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# The Role of Inherited Mutations in Colorectal Polyposis Syndromes

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## Key Words

Colorectal Cancer

Colorectal Polyposis

Adenomas

Hyperplastic Polyps

Hamartomatous Polyps

Familial Adenomatous Polyposis

*MUTYH*-Associated Polyposis

Polymerase Proofreading Associated Polyposis

*NTHL1*-Associated Polyposis

*MSH3*-Associated Polyposis

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## Abstract

Colorectal carcinoma (CRC) is the third most common cancer in men and the second most common cancer in women across the world. Most CRCs occur sporadically, but in 15-35% of cases, hereditary factors are important. Some patients with an inherited predisposition to CRC will be diagnosed with a 'genetic polyposis syndrome' such as Familial Adenomatous Polyposis (FAP), *MUTYH*-Associated Polyposis (MAP), Polymerase Proofreading Associated Polyposis (PPAP), *NTHL1*-Associated Polyposis, *MSH3*-Associated Polyposis or a hamartomatous polyposis syndrome. Individuals with  $\geq 10$  colorectal polyps have traditionally been referred for genetic diagnostic testing **aiming** to identify *APC* and *MUTYH* mutations which cause FAP and MAP respectively. Mutations are found in most patients with  $>100$  adenomas but in only a minority of those with 10-100

adenomas. The reasons that diagnostic laboratories are not identifying pathogenic variants include mutations occurring outside of the open reading frames of genes, individuals exhibiting generalised mosaicism and the involvement of additional genes. It is important to identify patients with an inherited polyposis syndrome, and to define the mutations causing their polyposis, so that the individuals and their relatives can be managed appropriately.

## 1. Introduction

Across the globe, colorectal carcinoma (CRC) is the third most common cancer in men and the second most common cancer in women (Ferlay *et al* 2013). An individual's lifetime risk of developing CRC is 5%, but this figure increases dramatically with age (reviewed in Fodde 2002). The incidence of CRC is generally high in developed countries, with a 20-fold difference in incidence rates between high- and low-risk geographical areas (reviewed in Fodde 2002). The difference is thought to largely result from environmental factors, in particular differences in diet (Fodde 2002).

The majority of CRCs occur sporadically, but in 15-35% of patients, hereditary factors are important (reviewed in Mishra and Hall 2012; Burt 2007). In approximately 5% of cases, the disease is caused by a highly penetrant dominantly inherited syndrome (reviewed in Mishra and Hall 2012). The most common is Lynch Syndrome, due to inherited defects in the mismatch repair (MMR) system. This accounts for 2-5% of cases of CRC (reviewed in Mishra and Hall 2012). Familial Adenomatous Polyposis (FAP), due to germline mutations in the *APC* gene, is responsible for <1% of the disease burden and non-syndromic familial presentations comprise 20% of cases (reviewed in Mishra and Hall 2012).

CRCs result from the progressive accumulation of genetic and epigenetic alterations which cause normal colonic epithelium to transform into adenocarcinoma

(Grady and Carethers 2008). Recent work based on gene expression profiling (Guinney *et al* 2015) suggests that CRC can be classified into 4 molecular subtypes: MSI Immune, Canonical, Metabolic and Mesenchymal. However, the traditional approach has been to categorise tumors into 3 groups: those with chromosomal instability (CIN), those with microsatellite instability (MSI) and those with a hypermethylated phenotype (CpG Island Methylator Phenotype or 'CIMP'). There is considerable overlap between the latter two groups.

## 1.1 Chromosomal Instability (CIN), APC and the Wnt-Pathway

The vast majority of CRCs develop from pre-existing adenomas. Such lesions are characterised by chromosomal instability, which is seen in 80-85% of colorectal tumors (reviewed in Grady and Carethers 2008). There are certain key genetic mutational events which occur, allowing the progression from normal epithelium, to dysplasia, and finally invasive malignancy. The loss of adenomatous polyposis coli (*APC*) gene function seems to be the initiating event, followed by mutations in *KRAS*, *SMAD4* and *p53* (Fodde 2002). In keeping with Knudson's two hit hypothesis of tumorigenesis, two mutational events are required to knock out the functioning of a tumor suppressor gene (Knudson 1971), whilst activation of an oncogene requires only one mutation.

### 1.1.1 Adenomatous Polyposis Coli (*APC*)

The *APC* gene is found on chromosome 5 (5q21). It consists of 8535 coding base pairs, encoding a 2843 amino acid multidomain protein. Exon 15 is responsible for more than 75% of the coding sequence of the gene and is the most common site for germline and somatic mutations (reviewed in Fearnhead *et al* 2002; reviewed in Fearnhead *et al* 2001). 95% of CRC-associated *APC* mutations are

nonsense or frameshift mutations, creating a truncated protein with abnormal function (Bodmer 1999).

The APC protein is a 312kDa tumor suppressor, which is involved in many cellular processes including intercellular adhesion, signal transduction, proliferation, apoptosis and migration. One of its major roles is in regulating cytoplasmic levels of  $\beta$ -catenin, thus negatively regulating Wnt signalling (Mishra and Hall 2012; reviewed in Fearnhead *et al* 2002; Fodde 2002; Fearnhead *et al* 2001).

### 1.1.2 The Wnt Pathway

The Wnt proteins are a family of signalling proteins which are involved in developmental events during embryogenesis and in adult tissue homeostasis (Logan and Nusse 2004). They have multiple effects within a cell, including triggering cell division, cell fate specification and differentiation (Logan and Nusse 2004).

Wnt proteins bind to Frizzled/ low density lipoprotein receptor-related proteins, which are found on cell surface membranes (Logan and Nusse 2004). This transduces a signal to intracellular proteins, including Dishevelled (Dsh), Glycogen Synthase Kinase-3 $\beta$  (GSK-3), AXIN, APC and  $\beta$ -catenin (Logan and Nusse 2004). In the absence of Wnt signalling,  $\beta$ -catenin levels are usually low: a complex composed of GSK-3, APC and AXIN targets it for ubiquitin-mediated degradation (Logan and Nusse 2004; reviewed in Fearnhead *et al* 2002; reviewed in Fearnhead *et al* 2001). When cells receive Wnt signals, the degradation pathway is inhibited. This allows  $\beta$ -catenin to accumulate in the cytoplasm and nucleus. In the nucleus, it complexes with one of the T cell factor (TCF) or lymphoid enhancer factor (LEF) transcription factors, to initiate transcription of a range of genes, including *c-myc* and *cyclin D1* (Logan and Nusse 2004; reviewed in Fearnhead *et al* 2002; reviewed in Fodde 2002; reviewed in Fearnhead *et al* 2001). *Myc* and *cyclin D1* are both

relevant to tumorigenesis as they have roles in proliferation, apoptosis and cell-cycle progression (reviewed in Fodde 2002).

In the normal intestinal epithelium, nuclear  $\beta$ -catenin expression is higher in the proliferative component, and APC levels are higher in post-replicative cells (reviewed in Fodde 2002). These findings support  $\beta$ -catenin signalling having a role in maintaining stem cell properties and controlling differentiation in the bowel (reviewed in Fodde 2002). As cells move along the crypt-villous axis, increasing levels of APC counteract  $\beta$ -catenin signalling and allow differentiation to occur (reviewed in Fodde 2002). *APC* mutations hence allow increased numbers of stem cells and reduced cellular differentiation (Fodde 2002).

