G396D / Y179C compound heterozygotes. The most frequently mutated alleles seen in our 207 MAP cases (in the 135 where there was mutational data available) were also the missense

mutations Y179C (32.5% of mutations) and G396C (34% of mutations). This is the first study to investigate if there is a correlation between *MUTYH* genotype and duodenal phenotype. We found that in concordance with the colorectum, Y179C homozygotes had a greater number of duodenal adenomas than patients with two truncating mutations, G396C homozygotes and G396D / Y179C compound heterozygotes. The number of adenomas and the overall Spigelman score at last endoscopy were significantly different between these groups. Cells with bi-allelic Y179C mutations appear to contain lower levels of the MUTYH protein than cells with bi-allelic G396D mutations (Parker et al. 2005), and Y179C slows a lesser ability to recognise an 8-oxo-G:A mismatch than G396D and has severely defective glycosylase DNA binding ability (Ali et al. 2008). We found that there was no significant difference between truncating and non-truncating mutations, a finding also reported by Nielsen et al (2009), which is surprising as a more severe phenotype may be expected in the truncating mutation group because of the greater effect of the truncating mutation on the protein activity. It has been proposed that a complete loss of MUTYH function might act to reduce cell survival and lead to less tumour formation than if some functional protein remains (Nielsen et al. 2009). The lack of separate analysis of bi-allelic combinations of other mutations may have biased this study, but was not undertaken because of the small numbers.

3.4.7 Extra-intestinal manifestations in MAP

As in cases with defective mismatch repair in HNPCC, a germline defect in the BER repair pathway might be anticipated to result in tumours in many organs. Vogt et al (2009) reported overall 49 malignant lesions other than CRC in 237 MAP cases (21%), suggesting that malignancies may not be common in MAP. This current study reported a 5% incidence of other malignant lesions (not including those with duodenal cancer).

3.4.8 Study limitations

The main shortcomings of this study were its retrospective design, and the fact there was no standardised method of surveillance (including use of a side –viewing endoscope) across the study centres. Some data was included before the introduction of high resolution endoscopes and some centres routinely use chromoendoscopy which has been found it improve adenoma detection rates in both MAP and FAP (chapter 2). In addition, only one patient in this study appeared to have undergone random biopsy, with no visible adenomas but dysplasia on histology. The importance of multiple random biopsies in the FAP duodenum was highlighted by Bulow et al (2004) who found 12% of adenomas diagnosed only histologically. The majority of surveillance in this study however was undertaken in the era of high resolution and magnifying endoscopes, so it can be argued that random biopsies are not required in the modern age of improved endoscopic optical diagnosis. Any bias from potential missed dysplasia in earlier endoscopies probably had a minimal effect on the overall results. There also appeared to be a lack of adherence to Spigelman surveillance protocols, which may reflect pressures on waiting times for surveillance procedures within different units.

Our study also did not compare the duodenal findings with the patient's colorectal disease or management. Biasco et al (2006) reported on the surgical procedures for colorectal adenomatous disease in FAP, with only 8% of patients with a proctocolectomy and ileo-anal anastomosis developing Spigelman stage IV disease compared to 50% of patients who had an ileo-rectal anastomosis that developed stage IV disease. The authors suggested that patients who underwent a proctocolectomy and ileo-anal annastomosis might be at lower risk of developing advanced duodenal adenomatosis because of a reduced biliary secretion of bile acids, and reduction in the secondary bile acid deoxycholic acid which has been described in patients who have undergone this surgical technique. More recently,

Serrano et al (2014) suggested that the speed of progression of polyposis in the colon predicts the speed in the duodenum, another factor that was not investigated in this current study.

3.4.9 Conclusions

In conclusion, this study has shown that in the MAP duodenum there is a cumulative incidence of developing adenomas of 30% by age 70 years, which is significantly lower than FAP. There remains however, a risk of duodenal adenocarcinoma development. Similar to the colorectum, there appears to be no strong correlation between the polyp burden and the risk of cancer. The findings of this study have important implications for clinical practice as the burden of adenomatosis may not be a sufficient indicator for determining the surveillance interval in patients. The Spigelman staging system may not be appropriate for long-tem use in MAP, but further prospective studies are needed to identify what factors are associated with progression of disease, and indeed if progression of disease follows a model that can be applied to clinical management. The underlying molecular genetic mechanisms and alterations involved in the adenoma to carcinoma sequence pathway may be of greater importance than the phenotypic expression of polyps within the duodenum in determining which patient will develop cancer. An emerging challenge will be to identify molecular biomarkers for risk stratification in those at risk of duodenal carcinoma in MAP and FAP that is necessary in order to fulfil the promise of ‘personalised medicine.’

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Chapter 4

Characterisation of the Somatic Mutational Spectrum in Duodenal Adenomas in MAP and FAP

4.1 Introduction

Patients with MAP carry biallelic inactivating mutations in the *MUTYH g*ene which increases the somatic mutation rate, generally resulting in an increase in G:C>T:A transversions. Previous research has shown that patients with MAP develop colorectal adenomas and carcinomas as a result of gaining secondary somatic mutations in drivers such as *APC* and *KRAS*. In one study, 100% of *KRAS* mutations in MAP colorectal adenomas were G to T transversions in codon 12 compared to 13% in sporadic adenomas (Jones et al, 2002). This form of genetic instability is distinct from microsatellite instability (MSI), which is apparently absent in MAP tumours (Lipton et al, 2003). FAP develops subsequent to somatic loss of the remaining wildtype allele of *APC*, which leads to deregulation of the WNT pathway. The “just right” hypothesis, discussed in section 1.2.8, whereby optimal levels of WNT activation drive cell growth without inducing apoptosis is thought to influence the number of polyps and growth of these polyps. This is governed by the combined nature and effects of the germline and somatic *APC* mutations.

In addition in one study, evaluation of the chromosomal instability (CIN) of MAP and FAP colorectal polyps suggested that up to 80% and 60% of MAP and FAP polyps respectively showed aneuploid changes, and that these changes detected at early stages of *MUTYH*-driven tumourigenesis may underlie accelerated tumour progression in MAP (Cardoso et al, 2006).

However for both MAP and FAP adenomas, especially in the duodenum, there remains a lack of any comprehensive data that describes the pattern of somatic changes, rate of mutation and the genes that are mutated.

4.1.1 Upper gastrointestinal adenoma-carcinoma sequence

Duodenal adenomas have been observed to progress to carcinoma following a pattern similar to colorectal adenomas, via an adenoma-carcinoma sequence (Brosens et al. 2005). Activation of the Wnt signalling pathway, by biallelic inactivating *APC* mutation is the initiating step. However, the molecular features of small bowel adenomas have not been extensively investigated and the genetic alterations that occur in duodenal tumours in FAP are poorly characterised. The same is true of MAP. Toyooka et al (1995) reported that somatic mutations in the "mutation cluster region" of *APC* were detected in 46% of duodenal adenomas, 67% of ampullary adenomas and 50% of ampullary cancers in FAP, with codons 1554-1556 the most frequently mutated. They also described somatic *APC* gene mutation in small duodenal adenomas of only 1mm size with mild dysplasia, suggesting that this is an early event. In the Toyooka series, *KRAS* mutations were not detected, but this was thought to be due to the majority of polyps having low or moderate grade dysplasia. *P53* mutations were not detected in any of the duodenal tumours analysed. In contrast, Gallinger et al (1995) found only 6% of periampullary duodenal tumours had *APC* mutations, but 37% had *KRAS* mutations.

Kashiwagi et al. (1996) described that in patients with FAP, *p53* expression was abundant in duodenal adenomas, and that *p53* expression increased with progression along the adenoma-carcinoma sequence, being demonstrated in 100% of duodenal carcinomas and 72% of duodenal tubulovillous/villous adenomas. In patients without FAP, p53 over-expression has been shown to correlate with more advanced pathology in tumours in the oral mucosa, oesophagus, stomach, colon and duodenum (Ogden et al. 1992; Hardwick et al. 1994; Joypaul et al. 1993; Kaklamanis et al. 1993; Scarpa et al. 1993). Detection of *p53* over-expression in the absence of a *p53* mutation was postulated to reflect the presence of an abnormal cellular environment, particularly DNA damage, and its may be an additional marker

of risk for malignancy in FAP (Kashiwagi et al. 1996). Further data from this group also demonstrated 3 adenomas of 25 (12%) had point mutations in *KRAS* codon 12 (Kashiwagi et al. 1997).

A comparison of ampullary and FAP-associated duodenal adenomas concluded that duodenal adenomas share morphologic and molecular features with colorectal adenomas, suggesting that they develop via similar mechanisms (Wagner et al. 2008). It also showed sporadic and FAP-related adenomas to have similar molecular features, regardless of their anatomical location. Wnt signalling pathway abnormalities occurred in sporadic non-ampullary polyps in 82%, in sporadic ampullary polyps in 77%, and in 67% of FAP polyps. *KRAS* mutations were observed infrequently, only seen in 18% of sporadic polyps and 9% of FAP polyps. Direct sequencing of *p53* exons 5 to 8, revealed no detectable *p53* mutations, and no *BRAF* mutations were found. In contrast to the studies by Kashiwagi et al (1996 and 1997) no significant molecular differences between adenomas of the ampullary and non-ampullary mucosa were identified.

SMAD4 mutations have been shown to play a role in polyp development in the upper intestine of mice (Takaku et al. 1998). Resnick et al. (1995) demonstrated that transforming growth factor alpha expression was greater in duodenal carcinomas than adenomas, and that epidermal growth factor receptor (EGF-R) expression correlated with the degree of dysplasia in duodenal adenomas.

These studies suggest that the transition of adenoma to carcinoma may be driven by other molecular factors that have yet to be fully elucidated.

4.1.2 Study aims

This study aims to determine the profiles of somatic mutations in MAP and FAP duodenal adenomas and the mutations rates and key drivers of tumorigenesis in each of these settings

4.2 Materials and Methods

4.2.1 Materials

4.2.1.1 General buffers, solutions, reagents and chemicals

Ethylenediaminetetraacetic acid (EDTA)

0.5M EDTA adjusted to pH8.0 with NaOH pellets. (Sigma, cat no: ED2SS)

Boric Acid (Sigma, cat no: B7901)

Tris-Borate-EDTA (TBE) buffer (5X)

0.445M Tris-HCL, 0.445M boric acid, 0.01M EDTA

Diluted to 1X for use in gel electrophoresis with dH_2O: 0.089M Tris, 0.089M boric acid and 0.002M EDTA

Tris-EDTA (TE) Buffer (1x)

10mM Tris-HCL pH8.0, 1mM EDTA pH8.0

Ethanol (Fisher Scientific, cat no: E/0600/DF17)

Propan-2-ol (Isopropanol) (Fisher Scientific, cat no: P/7490/17)

Sodium Acetate (Sigma, cat no: S2889)

DNA extraction buffer

Tris 10mM, EDTA 5mM and NaCl 100nm SDS 0.5% final volume 20ml

Sodium Dodecyl Sulfate (SDS) (Sigma, cat no: L4390)

AquaPhenol (Q-BIOgene, cat no: AQUAPH01)

Chloroform:Isoamyl alcohol 24:1 (Sigma, cat no: C0549)

Hi-Di™ Formamide (Applied Biosystems, cat no: 4311320)

4.2.1.2 Molecular biology solutions and reagents

Ethidium bromide (Sigma, cat no: E1510)

DNA loading solution

0.03g Bromophenol Blue (Sigma, cat no: B8026), 0.03g Xylene Cyanol (Sigma, cat no:X4126), 7.5g Ficoll 400 (Sigma, cat no: F9378) and 0.558g EDTA in 50ml dH_2O

1Kb plus ladder (250µg)

Suitable for sizing linear double stranded DNA fragments from 100 to 12kb. The ladder consists of 12 bands ranging in size from 1000bp to 12000bp in exact 1000bp increments as well as 7 bands from 100bp to 850bp (Invitrogen cat no: 10787-018).

Primers

All primers for use in PCR and sequencing were unless otherwise stated designed using Primer3 V. 0.4.0 (Rozen and Skaletsky, 2000) and synthesised by Eurogentec (Belgium) using standard purification and 10nm synthesis scale. All primers containing a FAM fluorescent label were synthesised on a 40nm scale. All primers were put through BLAT software (Kent, 2002) to ensure specificity.

Agarose (multipurpose) (Roche, cat no: 11388991001)

4.2.1.3 Molecular biology enzymes

Megamix Gold (Microzone, cat no: 2MMG)

Big Dye terminator v1 cycle sequencing kit (Applied Biosystems, cat no: 4337450)

Power SYBR® Green PCR master mix (Applied Biosystems, cat no: 4367659)

Shrimp Alkaline Phosphatase (SAP) (GE Healthcare, cat no: E70092Z)

4.2.1.4 Restriction enzymes

ExoI (New England Biolabs, Cat no: M0293)

4.2.1.5 Equipment and instruments

A GSTORM thermocycler for PCR reactions and incubations was used (Lab Tech). Horizontal gel tank apparatus were supplied by Biorad. Centrifugations were performed in a bench-top microcentrifuge Eppendorf 5415C (Eppendorf). Purified sequencing reactions, LOH and MLPA were analysed using an ABI Prism 3730 Genetic Analyser (Applied Biosystems). Relative quantification was completed on an ABI 7500 (Applied Biosystems). Following nucleic acid extraction, the resulting concentration was determined using 2µl of the DNA or RNA on a Nannodrop 8000 spectrophotometer (Thermo Scientific).

4.2.2 Methods

4.2.2.1 Patient samples

Fifty-two patients were recruited for this study, 32 were MAP patients and 20 were FAP patients. A blood sample (5mls EDTA) was taken from each patient and DNA was subsequently extracted as described below. This was to enable comparison with adenoma samples to facilitate identification of somatic mutations. Adenoma biopsy samples were taken at upper GI endoscopy at the University Hospital Llandough, Wales, UK and St Marks Hospital, London, UK. These samples were supplementary to the biopsies required for the patients' routine care. A section of each adenoma biopsy was formalin fixed and sent for histological analysis by a specialist GI pathologist at the University Hospital Wales, Cardiff, to assess histological classification, degree of dysplasia and the percentage of adenomatous material. The remainder was fresh frozen with liquid nitrogen and stored at -80C until the DNA was extracted. All samples had material available for follow-up validation experiments. For exome sequencing, we attempted to age and sex match

patients with MAP and FAP as far as was possible. Figure 4.1 provides an overview of the analysis performed on the adenoma samples.

