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Clinical and molecular aspects of MUTYH- and APC-associated polyposis

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Chapter 6

General discussion and future perspectives

Discussion and future perspectives

This thesis focuses on the further elucidation of the *MUTYH*-associated polyposis (MAP) syndrome that was first described in 2002 and investigates other causes of adenomatous polyposis. The knowledge of the genetic basis of polyposis syndromes and their typical clinical presentation is important for the identification and counselling of patients and the development of appropriate surveillance programs. By studying the literature and writing this thesis, it became clear that some observations in MAP patients still require further elucidation.

Clinical phenotype in MAP

Since 2002, more than 500 MAP patients have been described, of whom almost 300 are included in this thesis. There is a wide variety in the phenotype; some patients have hundreds of polyps, whereas others have only colorectal cancer (CRC) with no or only a few polyps (**chapter 1**). This variety in the phenotype might be explained to some degree by genotypic differences. Functional assays show that different mutations display variable influences on the severity of the disease. Of the two most common mutations, the p.G396D and the p.Y179C, the former has been shown to have a less deleterious effect than the latter in a number of studies.^{1,2} A large collaborative European study showed a milder clinical phenotype, marked by a lower CRC hazard, for the p.G396D homozygotes compared to the p.Y179C homozygotes (**chapter 2.4**). However, genotypes alone do not explain the wide range in polyp count and the age range at CRC diagnosis. Therefore, it is likely that there are (other) genetic modifiers and / or environmental factors that are responsible for the phenotypic differences.

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Extracolonic manifestations in MAP

A high prevalence of extracolonic manifestations is expected in MAP patients because oxidative stress is a common manifestation in all human tissues and the *MUTYH* protein repairs oxidative DNA damage. Several mouse studies have already shown the development of extracolonic tumours with a deficiency in *MUTYH*.^{3,4} In Chapter 2.3, we identified a significant twofold increase in the risk of extraintestinal lesions in general. More specifically, a significantly increased risk was observed for duodenal, ovarian, bladder and skin cancers. Nevertheless, compared with a high cumulative risk for developing intestinal cancer,^{5,6} organ systems outside the colon are relatively spared. The cause for this predilection for the bowel is unknown although some explanations can be hypothesised. First, oxidative stress leading to G:C>T:A transversions is more common in the digestive system, although this does not explain the absence of

tumours in the lung, where oxidative stress is also common. Second, *MUTYH* activity in an ulcerative colitis model was found to be necessary for eliciting the inflammatory response following acute oxidative stress. Authors theorised that subsequent chronic persistence of the inflammatory response might induce carcinogenesis in *MUTYH*-deficient humans.⁷ Finally, the *APC*-gene which is especially involved in colon carcinogenesis has more sequences (AGAA or TGAA motifs) than other genes which seem more dependent of *MUTYH* oxidative damage repair.

MUTYH testing in spouses and children

Given the 1-2% prevalence of *MUTYH* heterozygotes in the population, spouses of MAP patients might carry one (heterozygous) *MUTYH* mutation, which subjects their children to a risk (0.5-1%) of inheriting two *MUTYH* mutations. We show that the costs per quality adjusted life year (QALY) of genetic screening in families with MAP patients are acceptable according to international standards, and therefore, we recommend that genetic testing should be discussed with and offered to MAP families in clinical genetic practice (**chapter 3.2**). However, cost-effectiveness studies have some limitations. First, because economic considerations are never the only decision criterion, it is impossible to set a strict threshold for the acceptable costs per QALY. Second, psychosocial aspects of genetic counselling are not well accounted for. People may experience changes in their functional, emotional or social status after learning of their genetic predisposition. Griffith et al. stated that a cost-utility analysis is not suitable to account for the impact of genetic services on the individual, the family and society because of difficulties in measuring non-health benefits. There is a need for further research on the psychosocial impact of genetic services within a health-economics context.⁸ Currently, we recommend that counsellors should consider the psychosocial implications for anyone who is tested for *MUTYH* mutations as much as in any other genetic counselling procedure.