In addition to their roles in the initial stages of the adenoma-carcinoma sequence, *APC* mutations remain important throughout malignant progression. Nuclear  $\beta$ -catenin staining strongly correlates with tumor size and dysplasia, and high levels of nuclear  $\beta$ -catenin have been found at the invasive fronts of adenocarcinomas (reviewed in Fodde 2002). The APC protein is also involved in chromosomal stability at mitosis: it has an EB1-binding domain in its C-terminal end, which associates with the growing ends of cytoplasmic and spindle microtubules, as well as centrosomes. *APC* mutant cells are hence characterised by chromosomal instability which is observed in the majority of CRCs (reviewed in Grady and Carethers 2008; reviewed in Fodde 2002).

*APC* mutations can therefore be seen to have a key role in both initiating and promoting CRC: activation of the Wnt pathway affects cell proliferation, apoptosis, and possibly differentiation of intestinal stem cells (Fodde 2002) and at later stages of carcinogenesis, CIN resulting from *APC* mutations can accelerate tumor progression (Fodde 2002).

### 1.1.3 *KRAS, SMAD4 and P53*

Further genes involved in colorectal tumorigenesis include *KRAS*, *SMAD4*, and *p53*. The importance of *ras* gene mutations in colorectal carcinogenesis was first reported in 1987 (Bos *et al* 1987; Forrester *et al* 1987). The *K-ras* oncogene has been found to be mutated in 10-15% of adenomas <1cm, and in 30-60% of adenomas >1cm and carcinomas (reviewed in Brink *et al* 2003; reviewed in Fearon and Vogelstein 1990). The gene encodes a 21kDA protein located in the inner plasma membrane, with intrinsic GTPase activity. It is involved in the transduction of mitogenic signals (reviewed in Brink *et al* 2003). It is activated by a diverse spectrum of extracellular stimuli, such as growth factors, cytokines and hormones (reviewed in Brink *et al* 2003; Shields *et al* 2000). Once activated, it stimulates a multitude of downstream signalling cascades, including the Raf serine/threonine kinases, phosphoinositide 3-kinases (PI3Ks) and a family of GDP-GTP exchange factors (reviewed in Shields *et al* 2000).

Mutant *KRAS* has impaired GTPase activity, meaning that it is constitutively active (reviewed in Brink *et al* 2003). This can cause uncontrolled cell growth and proliferation. A *KRAS* mutation in a colonic epithelial cell which already has *APC* mutations results in a clonal expansion and increased risk of progression to cancer (reviewed in Vogelstein and Kinzler 2004).

The *SMAD4* gene is on chromosome 18q. It was first identified as a tumor suppressor gene in pancreatic cancer in 1996 (Hahn *et al* 1996). *SMAD4* mediates the TGF $\beta$  signalling pathway to suppress epithelial growth (reviewed in Miyaki and Kuroki 2003). The *SMAD4* protein acts as a trimer and forms complexes with additional *SMAD* proteins: receptor-phosphorylated *SMAD2* and *SMAD3* (Woodford-Richens *et al* 2001). These complexes then translocate from the cytoplasm to the nucleus and associate with DNA binding factors to facilitate the transcription of target genes, including cyclin-dependent kinase inhibitors such as

p15(ink4B) and the inhibitory SMAD7 (Woodford-Richens *et al* 2001). Loss of SMAD4 function may result in the loss of transcription of genes necessary for cell-cycle control (Woodford-Richens *et al* 2001). Cells may therefore become TGF- $\beta$  resistant and escape from TGF- $\beta$ -mediated growth control and apoptosis (Woodford-Richens *et al* 2001). *SMAD4* is mutated in a significant proportion of colorectal tumors, with the frequency of mutational events increasing with the progression of carcinogenesis: it is mutated in 0% of adenomas, 10% of 'intramucosal carcinomas', 7% of carcinomas without metastases and 35% of carcinomas with distant metastases (reviewed in Miyaki and Kuroki 2003).

In 1988, it was reported that 73% of CRCs, 47% of 'advanced adenomas' and 11-13% of 'early stage adenomas' had a deletion of part of chromosome 17 (Vogelstein *et al* 1988). This region was subsequently shown to include the *p53* gene (17p13.1) (Baker *et al* 1989). The p53 protein is a transcription factor which has a vital role in maintaining genomic stability (reviewed in Sarasqueta *et al* 2013). Following DNA damage, p53 activation causes arrest of the cell cycle to allow DNA repair (reviewed in Sarasqueta *et al* 2013). If the damage is too extensive, p53 can drive a cell towards senescence or apoptosis (reviewed in Sarasqueta *et al* 2013; reviewed in Vogelstein and Kinzler 2004). The functional loss of p53 is a key event in the malignant progression of a colorectal adenoma to CRC (reviewed in Iacopetta 2003; Vogelstein *et al* 1988).

## 1.2 Microsatellite Instability (MSI)

The human mismatch repair system (MMR) involves 7 key genes: *hMSH2*, *hMSH6*, *hMSH3*, *hMLH1*, *hPMS1*, *hPMS2* and *hMLF3*. Their protein products are able to recognise and repair nucleotide mismatches, which have escaped the normal editing function of DNA polymerase (reviewed in Grady and Carethers 2008). If such mismatches are not repaired, nucleotide transitions or transversions

result, allowing potentially oncogenic mutations to occur more frequently, leading to a 'hypermutable phenotype' (reviewed in Grady and Carethers 2008).

Lynch Syndrome (LS) is an autosomal dominant (AD) disease, which accounts for approximately 5% of cases of CRC (reviewed in Mishra and Hall 2012). Patients also have an inherited predisposition to a range of other malignancies, in particular endometrial carcinoma (Lynch *et al* 2015). LS occurs due to inherited mutations of *hMSH2*, *hMSH6*, *hMLH1* or *hPMS2* (reviewed in Grady and Carethers 2008). In 15-20% of sporadic colon cancers, inactivation of the mismatch repair (MMR) system occurs, either through methylation of *MLH1* or point mutations in *MLH1/MSH2*/other members of the MMR family (reviewed in Grady and Carethers 2008). This leads to microsatellite instability (MSI).

It is thought that certain key tumor suppressor genes drive the pathogenesis of MSI tumors, and these are different to those which are mutated in CIN tumors (reviewed in Grady and Carethers 2008). In around 85% of colorectal tumors with MSI, a repeat of 10 adenines undergoes a frameshift mutation in the *TGFBR2* gene. This allows tumor cells to escape the growth suppressing effects of TGF- $\beta$ 1 (reviewed in Grady and Carethers 2008). Another gene commonly mutated in MSI CRC is *BAX*, which plays a role in apoptosis. It is mutated in 50% of MSI CRC, allowing immortalisation of cells (reviewed in Grady and Carethers 2008). Interestingly, frameshift mutations within coding mononucleotide repeats are also seen in *APC* (reviewed in Lynch *et al* 2015).

MSI CRCs tend to have a certain clinical and pathological phenotype. They generally occur in the right side of the colon, and microscopically they have a mucinous appearance with large numbers of tumor-infiltrating lymphocytes (reviewed in Grady and Carethers 2008).

### 1.3 CpG Island Methylator Phenotype (CIMP)

Epigenetic regulation of gene expression can be achieved through methylation of CpG islands found in gene promoters, causing silencing of the downstream gene. Such silencing of tumor suppressors and/or DNA repair genes is a common feature of human neoplasia (reviewed in Hughes *et al* 2012). Widespread CpG island promoter methylation is referred to as the CpG island methylator phenotype (CIMP) and was first described in 1999 (Toyota *et al* 1999). The cause of CIMP remains to be elucidated but may potentially result from aberrant *de novo* methylation or through the loss of protection against *de novo* methylation (reviewed in Toyota *et al* 1999). Environmental factors, such as anthropometry and physical activity, smoking and alcohol may also play a role (reviewed in Hughes *et al* 2012).