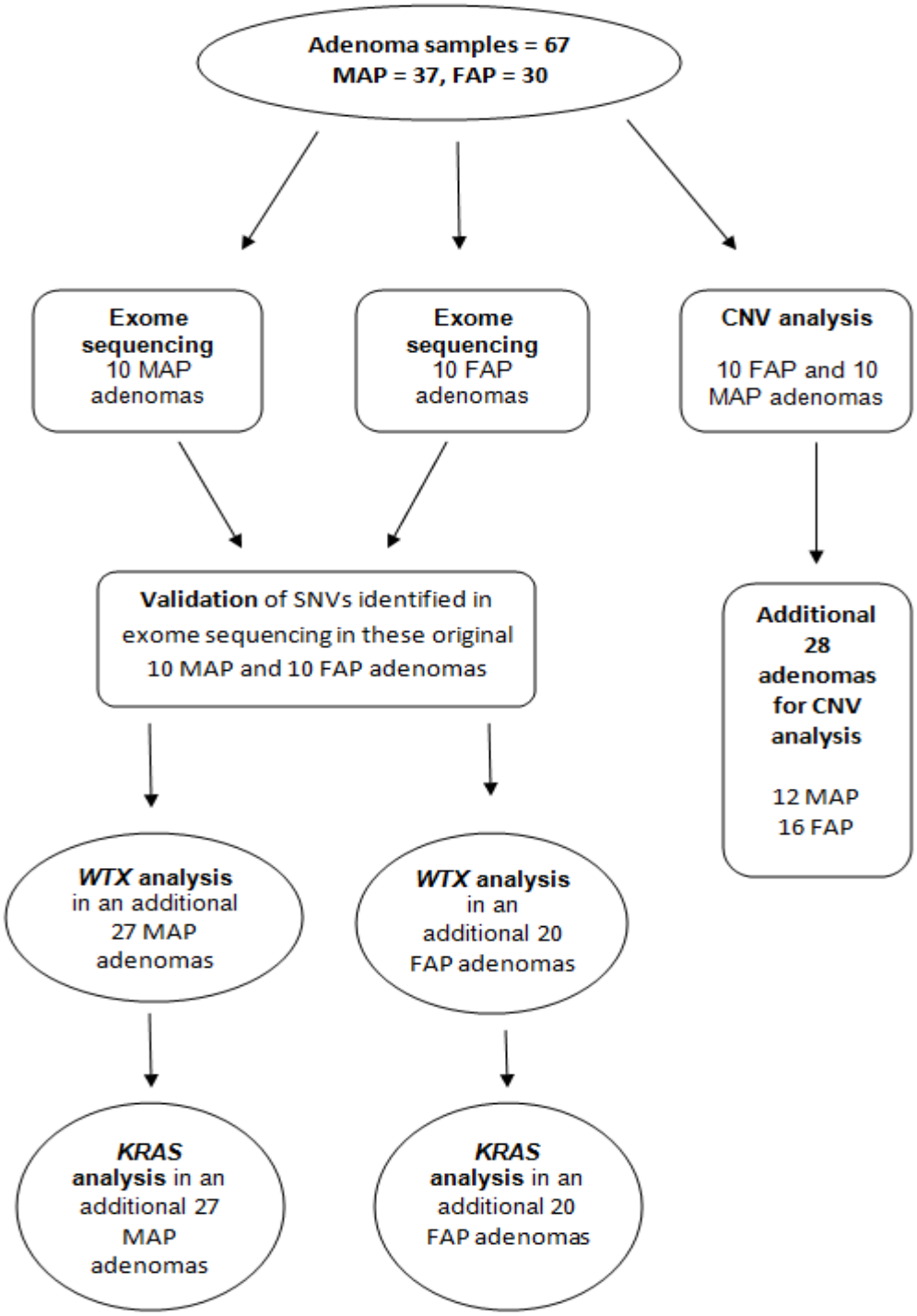


Figure 4.1 *– Flow chart of genetic analysis performed on the duodenal adenomas in the study*

4.2.2.2 Histology

A section from each adenoma was analysed to estimate what percentage (to the nearest 5%) of all the nuclei on the slide were epithelial adenoma nuclei in relation to the total number of nuclei comprising adenoma, non-neoplastic crypts, stroma / lamina propria / muscularis mucosae/ submucosa, lymphoid tissue and inflammatory cells.

4.2.2.3 DNA extraction

4.2.2.3.1 DNA extraction from peripheral blood

DNA was extracted from all blood samples by the All Wales Medical Genetics Service (AWMGS) using an automated DNA extraction facility (Hamilton).

4.2.2.3.2 DNA extraction from polyp tissue

This was carried out in a PCR clean environment. Once removed from the freezer, tissue for DNA extraction was finely chopped up in phosphate buffered saline using a homogeniser and transferred to a 1.5ml microcentrifuge tube containing: 400µl extraction buffer, 4µl proteinase K (20mg/ml) and 4µl Rnase (10mg/ml).This was then left to incubate overnight at 45°C in a hot block. The following morning, 400µl phenol was added to each tube and placed on a tube rotator for 20-30minutes. It was then centrifuged at 13,000 rpm for 4-6 minutes using a microcentrifuge. The aqueous (top) and interphase was transferred to a fresh tube containing 400µl of phenol, after which it was placed on a tube rotator for a further 20 minutes. It was then centrifuged at 13, 000 rpm for 4-6 minutes. The aqueous phase (only the top) was when transferred to a 2ml tube and 40µl of 3M Sodium acetate pH5.2 was added, mixed gently and contents spun to the bottom of the tube. 100% ice cold ethanol (1ml) was added to the tube, and this was left at -20°C for at least 20

minutes (I left it overnight). A precipitant (pellet) was not easily seen, so the tube was then centrifuged at 4°C at 13, 000rpm for 30 minutes. The ethanol was then decanted and pellets washed in 500µl of 70% ethanol for 10minutes on ice. The tube was the given a quick spin for 60 seconds, after which the ethanol was removed via pipette and the tube left open to dry for 30-60 minutes. The pellet was then re-suspended in 50µl of Tris 10mM and stored at 4°C overnight. Extracted DNA was stored at -40 until used for analysis.

4.2.2.4 Exome sequencing

The Beijing Genomics Institute (BGI), Hong Kong, completed Exome sequencing on constitutional DNA samples and DNA extracted from adenomas using the SureSelect Human 50Mb capture kit (Agilent). Twenty adenomas were sequenced from 9 patients together with matched blood DNA from each patient. Five patients had MAP (10 adenomas sequenced) and 4 had FAP (10 adenomas sequenced).

4.2.2.5 Validation of Single Nucleotide Variants (SNVs)

Putative SNVs identified by exome sequencing were validated using a standard PCR protocol described below followed by Sanger sequencing (performed by myself and Dr Laura Thomas). Primers (100uM) were purchased from Eurofins in 1.2ml deep well plates. Resulting PCR products were quantified with Quant-IT reagent (Life Technologies) and a 96 well plate fluorometer. 10ng/ul PCR products were purified and sequenced by Eurofins using Sanger sequencing technology. Putative *APC* (NM_000038) and *MUTYH* (NM_012222) germline mutations were validated in the 9 constitutional DNA samples in addition to 1415 putative somatic SNVs (including those in *APC*) in the 20 adenoma samples.

4.2.2.6 Primer design

Primers were designed by Primer 3 software (University of Massachusetts Medical School, U.S.A) and purchased from Eurofins (Luxembourg). The BLAT (BLAST –like Alignment Tool) algorithm to determine all possible regions to which a primer may bind was used to ensure primer specificity.

4.2.2.7 Polymerase chain reaction (PCR)

Direct sequencing allows the myriad of changes such as deletions, insertions and single base pair substitutions to be detected. The polymerase chain reaction allows the specific *in vitro* amplification of defined DNA target sequences within a source of DNA. This occurs in an exponential manner. Double stranded DNA templates are heat denatured and oligonucleotides bind specifically to complementary target sites on each strand. Thermostable DNA polymerases extend the primers in the 5' to 3' direction by incorporating dNTPs to create a complementary DNA strand. This cycle is repeated 20-40 times to produce up to 5μg DNA (Mullis et al, 1986).

PCR was completed with 12.5ul Megamix gold (Microzone), 2.5uM primers and 5ng/ul of DNA in a 25ul reaction. A standard cycling method was used: 95°C 5mins, 35x (95°C 1min, Ta 1min, 72°C 1min), 72°C 5mins. Annealing temperature was 58°C unless otherwise stated.

Reagent Volume	25µl	Cycle Conditions	x35 Cycles
DNA [5ng/µl]	1		
Megamix gold	12.5µl	95°C	5 minutes
		95°C	1 minute
Sense Primer [10uM]	0.25µl	60°C	1 minute
Antisense Primer [10uM]	0.25µl	72°C	1 minute
H_2O	11µl	72°C	5 minutes

4.2.2.7.1 Agarose gel electrophoresis

1.5% agarose gels were made by combining 1.5g multipurpose agarose with 100ml of 1X TBE and heated in a microwave for 2 minutes to melt the agarose. 100ml of 1.5% agarose gel was stained with 4µl of Ethidium Bromide, poured into the gel moulds and left to set prior to loading of PCR product. Unless otherwise stated, 4µl of PCR product was combined with 5µl of DNA loading dye and loaded onto the 1.5% multipurpose agarose gels. Gel tank equipment (Biorad) was set to 100Volts (0.4Amps) and run for 30 minutes. After running the gel, bands were visualised under UV with a GelDoc Imaging Station and Quantity One Software (Biorad).

4.2.2.8 DNA sequencing

4.2.2.8.1 ExoSAP PCR purification

This method of purification involves the enzymatic removal of excess nucleotides and primers from the PCR reactions. 1µl of SAP and *ExoI* was added to the PCR product to be purified in a 2:1 ratio. The PCR product was then incubated for 1 hour at 37ºC to activate the *ExoI* and then 80ºC for 15minutes to deactivate the *ExoI.*

4.2.2.8.2 Big Dye terminator reaction

The BigDye terminator mix was prepared using the following reagents and cycle conditions:

Reagent Volume	10µl	Cycle Conditions	x30 Cycles
DNA (PCR product)	1.5µl	96 ºC	5 minutes
BigDye	0.25µl		
Buffer	2µl	96 ºC	15 seconds
Primer [10µM]	0.16µl	50 ºC	15 seconds
H_2O	6.09µl		
		60 ºC	4 minutes

4.2.2.8.3 Isopropanol sequencing purification

This method removes unincorporated dyes by isopropanol precipitation. 40µl of 75% isopropanol was added to each well and mixed gently. The reaction was incubated at room temperature for 30 minutes followed by centrifugation at 4000rpm for 45 minutes. After centrifugation, the plate was inverted on to absorbent paper to remove the isopropanol and then placed inverted into the rotor bucket and centrifuged at 500rpm for 30 seconds. The plate was left to air dry in a dark box for 10 minutes and DNA was finally resuspended in 10µl of Hi-Di™ Formamide and analysed on an ABI 3730 analyser (Applied Biosystems).

4.2.2.9 Sequencing of WTX

The open reading frame (ORF) of *WTX* (NC_000023.10) was sequenced in an additional 47 adenomas (27 MAP adenomas and 20 FAP adenomas) using the above PCR conditions. Primers, which were all M13 tagged, and annealing temperatures are detailed in table 4.1. Sequencing was completed by Eurofins (Luxembourg) and analysed using Sequencher software (performed by Julie Maynard).

Gene/Primer name	Primer sequence (5'→3')	Annealing temperature (°C)	Product size (bp)
WTX			
WTX1F	TGT-AAA-ACG-ACG-GCC-AGT-AGT-GCC-TGG-AAG-CCT-GAG	65	511
WTX1R	CAG-GAA-ACA-GCT-ATG-ACC-GCA-GGG-TAA-CTC-AGG-CAA-AG		
WTX2F	TGT-AAA-ACG-ACG-GCC-AGT-GTT-CTG-GGA-AAG-GCA-GCT-C	54	448
WTX2R	CAG-GAA-ACA-GCT-ATG-ACC-GCC-TGG-CTC-TGA-CCC-TCT		
WTX3F	TGT-AAA-ACG-ACG-GCC-AGT-TAG-CAG-TAT-CCG-CCG-TCA-C	63	468
WTX3R	CAG-GAA-ACA-GCT-ATG-ACC-GGC-CAT-TGG-GTG-GGT-TTA		
WTX4F	TGT-AAA-ACG-ACG-GCC-AGT-CCC-ATA-GCC-CAG-AAA-CAG-G	44	466
WTX4R	CAG-GAA-ACA-GCT-ATG-ACC-GGG-CAG-TTT-CCC-ACA-GAT-ATT		
WTX5F	TGT-AAA-ACG-ACG-GCC-AGT-AAG-GAG-GTG-GGG-AGG-AGA-T	58	556
WTX5R	CAG-GAA-ACA-GCT-ATG-ACC-AAA-GGC-AGT-CAT-CTC-CAG-GT		
WTX6F	TGT-AAA-ACG-ACG-GCC-AGT-ACC-CCG-AGA-CAG-CTA-CAG-TG	52	420
WTX6R	CAG-GAA-ACA-GCT-ATG-ACC-CAT-AGG-CTT-CCC-TGC-CAT-AA		
WTX7F	TGT-AAA-ACG-ACG-GCC-AGT-TTG-TTG-TAT-TGG-GAG-CTT-CG	43	436
WTX7R	CAG-GAA-ACA-GCT-ATG-ACC-GGT-GGG-GAA-AGC-TGA-GGT-A		
WTX8F	TGT-AAA-ACG-ACG-GCC-AGT-CCG-TCT-TAG-AGT-ATC-AGA-TGA-GG	63	506
WTX8R	CAG-GAA-ACA-GCT-ATG-ACC-TCA-GAG-TCA-GAG-CTG-CAG-GA		
WTX9F	TGT-AAA-ACG-ACG-GCC-AGT-GGG-AAT-GCC-ACT-GTG-AGT-TT	63	466
WTX9R	CAG-GAA-ACA-GCT-ATG-ACC-TCT-CCT-GTT-GAG-GGC-CAT-AG		
WTX10F	TGT-AAA-ACG-ACG-GCC-AGT-AAC-ATG-CCT-TCA-ACA-ACT-ACC-A	58	475
WTX10R	CAG-GAA-ACA-GCT-ATG-ACC-CCA-ACT-GGT-TGG-GGC-TTA-T		
WTX11F	TGT-AAA-ACG-ACG-GCC-AGT-CTA-TGA-TTG-GCC-TGC-TTG-G	58	376
WTX11R	CAG-GAA-ACA-GCT-ATG-ACC-ACA-GGC-AGC-AGC-ACA-TCT-C		
WTX12F	TGT-AAA-ACG-ACG-GCC-AGT-CGT-CCC-TCA-CAC-CTA-CAC-CT	63	476
WTX12R	CAG-GAA-ACA-GCT-ATG-ACC-CTG-ATC-CCC-ATT-CAC-ATG-CT		

Table 4.1 *- Sequences of primers and PCR conditions used to screen WTX*

4.2.2.10 KRAS codon 12 and 13 sequencing

Codon's 12 and 13 of *KRAS* (NM_033360) were analysed by PCR and Sanger sequencing using the conditions described above, an annealing temperature of 58° C, and the following primer sequences: 5'-CCCTGACATACTCCCAAGGA-3' and 5'-CTCCTCCATCGACGCTTAAG-3'. Primers were designed by Primer 3 and purchased from Eurofins (Luxembourg). Sequencing was completed by Eurofins and analysed using Sequencher software (performed by Dr Laura Thomas).