MUTYH heterozygotes and possible genetic modifiers

In addition to biallelic *MUTYH* carriers, *MUTYH* heterozygotes may also have a higher CRC risk; they may lose function of the other normal *MUTYH* allele and develop tumours. It is also possible that one normal *MUTYH* allele is not enough for the repair of all the G:C>T:A transversions caused by oxidative damage. Meta-analyses of large case-control studies show that the CRC risk for *MUTYH* heterozygotes, whether or not significant, is only marginally increased in the population based studies (OR 1.1-1.3 in meta analyses).^{9, 10,11} In contrast, kin-cohort-based studies have shown a significant twofold, or at most threefold, increase in CRC risk in heterozygous family members

(**chapter 3.1**). These kin-cohort studies are possibly biased somewhat because families were selected from polyposis registries. Currently, unidentified genetic variants or environmental factors in such families might lead to a more severe polyposis phenotype and a predisposition for CRC (in combination with a heterozygous *MUTYH* mutation).

No evident genetic or environmental modifiers in MAP patients have yet been reported, although a higher CRC risk has been shown in patients with a simultaneous *MSH6* missense and monoallelic *MUTYH* mutation.¹² In contrast, pathogenic mutations in two different genes might also be mutually exclusive and lead to early apoptosis of the cancer cell. We found evidence for such a mechanism in a patient with a biallelic *MUTYH* mutation and a pathogenic *MSH6* mutation. This patient had an extremely mild clinical phenotype with only a few adenomas at 56 years of age. No second hit of *MSH6* was apparent in any of the adenomas because of a retained MSH6 nuclear expression and a lack of microsatellite instability (**chapter 4.3**). A possible explanation might be that in cells where a second hit of *MSH6* occurred, this hit led to a mutation load that was too high even for a cancer cell resulting in early apoptosis and no benefit for the cancer.

In conclusion, *MUTYH* heterozygotes might have a higher CRC risk when this genotype occurs in combination with other predisposing (genetic) CRC risk factors, although no evident genetic or environmental modifiers in MAP patients have yet been reported.

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Histology and molecular hallmarks in MAP CRC

Specific histology and molecular hallmarks can help identify patients with a heritable form of CRC. This is most important for Lynch syndrome, where microsatellite analysis and immune histochemistry (**chapter 1.1**) can lead to the diagnosis. We identified an overlap between MAP and MSI-high / Lynch CRCs, such as a preferential proximal location, mucinous histotype and increased presence of TILs. The above features should direct the pathologist towards a *MUTYH*-deficient aetiology of CRC as an alternative to a deficiency in mismatch repair (**chapter 4.1**). Furthermore, a distinctive molecular feature is the c.34G>T hotspot mutation in the *KRAS* gene, which is present in 65% of MAP carcinomas, but is uncommon in LYNCH carcinomas (**chapter 4.3**). In addition, in contrast to Lynch carcinomas, defective *MUTYH* does not seem to induce microsatellite instability because in most studies, an MSI-high phenotype is not found or may be present in a minority of MAP CRCs (**chapter 4.1**).^{5, 13, 14} Another distinction between carcinomas can be made on the basis of the type of genomic profile because genetic instability is a common feature in early carcinogenesis in sporadic cancer. In Lynch CRC's, few genomic chromosomal aberrations are reported. An analysis of

26 MAP carcinomas, using SNP-arrays and flow sorting, showed that these tumours have relatively few chromosome copy number changes, and instead contain many chromosomal regions of copy-neutral loss of heterozygosity (LOH) (71%) (**chapter 4.2**). This is in contrast to sporadic colon cancer, where physical loss is the main characteristic.

In conclusion, MAP CRCs have some specific molecular and histological features that differentiate them from sporadic CRCs and Lynch CRCs. In particular, a *KRAS* somatic c.34G > T mutation is an important hallmark of MAP CRCs, and *KRAS* C.34G>T somatic prescreening of tumour material can be used to identify patients with MAP (**chapter 4.3**). The role of the pathologist is especially important in atypical polyposis patients, i.e., patients with CRC with less than 10-15 adenomas because MAP is less often considered by the clinician.

