Universiteit
Leiden
The Netherlands

# HNPCC, molecular and clinical dilemmas

Wagner, A.

**Citation**
Wagner, A. (2005, April 27). *HNPCC, molecular and clinical dilemmas*. Macula, Boskoop. Retrieved from https://hdl.handle.net/1887/2719

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# Chapter 1.

# Introduction

## 1.1. Hereditary Non Polyposis Colorectal Cancer syndrome (HNPCC)

Hereditary Non-Polyposis Colorectal Cancer (HNPCC) or Lynch syndrome (MIM114500) is the most common genetic susceptibility for colorectal cancer. It accounts for 3-5% of all colorectal cancers in the Western world[73]. The HNPCC phenotype also includes other cancers, predominantly of the endometrium, but also ovarian, gastric, small bowel, biliary tract, urinary tract, skin and brain cancer may occur[5, 54, 158, 302, 303]. HNPCC is caused by germline mutations in the mismatch repair (MMR) genes *MSH2*, *MLH1*, *MSH6*, and *PMS2* (Table 1)[10, 26, 58, 152, 206, 218, 225]. The inheritance pattern is autosomal dominant, as shown by the 50% risk of children of an MMR gene mutation carrier of inheriting this predisposition to cancer. The Amsterdam criteria (Table 2) have been established by the International Collaborative Group on HNPCC (ICG-HNPCC) to allow clinical selection of HNPCC families. However, not all families fulfilling these criteria are bona fide HNPCC families. Viceversa, MMR gene mutations are also found in Amsterdam criteria negative families. Since loss of mismatch repair function causes microsatellite instability (MSI), a type of genetic instability in repetitive DNA sequences in the majority of HNPCC related cancers, this and aberrant immunohistochemical staining of MMR proteins are additional tools to identify HNPCC families on tumour material. As in the majority of cancer predisposition syndromes, (presymptomatic) diagnosis of HNPCC is of major importance for appropriate counselling, clinical surveillance and cancer prevention.

## 1.2. History

The first HNPCC family was reported in 1913 by A.S. Warthin (Figure 1)[324]. He described the family of his seamstress, known as Family G. Lynch et al. (Figure 1) described two additional HNPCC families and revisited family G in 1966 and 1971 respectively[185, 186].

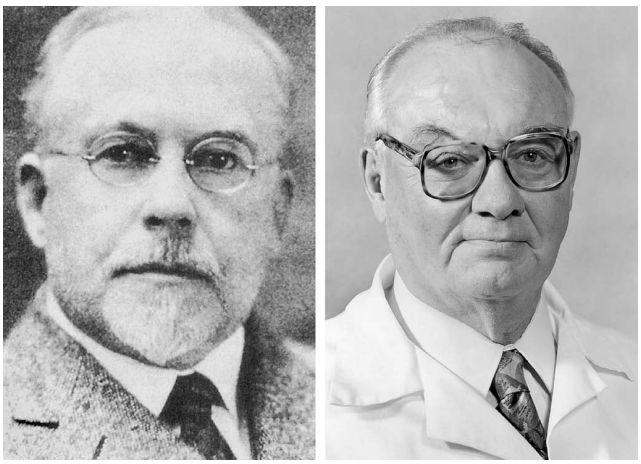

**Figure 1.**
A.S. Warthin (1866-1931), H.T. Lynch

**Table 1.**
Proven and "candidate" HNPCC genes.

| Gene | Bacterial Homologue | Chromosome position | cDNA(kb) | Number of exons | HNPCC associated |
|---|---|---|---|---|---|
| *MSH2* | MutS | 2p21 | 2,8 | 16 | yes |
| *MLH1* | MutL | 3p21-23 | 2,3 | 19 | yes |
| *MSH6* | MutS | 2p21 | 4,2 | 10 | yes |
| *PMS2* | MutL | 7p22 | 2,6 | 15 | yes |
| *PMS1* | MutL | 2q31-33 | 2.8 | 12 | possibly |
| *MLH3* | MutL | 14q24.3 | 4,7 | 12 | probably not |
| *MSH3* | MutS | 5q11-12 | 3,4 | 24 | probably not |
| *Exo1* | | 1q42-43 | 3 | 14 | probably not |
| *TGFβR2* | | 3p22 | 1,7 | 7 | probably not |

(www.expasy.org)

**Table 2.**
The Amsterdam criteria for the clinical diagnosis of HNPCC families[299,301].

| | |
|---|---|
| **Amsterdam criteria:** | 1: At least three relatives with colorectal cancer |
| | 2: One should be first-degree relative of the other two |
| | 3: At least two successive generations should be affected |
| | 4: At least one should be diagnosed before age 50 |
| | 5: Familial Adenomatous Polyposis should be excluded |
| | 6: Tumours should be verified by pathological examination |
| **Revised Amsterdam criteria:** | 1: At least three relatives with an HNPCC-associated cancer (colorectal, endometrial, small bowel, ureter or renal pelvis cancer) |
| | 2: One should be first-degree relative of the other two |
| | 3: At least two successive generations should be affected |
| | 4: At least one should be diagnosed before age 50 |
| | 5: Familial Adenomatous Polyposis should be excluded |
| | 6: Tumours should be verified by pathological examination |

Lynch recognised the autosomal dominant pattern of inheritance, and delineated HNPCC or Lynch syndrome. In 1986, autosomal dominant inheritance of colorectal cancer was also proven using segregation analysis in a cohort of 11 families[16].
International clinical criteria for HNPCC, the Amsterdam criteria, were formulated by the ICG-HNPCC in 1991 and subsequently updated in 1999 (Table 2)[299, 301].
In 1993 germline mutations in the MMR gene *MSH2* were found to be responsible for HNPCC (Table 1), followed by mutations in *MLH1*, *MSH6*, and *PMS2*[10, 26, 58, 152, 206, 218, 225].
The Bethesda guidelines to select HNPCC and HNPCC-like families for mutation analysis using MSI as a pre-screening tool were formulated in 1997 and updated in 2004 (Table 3)[248, 296]. International criteria for the diagnosis of MSI in colorectal cancer were formulated in 1998 (Table 4)[24]. More recently, immunohistochemical (IHC) analysis of the MSH2, MLH1, MSH6 and PMS2 protein in tumour sections has been added as an additional tool to direct mutation analysis[42, 43, 100, 160, 284]. Guidelines for screening of HNPCC risk carriers were proposed, and were proven to decrease overall mortality considerably[27, 117, 118, 238]. Since 1992, the Dutch Foundation for the Detection of Hereditary Tumours (Stichting Opsporing Erfelijke Tumoren/STOET) has been registering Dutch HNPCC family members and has played an important national and international role in evaluating the efficacy of cancer screening in HNPCC risk carriers. In 2001, the American Gastroenterological Association formulated recommendations on hereditary colorectal cancer and genetic testing[73]. Also, the Working Group on Oncogenetics of the Dutch Association of Clinical Genetics (Vereniging Klinische Genetica Nederland/VKGN) formulated guidelines for genetic testing, counselling and surveillance of HNPCC and HNPCC-like families[201].

### 1.3. The HNPCC genes and their protein products

As mentioned above, four MMR genes are known to date to cause HNPCC: *MSH2* and *MLH1* are responsible for the majority of the classical cases, whereas mutations in *MSH6* and *PMS2* account for more atypical kindreds (Table 1)(Figure 3).
*MSH2*. The *MSH2* gene was the first human HNPCC gene to be cloned[58, 152]. It resides on chromosome 2p21 and is the human homologue of the bacterial mismatch repair gene MutS. The *MSH2* gene contains 16 exons, with a total cDNA length of 2.8kb. In 2000, the crystal structure of the MutS protein was elucidated[149, 220], providing novel insights in the MMR function of the MutS and MSH2 protein. MutS proteins form a dimer with the general shape of two "opposing commas" or "praying hands" (Figure 2).

**Table 3.**
The Bethesda guidelines to select for MSI testing of colorectal tumours[248,296].

| | |
|---|---|
| **Bethesda guidelines:** | 1: Individuals with cancer in families that meet the Amsterdam criteria |
| | 2: Individuals with two HNPCC-related cancers, including synchronous and metachronous colorectal cancers or associated extracolonic cancers* |
| | 3: Individuals with colorectal cancer and a first-degree relative with colorectal cancer and/or HNPCC-related extracolonic cancer and/or a colorectal adenoma; one of the cancers diagnosed at age <45y, and the adenoma diagnosed at age <40y |
| | 4: Individuals with colorectal cancer or endometrial cancer diagnosed at age <45y |
| | 5: Individuals with right-sided colorectal cancer with an undifferentiated pattern (solid/scribiform**) on histopathology diagnosed at age <45y |
| | 6: Individuals with signet-ring-cell-type colorectal cancer diagnosed at age <45y*** |
| | 7: Individuals with adenomas diagnosed at age <40y |
| | * Endometrial, ovarian, gastric, hepatobiliary, or small bowel cancer, or transitional cell cancer of the renal pelvis or ureter.<br>** Solid/scribiform defined as poorly differentated or undifferentiated carcinoma composed of irregular, solid sheets of large eosinophilic cells and containing small gland like spaces.<br>*** Composed of >50% signet ring cells |
| **Revised Bethesda guidelines:** | 1: Colorectal cancer diagnosed in a patient who is less than 50y of age |
| | 2: Presence of synchronous, metachronous colorectal or other HNPCC associated tumours* regardless of age |
| | 3: Colorectal cancer with the MSI-H histology** diagnosed in a patient who is less than 60y of age |
| | 4: Colorectal cancer diagnosed in one or more first degree relatives with an HNPCC related tumour, with one of the cancers being diagnosed under age 50y |
| | 5: Colorectal cancer diagnosed in two or more first- or second degree relatives with HNPCC related tumour, regardless of age |
| | * Endometrial, stomach, ovarian, pancreas, ureter, renal pelvis, biliairy tract, brain (usually glioblastoma), smal bowel tumour and sebacous gland adenomas and keratoacanthomas<br>** Presence of tumour infiltrating lymphocytes, Crohn's-like lymphocytic reaction, mucinous/signet-ring differentiation or medullary growth pattern |

**Table 4.**
Selected markers for MSI analysis in colorectal cancer and criteria for the interpretation of MSI[24].

| **Reference panel** | **Alternative microsatellite markers** | | | |
|---|---|---|---|---|
| BAT25 | BAT40 | D18S58 | D13S175 | D17S787 |
| BAT26 | BAT34C4 | D18S61 | D17S588 | D7S519 |
| D5S346 | TGFßR2 | D18S64 | D5S107 | D20S100 |
| D2S123 | ACTC(635/636) | D3S1029 | D8S87 | |
| D17S250 | D18S55 | D10S197 | D13S153 | |
| **Interpretation** | **5 markers analysed** | **>5 markers analysed** | | |
| Number of markers displaying instability | >1 | ≥30% | MSI-High (MSI-H) | |
| | 1 | <30% | MSI-Low (MSI-L) | |
| | 0 | 0 | MSI-Stable (MSS) | |

Each MutS subunit contains 5 functional domains: domain I and IV are involved in DNA binding, domain V contains ATP-ase activity and links both MutS subunits, domain II and III connect the DNA binding and ATP binding domains of the MutS protein.