4.2.2.11 Loss of Heterozygosity Analysis (LOH)

LOH analysis of *APC* was completed on all adenomas in which a pathogenic somatic SNV in the *APC* gene could not be identified (6 adenomas). Fluorescently tagged (FAM) SNP and STS markers were used in a PCR reaction as described above. Markers were chosen to span chromosome 5 to determine the extent of loss. with 3 markers within the *APC* gene; D5S406 (5p15.32 – 9.6cM from *APC*), D5S1965 (5q22.2 – 200kb from *APC*), D5S346 (5q22.2), rs2019720 (*APC* promoter), rs1914 (*APC* intron 7) and nt5037 (*APC* exon 15). All reactions for microsatellite markers were carried out at an annealing temperature of 58°C. Fluorescently tagged PCR products were run on an ABI 3100 and analysed using Genotyper and Genescan software (Applied Biosystems). LOH was considered to be present if the resulting trace from the adenoma showed a peak height of 50% or less of the corresponding peak height for the matched constitutional DNA samples. All assays were carried out in duplicate (performed by myself with assistance from Dr Laura Thomas).

4.2.2.12 Array CGH

Microarray analysis was completed on forty-nine adenomas comprising 26 adenomas from FAP patients and 23 adenomas from MAP patients and matched blood samples (with assistance from Sian Jose) using the BlueGnome CytoChip ISCA 8x60k (v2.0) array (GRCh37), BG_Annotation_Ens70_20130319.db following the manufacturers protocol. Briefly, this involved labelling of sample and reference DNA (matched blood DNA) with Cy3 and Cy5 dyes, respectively. The labelled genomic DNA was cleaned up using Amicon Ultracel-30 membrane filters, followed by vacuum centrifugation to concentrate the samples. The samples were reconstituted in 9.5ul of 1xTE and the DNA concentration, dye incorporation and specific activity of each sample was determined using a Nanodrop spectrophotometer. The Cy3 and Cy5 labelled samples were then combined. Hybridisation was carried out following the addition of COT, blocking agent and hybridisation buffer to each labelled, combined sample. Gasket slides were loaded into an Agilent Microarray Hybridisation Chamber base and the samples dispensed into each gasket well. The array slide was then placed over the gasket slide and the hybridisation chamber cover was placed over the base and incubated in a pre-warmed hybridisation oven at 65°C, rotating at 20rpm for 16 hours to allow hybridisation. Finally the hybridised slides were washed to remove un-hybridised labelled DNA using the clear Hyb Wash System (Agilent). The slide was finally scanned at 3um scanning resolution using the Agilent microarray scanner. Array CGH results were analysed using CytoGenomics software (Agilent). For the 20 adenomas that have undergone exome analysis in addition to the arrayCGH analysis, ExomeCNV caller was used to validate the arrayCGH results to confirm the presence or absence of the CNV in addition to qPCR analysis.

4.2.2.13 Quantitative PCR (qPCR)

Relative quantification PCR (qPCR) can give an accurate comparison between the initial levels of template nucleic acid in different samples. qPCR analysis was used in addition to the exome data to confirm the CNVs identified by arrayCGH. qPCR was completed on the 7500 Real-Time PCR system (Applied Biosystems) using a SYBR Green PCR mastermix (Applied Biosystems) (performed by myself, Dr Laura Thomas and Dr Helena Leon-Brito).

qPCR was used to determine a relative increase or decrease in copy number of the genes of interest as determined by the delta delta ct method [ΔΔct] (comparative ct) given by the equation: t=0=2-ΔΔct. The ct values of the samples of interest are compared with that of a control (calibrator sample - DNA derived from normal mucosa). The ct values of both the calibrator and the samples of interest are then normalized to an appropriate endogenous housekeeping gene (Beta Actin) (Ponchel et al, 2003, Livak and Schmittgen, 2001). Primers were designed using Primer3 and purchased from Eurofins. Relative quantification was carried out using the following reagents and cycle conditions:

Reagent Volume	12.5µl	Cycle Conditions	x40 cycles
DNA (5ng/µl)	1µl	50°C	2 minutes
SYBR® Green	6.5µl	95°C	10 minutes
Sense Primer [2µM]	1µl		
Antisense Primer [2µM]	1µl	95°C	15 seconds
H_2O	3µl	60°C	1 minute

Three replicates were used per experiment and B Actin served as an endogenous control. Control reactions were used to eliminate the presence of contaminants in

the DNA samples and specificity of PCRs were determined by gel analysis and the addition of a dissociation step, producing a single peak upon analysis representing a specific reaction. All data was analysed with the ABI 7500 SDS System software (Applied Biosystems).

4.2.2.14 Bioinformatics Analysis

Bioinformatics analysis was carried out by Dr Kevin Ashelford. All samples were mapped and post-processed using a combination of BWA, samtools, picard, and GATK. Variants were then called by five separate variant calling methods. Finally the results were collated to identify consensus calls. Specifically, reads were mapped against human reference hg19 using BWA mem, version 0.7.4. Sorted indexed BAM files were then created with samtools version 0.1.19. Mappings from multiple runs were merged with picard MergeSamFiles version 1.108. Realignment around indels and recalibration of base quality values was achieved with Genome Analysis Toolkit (GATK) version 3.2.0 as per standard instructions with dbSNP version 138 to provide known variant sites. Read duplicates were flagged with Picard tool's MarkDuplicates tool, version 1.108. Single Nucleotide Variants (SNVs) were then called for each tumour-normal pairing using (i) GATK UnifiedGenotyper (version 3.3.0), (ii) samtools/bcftools (version 0.1.19), (iii) VarScan somatic (version 2.3.7), (iv) SomaticSniper (version 1.0.4), and (v) our own in-house script. GATK UnifiedGenotyper was run in conjunction with dbSNP version 138 as per standard instructions with VariantFiltration to provide basic filter annotations. Bcftools was applied to samtools mpileup output with parameter modifications (-q 15, -Q 10, -C 50, -m 3, -F 0.0002, -d 100000). Varscan somatic was applied to samtools view/mpileup output with parameter modifications (-q15, -Q 10). Somaticsniper was applied with parameter modifications (-q 15, -Q 15). In-house script simply called all variants regardless of quality down to a minimum of two reads based on samtools

mpileup output (with parameters -ABd100000). In all cases VCF outputs were annotated with ANNOVAR (July 2014 release) using UCSC KnownGene gene model, 1000 genomes (1000g2014sep_all), dbsnp 138, SIFT (ljb26_sift) and cosmic (cosmic70). Annotated VCF outputs from all five callers were then combined into a single spreadsheet using an in-house script.

Following the above process, the raw putative SNVs were subjected to further filtering to identify a final list of putative SNVs. This included passing of quality control filters, inclusion of SNVs with a MAF of <1% (0.01) or novel SNVs that have not been reported in dbSNP or 1000 genomes databases. Protein truncating or altering (nonsynonymous and stopgain) SNVs were also selected and synonymous SNVs were excluded. For inclusion SNVs had to be called by at least 3 of the 5 SNV callers. Germline variants were also excluded by virtue of the presence of the SNV in the data from the matched blood DNA. Metrics associated with each sample including yield of data (Gb), percentage of reads mapped, percentage of reads that are on target and on target mean depth of coverage can be found in table 4.2.

CNV analysis was performed on each tumour-normal pairing using exomeCNV version 1.4 (Sathirapongsasuti et al. 2011) run within R version 3.0.2 (http://www.r-project.org). The procedure was based on that outlined in the manual (https://secure.genome.ucla.edu/index.php/ExomeCNV_User_Guide) and can be briefly summarised as follows. Coverage data for both normal and tumour samples were generated from BAM files using the DepthOfCoverage command within GATK version 3.2.0. In R, log coverage ratios between tumour and normal coverage datasets were prepared and CNVs for each exon called individually using the exomeCNV command classify.eCNV (assuming a contamination rate of 0.5; minimum specificity and sensitivity both set at 0.9999 and option='spec'). Finally, exonic CNVs were combined into larger segments using the exomeCNV command,

multi.CNV.analyze, and the resulting outputs converted into spreadsheets and BED files using in-house scripts for subsequent exploration (Sathirapongsasuti et al. 2011).

Sample	Sample Type	Yield (Gb)	% Mapped	% On Target	On Target Mean Coverage
17	Germline	11.29	99.93	66.68	122.33
17A1	Adenoma	10.79	99.94	72.87	127.43
17A2	Adenoma	10.63	99.97	73.19	125.48
24	Germline	10.41	99.96	73.57	124.02
24A1	Adenoma	10.47	99.96	76.03	128.5
24A3	Adenoma	11.57	99.95	69.24	128.86
24A8	Adenoma	10.26	99.96	71.12	116.56
30	Germline	14.79	99.96	54.48	129.82
30A1	Adenoma	11.82	99.97	73.9	140.55
30A3	Adenoma	14.54	99.95	54.93	128.31
36	Germline	14.22	99.96	59.89	136.76
36A1	Adenoma	11.84	99.95	73.56	140.46
36A3	Adenoma	13.29	99.96	63.75	136.17
37	Germline	10.49	99.96	70.64	119.3
37A1	Adenoma	11.2	99.95	71.82	129.7
37A4	Adenoma	9.48	99.94	75.97	116.27
38	Germline	11.03	99.89	74.43	132.57
38A2	Adenoma	10.86	99.95	75.64	134.07
44	Germline	11.04	99.96	75.64	134.07
44A2	Adenoma	13.11	99.96	60.66	128.78
44A4	Adenoma	14.78	99.87	55.69	132.1
51	Germline	11.15	99.97	75.61	135.34
51A1	Adenoma	12.76	99.93	65.44	134.32
51A3	Adenoma	13.05	99.88	53.54	111.2
51A4	Adenoma	12.26	99.96	70.7	139.49
52	Germline	10.31	99.97	76.07	127.28
52A2	Adenoma	10.36	99.97	72.13	120
52A3	Adenoma	11.83	99.95	63.76	121.34
52A4	Adenoma	10.48	99.95	73.34	124.23

Table 4.2 *- Exome sample metrics for each sample (adenoma or matched blood)*

4.2.2.15 Statistical analysis

The nonparametric Mann Whitney U test was used to determine the differences in frequencies of SNVs in the two disease contexts. Fishers exact was used test to find a significant difference between G>T for MAP versus FAP. A *P* value of less than 0.05 was considered statistically significant. Statistical analysis for the study was performed using R (version 3.0.2) software.

4.3 Results

4.3.1 Patient and adenoma characteristics

23 patients (12 female: 11male) had duodenal adenomas at upper GI endoscopy (13 FAP, 10 MAP). The patient and adenoma characteristics for those that underwent exome sequencing are shown in table 4.3. Samples 17A2, 37A1 and 37A4 were from adenomas arising from the ampulla, the rest were duodenal adenomas.

Adenoma	Age (years)	Patient Sex	Size of adenoma	Histology	% Adenomatous Tissue	Spigelman Stage
17A1	38	F	15mm	TA LGD	80%	IV
17A2			20mm	TVA LGD	80%	
52A3	37	M	25mm	TVA LGD	80%	IV
52A4			15mm	TA LGD	60%	
52A2			8mm	TA LGD	50%	
51A1	42	M	10mm	TA LGD	50%	III
51A3			4mm	TA LGD	30%	
51A4			2mm	TA LGD	20%	
30A1	49	F	4mm	TA LGD	50%	III
30A3			8mm	TA LGD	40%	
24A1	59	M	15mm	TA LGD	40%	III
24A3			15mm	TA LGD	50%	
24A8			12mm	TVA LGD	90%	
37A1	63	F	25mm	TA LGD	40%	II
37A4			25mm	TA LGD	70%	
36A1	47	F	3mm	TA LGD	50%	II
36A3			8mm	TA LGD	50%	
44A2	42	M	5mm	TA LGD	60%	II
44A4			4mm	TA LGD	60%	
38A2	47	M	30mm	TA LGD	30%	II

Table 4.3 – *Patient and adenoma characteristics of the those samples that underwent exome sequencing (blue=FAP, red=MAP)*

4.3.2 APC and MUTYH Germline and Somatic Mutations

4.3.2.1 Germline APC and MUTYH Variants

Nine constitutional DNA samples were subjected to exome sequencing (matched with the 20 adenomas). Exome analysis confirmed the clinical and previous NHS molecular genetic diagnosis of 8/9 patients in this study. For one of the FAP patients, exons 4-5 of the *APC* gene were previously found to be deleted by diagnostic MLPA analysis (Cardiff and Vale NHS). For the remaining 3 FAP patients; 3 different monallelic *APC* germline mutations were identified (figure 4.2) and biallelic *MUTYH* germline mutations were identified in all 5 MAP patients (figure 4.3). PCR and Sanger sequencing confirmed the presence in the original constitutional DNA samples of all of these variants.

4.3.2.2 Somatic APC Variants

Twenty-five somatic mutations (table 4.4) in the *APC* gene were identified in the adenoma samples. For two of the FAP adenomas (17A1 and 52A2), no somatic changes at the *APC* locus were identified (SNVs or LOH). In sample 30A3 the only mutation identified was a missense variant. PCR and Sanger sequencing confirmed all somatic *APC* sequence changes identified by exome analysis. Monoallelic somatic *APC* mutations, SNV or LOH were identified in 8/10 of the FAP polyps (80%), 7 SNVs and 1 instance of LOH. Biallelic somatic *APC* mutations, 18 SNV stop gains and 2 cases of LOH, were identified in all of the MAP adenomas

4.3.2.3 LOH of the APC Gene.

APC LOH was detected in 3 adenomas (1 FAP and 2 MAP). LOH was only identified at markers; rs2019720 (*APC* promoter), rs1914 (*APC* intron 7) and nt5037 (*APC* exon 15). The LOH appeared to be copy neutral, as no CNVs were detected at the *APC* locus by array CGH, suggesting that the LOH was caused by mitotic recombination.