Survival

Specific histological and molecular genetic features may also reflect tumour behaviour and influence survival in CRC patients. Many studies have shown that patients with mismatch-repair-deficient tumours (associated with Lynch syndrome or sporadic micro-satellite instability (MSI)) have a more favourable clinical course and survival than those with sporadic CRC (**chapter 4.4**). The more favourable clinical outcome might be explained by the enhanced mutation rate associated with mismatch-repair-deficient tumours. In addition to favouring mutations in genes that are critical for oncogenesis, a very high mutation rate might hinder tumour growth and/or spread¹⁵. The continuous production of abnormal peptides, which are recognised as foreign by the host, might also lead to specific antitumor immune responses. This mechanism might also be present in MAP carcinomas because DNA damage repair is compromised. Indeed, a relatively higher number of TILs have been found in MAP CRCs (**chapter 4.1**). A better survival in MAP carcinomas was found for the first time in a European MAP cohort of 276 patients with matched controls (**chapter 4.4**).

Because of the activated immune system, selective pressure can be expected that favours the outgrowth of tumour cell clones with an immune-evasive phenotype. Supporting this theory, defects in the human leukocyte antigen (HLA) class I molecule (which present mutated proteins to the immune system) were found to be a frequent event in MAP CRCs (detected in 65%), similar to MMR-deficient colorectal tumours (**chapter 4.5**).

Attenuated polyposis

For some phenotypes, it remains necessary to have distinct diagnostic criteria in general practice because the genetic background is not uniform and is unknown in a relevant proportion. One such phenotype is *attenuated familial adenomatous polyposis* (AFAP). In several studies, different criteria that describe this phenotype have been used. The main criterion that can be identified from these studies is having less than 100 polyps at an older age. Because a large proportion of the general population will eventually develop one or more polyps at older ages (**chapter 1.1**), this criterion inadequately distinguishes patients with a possible inherited defect from sporadic cases. Using more precise criteria for defining AFAP in which age is also included as a factor, constitutional *MUTYH* and *APC* defects were found in 18 of 25 (72%) families (**chapter 2.5**). The problem with the definition of AFAP used in this study is that it is rather complex and not very useful in clinical practice. It is possible that in the future surveillance protocols can be solely based on the genetic defect and even on specific underlying mutations, when there is greater understanding of genotype-phenotype correlations.

In addition, increasing knowledge of the possible effect of genetic modifiers might lead to a more specific surveillance protocol.

Further causes of polyposis

In a substantial number of polyposis patients, the genetic predisposition still remains to be elucidated. After the finding that mutations in the *MUTYH* gene predispose patients to polyposis and CRC, it was hypothesised that enzymes that work with *MUTYH* to protect against oxidative DNA damage (such as *OGG1*, *NTH1*, *NUDT1*, and *NEIL1*, 2 and 3) represent candidate polyposis and/or CRC predisposition genes. Indeed, one case report described synchronous colon cancer at age 36 in a female patient with germline pathogenic mutations in *MUTYH* and *OGG1*.¹⁶ However, other studies that involved larger groups of polyposis and/or CRC patients did not find a significant association between the mutations in any of these genes and polyposis and/or CRC risk, also not in combination with variants in *MUTYH* (**chapter 5.2**).^{17,18,19,20}

With new techniques, previously undetected mutations can also be discovered in well known polyposis genes. Following the introduction of the technique of *Multiplex Ligation-dependent Probe Amplification* (MLPA)²¹, large exon deletions are readily and simply detected. With this advancement, *APC* deletions have been found in a considerable proportion of polyposis patients without detectable point mutations in *APC* or *MUTYH* (6-48%) (**chapter 5.3**)^{22,23} representing 8-12% of all *APC* mutations found (**chapter 5.3**).²³

We also found that mosaicism occurs in a significant number of *APC* mutations. We estimated that one-fifth of the de novo cases of *APC*-associated polyposis are mosaic. Due to parental *APC* mosaicism, the severity of manifestations in offspring may be underestimated when the parent only displays a mild phenotype. In addition, the recurrence risk for siblings of patients with apparently sporadic polyposis may be underrated (**chapter 5.4**). Because regular genetic testing might fail to detect somatic mosaicism, screening for germline mutations should preferably be conducted in affected children of apparently de novo cases (i.e. those with asymptomatic grandparents). In addition, we also advise DNA testing for siblings of patients in which polyposis coli apparently arose de novo because of a possible mosaicism that is confined to the parental gonads.