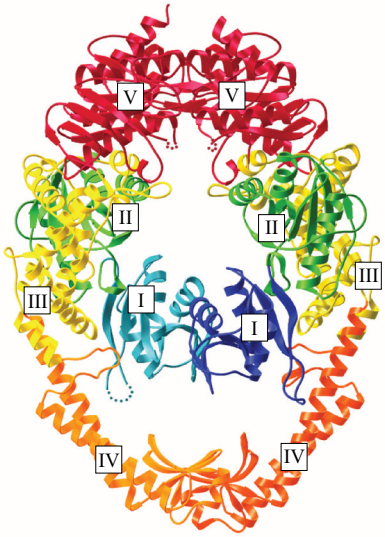


**Figure 2.**
The crystal structure of the MutS dimer:
Two MutS proteins are represented by ribbon diagrams in blue (domains I), green (domains II), yellow (domains III), orange (domains IV) and red (domains V). Domain I and IV are involved in DNA binding, domain V contains ATP-ase activity and links both MutS subunits, domain II and III connect the DNA binding and ATP binding domains of the MutS protein. In mismatch repair the DNA helix is positioned through the gap between domains I and IV.

Analogue to MutS, *MSH2* encompasses DNA binding domains, a domain containing ATP-ase activity, and a domain for protein-protein interaction with two other MutS-related proteins, MSH6 and MSH3. Accordingly, the MSH2 protein forms heterodimeric complexes of MutS-related proteins, MSH2-MSH6 (hMutS-$\alpha$) and MSH2-MSH3 (hMutS-$\beta$), to bind DNA at distinct but overlapping spectra of mismatches[7, 110]

Apart from its role in the repair of somatic mutations, the *MSH2* gene is likely to encompass additional functions. Martin et al.[195] described a significantly increased frequency of chromosomal aberrations in sperm cells derived from *MSH2* mutation carriers, suggesting a role for mismatch repair in meiosis. Possibly, this is due to interactions of *MSH2* with repair pathways involved in chromosomal recombination. Accordingly, Villemure et al.[308] reported compromised homologous repair in *MSH2* deficient tumour cell lines. Also, in *Msh2*-mutant mouse embryonic stem cells homologous recombination between non-isogenic DNA strands is highly enhanced[281].

*MLH1*. The *MLH1* gene was recognised to cause HNPCC by Bronner et al.[26] and Papadopoulos et al. in 1994[225]. It is the human homologue of the bacterial MMR gene *MutL* and resides on chromosome 3p21-23. This gene contains 19 exons, with a total cDNA length of 2.3 kb[88]. The aminoacid sequence of MLH1 encompasses a DNA binding domain, an ATP-ase activity domain and domains for protein-protein interaction. During mismatch repair, MLH1 forms heterodimers with other MutL-related proteins, PMS2, MLH3, and

PMS1[156, 161, 223]. The binding of a hMutS heterodimer to a mismatch triggers ATP-dependent steps that allow interactions with the hMutL heterodimers and completion of the repair process.

As for *MSH2*, the *MLH1* gene is likely to play additional roles in meiosis. In yeast, a complex of Mlh1 and Mlh3 is involved in meiotic recombination, possibly by stabilising the Holliday junctions[17].

*MSH6*. In 1995 Palombo et al.[222] and Drummond et al.[53] described a complex that binds GT-mismatches. This complex appeared to consist of the MSH2 protein and an unknown 160 kDa protein, they called GTBP (GT binding protein). One year later, both the group of Miyaki[206] and Akiyama[10] described germline mutations in the GTBP gene, now known as the *MSH6* gene, in HNPCC-like families. The *MSH6* gene maps close to *MSH2* on chromosome 2p21, and it encodes for a MutS-related protein homologous to MSH2. It is likely that *MSH2* and *MSH6* are the result of an ancient duplication event of the MutS gene of higher eukaryotes. The *MSH6* gene encompasses 10 exons, with a total cDNA length of 4.2 kb. As for *MSH2*, it includes 2 DNA-binding domains, ATP/GTP-binding sites, and a PWWP-domain for protein-protein interaction. The MSH6 protein forms heterodimers with MSH2 that specifically recognise GT mismatches.

*PMS2*. *PMS2* is located on chromosome 7p22 and contains 15 exons for a total cDNA length of 2.6 kb. Its protein product is a MutL homologue and forms a heterodimer with MLH1 during the mismatch repair process. Apart from its protein-protein interaction motifs, *PMS2* harbours sites for DNA-binding and an ATP-ase activity domain. To date, only few *PMS2* mutations have been reported in HNPCC patients. Nicolaides et al.[217, 218] described two distinct germline mutations of the *PMS2* gene in two unrelated HNPCC families. *PMS2* mutations were subsequently also found in HNPCC kindreds with central nervous system tumours, a condition also known as Turcot syndrome[45, 86, 205]. Two compound missense mutations of *PMS2* were detected in a Turcot patient without a family history of cancer, whereas a homozygous *PMS2* mutation was found in a family with brain tumours and café au lait spots, thus suggesting a recessive mode of inheritance[45, 46]. Several studies of in total more than 200 HNPCC and HNPCC-like families did not reveal any *PMS2* mutation[165, 169, 306, 319]. These data suggest that *PMS2* mutations are responsible for a small subset of HNPCC or HNPCC-like families, and are possibly preferentially associated with the Turcot variant of HNPCC. However, mutation analysis of *PMS2* has also been hampered by the presence of pseudogenes in the genome, so the current figures of the contribution of this gene to HNPCC may be an underestimate[46].

### 1.4. Candidate genes

A number of additional genes have been indicated as putative HNPCC genes. These include other members of the mismatch repair machinery like *PMS1*, *MSH3*, and *MLH3*, but also genes known to play important roles in cellular functions other than MMR like *TGFβRII*, the *SMAD* genes and *EXOI* (Table 1).

Nicolaides et al.[217, 218] described a germline nonsense mutation of the *PMS1* gene resulting in exon-skipping in a HNPCC family. No other *PMS1* mutations have been reported since. The *PMS1* gene maps to chromosome 2q31-33 and it encodes for a MutL-like protein that forms a heterodimer with MLH1 during mismatch repair. *PMS1* contains 12 exons for a total cDNA length of 2,8 kb.

The *MSH3* gene maps to chromosome 5q11-12 and encodes for a MutS –like protein. *MSH3* encompasses 24 exons and has a total cDNA length of 3.4 kb. MSH3 forms a heterodimer with MSH2 that recognises specific subsets of mismatches in DNA[325]. To date, *MSH3* mutations have only been found in somatic cells[107, 345].

The *MLH3* gene on chromosome 14q24.3 was identified and characterised by Lipkin et al. (2000).[161]. The gene contains 12 exons, and has a total cDNA length of 4.7 kb. As for PMS2 and PMS1, MLH3 complexes with MLH1 to form the third hMutL heterodimer involved in mismatch repair. Wu et al.[341] described nine missense mutations and one frameshift mutation in a cohort composed of 288 HNPCC-like families. Three of the index patients carrying the putative *MLH3* missense mutation were shown to carry an additional *MSH6* mutation and no *MLH3* mutations were detected in 39 Amsterdam criteria positive HNPCC families. Likewise, no pathogenic *MLH3* germline mutations were detected in three subsequent studies on 142 patients from families with familial colorectal cancer that tested negative for mutations in *MSH2* or *MLH1*[104, 163, 176]. Liu et al.[166] described a family with both an *MLH3* and *MSH2* missense mutations, both segregating with colorectal cancer in the corresponding kindred. Altogether, these findings suggest that germline mutations of *MLH3* are not likely to contribute to the development of HNPCC, or may at best represent cancer risks modifiers among carriers of mutations of the major MMR genes. Functional redundancy among hMutS (MSH2/MSH3 and MSH2/MSH6) and hMutL (MLH1/PMS2, MLH1/MLH3, and MLH1/PMS1) heterodimers may explain the differential role of *MSH2* and *MLH1* as main disease-causing genes in HNPCC when compared with other MMR genes.

The *Exonuclease 1 (EXO1)* gene encodes for an MSH2-interacting protein presumably involved in both mismatch repair and DNA recombination[258, 290]. Wu et al.[342] detected a splice site mutation in one out of 33 HNPCC families in addition to 13 missense substitutions of uncertain pathological significance in a cohort of 225 atypical HNPCC

families. About half of the tumours available displayed an MSI-High phenotype. Sun et al.[275] showed that two missense mutations of *EXO1*, E109K and L410R, interfere with the exonuclease activity, and three others, P640S, G759E and P770L, affect MSH2-binding. Jaghmohan-Changur et al.[116] tested a large series of European CRC patients and population controls to clarify whether EXO1 variants may indeed predispose to familial CRC. Several variants observed in patients were also observed in controls with similar frequencies, including the truncating variant described by Wu et al.[342]. Thus, no conclusive evidence was found for a role of *EXO1* as a colorectal cancer susceptibility gene.

Molecular studies in sporadic colorectal cancer have indicated that two distinct signal transduction pathways, TGF-β and Wnt signalling pathways, play rate-limiting roles during tumour initiation and progression. Therefore, specific members of these signalling cascades may be regarded as potential candidate genes for hereditary CRC syndromes. In 1998 Lu et al.[177] described a putative germline mutation in the *TGFβRII* gene encoding for the TGF-β type 2 receptor in a kindred with familial late-onset colorectal cancer. Mizuguchi et al.[208] showed evidence that this mutation is likely to be a rare polymorphism. Also, no germline mutations in this gene were detected in a cohort of 67 patients with colorectal cancer below age 55 years, and in a series of HNPCC families[305]. Hence, *TGFβRII* germline mutations seem not to be associated with HNPCC. Also, no germline mutations of three other TGFβ pathway genes, *SMAD2*, *SMAD3* and *SMAD4*, have been found in HNPCC families so far[249].

### 1.5. The HNPCC gene mutation spectra

A broad spectrum of germline mutations is characteristic of all HNPCC genes (mutation database on website: http://www.nfdht.nl). The majority of mutations in *MLH1* and *MSH6* are single nucleotide substitutions or small insertions and deletions[227, 315]. The pathogenicity of these mutations is not always straightforward. Nonsense mutations in *MLH1* as well as in other genes were shown to cause exon skipping, leading to several aberrant transcripts[273]. Also, missense mutations and polymorfisms, as well as intronic sequence variations, can be pathogenic by affecting splicing[167, 216]. Missense mutations can also influence the structure and/or function of the encoded protein. Several missense mutations were shown to affect protein-protein interaction of MSH2 with MSH3/MSH6, or the heterodimer function[83, 95]. Lipkin et al. described a *MLH1* missense mutation (D132H) causing susceptibility to MS-Stable colorectal cancer[164]. The establishment of the pathogenicity of a sequence variant still represents a main challenge as it often implies

analysis of large cohorts of affected and healthy individuals, cosegregation analysis of the alleged mutation with the disease phenotype within extensive, multi-generation pedigrees, and *in vitro* if not *in vivo* functional studies[55, 64, 216, 294].