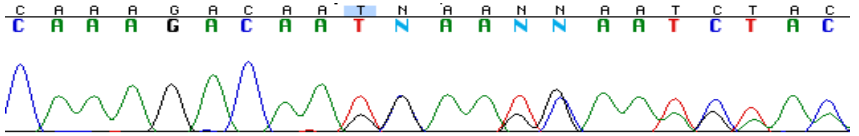
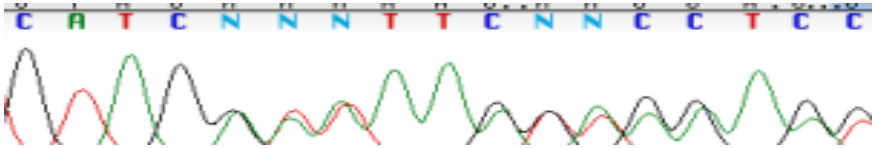
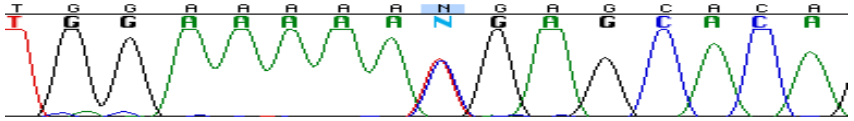
Sample	Gene	SNV/INDEL					Sequencing Validation
		Exon	c.	p.	Affect on protein	Position	
17	APC	4-5	n/a	n/a	Exon deletion	112178795	n/a
52	APC	15	3863 GA>A	G1288fsX16	Frameshift	112175152	
30	APC	15	3198 ACAAT>CAAT	R1067fsX59	Frameshift	112174489	
51	APC	5	637 C>T	R213X	Stopgain	112176592	

Figure 4.2. *– Germline APC validation*

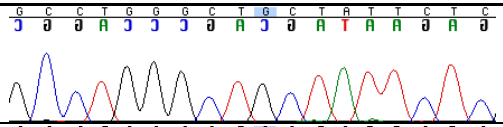
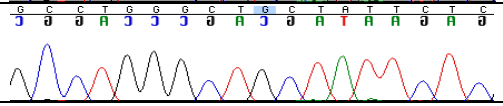
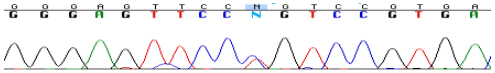
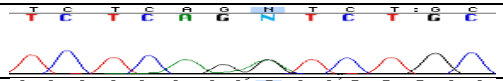
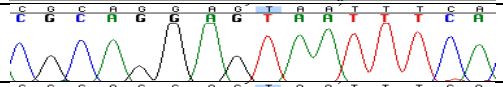
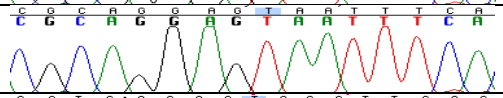
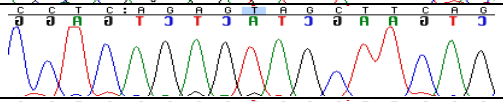
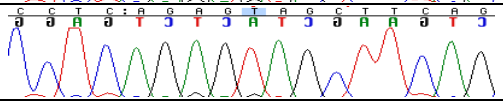
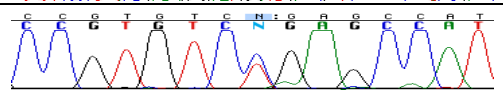
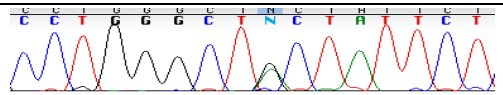
Sample	Gene	SNV/INDEL					Reported	Reported frequency	Sequencing Validation
		Exon	c.	p.	Affect on protein	Position			
24	MUTYH	7	536 A>G	Y176C	Nonsynonymous (HOM)	45798475	rs34612342	N/A	
	MUTYH	7	536 A>G	Y176C	Nonsynonymous (HOM)	45798475	rs34612342	N/A	
37	MUTYH	13	1214 C>T	P405L	Nonsynonymous (HET)	45797201			
	MUTYH	13	1187 G>A	G396D	Nonsynonymous (HET)	45797228	rs36053993	T=0.004/8	
36	MUTYH	14	1438 G>T	E480X	Stopgain (HOM)	45796892	rs121908381	N/A	
	MUTYH	14	1438 G>T	E480X	Stopgain (HOM)	45796892	rs121908381	N/A	
44	MUTYH	13	1240 C>T	Q414X	Stopgain (HOM)	45797175			
	MUTYH	13	1240 C>T	Q414X	Stopgain (HOM)	45797175			
38	MUTYH	9	739 T>C	R247X	Stopgain (HET)	45798112			
	MUTYH	7	526 G>A	Y176C	Nonsynonymous (HET)	45798475	rs34612342	N/A	

Figure 4.3 *– Germline MUTYH validation*

Sample	Gene	SNV/INDEL					Reported	Reported frequency
		Exon	c.	p.	Affect on protein	Position		
17A1	APC	No mutation identified						
17A2	APC	15	4691 T>G	L1564X	Stopgain	112175982		
30A1	APC	15	4660 G>GA	E1554fsX4	Frameshift	112175951		
30A3	APC	15	7504 G>A	G2502S	Nonsynonymous	112178795	rs2229995	A=0.0084/42 *
51A1	APC	15	4606 G>T	E1536X	Stopgain	112175897		
51A3	APC	15	4660 G>GA	E1554fsX4	Frameshift	112175951		
51A4	APC	LOH (nt5037)						
52A3	APC	15	4660 G>GA	E1554fsX4	Frameshift	112175951		
52A4	APC	15	4348 C>T	R1450X	Stopgain	112175139	rs121913332	N/A
52A2	APC	No mutation identified						
24A1	APC	15	4678 G>T	E1559X	Stopgain	112175969		
24A1	APC	15	LOH (rs2019720)					
24A3	APC	15	3502 G>T	E1168X	Stopgain	112174793		
24A3	APC	15	4654 G>T	E1552X	Stopgain	112175945		
24A8	APC	15	3502 G>T	E1168X	Stopgain	112174793		
24A8	APC	15	4654 G>T	E1552X	Stopgain	112175945		
36A1	APC	15	2962 G>T	E988X	Stopgain	112174253		
36A1	APC	15	LOH (nt5037)					
36A3	APC	15	3845 G>T	S1282X	Stopgain	112175136		
36A3	APC	15	472 6 G>T	E1576X	Stopgain	112176017		
37A1	APC	4	526 G>T	E176X	Stopgain	112111429		
37A1	APC	15	4660 G>GA	E1554fsX4	Frameshift	112175951		
37A4	APC	15	526 G>T	E176X	Stopgain	112111429		
37A4	APC	15	4660 G>GA	E1554fsX4	Frameshift	112175951		
38A2	APC	15	4381 G>T	E1461X	Stopgain	112175672		
38A2	APC	15	3460 G>T	E1154X	Stopgain	112174751		
44A2	APC	4	2311 G>T	E771X	Stopgain	112173602		
44A2	APC	15	4630 G>T	E1544X	Stopgain	112175921		
44A4	APC	4	44588 G>T	E1530X	Stopgain	112175879		
44A4	APC	15	3406 G>T	E1136X	Stopgain	112174697		

Table 4.4 – *Validated somatic mutations in **FAP** and **MAP** adenomas. *missense variant*

4.3.3 Validation of exome-wide somatic SNVs

Exome sequencing identified 1376 exome-wide somatic SNVs. Excluding the previously validated 25 *APC* somatic mutations, as described above, a further 1351 exome-wide somatic SNVs required confirmation by PCR and Sanger in the original DNA samples. The frequencies of putative and confirmed somatic SNVs (including *APC*) in each adenoma sample are shown in table 4.5. Details of validated individual SNVs identified in the adenoma samples can be found in appendix 4 of the electronic supplementary material. Of the 1351 exome-wide SNVs, 112 SNVs failed to amplify or could not be sequenced. Consequently, validation of 1239 exome-wide SNVs across the 20 adenoma DNA samples was undertaken. Of these, 881, including 25 *APC* mutations (71%) were confirmed. There were between 41 and 2 SNVs in each of the 10 FAP adenomas and between 158 and 6 SNVs in each of the 10 MAP adenomas, the majority being nonsense mutations (table 4.4). There is a statistically significant difference in the frequency of SNVs in the two disease contexts (p=0.035), with more SNVs identified in MAP than FAP adenomas. Polyp size also showed a significant positive correlation with number of SNVs for both FAP and MAP; FAP (correlation coefficient of 0.66; p-value = 0.03721) and MAP (correlation coefficient of 0.65; p-value = 0.04346).

4.3.4 WTX Gene Sequencing

Frequent somatic mutations of *WTX* have recently been observed in FAP and MAP colorectal adenomas (J Sampson, personal communication). Surprisingly, no *WTX* mutations were found on exome sequencing of duodenal adenomas in the current study. Therefore, the *WTX* ORF was Sanger sequenced in an additional 47 duodenal adenomas (20 FAP and 27 MAP) but again, no mutations were identified

Adenoma	Pre-Validation Exome-wide Somatic SNVs	Pre-Validation Total somatic SNVs (including APC)	Failed PCR/Seq	Not confirmed	Confirmed Exome-wide SNVs	Confirmed somatic *APC* SNVs	Post-Validation Total confirmed somatic SNVs	% validated	FAP/MAP	Adenomatous Tissue
17A1	25	25	5	2	21	0	21	84	FAP	80%
17A2	46	47	1	4	41	1	42	89		80%
52A3	65	66	6	33	26	1	27	40		80%
52A4	83	84	6	46	31	1	32	37		60%
52A2	27	27	2	19	6	0	6	22		50%
51A1	38	39	1	5	32	1	33	84		50%
51A3	54	55	9	35	12	1	13	22		30%
51A4	33	33	7	24	2	0	2	6		20%
30A1	37	38	7	12	17	1	18	46		50%
30A3	55	56	3	23	29	1	30	53		40%
24A1	27	28	1	3	23	1	24	85	MAP	40%
24A3	47	49	2	6	39	2	41	83		50%
24A8	92	94	6	6	80	2	82	87		50%
37A1	219	221	17	43	158	2	160	72		40%
37A4	102	104	10	30	62	2	64	61		70%
36A1	14	15	2	3	9	1	10	64		50%
36A3	131	133	5	10	116	2	118	89		50%
44A2	67	69	7	15	45	2	47	67		60%
44A4	14	16	2	6	6	2	8	43		60%
38A2	175	177	13	36	126	2	128	72		30%
Total	**1351**	**1376**	**112**	**361**	**881**	**25**	**906**	**66**		

Table 4.5 *– Total somatic SNVs identified and validated*

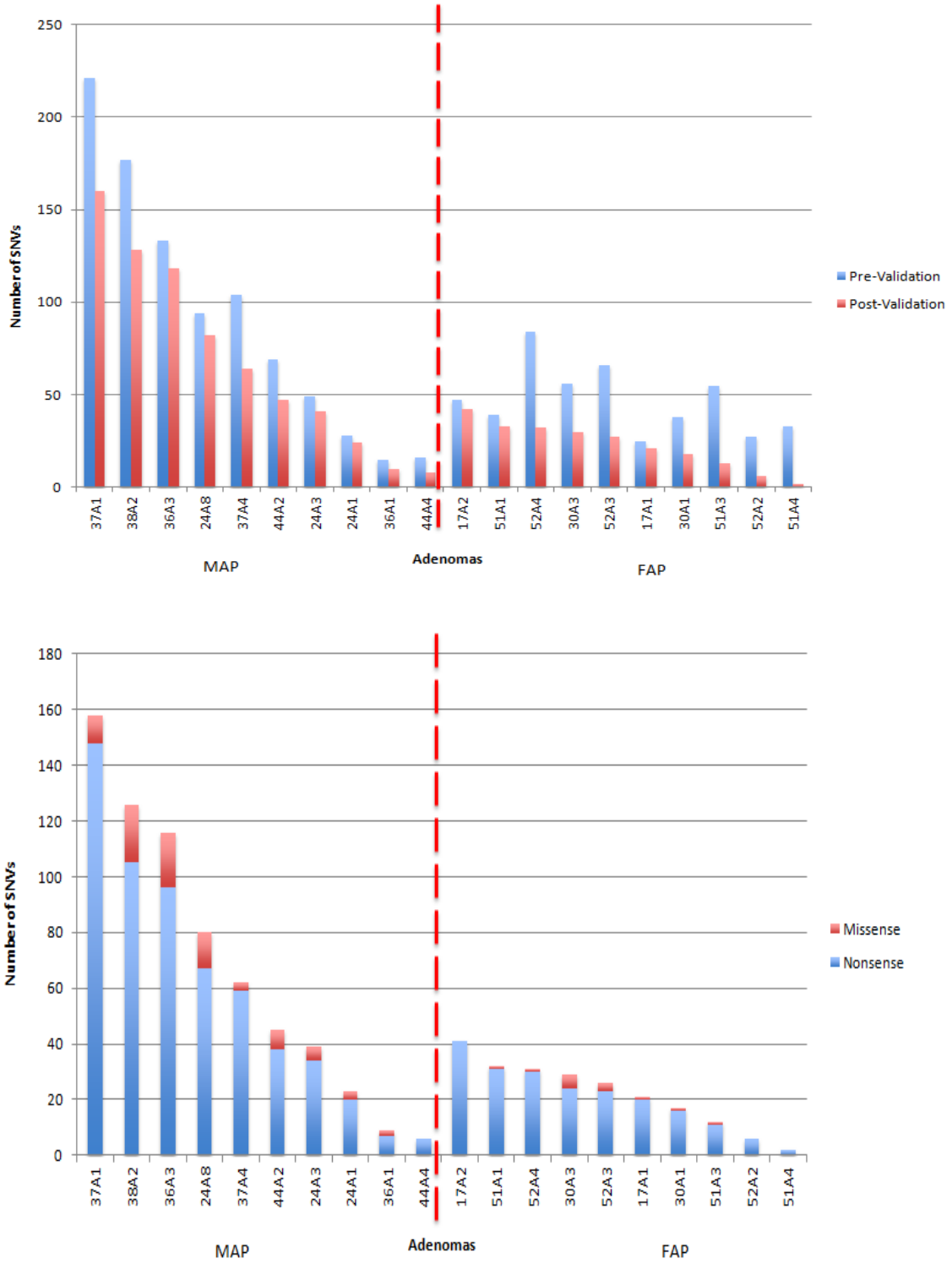


Figure 4.4 *– Comparison of total number of confirmed SNVs in MAP versus FAP adenomas.*

4.3.5 KRAS codon 12 and 13 sequencing

KRAS c.34G>A; p.G12S was identified in 4 of 10 MAP polyps and c.35G>A; p.G12D was identified in 1 of 10 FAP polyps. In order to investigate further the apparent excess of MAP-associated *KRAS* mutations, 27 additional MAP adenomas and 20 additional FAP adenomas were sequenced for codon 12 and 13 *KRAS* mutations. Additional mutations were found in 8 MAP and 3 FAP adenomas. In total 16 KRAS mutations were identified in 67 adenomas (24%; $p = 0.070$; table 4.6).

Adenoma	FAP / MAP	Change	Histology	Polyp size
4A1	FAP	c.37G>T; p.G13C	TA LGD	3mm
24A4	MAP	c.34G>A; p.G12S	TA LGD	8mm
29A2	FAP	c.35G>A; p.G12D	TA LGD	4mm
29A3	FAP	c.35G>A; p.G12D	VA LGD	4mm
38A4	MAP	c.34G>T; p.G12C	TA LGD	5mm
39A1	MAP	c.35G>T; p.G12V	TA LGD	9mm
39A4	MAP	c.35G>T; p.G12V	TA LGD	9mm
44A1	MAP	c.34G>T; p.G12C	TA LGD	5mm
54A1	MAP	c.34G>T; p.G12C	TVA LGD	8mm
54A9	MAP	c.34G>T; p.G12C	TA LGD	10mm
54A10	MAP	c.34G>T; p.G12C	TA LGD	12mm
52A4	FAP	c.35G>A; p.G12D	TA LGD	15mm
24A3	MAP	c.34G>A; p.G12S	TA LGD	15mm
24A8	MAP	c.34G>A; p.G12S	TVA LGD	12mm
37A1	MAP	c.34G>A; p.G12S	TA LGD	25mm
37A4	MAP	c.34G>A; p.G12S	TA LGD	25mm

Table 4.6 *- KRAS mutations detected in FAP and MAP adenomas (red=original adenomas exome sequenced)*

4.3.6 Frequency of G>T transversions

The number of G>T transversions was determined in each of the 20 adenoma samples and compared between FAP and MAP. There were significantly more G>T transversions in adenomas from MAP patients than FAP patients ($p<0.001$, figure 4.5a and b). The percentage of truncating SNVs calculated from the total number of SNVs per sample demonstrated a significantly higher proportion of truncating SNVs in the MAP adenomas than FAP adenomas ($p<0.0001$).