CRC which exhibit CIMP are thought to arise via the 'serrated pathway of neoplasia'. The precursor lesions are hyperplastic polyps, rather than adenomas, and an early event is a mutation of the *BRAF* oncogene (reviewed in Guarinos *et al* 2012). *BRAF* is a component of the MAPK signalling pathway. The pathway involves activation of cell membrane signalling molecules with subsequent stimulation of cytoplasmic protein kinases (Seger and Krebs 1995). The transmitted signals eventually activate cellular processes such as proliferation, differentiation and development (Seger and Krebs 1995). Activating mutations of *BRAF* increase its kinase activity, which drives the proliferation of malignant cells (reviewed in Bollag *et al* 2012).

Most CIMP CRCs have epigenetic silencing of *MLH1*, leading to microsatellite instability (reviewed in Hughes *et al* 2012), and may have silencing of tumor suppressor genes such as *p16* (Toyota *et al* 1999).

Typically, CIMP tumors are associated with older age, female sex and occurrence in the right side of the bowel (reviewed in Hughes *et al* 2012), as is seen with MSI CRC.

## 2. The Colorectal Polyposis Syndromes

Colorectal polyps are masses of tissue which are found projecting from the mucosa of the large bowel. They are classified according to their microscopic appearance, and include adenomas, hyperplastic polyps and hamartomatous polyps (Figure 1). Most polyps occur sporadically, but some are seen as part of a genetic 'polyposis syndrome' (Figure 2). Colorectal polyps are benign but are clinically significant as they may confer a risk of malignancy.

[Insert Figure 1]

**Figure 1: Haematoxylin and Eosin (H&E) microscopic images of colorectal polyps**

**A: Tubular adenoma with low grade dysplasia**

**B: Hyperplastic polyp: note the presence of glands in the centre of the image with a serrated/ 'tooth like' appearance**

**C: A Peutz-Jegher's polyp. The glands are separated by bundles of smooth muscle**

**D: A juvenile polyp. Cystically dilated glands are present in an oedematous and inflamed stroma**

**Image from Short *et al* 2015**

[Insert Figure 2]

**Figure 2: Diagram illustrating relationships between genes and signalling pathways involved in inherited colorectal cancer syndromes. Key: FAP:**

**Familial Adenomatous Polyposis; HMPS: Hereditary Mixed Polyposis**

**Syndrome; JPS: Juvenile Polyposis Syndrome; LS: Lynch Syndrome; MAP:**

***MUTYH*-Associated Polyposis; NTHL1: *NTHL1*-Associated Polyposis; PPAP:**

## **Polymerase Proofreading Associated Polyposis; PJS: Peutz Jegher's Syndrome.**

**Image from Short *et al* 2015**

### **2.1 Colorectal Adenomas**

Colorectal adenomas are common. They are found in between 1.72% and 63% of autopsies (Pendergrass *et al* 2008; Paspatis *et al* 2001; Correa *et al* 1977; Arminski and McLean 1964; Chapman 1963). In asymptomatic patients undergoing colonoscopies, adenoma prevalence is between 6.3% and 41% (Chung *et al* 2010; Rundle *et al* 2008; Lin *et al* 2006; Strul *et al* 2006; Soon *et al* 2005; reviewed in Giacosa 2004; Yamaji *et al* 2004; DiSario *et al* 1991). They occur more frequently in males than in females, and their prevalence increases with age (Chung *et al* 2010; Pendergrass *et al* 2008; Lin *et al* 2006; Strul *et al* 2006; Soon *et al* 2005; Yamaji *et al* 2004; Paspatis *et al* 2001; DiSario *et al* 1991; Correa *et al* 1977; Chapman 1963).

The significance of colorectal adenomas is that they are pre-malignant lesions. The majority of colorectal CRCs are thought to develop from pre-existing adenomas. The probability that an adenoma will become malignant depends upon its size, morphology and degree of dysplasia. Large villous lesions harbouring high grade dysplasia confer the highest risk (Terry *et al* 2002; O'Brien *et al* 1990; Shinya and Wolff 1979; Muto *et al* 1975).

Most colorectal adenomas occur sporadically. However, there are syndromes of colorectal polyposis, in which patients develop multiple polyps as a result of an underlying genetic mutation. These include Familial Adenomatous Polyposis (FAP), *MUTYH*-Associated Polyposis (MAP), Polymerase Proofreading-

Associated Polyposis (PPAP), *NTHL1*-Associated Polyposis/CRC and *MSH3*-Associated Polyposis/CRC.

### 2.1.1 Familial Adenomatous Polyposis (FAP)

FAP is a dominantly inherited Mendelian trait, in which patients develop hundreds to thousands of colorectal adenomas during adolescence or the third decade of life (reviewed in Fearnhead *et al* 2002; Bodmer 1999). All such patients will invariably develop CRC if they are left untreated (Bodmer 1999; reviewed in Fearnhead *et al* 2002).

The first case of histologically verified adenomatous polyposis was published in 1881 by Sklifasowski (reviewed in Bülow *et al* 2006). The following year Harrison-Cripps described ‘disseminated polypus of the rectum’ in two teenage siblings, both of whom had 20-30 colorectal polyps (reviewed in Bülow *et al* 2006; Harrison-Cripps 1882). In the late 1800s there were numerous case reports describing patients with multiple colorectal adenomas, and an association with colorectal malignancy was noted (reviewed in Bülow *et al* 2006). In 1925, Lockhart-Mummery stated that the ‘condition of multiple adenomata was invariably antecedent to carcinoma’ and that ‘the condition of multiple adenomata is often hereditary in a marked degree’ (Lockhart-Mummery 1925).

It is now known that FAP, and an attenuated form of the disease, AFAP, are due to germline or somatic mosaic mutations in *APC*. Over 1500 different mutations in *APC* have been identified to date (Kadiyska *et al* 2013). The majority of mutations are inherited. It used to be thought that approximately one quarter of cases occurred *de novo* (reviewed in Fearnhead *et al* 2001), but this is an overestimate, as this figure included apparent *de novo* patients who actually had *MUTYH*-Associated Polyposis (MAP). A third of all germline mutations occur at codons 1061 and 1309, with the remainder spread relatively uniformly between

codons 200 and 1600 (reviewed in Fearnhead *et al* 2001). The nature of the germline mutation determines the nature of the second somatic hit to *APC* (reviewed in Fearnhead *et al* 2002; reviewed in Fearnhead *et al* 2001). Germline mutations occurring between codons 1194 and 1392 tend to be followed by allelic loss of *APC* as a second hit (loss of heterozygosity, LOH), whereas germline mutations lying outside of this region tend to be associated with truncating mutations in the mutation cluster region (MCR) between codons 1286 and 1513 (reviewed in Fearnhead *et al* 2002). The reason for this may be related to the resultant level or functional characteristics of APC protein produced: it is proposed that to allow efficient tumorigenesis, the function of APC must be impaired sufficiently to allow a certain level of nuclear  $\beta$ -catenin accumulation, but that  $\beta$ -catenin levels must not be too great, or this can result in apoptosis (Albuquerque *et al* 2002).