As reported in chapter 2.1, we found that large genomic rearrangements significantly contribute to the HNPCC mutation spectrum[35, 232, 334]. Among the 4 MMR genes, *MSH2* has been shown particularly prone to genomic deletions and other genomic rearrangements[297, 334]. The *MSH2* locus on chr 2p21 contains a relatively high concentration of repetitive short interspersed elements (SINEs) like Alu repeats, which explain the high frequency of genomic rearrangements within this gene due to homologous but unequal recombination events[297, 334]. We detected a founder deletion in *MSH2*, responsible for a substantial part of the HNPCC in Mid-western American families[192, 315]. Several other founder-mutations in *MLH1* and *MSH2* have been described[33, 64, 65, 109, 210]. One of these (the Newfoundland exon 5 splice donor site mutation in *MSH2*) appeared to be a recurrent mutation also[49].

Green et al.[81] described a *MLH1* promotor mutation in a Newfoundland kindred. Germline mutations were also detected in the promotor region of *MSH2*[35, 262]. Notably, aberrant methylation of the *MLH1* promotor was found in normal tissue of patients with MSI-High tumours, indicative of a hereditary predisposition to aberrant promotor methylation[72, 80, 276]. *De novo* mutations of *MLH1*, *MSH2* and *MSH6* seem rare. Only one *de novo* mutation of *MSH2* has been described[143].

Biallelic germline mutations in the MMR genes have also been reported. Individuals homozygous or compound heterozygous for *MLH1*, *MSH2*, *MSH6* or *PMS2* mutations are at risk of childhood tumours, mainly haematological tumours, brain tumours and HNPCC related tumours[45, 46, 71, 200, 243, 320, 331]. We also diagnosed a boy being compound heterozygous for a frame shift and a missense mutation in *MLH1*. He developed a Wilms tumour and a glioblastoma at the age of 4 years. The majority of the MMR deficient patients have "café au lait spots", a main feature of neurofibromatosis type 1 (NF1). Also, other features of NF1 like neurofibromas or Lish noduli were occasionally reported. However, none of these cases fulfilled the clinical criteria for NF1. Mutational analysis of the *NF1* gene was performed in a child homozygous for a *MSH6* mutation[200], and was negative.

### 1.6. Molecular basis of tumour initiation and progression in HNPCC

HNPCC is caused by germline mutations in mismatch repair genes. The DNA mismatch repair process is best outlined for the bacteria Escherichia coli (E.coli). It repairs single base mispairs, small insertions and deletions caused by slippage of DNA-polymerases during DNA replication of repetitive stretches (mono-, dinucleotide, repeats

or other simple repetitive sequences). In E.coli, four proteins are essential for mismatch repair: MutS, MutL, MutH and MutU[209]. A MutS homodimer recognises and binds to the mismatch. It then forms a complex with a MutL homodimer, that (in the presence of ATP) juxtaposes the MutS and the MutH protein. MutH has endonuclease activity and recognises the newly replicated DNA because GATC sequences in this strand are transiently unmethylated. It binds the hemimethylated DNA at a GATC site and cleaves the unmethylated DNA strand thus introducing a single strand nick. Subsequently, UvrD helicase (MutU) unwinds the DNA thus allowing single strand exonucleases to make a gap of approximately 2 kb, from the nick past the mismatch. While the single strand binding protein stabilises the remaining DNA strand, DNA polymerase III fills in the gap. DNA ligase is required to link the repaired DNA to the pre-existing sequence[241]. Eukaryotic mismatch repair contains several mismatch repair pathways in which several MutS and MutL homologues are involved (Figure 3): MSH2, MSH3, and MSH6 are human homologues of MutS. MLH1, PMS1, PMS2, and MLH3 are homologues of MutL. No human MutH homologues have been detected so far.

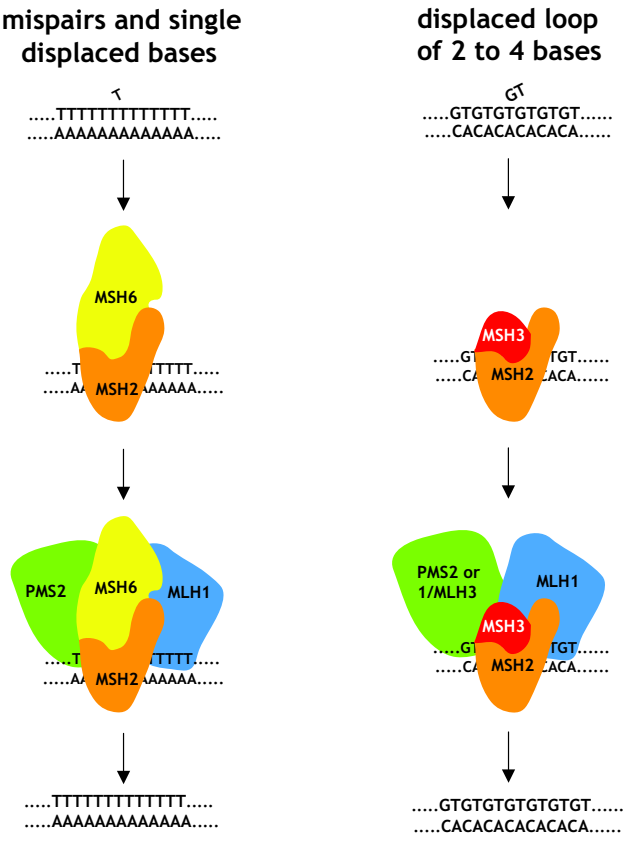


**Figure 3.**
Schematic representation of the eukaryotic mismatch repair (MMR) pathways:
Single mispairs and small base insertion/ deletions are preferentially recognised by MSH2/MSH6 heterodimers, whereas MSH2/MSH3 heterodimers recognise larger insertions/deletions.
After binding of the mismatch by MSH2/MSH6 or MSH2/MSH3, heterodimers of MLH1/PMS2, MLH1/PMS1 or MLH1/MLH3 are recruited.
Repair of the mismatch is now initiated.

A heterodimer of MSH2 and MSH6 (hMutS-α) recognises single base substitutions and small insertions/deletions-loops (IDL's; one to four nucleotides). After binding to mispaired DNA, this complex recruits a heterodimer of MLH1 and PMS2 (hMutL-α) and triggers mismatch repair. In addition to the mismatches recognised by hMutS-α, a heterodimer of MSH2 and MSH3 (hMutS-β) recognises larger IDL's (up to 12 base pairs). hMutS-β recruits hMutL-α or possibly a heterodimer of MLH1 and PMS1 or MLH3, again initiating mismatch repair[19, 121, 126, 138, 139, 193].

Apart from the repair of DNA-replication errors, the mismatch repair system is also involved in the repair of physical DNA damage, and in recombination and meiosis[17, 127, 129, 162, 255], either directly or through cross-talk with other repair systems. Yi Wang et al.[322] described a large (>2MDaltons) protein complex named BASC (BRCA1-associated genome complex), encompassing MSH2, MSH6, MLH1, PMS2, together with many other proteins involved in genomic recombination and repair (BRCA1, ATM, BLM, RAD50-MRE11-NBS1).

*How does MMR deficiency contribute to tumor initiation and progression?*

Cancer is a genetic disease involving mutations in multiple genes. The current concept of carcinogenesis is based on the assumption that a single cell may acquire a mutation that provides a selective growth advantage. From within the resulting clonal population a cell may acquire a second mutation, providing additional growth advantage, thus allowing further expansion. Repeated cycles of mutation followed by clonal expansion lead to a fully developed malignant tumour. Mutations of two classes of genes, proto-oncogenes, tumour-suppressor genes drive carcinogenesis[312].

Proto-oncogenes are involved in regulating proliferation and differentiation of normal cells. Mutations of proto-oncogenes result in a 'gain of function', and are dominant at the cellular level. Altered forms of these genes may evade cellular control and deregulate cell growth. Contrary to oncogenes, the function of tumour suppressor genes is to constrain cell growth. From this point of view, DNA repair genes involved in the maintenance of genome integrity, can be classified as tumour suppressor genes. Mutations of tumour-suppressor genes result in a 'loss of function', and are recessive at the cellular level as represented in the Knudson model (Figure 4)[134, 135]. This model was formulated based on observations on the incidence and distribution of sporadic and inherited retinoblastoma. Biallelic mutations of *RB1* cause retinoblastoma, an eye tumour. In the inherited form of retinoblastoma, the first 'hit' (mutation) is already present in the germline. For tumour formation, a second somatic mutation is needed. In view of the number of retina cells and the spontaneous mutation rate, a somatic mutation in at least one *RB1* allele in one retina

cell is highly likely to occur. In sporadic retinoblastoma, two independent somatic mutations must occur in the same cell.

The Knudson model illustrates how inherited and somatic mutations contribute to carcinogenesis and provides a rationale for the main clinical features of individuals with a genetic predisposition to cancer when compared with sporadic patients, e.g. age of onset, tumour multiplicity and multi-organ distribution.

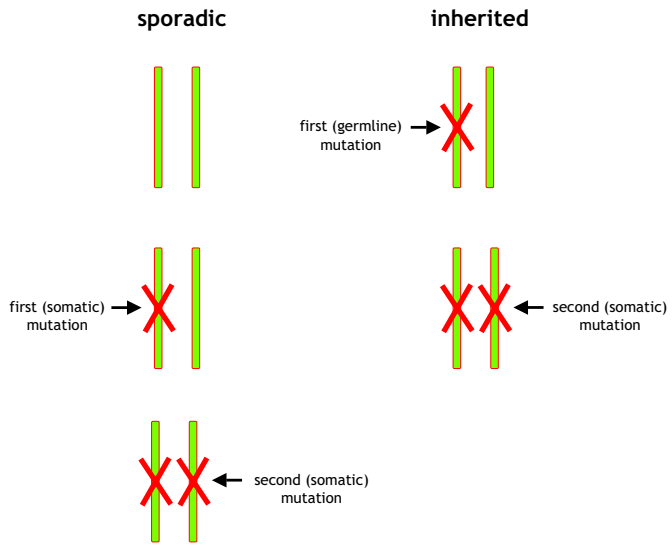


**Figure 4.**
Schematic representation of the Knudson model: The bars represent the alleles of a tumour suppressor gene, whereas the crosses symbolise mutations.
In sporadic tumours, all mutations are somatic, while in inherited tumours the first mutation is already present in the germline.

In addition to the inactivation of a tumour suppressor gene, 6-12 other regulatory genes must be activated or inactivated to allow progression towards malignancy[174]. Moreover, mutations at additional loci may be required to increase the chance of a second hit on a tumour suppressor gene. It has also been shown that haploinsufficiency, i.e. the gene dosage effect caused by a heterozygous mutation in a tumour suppressor gene may already trigger tumour initiation [60, 82].

Activation of oncogenes may result from chromosomal translocations, gene amplification or activating intragenic mutations. Inactivation of tumoursuppressor genes can be caused by point mutations, larger deletions or other genomic rearrangements, but also by epigenetic factors like methylation. Mutations can be acquired by replication errors, environmental agents, by normal reactive metabolites and, occasionally, by inheritance. The vast majority of the mutations that contribute to the development of cancer are somatic and are only present in the neoplastic cells of the patient. In contrast, germline mutations are present in all cells of the body including gametes, and thus may be passed to the next generation causing familial predisposition to cancer. Most cancer predisposing

genes are tumour-suppressor genes. In 2004, 291 genes involved in carcinogenesis have been reported. Ninety percent of these show somatic mutations whereas 20% are known to be mutated in the germline of hereditary cancer patients[70].