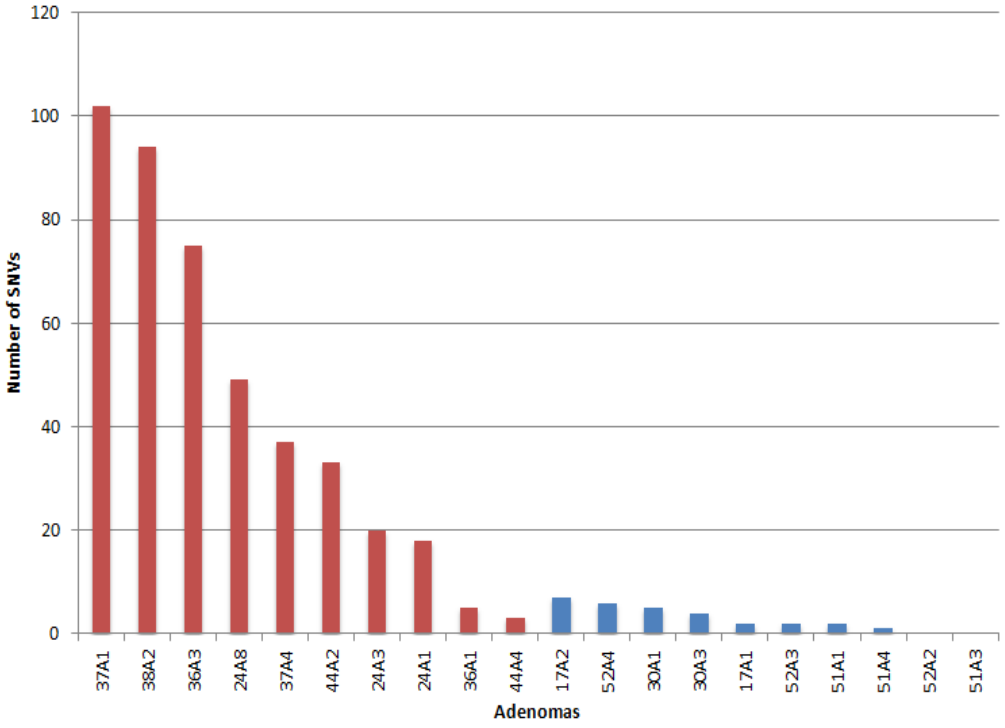


Figure 4.5a *– Number of G>T transversions in MAP and FAP*

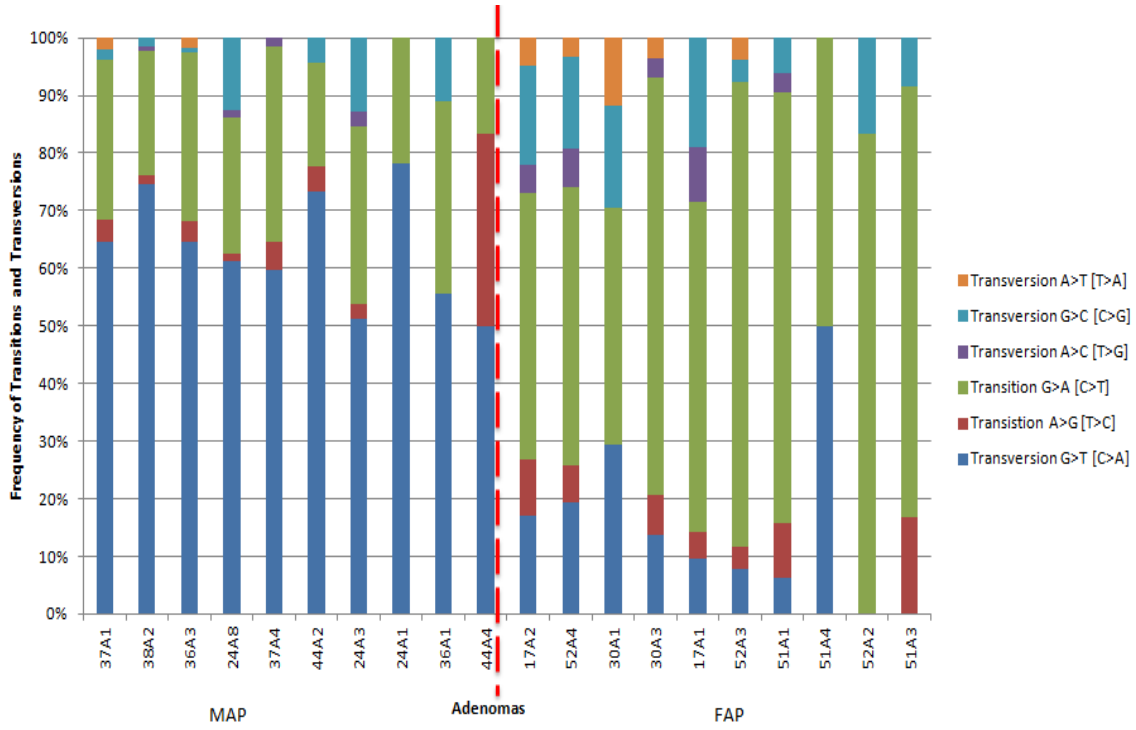


Figure 4.5b *–Differences between types of somatic mutations in MAP and FAP*

4.3.7 Frequently mutated Genes and common SNVs.

Of the 906 confirmed somatic SNVs (881 exome wide and 25 *APC*) identified in the exome data from the 20 adenoma DNA samples, there were 14 genes, which had more than one SNV; *APC* (25 SNVs), *PLCL1* (5 SNVs), *SYNE1* (5 SNVs), *KRAS* (5 SNVs), *CPSF6* (4 SNVs), *LRP1B* (4 SNVs), *DNAH5* (3 SNVs), *IGFN1* (3 SNVs), *DNAH2* (3 SNVs), *DCHS2* (3 SNVs), *PTCHD2* (3 SNVs), *SLC4A3* (3 SNVs), *ERBB3* (3 SNVs), *TTN* (3 SNVs) (table 4.6a). Additionally, recurrent mutations (i.e. the same SNV identified in between 2 and 5 individual samples) were detected in 9 of these genes (table 4.6b).

Chr	Position	ref	alt	gene. id	FAP samples	MAP samples
Chr 5				*APC**	7 mutations in 7	18 mutations in 10
Chr 2	198949617	G	A	*PLCL1*	17A2	
Chr 2	198949560	G	A		51A1	
Chr 2	198949768	G	T			37A1
Chr 2	198949880	G	T			37A1
Chr 2	198949880	G	T			37A4
Chr 6	152697533	T	C	*SYNE 1*	30A3	
Chr 6	152563464	G	T			37A1
Chr 6	152527386	G	A			36A3
Chr 6	152647170	G	A			36A3
Chr 6	152748947	G	T			38A2
Chr 12				*KRAS**	1 adenoma	4 adenomas
Chr 12	69656302	G	A	*CPSF6*		37A1
Chr 12	69656304	G	A			37A1
Chr 12	69656302	G	A			37A4
Chr 12	69656304	G	A			37A4
Chr 2	198949880	G	T	*LRP1B*		24A3
Chr 2	141298667	C	T			24A8
Chr 2	141298667	C	A			37A1
Chr 2	141777585	C	A			37A4

Chr 5	13735384	C	T	*DNAH5*	51A1	
Chr 5	13882892	C	A			36A3
Chr 5	13811890	G	A			38A2
Chr 1	201181973	C	T	*IGFN1*	52A5	
Chr 1	2011819731	C	T		52A3	
Chr 1	201181973	C	T		52A4	
Chr 17	7661866	G	A	*DNAH2*		24A3
Chr 17	7661866	G	A			24A8
Chr 17	7720915	G	A			44A2
Chr 4	155156598	G	A	*DCHS2*	51A1	
Chr 4	155158065	C	T		51A1	
Chr 4	11561594	C	T			24A8
Chr 1	11561594	G	T	*PTCHD2*		37A1
Chr 1	11584030	G	T			37A1
Chr 1	11591019	G	T			37A2
Chr 2	220500412	G	A	*SLC4A3*	52A5	
Chr 2	220500412	G	A		52A3	
Chr 2	220500412	G	A		52A4	
Chr 12	56487261	C	G	*ERBB3*	52A4	
Chr 12	56480320	C	A			24A3
Chr 12	56480320	C	A			24A8
Chr 2	179595059	A	G	*TTN*		37A1
Chr 2	179416845	C	A			38A2
Chr 2	179436284	C	A			38A2

Table 4.6a *– The most frequently mutated genes observed on exome sequencing (red = truncating mutation, black = missense mutation). Details of APC mutations are described in section 4.3.2 and KRAS mutations in 4.3.5*

chromosome	position	ref	alt	gene.id	number of times observed
chr5	112175951	G	GA	*APC*	5
chr12	25398285	C	T	*KRAS*	4
chr2	220500412	G	A	*SLC4A3*	3
chr1	201181973	C	T	*IGFN1*	3
chr5	112111429	G	T	*APC*	2
chr5	112174793	G	T	*APC*	2
chr5	112174697	G	T	*APC*	2
chr5	112175945	G	T	*APC*	2
chr2	198949880	G	T	*PLCL1*	2
chr12	69656302	G	A	*CPSF6*	2
chr12	69656304	G	A	*CPSF6*	2
chr12	56480320	C	A	*ERBB3*	2
chr17	7661866	G	A	*DNAH2*	2
chr2	141777585	C	A	*LRP1B*	2
chr2	141298667	C	T	*LRP1B*	2

Table 4.6b *– Recurrent somatic SNVs observed on exome sequencing*

4.3.8 Array CGH

Array CGH was completed on 48 adenomas, 26 adenomas from FAP patients and 22 adenomas from MAP patients. Six CNVs were detected in 3 adenomas, all of which were from MAP patients and included 1 copy number gain and 5 losses. No CNVs were identified in adenomas from FAP patients. The CNVs were detected on chromosomes 7, 8, 9 and 18 and ranged in size between 354kb and 157MB (table 4.7, figures 4.6a and b). All CNVs were validated by the Exome data and confirmed by qPCR analysis.