The incidence of FAP is approximately 1 per 8000, and it accounts for around 0.5% - 1% of CRC (Mishra and Hall 2012; reviewed in Fearnhead *et al* 2002; reviewed in Bodmer 1999). In addition to colorectal adenomas, FAP patients may develop extra-intestinal manifestations of their disease, for example congenital hypertrophy of the retinal pigment epithelium (CHRPE), duodenal and peri-ampullary tumors, desmoid tumors, papillary carcinoma of the thyroid, medulloblastoma, hepatoblastoma, osteomas and epidermoid cysts (Mishra and Hall 2012; reviewed in Fearnhead *et al* 2002).

### **2.1.2 *MUTYH*-Associated Polyposis (MAP)**

Prior to 2002, inherited defects of base excision had not been associated with any human genetic disorder (Al-Tassan *et al* 2002). That year, mutations in the *MUTYH* gene were shown to cause an inherited predisposition to colorectal tumors (Al-Tassan *et al* 2002).

The *MUTYH* gene is located on the short arm of chromosome 1 (1p32.1-p34.3) (Poulsen and Bisgaard 2008). It consists of 16 exons and encodes a protein of 535 amino acids, the MUTYH glycosylase (Poulsen and Bisgaard 2008). MUTYH glycosylase is part of the base excision repair (BER) system. It is involved in repairing DNA mismatches occurring as a result of oxidative DNA damage (Mazzei *et al* 2013). Each human cell metabolises approximately  $10^{12}$  molecules of oxygen per day (reviewed in Nohmi *et al* 2005). About 1% of oxygen metabolism results in the production of reactive oxygen species (ROS) which include superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen (reviewed in Nohmi *et al* 2005). ROS can damage DNA, producing 8-hydroxyguanine (8-oxodG). This frequently pairs with dAMP. Under normal circumstances, this mispairing would be repaired by MUTYH, to create C: 8-oxodG base pairs. Another enzyme, OGG1 will then remove the 8-oxodG. Hence the combined effects of MUTYH and OGG1 will prevent GC > TA transversions (Mazzei *et al* 2013).

Patients with biallelic germline *MUTYH* mutations are predisposed to mutations in genes including *APC* and *KRAS*. The clinical manifestation of this is MAP. MAP is an autosomal recessive (AR) disease, in which patients develop multiple colorectal adenomas. The mean age of diagnosis is 45-50 years, and patients typically have between 10 and 100 polyps (reviewed in Mazzei *et al* 2013; reviewed in Poulsen and Bisgaard 2008; Croitoru *et al* 2007; Nielsen *et al* 2007; Gismondi *et al* 2004; Wang *et al* 2004; Sampson *et al* 2003; Sieber *et al* 2003). Some patients do not develop polyps but present with cancer (Farrington *et al* 2005; Wang *et al* 2004; Enholm *et al* 2003). Although the majority of polyps are adenomas, hyperplastic polyps (HPPs) and sessile serrated adenomas (SSAs) are also seen (reviewed in Mazzei *et al* 2013; Boparai *et al* 2008; Lipton *et al* 2003a).

Patients with MAP have an increased risk of developing CRC. Malignancy has been reported with varying frequencies: one paper reported a prevalence of 19.47% at 50 years, and 42.89% at 60 years (Lubbe *et al* 2009); another found that

48% of patients with MAP developed CRC with a mean age of diagnosis of 49.7 years (Sampson *et al* 2003). It has been suggested that biallelic inactivation of *MUTYH* imparts an overall 93-fold excess risk and that all homozygotes/ compound heterozygotes will develop CRC by age 60 (Farrington *et al* 2005). Win *et al* (2014) estimated that males carrying biallelic *MUTYH* mutations had a 75.4% risk of developing CRC by age 70, and females had a 71.7% risk (Win *et al* 2014).

MAP may also have extra-colonic manifestations, although these are generally not part of the characteristic phenotype (Poulsen and Bisgaard 2008). The lesions which have been reported include duodenal adenomas and carcinoma, fundic gland polyps, stomach cancer, CHRPE, osteomas and breast cancer (reviewed in Venesio *et al* 2012; reviewed in Poulsen and Bisgaard 2008). The incidence of extra-intestinal malignancies is almost double that of the general population, with a significant increase in ovarian, bladder and skin cancers (reviewed in Venesio *et al* 2012).

By 2013, >300 *MUTYH* variants among MAP patients and/or controls had been described (Ruggieri *et al* 2013). The mutations observed in *MUTYH* vary according to the ethnic group studied, suggesting population specific ancestral variants (Dolwani *et al* 2007; Sieber *et al* 2003). In Caucasian populations, Y165C and G382D are the common mutations (Jones *et al* 2002; Sampson *et al* 2003; Enholm *et al* 2003; Sieber *et al* 2003). These account for 73% of all mutations reported and have been described in Swiss (Russell *et al* 2006), Italian (Gismondi *et al* 2004; Venesio *et al* 2004), French (Küry *et al* 2007), Swedish (Kanter-Smoler *et al* 2006; Zhou *et al* 2005), Canadian (Croitoru *et al* 2007), Australian (Kairupan *et al* 2005), Portuguese (Isidro *et al* 2004), Czech (Šulová *et al* 2007), British, American and Dutch populations (reviewed in Cheadle and Sampson 2007). There is evidence for strong founder effects for these mutations: it is suggested that they derive from ancestors who lived between 5-8000 years and 6-9000 years BC respectively (Aretz *et al* 2014).

Numerous other pathogenic variants in *MUTYH* have been reported, including g.1395delGGA in Italians (Gismondi *et al* 2004), E466X and Y90X in Asians (Sampson *et al* 2003; Jones *et al* 2002) and R231C in the Japanese (Miyaki *et al* 2005).

### 2.1.3 Polymerase Proofreading Associated Polyposis

#### (PPAP)

PPAP is a relatively recently defined clinical entity. *POLE* and *POLD1* code for DNA polymerases with exonuclease (proofreading) activity. Mutations in these genes are thought to cause a defect in correcting mispaired bases inserted during DNA replication (Palles *et al* 2013). In 2012, Palles *et al* undertook whole-genome sequencing of probands who had at least 10 colorectal adenomas by age 60, who had previously had known Mendelian cancer syndromes excluded in a clinical diagnostic setting. They also sequenced several affected relatives (Palles *et al* 2013). The group found that a genetic variant, *POLE* p.Leu242Val, was associated with multiple colorectal adenomas and carcinoma (Palles *et al* 2013). The trait showed dominant inheritance, with high penetrance (Palles *et al* 2013). Another variant, *POLD1* p.Ser478Asn, predisposed to colorectal tumors, endometrial cancer, and possibly brain tumors (Palles *et al* 2013).

In 2014, Valle *et al* sought to determine the prevalence of these mutations in 858 patients with unexplained familial/ early-onset CRC or polyposis. They didn't identify either mutation in any of their CRC cases. However, the *POLE* p.Leu424Val mutation was found in a polyposis family, in which case it had occurred as a *de novo* mutation in the proband. This accounted for 0.52% of the polyposis cases. The group also reported a novel variant, *POLD1* c.1421T>C (p.Leu474Pro) in a MMR-proficient family. This mutation occurs in the proofreading domain of a DNA polymerase and was predicted to be pathogenic (Valle *et al* 2014).