The MMR genes responsible for HNPCC when mutated in the germline, are classified as tumour suppressor genes. Loss of the wild type allele (loss of heterozygosity, LOH) has been observed in 44% of colorectal tumours from patients with a *MLH1* germline mutation[98]. Also, somatic mutations of *MLH1* occur[102]. LOH and somatic mutations of the wild type allele have been found less frequently in *MSH2*- than in *MLH1*-associated tumours[4, 98, 102].

Colorectal tumours progress through a series of clinical and histopathologic stages, ranging from normal epithelium to single crypt lesions (aberrant crypt foci) to small benign tumours (adenomatous polyps) and malignant cancer (carcinomas), the so-called adenoma-carcinoma sequence ("the Vogelgram", Figure 5). This stepwise progression results from a series of genetic changes that involve the activation of oncogenes and the inactivation of tumour suppressor genes[133, 310]. Current insights reveal that the single mutations among the adenoma-carcinoma sequence are indicative of the activation or inactivation of specific cellular regulatory pathways[312]. Signal transduction pathways involved in the development of colorectal cancer are Wnt/β-catenin, KRAS, TGF-β, and p53 pathways. A colorectal cell has to deregulate these signalling pathways to trigger adenoma formation and malignant transformation[40, 68, 78, 89, 151, 312]. The temporal order at which these mutations occur is also important: *APC* (triggering constitutive Wnt/β-catenin signalling) and *KRAS* mutations are generally involved in adenoma formation and growth, while mutations in the *p53* gene and in members of the TGF-β pathway are usually associated with malignant transformation.

Notably, although the general scheme of the adenoma-carcinoma sequence is common to hereditary and sporadic colorectal cancers, the somatic mutation profile of HNPCC-related tumours differs from other inherited and sporadic colorectal cancers at several points. In general, the vast majority of all colorectal cancers are characterised by aneuploidy and allelic losses (loss of heterozygosity, LOH), also referred to as chromosomal instability (CIN). Loss of mismatch repair function causes accumulation of DNA mismatches at an increased rate in coding and non-coding sequences, the so-called microsatellite instability (MIN/MSI). MIN tumours, both inherited and sporadic, are near-diploid. MSI is found in 12-18% of colorectal cancers[4, 114, 285] whereas HNPCC-related colorectal cancers show MSI in more than 90% of the cases[4, 67] As mentioned before, mutations of the MMR genes are present in the germline of HNPCC patients. In these cases, and in agreement with the Knudson hypothesis, the wild type allele is lost/inactivated by somatic mutations.

In contrast, more than 80% of sporadic MSI-High tumours are characterised by somatic hypermethylation of the *MLH1* promotor[103].

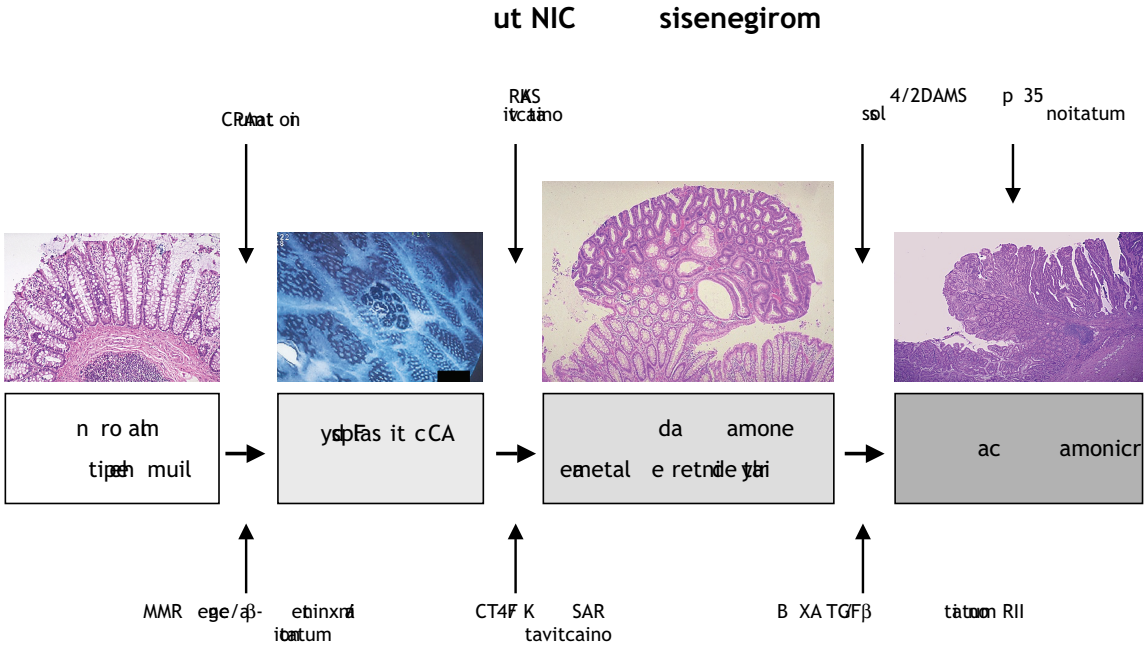


**Figure 5.**
The adenoma-carcinoma sequence:
The stepwise progression from normal epithelium to carcinoma results from a series of genetic changes that involve the activation of oncogenes and the inactivation of tumour suppressor genes. The different genes predominantly affected in CIN (upper part of the scheme) and MIN (lowers part of the scheme) tumours are depicted.

Members of the Wnt signal transduction pathway are mutated in both CIN and MIN tumours. The main tumour suppressing function of this signalling pathway is the regulation of β-catenin, a protein involved both in cell adhesion, when located at the cell membrane, and transcriptional regulation, when translocated to the nucleus (Figure 6). Several different extracellular Wnt ligands can bind and activate Frizzled and LRP6 receptors.

This ligand-receptor interaction prevents the formation of an intracellular multiprotein complex, the so-called 'destruction complex', composed of APC, β-catenin, GSK-3β, AXIN1 and AXIN2 (the latter also known as conductin). This complex earmarks β-catenin by Ser/Thr phosphorylation, thus triggering its ubiquitin-mediated proteolytic degradation[61]. In the absence of a functional APC protein, β-catenin accumulates in the cytoplasm and

eventually translocates to the nucleus where it acts as a transcriptional co-activator by associating with members of the T-cell factor/lymphoid enhancer (TCF/LEF) family. Downstream targets of the Wnt-signalling pathway are, among others, genes like *c-MYC* and *cyclinD1*, known to play a role in cell cycle regulation[61, 94, 283].

In the lower third of the colonic crypt, where intestinal stem and transient cells divide before migrating upwards and differentiate, Wnt/β-catenin signalling is responsible for stimulation of cell proliferation and inhibition of cell differentiation[132].

Loss of APC function constitutively activates signalling of β-catenin to the nucleus thus disturbing the equilibrium between proliferation and differentiation in the colonic crypt, and allowing clonal expansion, the first step in tumour formation. Specific activating β-catenin point mutations that render it resistant to proteolytic degradation are functionally equivalent to biallelic *APC* mutations[213].

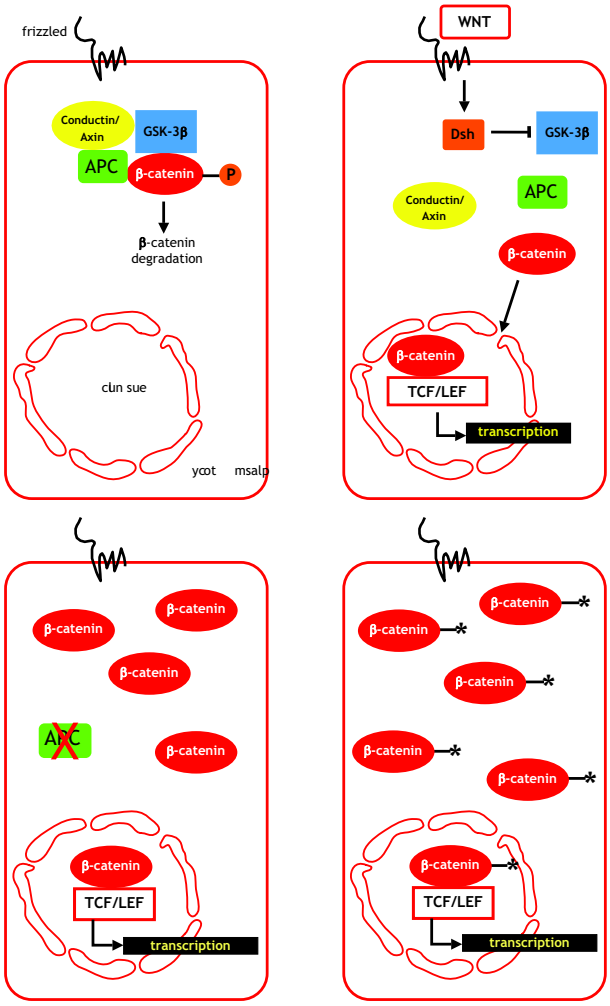


**Figure 6.**
A schematic representation of the Wnt-signalling pathway: The interaction of the WNT ligand with its receptor Frizzled prevents the formation of an intracellular multiprotein complex composed of APC, β-catenin, GSK-3β, AXIN1 and AXIN2 (the latter also known as conductin). This complex earmarks β-catenin by Ser/Thr phosphorylation, thus triggering its degradation. In the presence of a Wnt-signal β-catenin is not degradated and translocates to the nucleus, activating downstream targets.
In the absence of a functional APC protein or in the presence of a stabilising β-catenin mutation, β-catenin accumulates in the cytoplasm and eventually translocates to the nucleus where it acts as a transcriptional co-activator by associating with members of the T-cell factor/lymphoid enhancer (TCF/LEF) family.

Other members of the Wnt pathway such as AXIN1 have been found to be mutated in colorectal cancers with no *APC* mutations[170, 211, 270].Among CIN tumours, *APC* mutations are found in the vast majority of the cases, whereas gain-of-function β-catenin alterations are usually found in the minority of tumours with wild type *APC* [213,215, 267]. In MIN tumours, *APC* mutations are less common, though still detected at a considerably high incidence[142]. Somatic *APC* mutations were detected in 11 out of 19 (58%) MSI-High tumours from HNPCC patients, and were predominantly frameshifts within intragenic repeat sequences[105, 106]. In sporadic and HNPCC-related MSI-High colorectal tumours, mutations in β-catenin and in other components or downstream targets of Wnt-signalling occur more frequently[11, 170, 204, 260, 287].

Germline mutations of members of the Wnt signalling pathway underlie hereditary colorectal cancer syndromes. Germline *APC* mutations are responsible for Familial Adenomatosis Polyposis (FAP), a hereditary predisposition to the development of hundreds to thousands colorectal polyps (see section "Differential diagnosis"). *AXIN2* germline mutations were found in individuals with tooth agenesis and a predisposition to colorectal cancer[150].

In about 40% of both CIN and MIN colorectal cancers the *KRAS pathway* is activated by oncogenic *KRAS* mutations. KRAS belongs to the family of RAS proteins (KRAS, HRAS and MRAS) that are localised at the internal side of the cytoplasmatic membrane (Figure 7)[8, 151]. The activation in normal cells is triggered by the activation of growth factor receptors in the cell membrane. RAS is active when bound to GTP and inactive when bound to GDP.