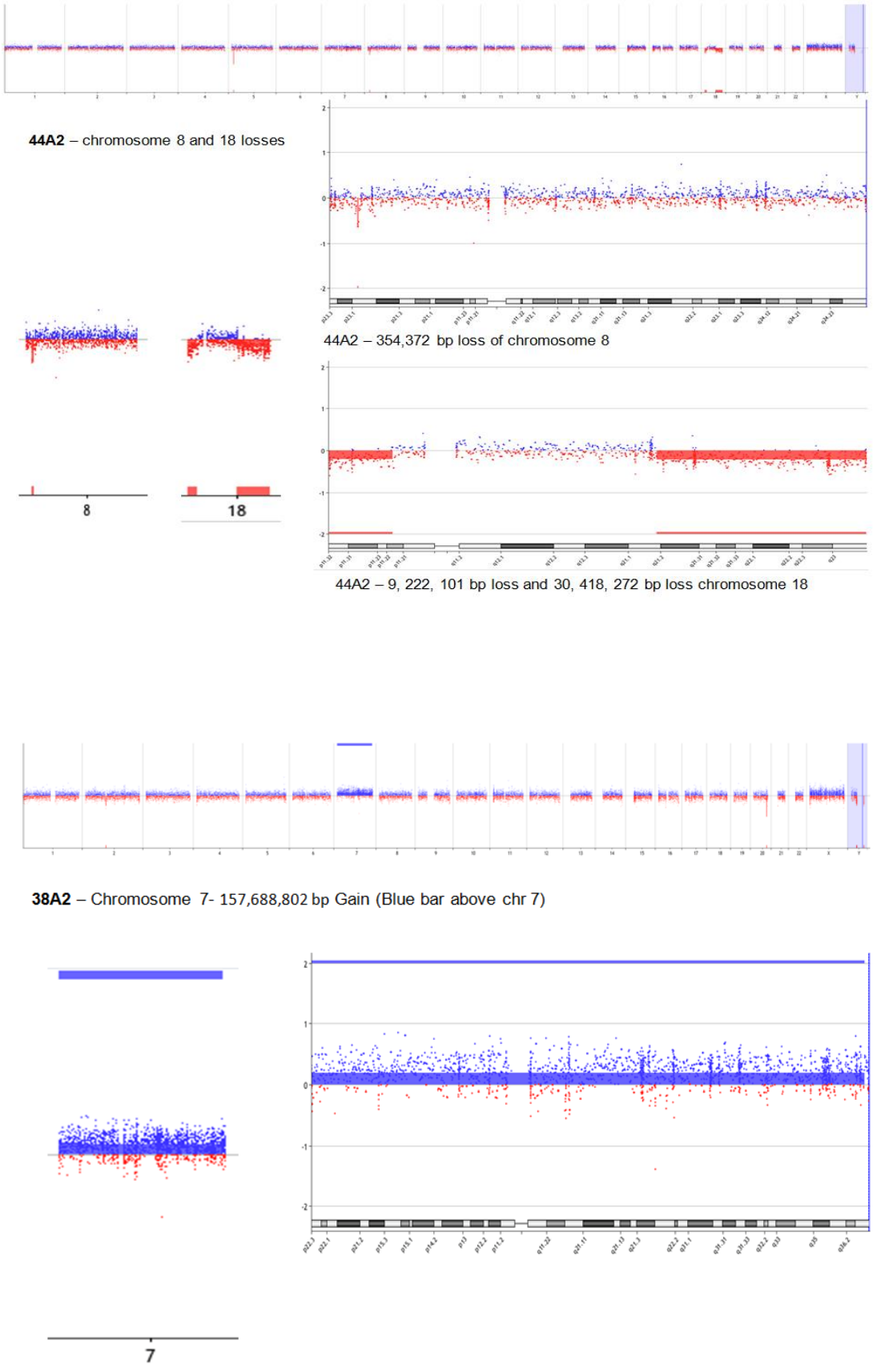


Figure 4.6a *– CNVs in MAP adenomas 44A2 and 38A*

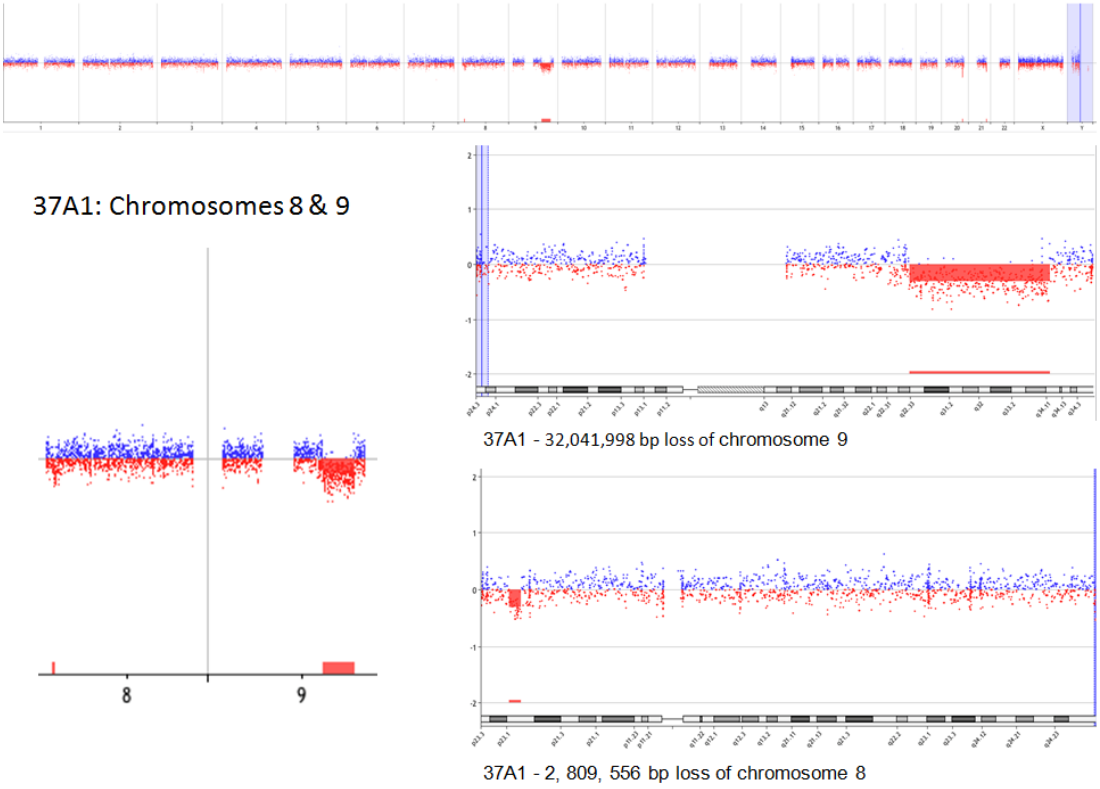


Figure 4.6b *– CNVs in MAP adenoma samples 37A1*

Chromosome	Location	CNV	Copy	Start	End	Size (bp)	No. of OMIM Genes	No of HGNC Genes	Adenoma Samples
7	7p22.3 – 7q36.3	GAIN	0.19	54,215	157,723,116	157,688,802	589	1, 243	38A2
8	8p.23.1	DEL	-0.30	6,805,940	9,615,505	2,809,556	15	74	37A1
	8p. 23.1	DEL	-0.62	7,691,931	8,046,302	354,372	3	15	44A2
9	9q22.32	DEL	-0.30	99,121,641	131,163,638	32,041,998	153	293	37A1
18	18p11.32	DEL	-0.20	148,993	9,371,093	9,222,101	24	38	44A2
	18q21.1	DEL	-0.22	47,594,529	78,012,800	30,418,272	70	104	44A2

Table 4.7 *Copy number gains and deletions in 3 MAP adenomas (OMIM: Online Mendelian inheritance in man; HGNC: Hugo gene nomenclature committee).*

4.4 Discussion

This is the first study that has explored the somatic mutational spectra of duodenal adenomas from patients with germline mutations in *APC* and *MUTYH***.**

4.4.1 Somatic mutations and LOH

Somatic 'second hits' in *APC* are known to be necessary for adenoma development in FAP, and bi-allelic somatic mutations in MAP. In this study exome analysis and subsequent validation of putative variants with Sanger sequencing detected somatic *APC* mutations in 8/10 (80%) FAP duodenal adenomas and bi-allelic somatic *APC* mutations in 10/10 (100%) MAP adenomas. Duodenal adenomas showed *APC* LOH in 3/20 (15%) of cases (2 MAP and 1 FAP) in this study LOH appeared to occur by mitotic recombination as there were no CNVs detected at the 5q locus by arrayCGH. Reports of LOH at the *APC* locus in the duodenum are very limited. One study on colorectal tumours detected no LOH in 13 CRCs from MAP patients (Johnson et al. 2005), and Lipton et al (2003) found no *APC* LOH in MAP colorectal adenomas. The incidence of LOH in FAP colorectal adenomas has been reported at between 22-40% (Lamlum et al. 1999, Crabtree et al. 2003, Will et al. 2008). Obrador-Hevia et al. (2010) reported LOH in 18% of colorectal FAP adenomas, with no associated loss of genetic material, similar to the current study. The current study suggests that *APC* mutations play a similar role in adenoma formation in the duodenum to the colorectum.

Somatic *APC* mutations were not identified in two FAP adenomas (17A1 and 52A2). The raw data (from bam files) were reviewed for the *APC* ORF in both adenomas, but no evidence of a pathogenic somatic *APC* mutation was detected. The amount of dysplastic tissue in these 2 samples was high (50% and 80%) and had low grade dysplasia. This finding compared is consistent with the data of the Cancer Genome

Atlas (TCGA) that reports frequencies of 60-80% for *APC* mutations in various colorectal tumours studies. One recent study (Obrador-Hevia et al. 2010) only found detectable second hits in *APC* that were not LOH in 6% of FAP colorectal adenomas, but only the MCR of the gene was analysed. One FAP adenoma (30A3) had a missense variant identified in *APC* (and no LOH) of unclear pathogenicity. Functional testing of this mutation has not been reported, so it is a variant of unknown significance (VUS) however usually somatic *APC* mutations are truncating. Alternative genetic mechanisms accounting for the somatic inactivation of *APC*, such as defects in gene expression and promoter hypermethylation may have occurred but were not examined as part of this study. Hypermethylation is more typically associated with carcinomas than pre-malignant lesions (Hiltunen et al. 1997; Brucher et al. 2006; Berkhout et al. 2007) and is rarely found in tumours from FAP patients (Schneikert and Behrens, 2007). This study found no 'third hits' in any of the adenomas. It has been proposed that some MAP tumours may have third somatic APC mutations, as has been reported in AFAP, (Spirio et al. 1998; Su, L-K. 2000; Sieber et al 2006) but this was not observed in MAP duodenal adenomas in this study.

For 16 of the 18 adenomas in which somatic *APC* mutations were identified, a distinct somatic *APC* mutation was detected indicating that each adenoma arose through an independent mutational event. Adenomas 37A1 and 37A4 from the same MAP patient were situated adjacently in the duodenum at the ampulla, and appear to have a common ancestral cell of origin. They share the same somatic *APC* mutations (c.526 G>T; pE176X and c.4660 G>GA; p.E1554fsX4), but the adenomas appear to have then diverged as 37A1 has additional exome-wide somatic mutations that are not present in 37A4, with differing SNVs and total numbers of mutations found. Adenoma 37A1 possessed a greater number of mutations and was larger and flatter in morphology. Adenoma size has been positively correlated with

risk of advanced dysplasia in the colorectum (Atkin et al. 2002; Matsuda et al, 2011), and in the duodenum (Lopez-Ceron et al, 2013). Although both polyps had the same histological findings from the random biopsies taken the greater number of mutations within this larger polyp might be associated with an increased risk of later higher grade dysplasia. Both 37A1 and 37A4 had a high depth of coverage on exome sequencing, indicating that the greater number of somatic hits in 37A1 is not likely to be a technical artefact.

4.4.2 Frequency of SNVs in duodenal adenomas

Exome analysis of duodenal adenomas from MAP and FAP patients has never previously been reported. In this study MAP adenomas were found to have a greater number of SNVs compared to FAP which was statistically significant (p=0.035). G:C>T:A mutations were the most commonly observed mutation in the MAP adenomas, and were significantly associated with MAP versus FAP adenomas (p<0.001). In addition, there was higher proportion of truncating SNVs in the MAP adenomas than FAP adenomas (p=0.007). This has also been reported in colorectal MAP adenomas (Al-Tassan et al. 2002; Jones et al. 2002), and is in keeping with the underlying mechanism associated with *MUTYH* dysfunction, whereby an 8-oxo-G mismatch is not recognised or excised during base excision repair resulting in a G:C>T:A transversion in the next round of DNA replication. The higher number of somatic mutations (including truncating mutations) in MAP adenomas supports the proposal that they may carry a higher risk of progression to cancer than FAP adenomas

A recent study by Rashid et al (2016), has explored the somatic mutational spectrum in FAP and MAP colorectal adenomas using exome sequencing and additional targeted sequencing. A higher mutation rate and greater frequency of

G>T transversions was observed in colorectal adenomas from MAP patients in comparison to those from FAP patients, similar to the duodenal findings in the current study. However, the pattern of driver gene involvement we found in the duodenum was significantly different from that reported in colorectal adenomas. In the colorectum, mutations were present in adenomas in important 'traditional' driver genes such as *TP53*, *NRAS*, *FBXW7, NF1, BCL9L, MAP3K5* and *PTEN*. Mutations in these genes are completely absent from the duodenal exome dataset.

Lack of *TP53* mutations in duodenal adenomas and carcinomas have been reported in FAP. Toyooka et al (1995) found no *TP53* mutations in 35 duodenal tumours, including two duodenal cancers, and Kashiwagi et al. 1997) reported only one *TP53* mutation in 25 duodenal adenomas studied. In the colorectum, *TP53* mutations have only been reported in 3-5% of colonic FAP adenomas (Kichuci-Yanoshita et al. 1992). Mutations in *TP53* act to promote the malignant transformation of adenomas, at a late stage, to malignancy. The results from the current study are in keeping with expectations for duodenal adenoma with low grade dysplasia. Lipton et al (2003) found three *TP53* mutations (all in carcinomas) in 14 colorectal cancers and 115 adenomas in MAP patients, but unlike *APC* and *KRAS* these were not biased to G>T changes. The role of *TP53* in MAP patient tumours is yet to be fully elucidated.

4.4.3 KRAS mutations

KRAS is a member of the RAS family of proto-oncogenes and is the most frequently mutated gene in all of human cancer (Pritchard and Grady, 2011) and mutations in codons 12 or 13 are found in 40% of CRCs (Downward, 2003). It acts as a downstream effector of EGFR that signals through *BRAF* to activate the MAPK pathway to promote cell growth and survival as discussed in section 1.4.3. *KRAS* mutations are not thought to be related to wnt pathway activation in FAP; there has

been no correlation found between β-catenin nuclear staining and the presence of *KRAS* mutations in FAP colorectal adenomas (Obrador-Hevia et al. 2010). Activating *KRAS* mutations are thought to usually occur after *APC* mutation in the adenoma-carcinoma sequence, but are still an early event in tumourigenesis (Vogelstein et al. 1998).

KRAS was the second most frequently mutated gene after *APC* in this study occurring in 24% of the adenomas; 32% of MAP polyps and 13% of FAP polyps were found to have a *KRAS* mutation this was not found to be statistically significant, (although was approaching significance), but this was likely due to the numbers of adenomas in the study. Previous studies of FAP duodenal adenomas have described *KRAS* mutations in 12%, with no relation to the grade of dysplasia of the adenoma (Kashiwagi et al. 1997), 9% (Wagner et al. 2008) and 10% (Obrador-Hevia et al. 2010) of duodenal adenomas. Schönleben et al, (2009), completed molecular analyses of *KRAS, HRAS, NRAS* and *BRAF* in duodenal adenomas and carcinomas. *KRAS* mutations at codons 12 and 13 were identified in 28.6% of adenomas and additionally 2 *BRAF* missense mutations, which have not been detected in this study. More recently, Sun et al (2014) detected *KRAS* mutations in 26.3% of sporadic duodenal adenomas. Data in the colorectum in FAP also shows a similar proportion of *KRAS* mutations within adenoma tissue of between 3 and 25% (Farr et al. 1988; Miyaki et al. 1990; Sasaki et al. 1990: Ando et al. 1992).

Rashid et al. (2016) also found *KRAS* G12C mutations in 12% of the MAP colorectal adenomas. This specific somatic *KRAS* mutation has previously been identified in 18% of colorectal MAP adenomas, and association seen with increased dysplasia, villous content and size of the adenoma (Jones et al. 2004). The G12C activating mutation is the result of a G:C to T:A transversion (Lipton, L. *et al*, 2003; Jones, S. *et al*, 2004), and was found to occur more frequently in MAP than in sporadic or FAP

associated tumours (p<0.0002). *KRAS* mutations have also been previously described in the MAP duodenum (Nielsen et al, 2006) although that study included only one duodenal carcinoma and one duodenal adenoma. In this study there appears to be selection for *KRAS* only and not for any other proto-oncogenes of the Ras family such as *NRAS* or *HRAS*. All *KRAS* mutations were all at codons 12 and 13 where the mutations prevent GTPase-activating proteins from hydrolysing the *KRAS*-bound GTP (Downward, 2003). No association of dysplasia grade with *KRAS* mutation,was found, but this may be due to the relatively small numbers of polyps analysed.

4.4.4 Recurrently mutated genes: duodenum and colorectum

When comparing our exome data from MAP and FAP duodenal adenonas with SNVs in MAP and FAP colorectal adenomas reported by Rashid et al (2016), we found, in addition to *KRAS,* mutations in 40 other genes common to both datasets. No specific SNVs were the same in both datasets. A list of these common genes can be found in appendix 5 of the supplementary electronic data.

Amongst the shared genes were *PLCL1* (Phospholipase C-Like 1) and *SYNE1 (*Spectrin Repeat Containing, Nuclear Envelope 1). Mutations in these genes were identified in 25% of the duodenal samples that underwent exome sequencing. *PLCL1* can act as an inhibitor of PPP1C in the proton pump inhibitor (PPI) pathway. *PLCL1* was one of the most commonly mutated genes in the duodenum and a novel finding in this study. It is involved in the Ca2+ signalling pathway of the PPI inhibitor pathway; Ca2+ is known to increase vesicular traffic to the membrane, thereby leading to more active proton pumps and increased acid secretion. Thus, inhibition or dysregulation of this pathway may lead to reduction in acid secretion in the parietal cells of the stomach, as with PPI medication. The profound reduction in

gastric acid secretion induced by PPIs leads to increased secretion of gastrin, and most PPI users have moderate hypergastrinaemia (Lamberts *et al.* 1988; Klinkenberg-Knol *et al.* 1994). Hypergastrinaemia has been associated with an increased risk of gastric carcinoids, and gastric and colonic carcinomas (Havu, 1986; Laine *et al.* 2000), although recent results for PPI use and colorectal cancer risk have shown no association (Robertson *et al*, 2007; van Soest *et al*, 2008). Long-term PPI use has been associated with an increased incidence of atrophic gastritis, a precursor of gastric adenocarcinoma (Uemura *et al*, 2001; Ye and Nyren, 2003), however there have been no reports of an association between duodenal cancer and PPI use. In addition, the increase in gastric pH encourages growth of the gut microflora, increasing bacterial translocation and affecting various immunomodulatory and anti-inflammatory effects. Susceptibility to *Salmonella, Campylobacter jejuni*, invasive strains of *Escheriscia coli, Clostridium Difficile, Vibrio Cholera* and *Listeria* has been shown to be increased by PPI use (Bavishi et al. 2011). Much less is known about the microbes that are present within the duodenum and the effects of PPI use, particularly because collecting samples for such studies is much more challenging. However, further research is needed, especially in light of the growing recognition of the composition of the duodenal microbiota and the association with gastrointestinal disorders; most duodenal microbiota studies have focused predominantly on small intestinal bacterial overgrowth, irritable bowel syndrome and coeliac disease. Immunological disruption may potentially be a mechanism for augmentation of the adenoma-carcinoma progression pathway in the duodenum in MAP and FAP patients and the role of *PLCL1* may be of significant future interest.