Valle's group sought to further characterise the phenotypic spectrum of patients carrying germline *POLE/POLD1* mutations (Bellido *et al* 2016). They sequenced the entire exonuclease domains of *POLE/POLD1* in 544 CRC cases from 529 families, including those from Valle's original paper (Valle *et al* 2014). Although no additional *POLE* mutations were identified, 4 of 6 novel/rare nonsynonymous *POLD1* variants detected were believed to be pathogenic: p.D316H, p.D316G, p.R409W and p.L474P. The group reviewed the phenotypic data from all 69 carriers of *POLE/POLD1* mutations that had been reported to date. They observed that the associated phenotype was characterised by attenuated/ oligo- adenomatous polyposis, with >80% of *POLE* and >60% of *POLD1* mutation carriers being diagnosed with  $\geq 2$  adenomas, with an average of 19 lesions. CRC was diagnosed in 60-64% of carriers, and brain tumors in 5.8%. Gastroduodenal (mostly duodenal) adenomas were identified in 57.1% of carriers who underwent gastroduodenoscopies. For patients harbouring *POLD1* mutations, the phenotypic spectrum was extended to include endometrial tumors (57.1% of carriers) and breast tumors (14.3% of carriers).

#### **2.1.4 NTHL1-Associated Polyposis**

A further gene involved in the pathogenesis of colorectal neoplasia is *NTHL1*. In 2015 Weren *et al* carried out whole exome sequencing on 51 patients with multiple colorectal adenomas +/- CRC, who had tested negative for *APC* and *MUTYH* mutations (Weren *et al* 2015). The group found that 7 individuals were homozygous for a *NTHL1* nonsense mutation, c.268C>T, which triggers nonsense-mediated decay (NMD). The patients harbouring the mutations all had multiple colorectal adenomas, ranging from 8-50, and 4 also had multiple CRCs. All 3 affected women developed complex endometrial hyperplasia or endometrial cancer.

*NTHL1* is a base excision repair gene, and homozygous mutations cause an increase in C>G>T:A changes in genes such as *APC*, *p53*, *KRAS* and *PI3K* (Weren *et al* 2015).

### 2.1.5 *MSH3*-Associated Polyposis

The most recently identified polyposis syndrome is *MSH3*-Associated Polyposis (Adam *et al* 2016). Adam's group performed whole exome sequencing on germline DNA extracted from 102 unrelated individuals with unexplained adenomatous polyposis. They found two different individuals with different compound heterozygous mutations in the mismatch repair gene, *MSH3*. Both index persons had an affected sibling carrying the same mutations. The mutations were associated with tumors which displayed Elevated Microsatellite Alterations At Selected Tetranucleotide Repeats (EMAST), a type of microsatellite instability.

The phenotypic spectrum in *MSH3* mutation carriers was reported to include colorectal and duodenal adenomas, CRC, gastric cancer and an early onset astrocytoma (Adam *et al* 2016).

## 2.2 Hyperplastic Polyps (HPPs)

HPPs are a frequent finding, seen in between 1% and 73% of autopsies (Paspatis *et al* 2001; Williams *et al* 1982; reviewed in Correa *et al* 1977). In asymptomatic individuals undergoing colonoscopy, HPPs are observed in 21% - 34% of cases (Forsberg *et al* 2012; DiSario *et al* 1991). They are more common in males than females, and their prevalence increases with age (Williams *et al* 1982; Williams *et al* 1980; Correa *et al* 1977).

Until approximately 1990, hyperplastic (or 'metaplastic') polyps were regarded as a homogeneous group of tumors with no malignant potential (Rosty *et al* 2013a). Since that time, it has been increasingly recognised that hyperplastic

lesions are not a single entity – they differ in their morphology and their clinical significance, in particular their risk for progressing to carcinoma. In 2010, the World Health Organisation published a classification system which subdivides hyperplastic lesions into 3 groups based upon their microscopic appearance: Hyperplastic Polyps (HPPs), Sessile Serrated Adenomas/Polyps with or without cytological dysplasia (SSA) and Traditional Serrated Adenomas (TSA) (reviewed in Rosty *et al* 2013a; reviewed in Leggett and Whitehall 2010).

It is now known that hyperplastic lesions may be the precursors to CRC developing along the serrated pathway of carcinogenesis, which accounts for approximately 10% of CRCs (Yamane *et al* 2014). In contrast to the CRC which follow the traditional ‘adenoma carcinoma’ pathway, tumors arising from the serrated pathway tend not to display CIN, but instead exhibit MAPK pathway activation, through *BRAF* mutations, and they commonly develop CIMP (Yamane *et al* 2014; Rosty *et al* 2013a; Leggett and Whitehall 2010).

## 2.2.2 Serrated Polyposis Syndrome

Although most HPPs are sporadic lesions, there is a condition in which patients develop multiple and/or large lesions. One of the first descriptions was in 1980 by Williams *et al* (Williams *et al* 1980). They observed 7 patients, with a mean age of 37.4 years, who each had at least 50 lesions throughout their large bowel. At that time, the authors concluded that ‘it is impossible to deduce whether or not ‘metaplastic polyposis’ is a distinct entity. There is no good evidence that it is familial in this small series, but the appearance of numerous metaplastic polyps of an unusually large size and configuration, predominantly in young males, might suggest a specific ‘disease’.

As increasing evidence came to light that there seemed to be a syndrome in which patients developed numerous hyperplastic lesions throughout their large

bowel, it was named 'hyperplastic polyposis syndrome' (HPS). In 2000, a definition of HPS was proposed by Jass and Burt in the World Health Organisation classification of tumors (Jass and Burt 2000). This definition was modified in 2010, and the disease was officially renamed Serrated Polyposis Syndrome (SPS). It appears in the 2010 World Health Organisation classification of tumors of the digestive system (Snover DC *et al* 2010). In order to meet the diagnostic criteria for SPS, patients must fulfil at least one of the following criteria:

1. At least 5 serrated (hyperplastic) polyps proximal to the sigmoid colon, 2 of which are >10mm diameter
2. Any number of serrated polyps proximal to the sigmoid colon in an individual who has a first degree relative with serrated polyposis
3. More than 20 serrated polyps of any size distributed throughout the colon

(reviewed in Rosty *et al* 2013a; reviewed in Guarinos *et al* 2012; reviewed in Leggett and Whitehall 2010; Snover DC *et al* 2010)

It was thought that SPS affected 1 in 3000 asymptomatic individuals between the ages of 55 and 64 years (reviewed in Rosty *et al* 2013b). However, most studies report a broad age distribution of the disease (17 to 85 years) with a mean age of diagnosis of 47.7-56 years, so it is possible that the prevalence of SPS in the general asymptomatic population is higher than 1/3000 (Rosty *et al* 2013b). Recent data suggest that the prevalence may be as high as 1/151 patients who have a colonoscopy following a positive faecal occult blood test (reviewed in Rosty *et al* 2013a).

SPS shows no sex predilection, and the mean age of diagnosis is 55 years (Guarinos *et al* 2012; Kalady *et al* 2011). As well as hyperplastic lesions, up to 85% of patients also have conventional adenomas present in the bowel (Rosty *et al*

2013a; Rosty *et al* 2012). Patients with SPS have an increased risk of developing CRC, which generally occurs between 50 and 60 years of age (reviewed in Guarinos *et al* 2012). Malignancy is associated with a larger number of polyps, the presence of dysplasia (reviewed in Guarinos *et al* 2012; Yeoman *et al* 2007) and the presence of conventional adenomas in addition to HPPs (Rosty *et al* 2013b). The incidence of CRC in SPS patients varies from 14% to 58% (reviewed in Rosty *et al* 2013b; Yeoman *et al* 2007; Hyman *et al* 2004; Lage *et al* 2004) and the incidence is greater in females than in males, with a ratio of 2.4:1 (Rosty *et al* 2013b). When carcinoma develops in an SPS patient, it is likely to have a proximal location: 64% of CRC are identified proximal to the descending colon (Rosty *et al* 2013b). Interestingly, a large proportion of CRCs seen in patients with SPS do not develop through the 'serrated pathway of carcinogenesis' driven by *BRAF* mutation (Rosty *et al* 2013b). The tumors show various molecular changes, including those more likely to be associated with the traditional adenoma-carcinoma pathway, for example  $\beta$ -catenin activation and/or overexpression of p53 (Rosty *et al* 2013b).