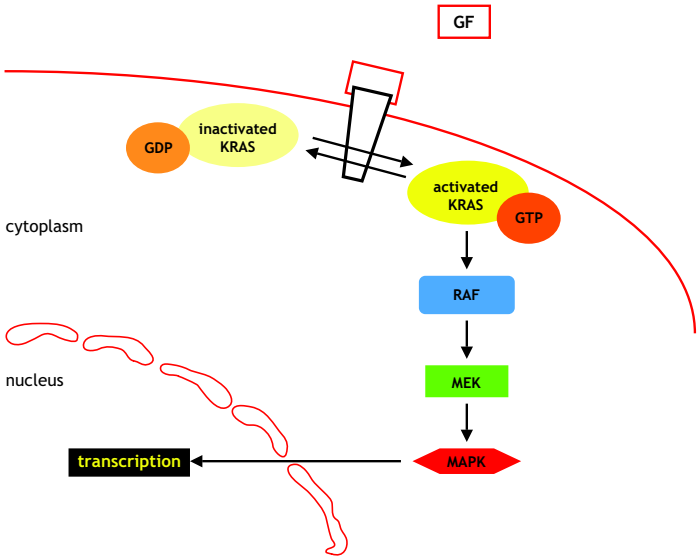


**Figure 7.** A schematic representation of the KRAS-signalling pathway: Activation of growth factor receptors in the cell membrane activates RAS. Through other members of the pathway like RAF, MEK, and MAPK, RAS signalling results in the transcription of target genes.

GTP is normally dephosforylated to GDP by GAPs (GTP-ase activating proteins). Through other members of the pathway like RAF, MEK, and MAPK, RAS signalling influences cell shape, motility and growth. Also, RAS activation up-regulates vascular endothelial growth factor (VEGF), important in vascularisation of tumours[89]. *KRAS* mutations in colorectal adenoma and carcinoma lock KRAS in the GTP-bound form by interfering with its interaction with GAP. Mutations in the RAF gene *BRAF* are frequently found in sporadic but not in HNPCC related MSI-High tumours[48, 197].

The TGFβ-receptor pathway (Figure 8) is deregulated in both CIN and MIN tumours by mutations in different genes, respectively *SMAD2/4* and *TGFβIIR*[22, 151, 252]. A polyA tract in *TGFβIIR* represents a mutational hotspot in MSI-High tumours. Normally TGFβ binds TGFβ type 2 receptor (directly or via TGFβ type 3 receptor), which complexes with TGFβ type 1 receptor thus triggering its phosphorylation. TGFβ type 1 receptor phosphorylates SMAD2 or SMAD3, which bind to SMAD4. The heterodimer moves to the nucleus and induces transcription of specific target genes. TGFβ signalling induces cell cycle arrest in G1, differentiation, and apoptosis in normal cells.

Also, members of the TGFβ-receptor-pathway have been found to be mutated in the germline in men. In 1998 Lu et al. [177] described a germline mutation in the *TGFβ type2 receptor* in a family with familial late onset colorectal cancer. Intriguingly, Mizugucho et al.[208] showed *TGFβIIR* germline mutations to cause Marfan syndrome, a connective tissue disease. They also make likely that the mutation described by Lu et al. is a rare polymorphism. Germline mutations of *SMAD4* cause juvenile polyposis (see section "Differential diagnosis").

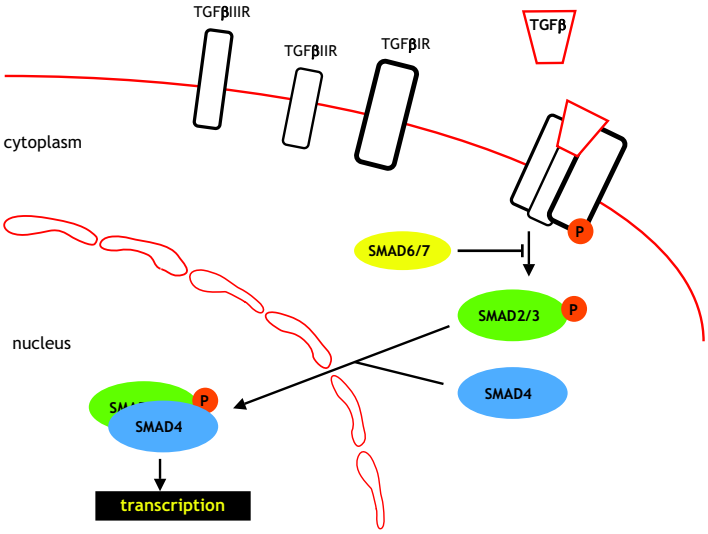


**Figure 8.**
A schematic representation of the TGFβ-signalling pathway: TGFβ binds TGFβ type 2 receptor (directly or via TGFβ type 3 receptor), which complexes with TGFβ type 1 receptor thus triggering its phosphorylation. TGFß type 1 receptor phosphorylates SMAD2 or SMAD3, which bind to SMAD4. The heterodimer moves to the nucleus and induces transcription of specific target genes.

The p53-pathway controls cellular responses to genotoxic damage, in particular apoptosis or cell cycle arrest to allow DNA repair (Figure 9). *TP53* mutations occur in 75% of CIN tumours, but not in MIN tumours. However, about 42% of MIN tumours carry mutations in the *BAX* gene[266], another member of the p53 pathway. P53 is normally activated by DNA damage (for example by hypoxia or ionizing radiation), aberrant growth signals (for example resulting from expression of the oncogenic *RAS* or *MYC*), chemotherapeutic drugs, UV-light, and/or protein-kinase inhibitors[311]. The p53 proteins form a tetramer able to bind DNA. The 5' side of the protein (the acid domain) can act as a transcription factor that can increase transcription of growth inhibiting genes like *BAX*, thus triggering apoptosis, or *p21*, causing G1 cell cycle arrest by inhibition of cyclin dependent kinases (CDK)[89, 151]. Also, p53 upregulates thrombospondin-1, an angiogenesis inhibitor, and is involved in the initiation of DNA repair[89].

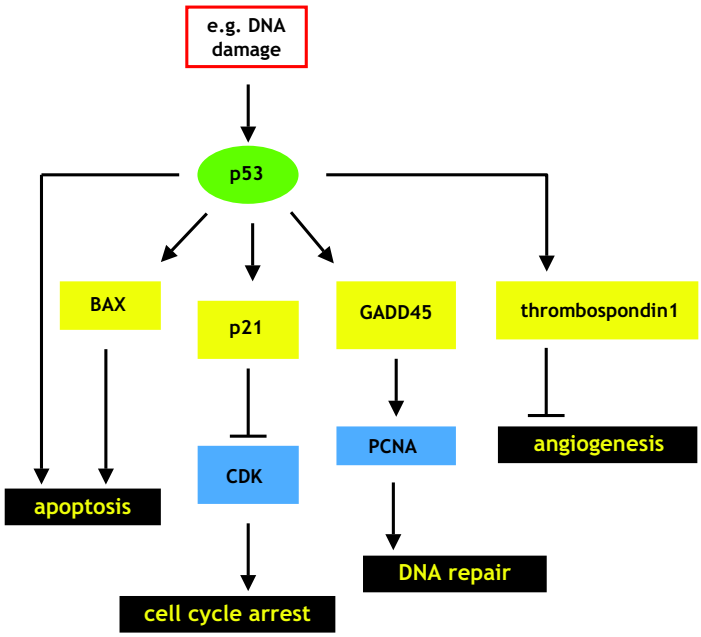


**Figure 9.**
A schematic representation of the p53-pathway:
P53 is normally activated by DNA damage, aberrant growth signals, chemotherapeutic drugs, UV-light, and/or protein-kinase inhibitors. The p53 proteins form a tetramer able to bind DNA. The 5' side of the protein (the acid domain) can act as a transcription factor that can increase transcription of growth inhibiting genes like *BAX*, thus triggering apoptosis, or *p21*, causing G1 cell cycle arrest by inhibition of cyclin dependent kinases (CDK). Also, p53 upregulates thrombospondin-1, an angiogenesis inhibitor, and is involved in the initiation of DNA repair.

*TP53* germline mutations cause the Li Fraumeni syndrome, a cancer syndrome with childhood cancer (sarcomas and brain tumours), early onset breast cancer in addition to other tumours, among which, more occasionally, also colorectal cancer[207, 271].

By overcoming or modulating the different regulatory pathways as described above, tumour cells acquire the necessary qualities for local invasion and metastasis: autocrine growth signals (e.g. Wnt signalling and RAS pathway), insensitivity to growth inhibition (e.g. TGFβ pathway), resistance to apoptosis (e.g. MMR, p53 pathway), limitless

replicative potential (p53 pathway), sustained angiogenesis (e.g. p53 and RAS pathway), and tissue invasion and metastasis (e.g.Wnt signalling). In HNPCC, deregulation of the described pathways occurs predominantly through mutation of pathway members like *TGFβR2* and *TCF4*, that have been shown to encompass intragenic repeat sequences prone to replication errors normally repaired by MMR[228]. This accumulation of mutations at increased rate is often referred to as the mutator phenotype. Although the role of the mutator phenotype in tumour progression is generally accepted, different models have been advocated for the mechanisms underlying tumor initiation due to loss of MMR function. In a normal cell, excessive mutation load triggers apoptosis. MMR deficient cells have been shown to be resistant to apoptosis and the latter is more likely to represent the true selective advantage that enables the initial clonal expansion of MMR deficient cells. Accordingly, MMR genes have been shown to play a central role in 'sensing' the presence of DNA mismatches and the activation of either repair or apoptotic machinery[59, 127, 228].

The vulnerability of specific genes involved in carcinogenesis for MMR deficiency due to the repetitive nature of their coding sequences may at least partly explain the difference in tumor phenotypes in *MSH2*, *MLH1* and *MSH6* mutation carriers, the so-called genotype-phenotype correlation (see section "Cancer risks"). MSH6 is mainly involved in the recognition of single nucleotide mismatches, wheraes MLH1 and MSH2 are also important in the repair of larger mispairs or loops. Loss of the different MMR proteins is therefore likely to result in the accumulation of mispairs in respectively mononucleotide repeats and both mono- and multiple-nucleotide repeats. Regulatory genes in different tissues may be more or less prone to deficiency of a particular MMR pathway, depending on the nature of their repetitive sequences[228]. The observation of different MSI patterns in HNPCC related colorectal and endometrial cancer supports this theory[145].

### 1.7. Cancer risks

Many studies have been performed on cancer risks in HNPCC, though only a few have addressed proven *MLH1, MSH2* and *MSH6* mutations carriers. A summary of the results of these studies is presented in Table 5[5, 54, 101, 158, 302, 303, 314].