The explanation for the relatively high prevalence of mosaicism lies in the fact that *APC* mutations often arise de novo (one third of cases). This is in contrast to *MAP*, in which de novo mutations have not been reported until now.

In conclusion, no other highly penetrant genes involved in repairing oxidative damage have been found responsible for CRC, although it was found that deletions in the *APC* gene are relatively common, and mosaicism occurs in a significant number of *APC* mutations.

Future perspectives

Genetic modifiers and environmental factors

Genetic modifiers and environmental factors might explain part of the variation in phenotype for *MUTYH*- and *APC*-associated polyposis phenotypes. In addition, they might also explain why family-based studies seem to find an almost twofold higher CRC risk for *MUTYH* heterozygotes compared with CRC–population-based studies. Recently, Wijnen et al. showed that two loci that were previously identified as low CRC risk variants in the general population significantly influence the CRC risk in Lynch patients.²⁴ It would be interesting to determine the role of these low CRC risk variants in *MAP* patients and *MUTYH* heterozygotes. In addition, well known cancer risk factors, such as obesity, diabetes, smoking and/or chronic bowel inflammation, might contribute to the CRC risk and the development of polyps.^{25–28} Notably, common intestinal flora (such as *Bacteroides Fragilis*) were also found to promote inflammation-induced colonic tumour formation.²⁹ The presence of such bacteria in homozygous *MUTYH* mutation carriers might be an important external modifier of CRC risk given the role of *MUTYH* in the repair of oxidative injury and its possible role in eliciting the inflammatory response.⁷

Surveillance guidelines and treatment

It may be appropriate to initiate surveillance later for p.G396D homozygotes because these patients have a later mean age of CRC diagnosis than the p.Y179C homozygotes (**chapter 2.4**). Ideally, verification with long-term prospective studies or larger studies should be undertaken to extend the evidence base for the refinement of clinical guidelines. Unfortunately, in practice, this will be difficult because only approximately 1% of newly diagnosed CRC patients are MAP patients; therefore, a very large prospective cohort is necessary to obtain numbers that are large enough to analyse and provide relevant results. In addition, newly diagnosed *MUTYH* family members will be under surveillance for CRC and are less likely to develop CRC.

Future research should also be aimed at elucidating the nature of polyp development over time because there is little detailed information about the natural course of MAP disease, and this can have implications for surveillance guidelines. The pace at which polyps develop might depend on age, genetic modifiers or external factors. For example, an unexplained strong acceleration in adenoma development beyond 52 years of age has been reported in one patient.³⁰ Furthermore, an accelerated adenoma–carcinoma sequence might be present in MAP patients who manifest CRC and few or no polyps.

An option for the prevention of carcinoma development in the future might be vaccination aimed at frequently mutated proteins, such as *KRAS*, especially because the activated immune response found in MAP carcinomas indicates that the immune system is able to recognise tumour antigens during cancer development. In addition, the efficacy of commonly used chemo/radiotherapies for MAP patients should be investigated. For example, for Lynch patients, it has been well documented that chemotherapy (5-FU) anti EGFR is less effective on CRC.^{31 32}

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Pathogenicity of mutations

Although the two most common missense mutations have been thoroughly analysed, the pathogenicity of many of the prominent missense mutations have not yet been determined, and the reporting of discovered mutations in online databases is important. Variants in the *MUTYH* gene might have an effect on different levels of *MUTYH* functioning. Analyses should assess the effect of *MUTYH* variants on the stability and expression of the protein, the recognition and binding of the 8-oxoG adenine mismatch, the rate and consequences of base removal in a vivo setting. Mutations can be reported in the LOVD database: http://chromium.liacs.nl/LOVD2/colon_cancer/home.php.³³