SYNE1 is a cytoskeletal protein and methylation of the *SYNE1* gene promoter has been identified by a number of studies in a range of colorectal lesions including carcinomas, tubular adenomas, villous adenomas and sessile serrated adenomas

with or without dysplasia, indicating that deregulation of this gene may be associated with tumour progression (Schuebel et al, 2007; Dhir et al, 2011).

We identified a single truncating mutation in *ATRNL1* in one FAP adenoma. Rashid et al (2016) only identified missense mutations in this gene. *ATRLN1* may play a role in melanocortin signalling pathways that regulate energy homeostasis, but little is known about this gene.

One missense mutation was found in the *KMT2C/MLL3* gene in a MAP duodenal adenoma whereas several *MLL3* truncating mutations were detected in colon adenomas (Rashid et al, 2015). *MLL* genes encode histone methyltransferases, the chief protein components of chromatin, which act as spools around which DNA winds and *MLLs* are required for the correct expression of a variety of genes. Deletion of the region harbouring the *MLL3* gene was noted to be the most frequently recurrent chromosomal abnormality in acute myeloid leukaemia (Ruault et al. 2002). Exome, sequencing has revealed a significant role of *MLL3* in solid tumours (Ford and Dingwall, 2015). Inactivating mutations have been described in pancreatic ductal cell carcinoma and bile duct carcinoma, aggressive cutaneous squamous cell carcinoma, hepatocellular carcinoma and gastric adenocarcinoma (Balakrishnan et al, 2007; Biankin et al. 2012; Ong et al. 2012; Fujimoto et al. 2012; Zang et al. 2012; Pickering et al. 2014; Li et al 2014).

4.4.5 SMAD4 mutations

Interestingly, *SMAD4* mutations were not observed in the colorectal dataset described by Rashid et al (in press), but were seen in our duodenal dataset. S*MAD4* is a postreceptor signalling pathway gene, and a member of the TGFβ superfamily.

Deregulation of TGFβ signalling, which is generally considered a tumour-suppressor pathway in the colon, occurs in the majority of colorectal cancers (Chittenden et al. 2008). Bi-allelic inactivation of *SMAD4* occurs in a significant proportion of advanced CRCs (Koyama et al. 1999) and it location on 18q results in vulnerability to deletion as it is found in the region commonly deleted in CRC. Mutations of *SMAD4* and *BMPR1A* cause juvenile polyposis syndrome. In mouse models *SMAD4* mutations have been associated with adenoma-carcinoma progression (Takaku et al. 1998), but no study has reported somatic *SMAD4* mutations in FAP colorectal or duodenal adenomas. Lipton et al (2003) reported that MAP colorectal adenomas and carcinomas were found not to harbour somatic mutations in genes frequently mutated in MSI tumours such as *BRAF*, *SMAD4* and *TGFβIIR.*

4.4.6 WTX mutations

Another important difference between the mutational spectrum in the colorectum and the duodenum appears to be the presence / absence of mutations in the WTX gene (*APC* membrane recruitment protein 1; *AMER 1, FAM123B*). The role of *WTX* mutations has been reported in advanced colorectal cancers, but until recently had not been investigated in the early stages of colorectal tumour development. There were no truncating mutations of *WTX* found in any of the 67 duodenal adenomas sequenced in this study. This is in contrast to the findings in colorectal adenomas according to Rashid et al (2016) who identified truncating *WTX* mutations in ~10% of colorectal adenomas in patients with FAP and MAP, second in frequency only to *APC*. *WTX* is on the X-chromosome, however mutations were found in adenomas from both males and females suggesting X-inactivation (lionization) may cause functional loss in females.

WTX was first described as a gene involved in kidney Wilm's tumour development (Rivera et al. 2007). It has a role in the regulation of the wnt pathway, TP53 signalling and in the localisation of the tumour suppressor protein WT1 (Kim et al 2012; Rivera et al. 2009; Moisan et al. 2011). Sclerosing skeletal dysplasia has been linked to germline *WTX* mutations and1 of 25 reported cases in one study had early onset CRC (Jenkins et al. 2009).

Major et al. (2007) has shown that *WTX* forms a complex with β-catenin, *AXIN1*, β-transducin repeat containing protein 2 and *APC,* acting to promote ubiquitin post-translational modification and degradation of β-catenin. A further recent study (Sanz-Pamplona et al. 2015) has shown variants resulting in early stop-codons in *WTX* in approximately 10% of CRC tumours. Tumours lacking this tumour suppressor gene exhibited inhibition of the canonical wnt pathway. Losses of *WTX* by other mechanisms apart from mutation, such as methylation and copy number aberrations, were also reported.

Therefore mutations in *WTX* appear to be associated with both early and late stage colorectal tumours. By comparison we did not identify any WTX mutations in the early stage duodenal tumours in the current study. The reasons for this, and any consequences in terms of tumour progression, are unclear.

4.4.7 Other frequently mutated genes in duodenal adenomas

Other genes that were frequently mutated were *CPSF6, LRP1B, DNAH5, DNAH2, IGFN1, DCHS2, PTCHD2, SLC4A3, ERBB3* and TTN (table 4.6a).

Cleavage and polyadenylation specific factor 6 (*CPSF6*) was mutated in two MAP polyps. It is involved in the production of mature messenger RNA for translation,

forming part of the larger process of gene expression. The protein encoded by this gene is one subunit of a cleavage factor required for 3' RNA cleavage and polyadenylation processing. The interaction of the protein with the RNA is one of the earliest steps in the assembly of the 3' end processing complex and facilitates the recruitment of other processing factors. Common forms of both alpha- and beta-thalassemia (one of the world's most common hereditary diseases) are both associated with point mutations within the polyadenylation signals of alpha-globin and beta-globin genes, respectively (Higgs et al., 1983; Orkin et al., 1985), leading to the generation of abnormal haemoglobin.. Rasaiyaah et al (2013) have reported that HIV-1 has evolved to use the CPSF6 protein to cloak its replication, allowing evasion of innate immune sensors and induction of a cell-autonomous innate immune response in primary human macrophages thus playing a critical role in HIV-1 replication.

Low density lipoprotein receptor-related protein B1 (*LRP1B*) belongs to the low density lipoprotein (LDL) receptor gene family. Mutations were found in 4 MAP adenomas. These receptors play a wide variety of roles in normal cell function and development due to their interactions with multiple ligands (Liu et al. 2001). *LRP1B* is preferentially inactivated in non-small cell lung cancer (NSCLC), and is thought to play an important role in tumorigenesis in this particular type of lung cancer (Liu et al. 2000).

Dyneins are microtubule-associated motor protein complexes composed of several heavy, light, and intermediate chains. The axonemal dyneins, found in cilia and flagella, are components of the outer and inner dynein arms attached to the peripheral microtubule doublets. Dynein, Axonemal, Heavy Chain 5 (*DNAH5*) encodes a protein that functions as a force-generating protein with ATPase activity, whereby the release of ADP is thought to produce the force-producing power stroke.

Mutations in this gene cause primary ciliary dyskinesia type 3, as well as Kartagener syndrome (the classic triad of sinusitis, situs inversus and bronchiectasis), which are both diseases due to ciliary defects (Olbrich et al. 2002; Failly et al. 2009). *DNAH2,* which was also found to be mutated in three MAP adenomas, has been found to be expressed primarily in testes and the trachea (Chapelin et al. 1997) however no disease association has been described to date.

Immunoglobulin-Like And Fibronectin Type III Domain Containing 1 (*IGFN1*) mutations were found in three adenomas form the same FAP patient. It has been found to be expressed in skeletal muscle in mice (Beatham et al. 2004), but little is known about this gene in relation to human disease. This same patient also had three mutations of Solute Carrier Family 4 (Anion Exchanger), Member 3 (*SLC4A3*) whose gene product (AE3) is a plasma membrane anion exchange protein of wide distribution. It mediates at least a part of the chloride-bicarbonate exchange in cardiac myocytes (Kopito et al. 1989). Reduced activity of AE3 in the brain contributed to promoting neuron hyperexcitability and the generation of seizures (Vilas et al. 2009).

Dachsous Cadherin-Related 2 (*DCHS2*) encodes a calcium-dependent cell-adhesion protein. The *DCHS2* gene is expressed in the cerebral cortex and thus is a potential candidate for affecting age of onset in Alzheimers disease (Kamboh et al. 2012), although no studies have linked this gene to tumourigenesis.

Patched Domain Containing 2 (*PTCHD2*) functions as part of the Hedgehog (Hh) signaling pathway. The mechanism of Hh signalling is complex and remains incompletely understood (Briscoe and Therond, 2013) but it is essential for normal embryonic development and plays critical roles in adult tissue maintenance, renewal and regeneration particularly in normal mammalian gastrointestinal development. Aberrant Hh signalling is responsible for the initiation of a growing number of

cancers including, classically, basal cell carcinoma (Chidambaram et al. 1996), medulloblastoma (Cowan et al. 1997), and rhabdomyosarcoma (Berman et al. 2002); more recently overactive Hh signalling has been implicated in pancreatic, lung, prostate, ovarian, and breast cancer in addition to CRC (Watkins et al. 2003; Thayer et al. 2003; Lees et al. 2005). This finding in MAP adenomas is therefore of interest, potentially as an alternative pathway that may predispose to carcinoma development.

V-Erb-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 3 (*ERBB3*) is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases. Amplification of this gene and/or overexpression of its protein have been reported in numerous cancers, because of uncontrolled cell division that ensues when EGFR is constantly activated (Lynch et al. 2004). However we did not identify any potentially activating mutations in our dataset which would be more likely to lead to loss of function. Cancers related to *ERBB3* mutations include prostate, bladder, and breast (Koumakpayi et al. 2006; Hanrahan et al. 2014; Perez-Nadales and Lloyd, 2004). Although ERBB3 has limited inherent kinase activity, ligand stimulation promotes its dimerization with active kinases like ERBB2 and EGFR that can phosphorylate ERBB3 which then promotes transformation. Thus genomic alterations in ERBB3 may be a key means of promoting oncogenic signalling despite the protein lacking robust enzymatic activity (Hanrahan et al. 2014). Again, this is an interesting finding within the duodenal adenoma dataset.

Titin (*TTN*) or connectin, encodes for a giant muscle protein expressed in cardiac and skeletal muscles which plays a key role in muscle assembly, force transmission and maintenance of resting tension (Itoh-Satoh et al. 2002). Mutations in *TTN* are associated with familial hypertrophic cardiomyopathy (Satoh et al. 1999), dilated cardiomyopathy (Gerull et al. 2002) and muscular dystrophy (Hackman et al. 2002).

However this data must be interpreted with caution as it has been previously reported that *TTN* is frequently mutated due simply to its size (Sanders et al. 2012). *TTN* also appears to be a gene frequently affected by rare and sometimes functional variants in the general population (Shyr et al. 2014).

4.4.8 Array CGH

The molecular mechanism responsible for CIN is not fully understood but this instability is thought to occur at an early stage of colorectal tumourigenesis (Shih et al. 2001). Mutations in genes involved in the accurate mitotic segregation of chromosomes, including *hBUB1* (Cahill et al. 1998), *APC* (Fodde et al, 2001) and *hCDC4* (Rajagopalan et al. 2004), has been suggested as a possible cause of CIN. However the incidence of mutations found in checkpoint genes and/or other CIN candidate genes is low (Cahill et al. 1998; Wang et al. 2004) and *APC*-mutant polyps do not show major aneuploidy changes (Sieber et al. 2002).

ArrayCGH was performed in this study to determine whether copy number losses or gains occurr in duodenal adenomas. Six CNVs (1 gain and 5 losses) were detected in 3 adenomas (15%), all of which were from MAP patients. CNV analysis has not been reported previously in duodenal adenomas, however, Berkhout et al, (2007), investigated chromosomal and methylation alterations in sporadic and FAP-associated duodenal carcinomas. They identified CNVs in 4 of the 5 FAP-associated carcinomas. CNVs, particularly gains were detected on chromosomes 8, 17 and 19. In the current study one adenoma showed two deletions on chromosome 8, which were in chromosomal locations characterised by locus control regions (LCRs) and pseudogenes, indicating that they may be a common site for rearrangement.

Cardoso et al (2006) studied chromosomal instability in colorectal adenomas, identifying CNVs in 80% and 60% of MAP and FAP adenomas respectively. These CNVs included gains on chromosome 7 which we have also detected, as have other recent studies (Voorham et al, 2012; Loo et al, 2013). Jones et al (2007) found a small number of large scale genetic changes by arrayCGH in colorectal adenomas in FAP and MAP patients. Their results were in agreement with the results of Cardoso et al (2006), with the exception that chromosome 7 gains were not commonly observed. They reported a small excess of changes in MAP, compared with FAP colorectal adenomas, with all the relatively high level copy number change occurring in MAP polyps.

The current study detected losses on chromosome 18 which are commonly associated with colorectal carcinomas and have also been identified in duodenal carcinomas (Blaker et al, 2002; Berkhout et al, 2007; Voorham et al, 2012; Loo et al, 2013). Chromosome 18 CNVs typically involve terminal regions and so may be indicative of structural rearrangements. We also identified a deletion on chromosome 9, which has not previously been reported in the duodenum or colorectum. Two of the CNVs identified in this study were at chromosome 8p.23.1, a fragile site within the chromosome. Bartkova et al (2005) found an elevated breakage rate at fragile sites, specifically chromosome 8p.23.1/8p21.3, 9q32 and 11p15.1 in colorectal tumours. Jones et al (2007) found no changes arising from chromosome breakage at this site in their FAP and MAP colorectal adenomas, but this study provides some evidence in support of the hypothesis that some copy number changes may result from this ‘different’ type of CIN.

Importantly, CNVs were only identified in 15% of samples in this study, a relatively low frequency in comparison to the previously reported frequency of CNVs in colorectal adenomas. The low CNV rate could suggest that we have not identified all

CNVs that are actually present, perhaps in samples with low levels of adenomatous material. However, the arrayCGH technique utilised in this study was also used in previous CNV analyses of colorectal adenomas when a higher frequency of CNVs was found (Roger et al, 2013). Furthermore, in this study CNVs were detected in adenomas with low levels of neoplastic cells (30%) and confirmed by independent techniques. This suggests that cellular heterogeneity is unlikely to account for the low frequency of CNVs detected in this study. To ensure that adenomatous material is selected for analysis, macrodissection or laser capture microdissection could be incorporated into the arrayCGH protocol to sample the neoplastic portion of the adenoma, as reported by Cardoso et al, 2006. However, this usually requires working with formalin fixed paraffin embedded (FFPE) samples which can have major problems with DNA quality.