Colorectal cancer risk at age 70 years in *MLH1* and *MSH2* mutation carriers ranges between 65-100% in male and 30-83% in female carriers[5, 54, 158, 302, 303]. In the Netherlands, the Working Group on Oncogenetics of the Dutch Association of Clinical Genetics (Vereniging Klinische Genetica Nederland/VKGN) advises a lifetime risk of colorectal cancer of 60-90% for counselling purposes in high risk families. The mean age at diagnosis of colorectal cancer is younger when compared to sporadic cases (41-52 years vs.67 years) (Table 5)[5, 54,

[158, 302, 303]. The risk at age 70 years of male and female *MSH6* mutation carriers is 69% and 30% respectively, being significantly lower in female *MSH6* compared to male *MSH6* mutation carriers, and in *MLH1* and *MSH2* mutation carriers of both sexes. The age at diagnosis in both male and female *MSH6* mutation carriers is an average 5-10 years delayed when compared with *MSH2* and *MLH1* mutation carriers of both sexes (Table 5 and 6)[101, 314]. Only one third of sporadic colorectal cancer develops proximal to the splenic flexure, whereas approximately two thirds of HNPCC-associated colorectal cancers do. Multiple colorectal cancers occur frequently in HNPCC. Synchronous colorectal cancers have been reported at a frequency of 7.4% and 6.7% in *MLH1*- and *MSH2*- associated colorectal cancer, vs. 2.4% in sporadic colorectal cancer. The annual rate of a second colorectal cancer is 2.1% and 1.7% in *MLH1* and *MSH2* mutation carriers respectively, vs. 0.33% in sporadic patients[159, 180, 198, 199, 302].

Endometrial cancer risk at age 70 years ranges between 25-61% in female *MLH1* and *MSH2* mutation carriers. The risk in *MSH2* carriers has been suggested to be somewhat higher than in *MLH1* carriers[5, 54, 158, 302, 303]. The VKGN advices a lifetime risk of 30-40% for counselling purposes in high risk families. Female *MSH6* mutation carriers are at higher risk of endometrial cancer when compared to *MSH2* and *MLH1* mutation carriers (71% at age 70), though age at diagnosis is delayed by an average 5-10 years (Table 5 and 6)[101, 233, 314].

Other extracolonic tumours. Tumours of the stomach, ovary, urinary tract, small bowel (including Papilla Vateri), biliary tract, skin and brain are part of the tumour spectrum of HNPCC. Associations with other tumours are also incidentally reported, like pancreatic cancer[184, 199], laryngeal cancer[183], fibrous histiocytoma[264], prostate cancer [269], and breast cancer[245]. Of note, no consensus exists whether breast cancer is part of the HNPCC tumour spectrum[44, 214, 245, 304].

The cumulative risk at age 70 years of all extracolonic tumours (except endometrial cancer) usually does not exceed 10% among *MSH2* and *MLH1* mutation carriers (Table 5)[5, 54, 302, 303]. Vasen et al.[303] and Lin et al.[158] described a higher risk of extracolonic cancers in *MSH2* compared to *MLH1* mutation carriers.The risk of *MSH6*- associated other extracolonic tumours are at present largely unknown. The frequency of the extracolonic tumours in HNPCC must be evaluated in relation to their incidence rates in the general population[186, 226, 259]. Also, environmental factors and other genetic factors may influence the incidence of extracolonic tumours in HNPCC. Preliminary data suggest earlier ages at diagnosis of extracolonic tumours in HNPCC when compared to sporadic cases, in particular among *MLH1* and *MSH2* mutation carriers (Table 6). This phenomenon adds to clinical recognition of *MLH1* and *MSH2* mutation families.

**Table 5.**
Cumulative lifetime risks of colorectal (CRC), endometrial (EC), stomach (ST), ovarian (OV), urothelial cell (UR), small bowel (SMB), biliary tract (BIL) cancer and brain tumours (BT) in *MLH1*, *MSH2* and *MSH6* mutation carriers compared to the population risks[5,54,101,158,302,303,314].

| Publication | Gene | Cumulative life time risk (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | CRC | | EC | ST | OV | UR | SMB | BIL | BT |
| | | M | F | | | | | | | |
| Vasen et al. '96(a) n=240 (at 75 yrs) | *MLH1* | 92 | 83 | 42 | | | | | | 3.35 |
| | *MSH2* | | | 61 | | | | | | |
| Dunlop et al. '97 n=67 (at 70 yrs) | *MLH1* *MSH2* | 74 | 30 | 42 | | | | | | |
| Lin et al. '98 n=105* (at 60 yrs) | *MLH1* | 94 | 63 | | | | | | | |
| | *MSH2* | 96 | 39 | | | | | | | |
| Aarnio et al. '99 n=360 (at 70 yrs) | *MLH1* *MSH2* | 100 | 54 | 60 | 13 | 12 | <4 | <4 | <4 | <4 |
| Vasen et al. '01 n=676** (at 70 yrs) | *MLH1* | 65 | 55 | 25 | 2.1 | 3.4 | 1.3 | 7.2 | | 0 |
| | *MSH2* | 75 | 55 | 37 | 4.3 | 10.4 | 5.4 | 4.5 | | 1.2 |
| Wagner et al. '01 n=34 (at 80 yrs) | *MSH6* | 32 | | | | | | | | |
| Hendriks et al. '03 n=146 (at 70 yrs) | *MSH6* | 69 | 30 | 71 | | | | | | |
| Population | | 5 | | 1.5 | 1 | 1.5 | <1 | <0.1 | <1 | 0.5 |

n=number of carriers;* 49 *MSH2*/56 *MLH1* mutation carriers;** 311 *MSH2*/356 *MLH1* mutation carriers

**Table 6.**
Mean age of onset (in years) of colorectal (CRC), endometrial (EC), stomach (ST), ovarian (OV), urothelial cell (UR), small bowel (SMB), biliary tract (BIL) cancer and brain tumours (BT) in *MLH1*, *MSH2* and *MSH6* mutation carriers compared to the age of onset of these tumours in the population[5,54,101,158,247,302,303,314].

| Publication | Gene | Mean age of onset (YRS) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | CRC | EC | ST | OV | UR | SMB | BIL | BT |
| Vasen et al. '96 | *MLH1/ MSH2* | 41-44 | 48-49 | | | | | | |
| Dunlop et al. '97 | *MLH1/ MSH2* | 47-50 | 54 | 59 | | | | | |
| Aarnio et al. '97 | *MLH1/ MSH2* | | | 57 | | | | | |
| Lin et al. '98 | *MLH1/ MSH2* | 45-52 | | | | | | | |
| Rodrigues et al. '98 | *MLH1/ MSH2* | | | | | | 49 | | |
| Aarnio et al. '99 | *MLH1/ MSH2* | | | | 45 | | | | 53 |
| Vasen et al. '01 | *MLH1/ MSH2* | 43-44 | | 68 | 43 | 50 | 54 | | 56 |
| Parc et al. '03 | *MLH1/ MSH2* | 43-48 | 46 | 49 | 40 | 49-53 | 45 | 43 | 63 |
| Vasen et al. '01 | *MSH6* | 50 | | | | | | | |
| Wagner et al. '01 | *MSH6* | 55 | 55 | | | | | | |
| Hendriks et al. '03 | *MSH6* | 56 | 55 | | | | | | |
| Population | | 67 | 55-65 | 60-70 | >50 | 60-70 | 60 | >50 | >45 |

## 1.8 HNPCC tumours: histopathologic features

The main precursors of colorectal cancer are adenomatous polyps and flat adenomas[326]. In agreement with the underlying MMR genetic defect, polyps from HNPCC patients seem to progress to invasive cancer more rapidly than in sporadic or even FAP patients[119]. Specific pathologic characteristics of HNPCC colorectal tumours have been identified, but none of them are pathognomic: poor differentiation, presence of mucinous and signet cells, medullary features, peritumoural lymphocytic infiltration, Crohn's like reaction, and tumour infiltrating lymphocytes (TIL) mixed with tumour cells[181, 198, 346].
Most HNPCC-associated endometrial cancers are of the endometroid subtype[43].
Among other extracolonic HNPCC-associated neoplasms, gastric cancers are generally of the intestinal type[6], whereas ovarian cancers are adenocarcinomas, most commonly of the serous or mucinous type[5, 180]. With respect to tumours of the urinary tract, transitional cell carcinomas are associated with HNPCC, localised in the ureter and renal pelvis, though not in the bladder[265, 328]. Small bowel cancers are adenocarcinomas[180, 182, 247].
The skin tumours characteristic of the Muir-Torre allelic variant of HNPCC are predominantly sebaceous adenomas and adenocarcinomas. In 1995, Hamilton[86] recognised that HNPCC-related brain tumours are predominantly glioblastomas. The latter observation was subsequently confirmed by Vasen et al.[300] and Aarnio et al.[5].

## 1.9 Microsatellite Instability (MSI)

Microsatellite instability (MSI) is defined as the type of genomic instability associated with defective DNA mismatch repair in tumours. MSI provides an indication of the presence of genetic instability in a given tumour by comparing the size of a subset of simple repeated sequences occurring throughout the genome (mono-, di-, tri-, and, less frequently, tetranucleotide repeats) between normal and tumour DNA from the same individual. Slippage of DNA polymerases during replication of such simple sequence repeats often causes expansions and contractions of their alleles which are efficiently repaired by the MMR machinery. In MMR-deficient cells these errors are not properly corrected and accumulate at each cell division. Notably, MSI is rarely caused by processes other than defective mismatch repair, e.g. reduced replication fidelity by polymerase alterations, or imbalance in deoxynucleoside triphosphate pools[128].
MSI was initially described in a subset of colorectal cancers in 1993[4, 114, 285]. Initially, some authors used the term replication errors (RER), though in 1998 the National Cancer Institute Workshop on HNPCC recommended the use of the term MSI and established "MSI golden standards" that are currently employed in research and diagnostic laboratories worldwide[24] (see also chapter 1.14).

More than 90% of the HNPCC-associated colorectal cancers displays MSI[4, 67], compared to 12-18% of the sporadic tumours[114, 285]. MSI is also found in ~80% of adenomas of variable size from HNPCC patients[112]. With respect to MSI analysis of colorectal cancers, the NCI workshop recommended five most informative markers, and has formulated guidelines for MSI interpretation (Table 4 and chapter 1.14)[24].
No international criteria have been formulated for MSI analysis of endometrial cancer. However, based on studies with different microsatellite markers including those commonly used for colorectal cancer, at least 75% of the endometrial cancers from *MSH2* and *MLH1* mutation carriers displays MSI[43, 111, 246], compared to only 15-30% of the sporadic tumours[97, 246, 339]. Notably, *MSH6*-related endometrial cancers predominantly show instability at mononucleotide markers[43].
Insufficient data are yet available on MSI analysis of other HNPCC-related extracolonic tumours. However, in agreement with its molecular-genetic basis and based on studies with different microsatellite markers and on incidentally tested tumours in HNPCC families, MSI seems to represent the common denominator of virtually all HNPCC-related cancers: 75% of the HNPCC related gastric cancer displayed MSI vs 15-39% of sporadic gastric cancers[6, 51, 263, 278]; 5/5 HNPCC-related ovarian tumours were MSI-High vs. an overall frequency of 17% of MSI in ovarian tumours[38, 66, 111]; 21-31% of upper tract urothelial carcinomas exhibit MSI (Low and High) whereas incidentally tested HNPCC-related carcinomas of the same histological type were positive for MSI[21, 90, 314]; 3/3 HNPCC-related pancreatic ductal adenocarcinomas (Papilla Vateri) showed MSI vs. 26/100 sporadic tumours[343]. Entius et al.[56] detected MSI in 6 out of 10 sebacous gland tumours of possible Muir-Torre patients. Moreover, MSI is detected in a subset of early onset gliomas[124, 155]. Other tumour types thought not to belong to the HNPCC spectrum, like breast and other relatively common cancers, often display MSI in proven MMR mutation carriers, suggesting a role of MMR-deficiency in the development of these particular tumours[44, 245, 264, 269].