Lung cancer and other extracolonic manifestations

Given that oxidative stress is also commonly found in tissues that are exposed to cigarette smoke and assuming that at least some of the MAP patients smoke or have smoked, a higher prevalence of lung cancer is expected; however, this is not observed (**chapter 2.3**). In addition, no biallelic *MUTYH* mutations were found in a cohort of lung cancer patients.³⁴ Notably, a Japanese study found that the *MUTYH* polymorphism Q338H (previously Q324H) in the biallelic state had a significant association with lung cancer risk in smokers (adjusted OR 3.82, P= 0.022).³⁵ It is possible that future research might clarify the role of *MUTYH* polymorphisms in patients with lung cancers. In this regard, the contribution of the underlying *MUTYH* defect in reported extracolonic cancers has not been elucidated. Histological characteristics of these cancers, such as lymphocytic infiltration, and molecular research into somatic mutations, e.g., in *APC* and *KRAS*, can help in determining the role of *MUTYH* in the development of these lesions.

Where are all the MAP patients (p.G396D homozygotes)?

Based on the Hardy-Weinberg (HW) equilibrium and the reported p.G396D and p.Y179C heterozygotes in the population (1% and 0.4%, respectively), six times more p.G396D than p.Y179C homozygotes would be expected (HW equilibrium: 1/40,000 p.G396D homozygotes, 1/250,000 p.Y179C homozygotes and 1/100,000 p.Y179C/p.G396D heterozygotes). However, in CRC-population-based cohorts, only 3 times more p.G396D than p.Y179C homozygotes are found,³⁶ and in polyposis-based cohorts, 3-4 times more p.Y179C than p.G396D homozygotes are found.

An explanation for this might be that the p.G396D homozygotes may have a very mild phenotype with no or only a few polyps and therefore, they receive medical attention as often as do the p.Y179C homozygotes. Indeed, we did find that p.G396D homozygotes had fewer polyps than the p.Y179C homozygotes (**chapter 2.4**), but so far, p.G396D homozygotes have not been identified in patient cohorts with few (i.e. less than 10) polyps and no CRC or in healthy controls (**chapter 1.2**).

Heterozygous MUTYH mutations

The relatively high number of *MUTYH* heterozygotes in the population (1,5%)¹³ illustrates a possible evolutionary advantage of single *MUTYH* mutations as has been shown for carriers of sickle cell disease and hemochromatosis.³⁷⁻⁴⁰ It could be hypothesised that cells of *MUTYH* heterozygotes are better protected against infections. During oxidative stress, oxidative DNA repair could be more compromised. Subsequently, infected cells might undergo apoptosis sooner and prohibit the spread of viruses or

bacteria. Moreover, there is little evolutionary pressure for the extinction of *MUTYH* mutations in the population because CRC in MAP manifests in the majority of patients after reproduction has taken place.

Population screening

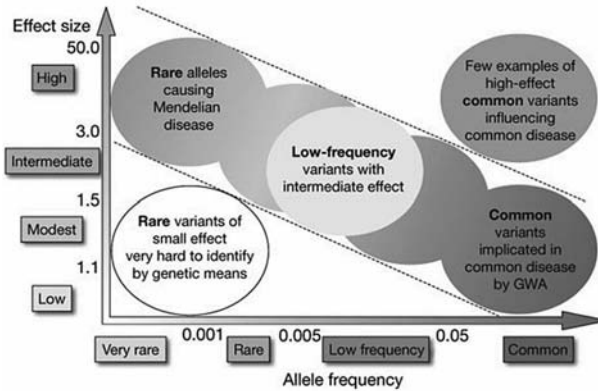
Compelling evidence shows that CRC screening programmes can save lives at a cost that is similar to or less than that of the existing breast cancer screening programmes.⁴¹ Screening strategies for CRC in the general population include immunochemical and guaiac-based faecal occult blood tests (FOBT).⁴² An emerging new promising method for the early detection of colorectal neoplasia is faecal DNA testing.⁴³ An additional application of this technique might be the detection of heritable polyposis and CRC syndromes by including screening for mutations in *APC*, *MUTYH*, mismatch repair and other high and low penetrance CRC-associated genes. Subsequently, high risk CRC patients can be detected, and the incidence of colon and other cancers can be reduced in index patients and their family members through surveillance. In addition to the high cost of this technique, ethical concerns have been raised for detecting a heritable form of CRC through population screening.