SPS is thought to be a genetic disease, but the mode of inheritance is unclear (reviewed in Guarinos *et al* 2012). There are papers which report germline mutations in the Wnt inhibitor *RNF43*, an E3 ubiquitin ligase, in individuals and families with features of SPS (Yan *et al* 2017; Taupin *et al* 2015; Gala *et al* 2014). However, Buchanan *et al* note that mutations in *RNF43* may account for only a small proportion of SPS, and that additional genetic risk factors are yet to be identified (Buchanan *et al* 2017).

### **2.3 Hamartomatous Polyps**

Hamartomas are overgrowths of the tissue which is native to the site of the lesion. Polyposis syndromes which are characterised by hamartomas include Peutz-

Jegher's Syndrome (PJS), Juvenile Polyposis Syndrome (JPS) and Cowden's Disease.

### 2.3.1 Peutz- Jegher's Syndrome (PJS)

In 1921, a Dutch physician, Dr. Peutz, first described the combination of gastrointestinal polyps and mucocutaneous pigmentation (Peutz 1921). In 1949, Dr. Jeghers published an article describing 10 patients who had a combination of pigmentation of the oral mucosa/lips/digits, and intestinal polyps (Jeghers *et al* 1949). The observations made by Peutz and Jeghers led to the definition of an AD syndrome characterised by gastrointestinal polyposis and mucocutaneous pigmentation, now known as Peutz-Jeghers Syndrome (PJS) (Westerman *et al* 1999).

PJS is inherited in an AD manner and has variable penetrance. It is a rare condition, with a prevalence of approximately 1 in 200 000 (reviewed in Omundsen and Lam 2012). The disease is characterised by hamartomatous polyps throughout the gastrointestinal tract and mucocutaneous pigmentation. Patients present at a median age of 11 years, and this is often as a result of a complication of their GI polyps, for example intussusception, small bowel obstruction, rectal bleeding or volvulus (reviewed in Omundsen and Lam 2012).

Approximately 50% of cases of PJS are caused by germline mutations in the nuclear serine threonine kinase gene *LKB1/STK11* (Jenne *et al* 1998; reviewed in Omundsen and Lam 2012). This gene regulates cell polarisation, growth and metabolism. Most mutations are small insertions or deletions, resulting in a truncated protein with no kinase activity (reviewed in Omundsen and Lam 2012).

Patients with PJS are at increased risk of developing cancer, both at gastrointestinal and extra-intestinal sites. The most common tumors are CRCs, but there is also an increased risk of other gastrointestinal (GI) carcinomas

(oesophageal, gastric, small bowel and pancreas), breast cancer, ovarian cancer, cervical cancer and sex cord tumors (Hizawa *et al* 1993; reviewed in Omundsen and Lam 2012).

### **2.3.2 Familial Juvenile Polyposis Syndrome**

In 1914, Hertz described four family members who had rectal polyps in childhood, the youngest being only 8-years-old (Hertz 1914). That report is regarded as the first instance of juvenile polyposis in the medical literature (Calva and Howe 2009).

Juvenile polyps are common hamartomatous lesions which occur in the large bowel. They are usually solitary and sporadic. If multiple juvenile polyps are present, the patient may have Juvenile Polyposis Syndrome (JPS). JPS is an AD disease with variable penetrance and which has an incidence of 1 per 100 000 births (reviewed in Omundsen and Lam 2012). Patients can present in infancy with GI bleeding, intussusception, rectal prolapse or a protein losing enteropathy. Around 15% will have an associated congenital birth defect, such as gut malrotation, cardiac and cranial abnormalities, cleft palate, polydactyly or genitourinary defects. If presentation is as an adult, the patient is likely to suffer from GI bleeding (reviewed in Omundsen and Lam 2012).

For a diagnosis of JPS, patients must fulfil one of the following criteria:

1. More than 5 juvenile polyps of the colon or rectum
2. Juvenile polyps in other parts of the GI tract
3. Any number of juvenile polyps and a positive family history

(reviewed in Omundsen and Lam 2012)

Germline mutations in *SMAD4* and *BMPR1A* are seen in JPS, and it is suggested that *ENG* mutations may also have a role (Sweet *et al* 2005), although this is not certain (Howe *et al* 2007). All of these genes are involved in transforming growth factor  $\beta$  (TGF $\beta$ ) signalling. The TGF $\beta$  family of cytokines are growth inhibitors, and loss of sensitivity to these factors promotes tumorigenesis (reviewed in Fleming *et al* 2013). As such, patients with JPS are at increased risk of developing CRC (Rozen and Baratz 1982; Järvinen and Franssila 1984; Giardiello *et al* 1991). In addition to colorectal malignancies, patients are also at risk of developing gastric and duodenal cancer (reviewed in Omundsen and Lam 2012).

### 2.3.3 Cowden's Syndrome

In 1963 Lloyd and Dennis reported a 20-year old female with multiple pathologies, including multiple thyroid adenomas, extensive fibrocystic change of both breasts and 'space occupying lesions in the liver and bone' (Lloyd and Dennis, 1963). At that time, it was noted that 'whether this case represents a new familial disease....has not been established' (Lloyd and Dennis, 1963), although they named the syndrome 'Cowden's Disease'. It is now established that Cowden's Syndrome is an AD disease, affecting approximately 1 in 200 000 births. It is commonly diagnosed in the second decade of life, but age of onset may vary from 4 to 75 years (reviewed in Lam-Himlin *et al* 2014). Patients develop multiple hamartomas in multiple organ systems. Lesions can be found in the skin, GI tract, breast, thyroid gland and central nervous system (reviewed in Omundsen and Lam 2012; Hanssen and Fryns 1995).

80% of patients have a germline mutation in the *PTEN* gene (reviewed in Omundsen and Lam 2012). *PTEN* is a tumor suppressor: its product is a phosphatase which negatively regulates the phosphatidylinositol 3-kinase-AKT (PI3K) and mammalian target of rapamycin (mTOR) signalling pathways, which are

involved in cell growth and proliferation, cell cycle progression and apoptosis (reviewed in Lam-Himlin *et al* 2014). *PTEN* mutations are involved in the pathogenesis of several carcinomas, including breast, endometrial, thyroid, large bowel and kidney (reviewed in Lam-Himlin *et al* 2014). As such, patients with Cowden's Syndrome are at increased risk of these malignancies (Hanssen and Fryns 1995).

## 2.4 Hereditary Mixed Polyposis Syndrome (HMPS)

Hereditary Mixed Polyposis Syndrome (HMPS) is a relatively recently defined entity. In 1971, Kaschula described an 11-year-old girl who had profuse diarrhoea mixed with blood and mucus. She was found to have polyps throughout her large bowel, and the polyps had both adenomatous and juvenile morphologies (Kaschula 1971). Over a decade later, in 1987, the term 'mixed familial polyposis syndromes' was used as the title of a report by Sarles *et al* (Sarles *et al* 1987). This article described 3 patients, including a father and son, who all had multiple polyps of different histopathological types.