### 1.10 Prognosis

The prognosis of colorectal cancer patients from HNPCC families appears to be more favourable than that of sporadic CRC patients, with overall 5 years and 10 years survival rates of 65% vs. 44%, and 68% vs. 37%, respectively[181, 254, 327]. It has been hypothesized that this is caused by a higher sensitivity to chemotherapy of mismatch repair deficient tumours[99, 115]. However, a recent study did not show benefit of fluorouracil-based adjuvant chemotherapy in patients with MSI-High tumours[242]. Also, MMR-deficient cells are tolerant to alkylating chemotherapeutics. Another possible explanation for the increased survival in HNPCC-associated colorectal cancer is the

enhanced immune-response to the highly genetically unstable MSI tumours[250, 251]. Chang et al.[34] assumed that the abundant presence of immune cells in MMR-mutant tumours increases oxidative stress. Vulnerability of MMR-deficient cells to oxidative stress may cause cell cycle arrest and lead to a more favourable prognosis.
No difference in survival of endometrial cancer was found between HNPCC-related and sporadic patients[23].

### 1.11 Screening

An overview of the current screening advices in HNPCC and in other families reminiscent of HNPCC is presented in Table 7. In the Netherlands, members from these families are registered by the Dutch Foundation for the Detection of Hereditary Tumours (Stichting Opsporing Erfelijke Tumoren/STOET). This foundation sends timely notification to their gastrointestinal specialists to perform the colonoscopic screening and, reversely, receives information on the screening results. Hence, the foundation plays a central role in the optimisation of screening protocols for hereditary colorectal cancer families.
Colonoscopy is the technique of first choice for colorectal screening. It is a powerful tool in the detection and treatment of premalignant adenomas or early colorectal carcinomas in at-risk individuals. Regular colonoscopy was reported to reduce the colorectal cancer rate by 62%, and to decrease the overall mortality by about 65%[117, 118, 238]. These figure can be expected to improve with the development of high-resolution and -magnification techniques like magnifying endoscopy[141]. Currently, healthy MMR gene mutation carriers are advised to undergo a colonoscopy every 1-2 years from the age of 20-25 years onward[27, 87]. In female *MSH6* mutation carriers, the age to start colonoscopic screening may be postponed to 30 years[101]. In Amsterdam criteria positive families without a MMR gene mutation, colorectal screening advices are the same as for mutation positive families, and apply to all 1st-degree relatives of individuals diagnosed with an HNPCC related tumour and to all 2nd-degree relatives whose parent died at young age. Screening advices to affected relatives are weighted carefully, taking into account the prognosis due to the former tumour. Clear disadvantages of colonoscopy are the burden of this procedure for the patient, the risk of perforation of the colorectum (0.1%), the risk of bleeding, and the costs. Among 42 healthy Dutch MMR gene mutation carriers, 88% had colonoscopic screening every 1-2 years. In chapter 3.4, we discuss the appreciation of colonoscopy in these mutation carriers. In individuals with a contra-indication for colonoscopy, barium enema and/or faecal occult blood testing are, though less sensitive, alternatives[29, 337]. Testing for genetic markers TP53, BAT26, APC and KRAS in stool may represent a future non-invasive screening protocol to preselect patients for colonoscopy[9,

[50, 157, 268, 292, 293]. Virtual colonoscopy is also considered a potential non-invasive screening tool[79]. Using computer tomography (CT), 80-90% of the polyps larger than 6 mm can be detected. Because of the radiation load in CT scanning, magnetic resonance imaging (MRI) is a more patient-friendly alternative. However, since detection rates of smaller and flat polyps are rather poor in both CT and MRI, virtual colonoscopy is not likely to replace conventional colonoscopy in the near future. Moreover, when a polyp is detected by virtual colonoscopy, conventional colonoscopy has to be performed anyhow to allow polypectomy. No consensus exists on prophylactic colectomy in mutation carriers as a standard procedure, primarily because the combined colonoscopy and polypectomy are a highly reliable and effective surveillance and preventive strategy[137]. However, at time of diagnosis of colorectal cancer or polyps, subtotal colectomy may be considered in MMR gene mutation carriers[87, 187].

Yearly gynaecological examination is advised to female MMR gene carriers starting from the age of 30-35 years. This examination includes endometrial aspirate or vaginal ultrasound of the uterus and ovaries, and CA125 measurements in blood (CA 125 is a tumour marker of ovarian cancer)[27, 87, 298]. However, the value of this screening is disputed and adequate management of early symptoms remains very important[52, 244]. Prophylactic hysterectomy is not routinely offered, but in female carriers already scheduled for a colectomy and in female *MSH6* carriers, this procedure may be considered. In case of a family history of ovarian cancer, prophylactic oophorectomy may also be considered in view of the limited value of screening and the poor prognosis of this tumour type[87].

Advises for regular screening for other HNPCC related cancers are tailored based on the individual family history[27, 87, 298]. In families with two or more gastric cancers, gastroscopic screening is recommended from the age of 30-35 years every 1-2 years[27, 87]. Of note, gastroscopic screening had no beneficial effect in Finnish *MLH1* mutation carriers at a follow-up of 3-4 years[239]. However, limitations of this study are the size of the patient cohort, the genetic homology (the majority of the patients carried a Finnish founder mutation in *MLH1*) and the short follow-up period. Helicobacter Pylori may also represent a confounding factor. In HNPCC families with familial or early onset gastric cancer relatives should be analysed for H. Pylori infection. Urinary tract screening is also advised from the age of 30-35 years by cytological urine sediment analysis every 1-2 years. The sensitivity of this technique can be improved by molecular methods like microsatellite analysis and immunoassays on the urine sediment[37, 274], though this is not generally applied.

**Table 7.**
Screening advises in HNPCC(-like) families[27,29,30,73,201]. These advices apply to healthy first degree relatives of individuals with an HNPCC related tumour. Of note, if a parent died young of a not HNPCC related cause, a second-degree relative must be considered as first-degree.

| Clinical criteria | Site | Technique | Age of Onset (yrs) | Interval (yrs) | Remarks |
|---|---|---|---|---|---|
| *MLH1/MSH2/ MSH6* mutation carrier | colon | colonoscopy | 20-25* or 5 yrs before earliest crc in family | 1-2 | Consider colectomy with ileorectal anastomosis at diagnosis of cancer or in case of multiple recurrent polyps. In female *MSH6* carriers the age of onset of screening may be postponed to 30 years. |
| | uterus | gynaecological examination & intravaginal ultrasound or endometrial aspirate | 30-35 | 1 | Consider prophylactic hysterectomy in *MSH6* mutation carriers. |
| | ovary | gynaecological examination & intravaginal ultrasound & CA125 in blood | 30-35 | 1 | Consider prophylactic oophorectomy if 2 or more relatives have ovarian cancer. |
| | stomach | gastroduodenoscopy | 30-35 | 1-2 | depending on family** |
| | small bowel | gastroduodenoscopy | 30-35 | 1-2 | depending on family** |
| | urinary tract | urine cytology & sediment | 30-35 | 1 | depending on family** |

| Clinical criteria | Site | Technique | Age of Onset (yrs) | Interval (yrs) | Remarks |
|---|---|---|---|---|---|
| MMR gene mutation negative/ unknown & AC positive | colon | colonoscopy | 20-25 | 1-2 | or starting 5 years before the youngest crc case in the family in crc-only families |
| | uterus | gynaecological examination & intravaginal ultrasound or endometrial aspirate | 30-35 | 1 | depending on family** (not if only crc occurs in the family) |
| | ovary | gynaecological examination & intravaginal ultrasound & CA125 in blood | 30-35 | 1 | depending on family** |
| | stomach | gastroduodenoscopy | 30-35 | 1-2 | depending on family** |
| | small bowel | gastroduodenoscopy | 30-35 | 1-2 | depending on family** |
| | urinary tract | urine cytology & sediment | 30-35 | 1 | depending on family** |
| 1 first-degree relative with crc <50 yrs or 2 first-degree relatives with crc | colon | colonoscopy | 45-50 or 5 years before youngest crc patient in family | 5 | |
| Others | colon | FOBT | 50 | 1 | on request Fecal Occult Blood Testing (FOBT) is less invasive but also less sensitive than other screening tools. Colonoscopy is the most sensitive tool. |
| | | sigmoidscopy | | 5 | |
| | | sigmoidscopy & FOBT | | 5 | |
| | | double contrast barium enema | | 5-10 | |
| | | colonoscopy | | 10 | |

** If 2 or more relatives are affected with stomach, small bowel or urinary tract cancer (If only 1 relative is affected at an early age, screening the first degree relatives can be considered in view of additional riskfactors in this branch of the family).
crc = colorectal cancer; AC = Amsterdam criteria

## 1.12 Prevention

It is generally believed that diet has a profound impact on colorectal cancer risk. However, experimental evidence has been hard to obtain and it is unlikely that the colorectal cancer risks of MMR gene mutation carriers can be significantly modulated by dietary factors[131, 236]. No difference in meat consumption was seen in sporadic colorectal adenoma cases and HNPCC cases in the Netherlands[313]. A study on the prevention of polyps in HNPCC carriers by resistant starch supplements is in progress[28]. Nevertheless, a balanced diet with adequate fruit, vegetable and cereals intake, restricted alcohol consumption, no smoking, weight control, and regular physical exercise are reasonable advises.

Chemoprevention studies in HNPCC carriers are limited[309]. Studies on calcium carbonate were not conclusive[91]. A international study (CAPP2) on the effect of aspirin (alone and in combination with resistant starch) in HNPCC carriers is ongoing[28]. NSAIDs (non-steroidal anti-inflammatory drugs), including aspirin, have been consistently associated with a reduced risk of colorectal cancer[28, 236]. Aspirin inhibits the conversion of arachidonic acid to prostaglandins by cyclo-oxygenase 1 and 2 (COX1 and COX2). Because the adverse effects of long-term aspirin use are mainly caused by COX1 inhibition, novel NSAIDs, e.g. Celecoxib, were developed that selectively inhibit COX2. However, also selective COX2-inhibitors may increase the risk of cardiovascular events[213], making them unsuitable for chemoprevention purposes. Also, the spectrum of antineoplastic actions of NSAIDs is broad and includes inhibition of angiogenesis, induction of apoptosis, and cancer growth inhibition by interference with several signal transduction pathways[108, 288]. Both Celecoxib and Sulindac were reported to cause adenoma regression in patients with familial adenomatous polyposis (FAP)[41, 74, 91, 108, 288]. Also, a significant reduction in duodenal polyposis was seen in FAP carriers after treatment with Celecoxib[231]. However, no effect was observed in FAP carriers ranging from 8 to 25 years of age using standard doses of sulindac[75]. A study (CAPP1) on the effects of aspirin in FAP mutation carriers is about to report its results[28]. More insights in the molecular genetic mechanisms underlying tumour initiation and progression in HNPCC are expected to lead to the identification of new safe targets for tailor-made chemoprevention. For an overview of the status quo of chemoprevention in colorectal cancer see Hawk et.al.[92].