Hidden mutations in CRC-predisposition genes and candidates genes

Finally, the search for unknown mutations in additional and already identified polyposis/CRC-associated genes continues. For example, a role for multiple, rare, non-synonymous *APC* variants that promote the development of colorectal adenomas has been proposed based on a high incidence of these variants in *APC*- and *MUTYH*-negative patients when compared to FAP or MAP patients (17% versus 10%, $p=0.01$)⁴⁴. In addition, we anticipate the identification of further mosaic cases in apparently sporadic patients without an identified germline mutation based on advanced mutation detection methods and by analysing tumour DNA in addition to lymphocyte DNA. Additional mutations in non-scanned parts of the *APC* and *MUTYH* genes might be discovered. Moreover, a reduced mRNA expression can be caused by cryptic germline mutations and mutations in associated regulatory regions that are not detectable by standard methods of mutation analysis.

In addition to genes with an almost complete penetrance that lead to a clear Mendelian inheritance, low penetrance genes, when combined, might provide a partial explanation for the remaining CRC/polyposis families (Figure 1).

Figure 1: Feasibility of identifying genetic variants by risk allele frequency and strength of genetic effect (odds ratio). From: TA Manolio et al. Nature 461, 747-753 (2009)



In CRC studies, several low penetrance alleles and chromosome locations have been discovered, including the 8q24 locus, 10p14, 8q23, 11q23, *BMP4*, *CDH1*, *RHPN2*, and 20p12.3⁴⁵. These loci might be involved in the unresolved familial CRC cases. Indeed, a clustering of low-risk genetic factors was recently shown as a partial explanation of the excess risk in these families.⁴⁶

Next generation sequencing includes exome or whole genome sequencing and will enable the detection of rare variants, which are not easily detected with SNP-array studies. The variants identified might also reveal several biological pathways and generate hypotheses for further research. The currently ongoing 1000 genomes project (<http://www.1000genomes.org>) aimed at sequencing 1000 individuals will further add to the publicly available catalogues of genetic variants.

Finally, another ongoing project is the Expression Project for Oncology (expO) of the International Genomics Consortium (IGC). This project involves gene expression analyses on a clinically annotated collection of de-identified tumour samples of more than a thousand frozen cancer specimens (<http://cancergenome.nih.gov/>). This open and free publicly-available project will likely accelerate genetic discoveries. Indeed, analysis of the distinct tumour profiles and the detection of specific G>T transversions in the *APC* gene led to the discovery of the *MUTYH* gene in 2002.

In conclusion, this thesis describes several new insights into polyposis, particularly *MUTYH*-associated polyposis. This recessive inheritable disease represents

approximately 10-20% of all polyposis patients with a wide variety in the phenotype and significant genotype-phenotype correlations. Organ systems outside the gastrointestinal tract seem to be relatively spared in MAP patients. Colorectal carcinoma in MAP patients have some specific molecular and histological features, which are similar to MSI-high and Lynch-associated CRCs, such as a preferential proximal location, mucinous histotype, and increased presence of TILs. These TILs could be associated with higher activated immune response, which leads to reduced tumour growth and reduced metastasis. Indeed, better survival in MAP carcinomas was found in a European MAP cohort. Furthermore, it was shown that *APC* deletion and *APC* mosaicism represent a substantial number of the discovered *APC* mutations. The remaining *APC* and *MUTYH* negative polyposis patients might have the following: mutations in high penetrance CRC associated genes that are yet to be discovered, mutations in non-scanned parts of the *MUTYH* or *APC* genes, or a combination of low penetrance alleles (Figure 1).

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