Molecular alterations associated with the adenoma to carcinoma transition are well documented in the colorectum but data to support a similar pathway in the duodenum is limited. It is known that colorectal adenomas in MAP patients develop at a later age, perhaps due to the requirement for two somatic *APC* mutations in addition to the biallelic *MUTYH* germline mutations, whereas FAP patients only require one additional somatic *APC* mutation for adenoma development (Nieuwenhuis et al, 2012). However, it has also been suggested that the genetic changes (SNVs and CNVs) associated with MAP tumours in the colorectum may underlie accelerated cancer progression (Cardoso et al, 2006; Nieuwenhuis et al, 2012). The exome and arrayCGH data presented here supports the idea that MAP duodenal adenomas have a greater burden of genetic changes than FAP duodenal polyps and may therefore also have faster tumour progression.

4.4.9 Study limitations

A potential limitation of this study was the use of biopsy samples of adenoma tissue, thus the sample that underwent exome sequencing was a biopsy of adjacent tissue (or of the polyp was small, a divided portion of the sample taken), rather than the same piece of tissue that was histologically confirmed as an adenoma. The routine clinical management of patients undergoing upper GI surveillance differs from colorectal surveillance, where polyps are removed in their entirety. This risk is too high in the duodenum and so adenomas are not routinely removed unless thought to be high risk as discussed in previous chapters. Most studies on colorectal adenomas have been able to use much larger tissue samples from intact polyps. The possibility of sampling error cannot be entirely disregarded. Frozen sections to confirm dysplastic material could have been undertaken, but at the potential cost of loss of a proportion of the sample, and consequently DNA for analysis. However, all samples taken for histology were confirmed adenomas, suggesting a degree of diagnostic accuracy. The fact that the vast majority of *APC* somatic mutations in each sample that underwent exome sequencing were identified further supports this. Although there was no standardised method of estimating the proportion of adenomatous tissue in each biopsy, the adenoma biopsy slides from St Mark's Hospital were reviewed by the same GI pathologists (MM and GT) in Cardiff to ensure no discordance in reporting.

The number of patients with adenomas, especially with MAP, in this study was not high, but this is a reflection of the nature of a rare manifestation of a rare disease. However, studies of MAP adenomas in the colorectum of patients usually include only a small number of patients, for example 11 patients (Jones et al. 2007), 22 patients (Lipton et al. 2003) and 5 patients (Jones et al. 2004). The age matching and sex matching of MAP and FAP patients was limited by the patients that had adenomas at GI endoscopy, and because the average age of the MAP patients was

higher, likely because of a greater age of diagnosis; the oldest FAP patient in this study was 69 years old and the amount of dysplastic adenomatous tissue in the samples was only estimated at 50%.

Only 10 samples underwent whole exome sequencing, and 47 samples were used for validation. Rashid et al (2015) whole exome sequenced 14 adenomas, with 55 samples used for validation and the numbers in this current study are comparable, given the lower incidence of adenomas in the duodenum in both conditions. However, the aim of this study was to act as a pilot study to inform further larger studies of duodenal polyps.

The next generation sequencing technique itself could have led to missing 'hidden' mutations, as it did not analyse any non-coding regions, removing introns, promoters and untranslating regions (UTRs; acting as gene regulating regions). One study on retinal dystrophies (Eisenberger et al. 2013) suggests UTR inclusion and quantitative analysis should be part of a comprehensive NGS approach due to the significant increase in diagnostic yield reported. This study incorporated CNV analysis, and >100x coverage seen in all adenoma samples is likely to have detected the most important driver mutations.

Validation with Sanger sequencing is restricted by a limit of detection of 15-20% mutant alleles (Tsiatis et al. 2010). This means that low frequency alleles in heterogeneous tumour samples may be missed if they occur at a rate of less than <20%. Thus, some groups are endeavouring to develop comprehensive characterisation of genomic alteration occurring within individual tumours without the need for Sanger sequencing validation (Frampton et al. 2013). Techniques such co-amplification at lower denaturation temperature PCR (COLD-PCR) can be used to detect these low level mutations that would likely have been missed using

conventional methods that do not enrich for variant sequence DNA. This study did not use COLD-PCR, however the *APC* mutations were seen at such a high frequency when validated by Sanger sequencing, that this acted as a benchmark to assume that other mutations in different genes (in a targeted manner given the exome data available) were highly likely to have been detected.

Multiplex ligation-dependent probe amplification (MLPA) was not completed as part of this study and thus, small CNVs at 5q21-22 may have been missed through the arrayCGH technique that was used. Cellular heterogeneity is unlikely to contribute to the inability to detect these mutations, as exome-wide somatic SNVs were detected in both tumours lacking *APC* mutations through exome analysis and Sanger sequencing.

Initially the GATK pipeline was used to identify mutations in *APC* and *MUTYH* in this study. This pipeline is able to identify a range of mutation types, including frameshift mutations in addition to SNVs. However, most indels identified during next generation sequencing are artefacts of the sequencing process and, due to the high volume of variants identified following exome sequencing, a somatic caller merging approach was used to filter variants to a manageable number in order for validation. Therefore, only SNVs were identified in the exome-wide data leading to a trade – off between sensitivity and specificity.

4.4.10 Conclusions

This study found that, MAP adenomas carry a higher mutational load (SNVs and CNVs) than FAP adenomas. This finding would be consistent with MAP duodenal adenomas having greater risk of progressing down the adenoma-carcinoma pathway than the FAP adenomas. If so, the Spigelman grading system that is based upon risk of progression to duodenal cancer in FAP may not be appropriate for MAP. In future, the system should perhaps be modified to take into account the underlying mutational spectrum in adenomas. Such a change would require confirmation of the findings of the initial studies reported here, a better understanding of the natural history of duodenal polyposis in MAP (informed by studies such as the European Prospective Study described in Chapter 3) and studies correlating mutational status and tumour progression.

Chapter 5

General Discussion and Future Prospects

5.1 General Discussion

Duodenal polyposis and carcinoma has become a major health problem in patients with FAP, and emerging data suggests this is also true in MAP. However detailed information about the burden of duodenal polyposis and progression of disease in MAP has remained undefined. The work described in this thesis has focused on determining whether techniques for enhanced adenoma detection in MAP can give a more accurate picture of the presence and extent of duodenal disease, investigating the natural history and progression of duodenal adenomas in MAP and to examine and compare the somatic mutational spectrum of MAP and FAP adenomas.

These issues have received little attention thus far in MAP. Despite the significant implications for patients who undergo regular endoscopic surveillance, there is little evidence on which to base surveillance protocols or a decision about which lesions may benefit from an aggressive treatment approach. Radical prophylactic surgery is associated with high mortality rates, and duodenal cancer has a poor outcome.

5.1.1 Benefit of enhanced duodenal adenoma detection rates in MAP and FAP

Previous studies have demonstrated the benefit of enhanced detection rates of adenomas by use of chromoendoscopy both in sporadic colorectal disease (Brown et al. 2007) and in groups at high risk of colorectal cancer (Matsumoto et al. 2009). By applying this technique to the duodenum in MAP the study described in chapter 2 demonstrated that chromoendoscopy increased the number of adenomas detected in MAP and that this resulted in a clinically significant change in the Spigelman score for those patients. However, despite the positive clinical impact that this may have on patients if it were to be employed on a routine basis, there are significant time and resource implications. Chromoendoscopy takes longer than conventional

endoscopy, requires appropriate training, and by increasing the Spigelman stage leads to patients requiring more frequent surveillance endoscopies. In their prospective study of duodenal adenoma progression in FAP, Saurin et al (2004) found a higher rate of progression to high grade dysplasia compared to previous studies, thought to be due to the use of different methodology of duodenal examination, which included the routine utilisation of chromoendoscopy but also general anaesthesia. They concluded that these methods better identified neoplastic lesions.

In the work reported in this thesis, the lower number of adenomas observed in MAP as compared to FAP resulted in significant upstaging when chromoendoscopy was employed, suggesting that the accurate identification of polyps is of importance for subsequent clinical management at this present time using Spigelman staging in its current form.

Whether there is any additional long-term benefit in detecting 1-2 small adenomas in MAP using chromoendoscopy remains to be seen. Polyp size in addition to multiplicity is a component of the Spigelman score, but the Spigelman stage may be a poor indicator of duodenal cancer risk in MAP, as one of the limitations of the staging system is that it confers equal weighting to each of the components used to stage duodenal adenomatosis. In the recent study reported by Lopez-Ceron et al. (2013) in FAP that the only endoscopic feature that predicted advanced histology of a duodenal adenoma was size greater than 1cm. Further studies are required to determine how best to optimise the Spigelman classification to order to facilitate accurate risk stratification in MAP patients.

Emerging molecular imaging techniques such as confocal endoscopy which allows real time visualisation of mucosal surfaces with immediate histological images and

observation of the cellular and vascular networks are exciting developments that may push the boundaries of polyp detection and assessment. Neoplastic changes can be predicted with 97.4% sensitivity and 99.4% sensitivity when compared with histopathology (Kiesslich et al. 2004; Hurlestone et al 2008). Given the complications associated with adenoma resection in the duodenum, especially at the ampulla, and sampling error on biopsy, prospective studies are required to define its use in the evaluation of duodenal disease not just in MAP but in FAP as well. This would lead to very prolonged procedures, with patients requiring deep sedation or general anaesthesia, but this may be moderated by a need for less frequent surveillance procedures.

5.1.2 A European cross-sectional study duodenal adenomas in MAP

Previously, it has been reported that there was a risk of duodenal adenoma development in MAP of 1.8% to 25%. However, this data is from case reports, anecdotal reports and retrospective case note studies. No data on the progression of adenomas or lifetime risk of adenomas has ever been reported. Although the current study was retrospective, detailed information was collected on 207 patients undergoing upper GI surveillance for MAP. The study detailed in chapter 3 has shown that in the MAP duodenum there is a cumulative incidence of developing adenomas of 30% by age 70 years, which is significantly lower than described in FAP. There remains however, a significant risk of duodenal adenocarcinoma development, and the cumulative incidence of developing duodenal adenocarcinoma by age 70 years in this study was 2.3%.

This study is consistent with a small number of anecdotal reports that patients may develop duodenal adenocarcinoma in MAP on a background of a low number of polyps when compared to FAP.

The lack of a strong correlation between duodenal polyp burden and risk of cancer highlighted in this study has implications for the subsequent management of this group of patients. As in the chromoendoscopy study (chapter 2), it can be concluded that the Spigelman staging system may not be appropriate for long-term use in MAP, and a high risk FAP Spigelman stage IV may not be equivalent to a 'high risk' MAP duodenum. Future prospective studies of the phenotypic manifestation of duodenal disease in MAP are vital in order to learn more about the natural history of duodenal adenoma progression and which patients are at high risk of carcinoma development.

As with previous studies on FAP, this study demonstrated down-staging of disease with endoscopic intervention in the short term, but previous work has shown that polyp recurrence is the norm. Longer-term studies are needed to determine if there is a similar risk of adenoma recurrence in MAP following endoscopic therapy.

This thesis presents data showing that homozygotes for Y179C mutations had a greater number of duodenal adenomas than patients with two truncating mutations, G396C homozygotes and G396D / Y179C heterozygotes, suggesting a more severe phenotype in Y179C homozygotes that is also observed in the colorectum. In FAP several studies have shown that the severity of colonic polyposis is correlated with the site of the mutation in the *APC* gene, with mutations between codons 1250 and 1464, especially those with a mutation at codon 1309, associated with a severe form of FAP. Several authors have proposed to use the outcome of genetic testing to guide the type of surgical procedure in patients with a relatively polyp-free rectum (Vasen et al. 1996, Bulow et al. 2000; Nieuwenhuis et al. 2007). The strength of genotype-phenotype correlation in MAP needs further investigation, but there may be potential to use genotype as one factor guiding surveillance in the future.

5.1.3 Somatic mutations in MAP and FAP duodenal adenomas

Investigating the somatic mutational landscape in MAP and FAP duodenal adenomas established significant differences between the numbers of mutations – both SNVs and CNVs - seen in the MAP versus FAP adenomas. Furthermore, our data pointed to differences in the spectra of genes mutated in the duodenum compared to the colorectum. The significance of some of the novel mutations identified within the duodenum for tumorigenesis is difficult to assess and will require larger studies for confirmation. The absence of *WTX* mutations and prevalence of *PLCL1* mutations in duodenal adenomas do however appear to be robust findings and deserve further investigation.

Importantly, the study described in chapter 4 did not investigate all types of genetic and epigenetic changes that could lead to deregulation of cellular pathways and hence cellular growth, for example, gene expression changes were not assessed and further studies are required to gain a more complete picture of the genetic and epigenetic basis of duodenal tumorigenesis in MAP and FAP.

The results from this thesis are consistent with previous observations that patients can develop duodenal cancer in MAP on a background of minimal polyposis, because each adenoma is on average more ‘genetically unstable’ than is the case in FAP. Thus Spigelman staging should perhaps be modified to take into account the underlying mutational spectrum in adenomas to more accurately identify which patients are at highest risk of developing duodenal cancer.

From the data presented in this thesis it also appears that duodenal disease in MAP and FAP is heterogeneous even at an early stage of tumorigenesis, and a major challenge remains to identify the sub-groups or individual patients that would benefit from an enhanced surveillance strategy or early definitive prophylactic management

until medical therapies to target the initiating events of adenoma development before they become mutationally diverse are available.

5.2 Future Prospects

Long term prospective studies are required to further define the natural history of duodenal adenomas in MAP, in combination with further studies of the use of newer endoscopic technologies which may increase the yield of adenoma recognition and advanced neoplasia detection. Further investigation of the somatic mutational spectrum in larger numbers of adenomas and carcinomas is also needed. This would help to further define which common driver genes may be associated with tumour progression in the duodenum in FAP and MAP. These could prove to be important targets for therapeutic intervention.

Results from this body of work suggest it is unlikely that any one biomarker will identify all patients at risk of duodenal cancer. Genetic biomarkers including mutations and changes in gene expression or methylation status need to be assessed in conjunction with endoscopic and host and microbiome factors to establish biomarkers that can guide patient management. .

As next generation sequencing techniques advance, analysis of the ‘miRNAome’ and transcriptome by RNA-sequencing in MAP and FAP could identify novel mechanisms of tumorigenesis mediated via gene expression changes, and potential miRNA biomarkers. RNAseq could be used to identify differential expression (with or without changes in copy number) between normal mucosa and duodenal adenomas/carcinoma and also differential expression between duodenal adenomas and colorectal adenomas from the same patient to give insights into the effects of the different gastrointestinal environments on tumour development.

The identification of germline modifier genes in MAP and FAP as well as specific environmental agents that promote adenoma and carcinoma development in the duodenum will also enhance our understanding of these diseases and their progression. Investigation of the exome and transcriptome in the germline of patients with duodenal adenomas versus those who do not develop duodenal disease may reveal germline factors modifying the risk of duodenal polyposis. The effects of bile (and thus pH) and gut organisms such as helicobacter pylori and how these relate to germline genetic variants warrant further study.

Duodenal polyposis remains an under-researched area but as colorectal cancer is prevented or better treated in more patients with the polyposis syndromes, duodenal disease is emerging as an important area of clinical need. The work reported in this thesis provides some early insights into the clinical and genetic characteristics of duodenal polyposis and a basis for future studies.

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