In HMPS, patients develop multiple polyps with mixed morphologies. This is an AD disease, and patients may have adenomas, hyperplastic polyps and hamartomatous polyps. There is a high risk of developing CRC (Jaeger *et al* 2012). The disease is caused by a duplication spanning part of the *SCG5* gene and a region upstream of the *GREM1* locus. This duplication causes increased expression of *GREM1*, which acts as a bone morphogenetic protein (BMP) antagonist. The subsequent reduction in BMP signalling is thought to play a role in tumorigenesis (Jaeger *et al* 2012).

### 3. Clinical Management of Patients with Colorectal

#### Polyposis

It has been common clinical practice for patients with >10 colorectal adenomas to be referred to a regional genetics centre for genetic counselling and for consideration of diagnostic analysis of the *APC* and/or *MUTYH* genes. The diagnostic testing carried out will depend upon the individual's phenotype and their family history.

Up to 90% of patients with a phenotype of typical FAP have a pathogenic *APC* germline mutation identified through sequencing of coding exons and deletion/duplication analysis via multiplex ligation-dependent probe amplification (MLPA) (Spier *et al* 2012). Of those with a phenotype of AFAP, *APC* or biallelic *MUTYH* germline mutations are detected in only 20-50% of cases (Spier *et al* 2012).

#### 4. 'Unexplained' Colorectal Polyposis

Since 1991, when *APC* was recognised as the causative gene of FAP (Grodén *et al* 1991; Joslyn *et al* 1991; Kinzler *et al* 1991; Nishisho *et al* 1991), several screening and diagnostic strategies have been developed to identify pathogenic *APC* mutations (Scott *et al* 2001) in patients with multiple colorectal adenomas. These have included denaturing gradient gel electrophoresis analysis (DGGE) (Scott *et al* 2001; Olschwang *et al* 1993), ribonuclease protection analysis (Miyoshi *et al* 1992), single strand conformation polymorphism analysis (SSCP) (Cottrell *et al* 1992; Grodén *et al* 1993), heteroduplex analysis (HA) (Cottrell *et al* 1992) and the protein truncation test (PTT)/ in vitro synthesised protein assay (IVSP) (Powell *et al* 1993). The characterisation of a genetic mutation identified through screening requires DNA sequencing. Sanger sequencing has been the gold standard of sequencing for several decades, and until very recently was the main approach used for the molecular diagnosis of colorectal polyposis. In the last few

years, next-generation sequencing (NGS) technologies have progressively replaced Sanger sequencing.

Genetic diagnostic protocols used in patients with polyposis typically include sequencing of *APC* and/or *MUTYH* and dosage analysis of the genes using a technique such as MLPA. These approaches could miss pathogenic variants located in promoter regions, deep within introns (Spier *et al* 2012) or in untranslated regions (UTRs), which may have effects on gene expression mediated through effects on transcription, mRNA splicing or mRNA stability. Similarly, diagnostic protocols may miss low frequency variants in patients with somatic mosaicism. Most Many diagnostic protocols would also not identify epigenetic phenomenon such as promoter methylation, they would not detect mutations in genes which are established but rare causes of polyposis (e.g. *POLE/POLD1*) and they would also not identify novel polyposis genes.

#### **4.1 Promoter Variants and Allelic Imbalance (AI)**

The *APC* gene has two promoter regions, 1A and 1B (reviewed in Rohlin *et al* 2011). The major transcript is initiated by the major promoter, 1A (reviewed in Charames *et al* 2008). It is possible that genetic variants occurring in these promoters could lead to reduced gene expression, therefore predisposing to tumor formation. Such variants might not be identified through standard genetic diagnostics as the promoter is not typically included in diagnostic sequencing protocols.

There are several reports of *APC* promoter mutations in the literature. However, these typically describe deletions, which would be detected through diagnostic MLPA (Yamaguchi *et al* 2016; Pavicic *et al* 2014; Rohlin *et al* 2011; Charames *et al* 2008). There is a paucity of literature describing *APC* promoter point mutations or methylation as a cause of colorectal polyposis.

Allelic imbalance (AI) refers to a situation in which the two alleles of a given gene are expressed at different levels in a given cell (Wagner *et al* 2010). It can occur due to epigenetic inactivation of one of the alleles, or because of genetic variation in regulatory regions (Wagner *et al* 2010). AI can involve complete inactivation of one allele, for example in parent-of-origin imprinting, when a specific allele at a given locus is silenced through epigenetic mechanisms depending on whether it was inherited from the mother or father (Wagner *et al* 2010). Allele expression can also be partially reduced, which can occur when different alleles have differing affinities for transcription factors (Wagner *et al* 2010), or through *cis*- acting genetic variants, for example in promoters (Wagner *et al* 2010).

As well as being important in normal phenotypic variation, AI can also contribute to tumorigenesis. There is some evidence that *BRCA1* and *BRCA2* AI plays a role in the pathogenesis of ovarian and breast cancer (Chen *et al* 2008; Shen *et al* 2011). Whilst only a small number of studies have considered *APC* AI in the context of colorectal neoplasia, those which have been performed have found it may make an important contribution. As early as 1993, Powell *et al* (Powell *et al* 1993) used an allele-specific expression assay to show that 3/11 *APC* NMI patients with clinical FAP had significantly reduced expression of one *APC* allele. In 1999 Laken *et al* (Laken *et al* 1999) used monoallelic mutation analysis (MAMA) to reveal that 7/9 *APC* NMI patients had reduced/no expression from one of their *APC* alleles. More recently Yan *et al* (2002) identified a patient with colorectal tumors who was known to have reduced levels of the APC protein. The group quantified the relative levels of mRNA transcripts from each *APC* allele using Digital-SNP. They found that gDNA yielded the expected 50% allelic ratio, but that cDNA from lymphoblastoid cells showed a skewed distribution, with a ratio of approximately 66% (Yan *et al* 2002). Linkage analysis showed that the allele whose mRNA was expressed in lower amounts was the one linked to disease (Yan *et al* 2002). Further work confirmed that the skewed allelic ratio was also present in 4 affected family

members, but that the ratio was normal in 24 unrelated unaffected individuals. The group continued to investigate expression levels of *APC*: in four patients with clinical FAP who had no abnormalities with the in vitro synthesised protein assay (IVSP) or allele sequencing, one was found to have an abnormal 71% allelic ratio in cDNA. When tumors from the patients with AI were studied, 30/38 had LOH of *APC*, and in 29 of these cases, it was the normal allele which had been lost. Yan *et al* concluded that an allele which causes a decrease in transcript levels can result in a predisposition to severe disease, but that there needs to be a second hit to the normal allele for a disease to manifest (Yan *et al* 2002). Interestingly the cause of the decreased expression was not determined: the sequences of the coding regions, promoter and 3'UTR were normal, so the group assumed that the pathogenic variant must lie within an intron or upstream of the gene (Yan *et al* 2002). These early findings regarding *APC* AI have been supported by Castellsagué *et al* (2010). Of 23 *APC/MUTYH* NMI polyposis families who were heterozygous for rs2229992, 2 were shown to harbour *APC* AI. The AI in one family was suggested to result from promoter variants (Castellsagué *et al* 2010).

## 4.2 Intronic Variants

Intronic mutations may affect RNA splicing and introns are not screened as part of most routine genetic diagnostic protocols. In 2000 Su *et al* sought to identify novel intragenic rearrangements of *APC* in patients with a clinical phenotype of FAP or AFAP. They found four germline *APC* mutations, one of which was a deletion 27-1627bp downstream of exon 14, which was replaced with a novel sequence of about 180bp. The deletion was completely within intron 14, but it affected the splicing of exon 14 (Su *et al* 2000). In 2010 Tuohy *et al* used Southern Blot analysis of the *APC* gene to identify a 1.4kb deletion within intron 14 in a family with AFAP. Subsequent PCR amplification from exon 13 to exon 15 of cDNA showed that the

intronic deletion resulted in abnormal splicing, and that exon 14 was deleted. This caused a frameshift and protein truncation at codon 673 of the normal reading frame resulting in a truncated product that lacked all of the  $\beta$ -catenin, microtubule and EB-1 binding domains (Tuohy *et al* 2010).

In 2012 transcript analysis in a sample of 125 mutation negative patients with colorectal adenomatous polyposis found that 8% had a reproducible aberrant transcript pattern, suggesting an intronic mutation at a genomic level (Spier *et al* 2012). 80% of these were found to have transcript insertions between two exons originating from exonised sequences deep within the corresponding intron (Spier *et al* 2012). All pseudoexons were predicted to result in out-of-frame transcripts with premature stop codons (Spier *et al* 2012). In those patients who had insertions, the underlying genomic mutations were identified: they comprised 3 different heterozygous point mutations (c.532-941G>A, c.1408+731C>T, c.1408+735A>T) which activated cryptic splice sites. A pre-existing complementary cryptic splice site was predicted at the other end of the insertion (Spier *et al* 2012).

### 4.3 Untranslated Region (UTR) Variants

A pre-RNA molecule undergoes several steps of processing before it becomes a functional mRNA molecule (Mignone *et al* 2002). Mature mRNA consists of a 5' untranslated region (5'UTR), a coding region, and a 3' UTR (Mignone *et al* 2002). UTRs, particularly 3'UTRs, have multiple roles in the post-transcriptional regulation of gene expression, including effects on mRNA transport out of the nucleus, translation efficiency, subcellular localisation and mRNA stability (reviewed in Mignone *et al* 2002). In addition to normal physiological intracellular effects, there is increasing evidence that UTR variants can be involved in disease, for example an expanded number of trinucleotide repeats in the 3'UTR of the *DMPK* gene is thought to play a role in the pathogenesis of in myotonic dystrophy (Conne *et al*

2000), and a somatic 5'UTR variant has been reported to reduce translation efficiency of *BRCA1* in a highly aggressive sporadic breast cancer (Signori *et al* 2001).

In the context of colorectal neoplasia, Wilding *et al* have shown that in microsatellite unstable cancer, deregulation of mRNA stability due to mutations in regulatory 3'UTR sequences can lead to a marked difference in gene expression profiles when compared to microsatellite stable tumors (Wilding *et al* 2010).

#### 4.4 Mosaicism

A mosaic is an individual who has at least two genetically different cell lines despite developing from a single zygote. Mosaic mutations may be missed with standard mutation diagnostic techniques, for example if they occur at a low frequency within the individual or if they do not occur in the part of the body which is being analysed.

It is reported that somatic mosaicism can occur in 10-20% of sporadic cases of FAP (reviewed in Rohlin *et al* 2009; Hes *et al* 2008; Aretz *et al* 2007). The timing at which an *APC* mutation occurs will have an important bearing on the patient's phenotype: if it arises in a single colonic epithelial stem cell, the only consequence will be adenomatous polyps in the segment of the colon that becomes populated with descendants of the stem cell (Tuohy and Burt 2008). At the other extreme, if the mutation occurs early in embryogenesis, it may be found in all three germ cell layers. This would result in multiple clinical manifestations seen throughout multiple organ systems, potentially including mutations in reproductive cells, which could then be passed on to future generations (Tuohy and Burt 2008).

Depending on the frequency of the mutation, it is likely that a significant proportion will be missed using current diagnostic sequencing protocols and DNA extracted from whole blood. Rohlin *et al* (2009) carried out a study to evaluate the

different mutation screening/diagnostic techniques in terms of their sensitivity in detecting mosaicism. They looked at Sanger sequencing, single-strand conformation polymorphism (SSCP)/heteroduplex analysis (HA), the protein truncation test (PTT), denaturing high performance liquid chromatography (DHPLC) and massively parallel sequencing. A total of 9 mutations were addressed – 8 in *APC* and 1 in *NF2*. The group constructed 7 artificial mosaics in *APC* through serial dilutions of DNA, with a non-mosaic heterozygous mutation being defined as 100%. The two remaining samples were from naturally occurring mosaics. All of the dilutions of all of the mutations were analysed with SSCP, DHPLC and Sanger sequencing. Three were included in the PTT assay (these were mutations in exon 18, which is readily screened by PTT). Only four artificial mosaics, at various concentrations, and both natural mosaics underwent massively parallel sequencing, due to cost limitations. The group found that SSCP and DHPLC were able to detect mutant alleles at frequencies between 5% and 25%, whereas Sanger sequencing required frequencies between 15% and 50% for detection. The mutations included in the PTT assay were detected at frequencies between 10 and 100%. The Genome Sequencer FLX was used for massively parallel sequencing, and this achieved coverage between 648 and 8313 reads. Mutations frequencies as low as 1% could be detected, but this required a high coverage (Rohlin *et al* 2009).

The results from this study showed that Sanger sequencing, which has been commonly used in a diagnostic setting, was the least sensitive method at detecting mosaics. Dependent upon the type of mutation being analysed, this technique may require mutation frequencies as high as 50% in order for them to be detected (Rohlin *et al* 2009).

Genetic testing in the diagnostic setting is usually carried out on DNA extracted from whole blood. For some patients, a mosaic mutation may only be present in colonic tissue so will not be detectable through testing blood-derived DNA (Jansen *et al* 2017).

## 4.5 The Involvement of Additional Genes

Standard diagnostic protocols carried out in the context of adenomatous colorectal polyposis typically examine the *APC* and *MUTYH* genes, although an increasing number of laboratories are screening DNA for the recurrent mutations in *POLE* and *POLD1*. It is only in recent years that the pathogenic effects of mutant *POLE*, *POLD1*, *NTHL1* and *MSH3* have been discovered. It is therefore feasible that there may be further genes involved in the development of heritable colorectal polyposis, which are yet to be identified.

Furthermore, mutations in genes such as *APC*, *MUTYH*, *POLE*, *POLD1*, *NTHL1* and *MSH3* are all highly penetrant. It may be the case that some patients with unexplained colorectal polyposis have a phenotype which results from the complex interplay of several low/moderate penetrance genetic variants.

## 5. Summary

Colorectal carcinoma (CRC) is the third most common cancer in men and the second most common cancer in women (Ferlay *et al* 2013). Most CRCs occur sporadically, but in approximately a third of patients, hereditary factors are important (reviewed in Mishra and Hall 2012; Burt 2007). Some patients with an inherited predisposition to CRC will be diagnosed with a 'genetic polyposis syndrome' such as FAP, MAP, PPAP, *NTHL1*-Associated Polyposis, *MSH3*-Associated Polyposis or a hamartomatous polyposis syndrome. It is important to identify these patients, and to define the mutations causing their polyposis, so that the individuals and their relatives can be managed appropriately.

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