The mutator phenotype characteristic of HNPCC tumours causes frameshift mutations in a broad spectrum of genes leading to novel peptides that can be recognised by T cells and trigger an immune response. The characterisation of peptides specific for MSI-positive colorectal cancer cells fuels hope for the development of a prophylactic vaccine for HNPCC carriers[250].

### 1.13 Differential diagnosis

The Muir-Torre Syndrome. In 1966 Muir[212] described a syndrome with multiple primary tumours of the colon, duodenum, larynx, and skin. In 1968 Torre[291] presented an additional patient with multiple sebaceous adenomata and colon cancer. In the 1980's Lynch et al.[69, 188, 190] recognised the Muir-Torre syndrome as a clinical variation of HNPCC. Since then, germline mutations of *MSH2* and in a lesser extend of *MLH1* have been found in Muir-Torre families[140, 144].

The Turcot Syndrome. Another clinically defined syndrome that appeared to be an allelic variant of HNPCC is Turcot syndrome. In 1959 Turcot[295] described two cases (a brother and sister) with central nervous system tumours and polyposis coli. In 1995 Hamilton[86] recognised that this association could derive from two distinct types of germline defects: mutations in the APC gene that cause Familial Adenomatous Polyposis (FAP) and mismatch repair gene mutations (*MLH1* and *PMS2*). The brain tumours in FAP are predominantly medulloblastoma, while the HNPCC-associated brain tumours are glioblastoma[5, 300]. Wang et al.[321] demonstrated *NF1* somatic mutations in MMR-mutant cell lines, possibly providing a molecular basis for the development of brain tumours in a subset of MMR gene mutation carriers.

Familial Adenomatosis Polyposis (FAP) and Attenuated FAP (AFAP). In the majority of the cases, the clinical diagnosis of Familial Adenomatosis Polyposis is facilitated by the presence of hundreds to thousands colorectal adenomas. More than 95% of FAP patients carries a disease-causing *APC* mutation[147]. MMR gene defects play no apparent role in *APC* mutation negative FAP families[96].

An atypical variant of FAP, attenuated FAP (AFAP), characterised by reduced polyp multiplicity (between 5-10 and 100) and a delayed age of onset, is more reminiscent of the HNPCC phenotype[32]. AFAP is caused by specific *APC* mutations, usually located at the extreme 5' or in the 3' half of the gene[63], or by biallelic *MYH* mutations[15, 122]. The latter form is now referred to as MAP, *MYH*-associated polyposis. Wang et al.[318] described two patients with early onset colorectal cancer and respectively zero and three polyps, both homozygous for *MYH* mutations. In these cases family history and MSI/IHC tests are helpful to differentiate diagnoses. In case of a MSI stable phenotype, MMR-gene defects become unlikely and analysis of *APC* and *MYH* should be considered[15, 122].

Familial colorectal cancer. Colorectal cancer is a common type of cancer and random familial clustering of CRC cases is seen in about 15% of the families of patients with colorectal cancer. For example allelic variants of high risk colorectal cancer susceptibility genes can contribute to less penetrant forms of CRC predisposition[164]. The guidelines depicted in Figure 10 will facilitate differential diagnosis between bona fide HNPCC

families and those kindreds with a clustering of colorectal cancers due to other hereditary or non-hereditary factors. Detailed phenotypic characterisation will continue to be of great importance for the subclassification of the remaining unresolved colorectal cancer families. Until the moment additional molecular genetic markers will be available for most of the familial colorectal cancer syndromes, screening advises will mainly be based on accurate family history data (Table 7)[29, 30].

Crohn's disease and colitis ulcerosa. Inflammatory bowel disease is a known risk factor for colorectal cancer. *NOD2* has been identified as a low risk susceptibility gene for Crohn's disease[221]. The pathological features of HNPCC related colorectal tumours can include a Crohn's like reaction with tumour infiltrating lymphocytes (TIL) mixed with tumour cells[181, 198]. However, Crohn's disease and colitis ulcerosa are distinct clinical entities, which can easily be differentiated from HNPCC by symptoms and a detailed family history.

Hyperplastic polyposis, mixed polyposis and serrated adenomatosis. Hyperplastic polyposis is a loosely defined syndrome characterised by the occurrence of multiple hyperplastic polyps in the colorectum. Hyperplastic polyps are generally considered less prone to malignant transformation when compared with adenomatous polyps. However, dysplasia has been described in hyperplastic polyps and, especially in cases with high polyp multiplicity's, patients are at increased risk of colorectal cancer[93, 136, 153, 237].

A few families have been described with a combination of different types of colorectal polyps, atypical juvenile polyps, adenomas and hyperplastic polyps[256, 330]. These families and are classified as affected by Hereditary Mixed Polyposis Syndrome (HMPS). The disorder was mapped to chromosome 6q in 1996, though its genetic basis is yet to be elucidated[286].

Occasionally, polyps may display both hyperplastic and adenomatous features. These polyps are usually referred to as serrated adenomas. It has been claimed that these adenomas arise through a distinct MSI-Low pathway[120, 257].

As the above entities have overlapping clinical features with attenuated or atypical HNPCC and FAP cases, their diagnosis should always be excluded in families with hyperplastic, mixed or serated polyposis.

The hamartomatous polyposis syndromes. In juvenile polyposis, the Peutz-Jeghers syndrome, and the Cowden syndrome, colorectal hamartomatous polyps and cancer occur at increased frequency[76, 338]. Hamartomatous polyps can be pathologically distinguished from HNPCC-related adenomas. Hamartomatous polyposis are often considered as non-neoplastic. However, they may sometimes harbour dysplasia and their true neoplastic potential is yet unknown. Nevertheless, an increased cancer risk both within and outside the gastrointestinal tract exists in these syndromes. Other clinical features of

hamartomatous polyposis syndromes, like early age at diagnosis of juvenile polyposis, the mucocutanous pigmentations in Peutz-Jeghers syndrome and the tricholemmoma in Cowden syndrome, may help to differentiate them from HNPCC. Finally, mutation analysis of the respective causative genes (*SMAD4* and *BMPR1A* for juvenile polyposis, *LKB1/STK11* for Peutz-Jeghers syndrome, and *PTEN* for Cowden syndrome) may indisputably resolve these syndromes from HNPCC.

Hereditary breast-ovarian cancer. Germline *BRCA1* and *BRCA2* mutations cause an increased risk for breast and ovarian cancer[202, 340]. In familial ovarian cancer families both *BRCA1/BRCA2* susceptibility and HNPCC should be considered. Apart from mutation analysis of *BRCA1/BRCA2* and the MMR genes, MSI and IHC analysis may help to distinguish the two entities. However, data on MSI and IHC testing in ovarian cancer are limited.

Familial gastric cancer. Familial gastric cancer has been linked to two genetic predispositions, *E-cadherin* mutations and HNPCC[31]. Pathologically, *E-cadherin* mutations give rise to diffuse gastric cancer, while HNPCC-associated gastric cancer tends to be of the intestinal type. Once again, detailed family history, clinical data, and MSI/IHC tumour analysis can help to direct further molecular analysis and formulate a more accurate diagnosis[84, 230].

Neurofibromatosis type 1 (NF1). Though individuals with NF1 have an increased risk of colorectal cancer, the phenotype is generally easy to distinguish by the occurrence of café au lait spots, freckling and neurofibromas. However, children with biallelic MMR gene mutations develop features of neurofibromatosis type 1 in addition to other malignancies[45, 46, 71, 200, 243, 320, 331]. Hence, in children with features of NF1 and a family history of HNPCC-related tumours or a more recessive inheritance pattern, one should consider MMR gene mutation analysis.

### 1.14. Molecular diagnostics of HNPCC

The molecular diagnosis of HNPCC in a given kindred implies several steps and analytical procedures. Rather than directly applying mutation detection techniques to identify HNPCC-causing lesions in MMR genes, most diagnostic centres initiate their search by investigating tumour material (when available) to assess genetic instability and loss of specific MMR protein expression by MSI (microsatellite instability) and IHC (immunohistochemistry) analysis respectively. They allow confirmation of the MMR defect in the tumour (MSI and IHC) and the identification of a specific gene whose protein expression has been lost in the tumour (IHC). The latter gene will then represent the first candidate gene to be analysed for the presence of germline mutations in non-neoplastic

(usually blood) patient material. Here, each technical approach will be described separately.

Microsatellite instability (MSI) is defined as the type of genomic instability associated with defective DNA mismatch repair in tumours. Analysis of MSI is performed by PCR amplification of specific mono- and dinucleotide microsatellite repeats and by comparing the size of their alleles between normal and tumour DNA. Tumour-specific changes in allele sizes are indicative of genetic instability due to loss of MMR function. MSI analysis is usually performed on paraffin embedded tumour samples with or without microdissection of the parenchymal cells. The National Cancer Institute recommended five most informative markers with respect to colorectal cancers, and has formulated guidelines for MSI interpretation (Table 4)[24]. According to the degree of MSI detected, tumours are subdivided in high-frequency MSI (MSI-H), if two or more of the five markers show instability, and low-frequency MSI (MSI-L), if only one of the five markers shows instability. Tumours with no instability in any of the markers are considered to be microsatellite stable (MSS). The implications for MSI-L are not yet clear. It is suggested that if enough markers are tested, all colorectal tumours display some degree of instability[146]. The resolution between microsatellite high (MSI-H) and low frequency MSI (MSI-L) can only be accomplished if a greater set of markers (especially including mononucleotide markers like BAT40) is utilised (Table 4)[24, 296]. The use of additional mononucleotide repeats is also useful when dealing with HNPCC due to *MSH6* germline mutations, known to be associated with preferential microsatellite instability at mononucleotide repeats[43, 101]. The same NCI markers are also employed for the prediction of a MMR gene defect in endometrial cancers[43], though little data are yet available on their reliability for other HNPCC-related tumours.

Immunohistochemical analysis (IHC) is a rapid and inexpensive method for identifying MMR-gene alterations[160]. As for MSI, IHC is also performed on histological sections from paraffin embedded tumours with antibodies specifically raised against the main MMR proteins: MSH2, MLH1, MSH6 and PMS2[42, 43]. Absence of staining for a specific protein is likely to result from the 2$^{nd}$ somatic hit at the MMR gene where the germline mutation is present. Therefore, IHC represents a useful tool to direct mutation analysis and enhance cost-effectiveness of the mutation detection procedure. Normal staining of a given MMR protein, however, does not exclude the presence of a mutation of the corresponding gene[160, 317].

Tissue Microarrays (TMAs) represent a powerful novel technology for high-throughput analysis of protein expression in a large number of tissue (tumour) samples. Hundreds of tissue cores are arranged on a single slide, and then analysed by a single IHC reaction[123].

TMA's can significantly enhance the processivity of IHC, although their systematic use in a diagnostic setting has not been evaluated yet.

In order to detect mutations in genomic DNA, a large number of protocols are available[280]. However, since the comprehensive description of these diverse technologies is outside the scope of this thesis, I will here only briefly describe the HNPCC mutation detection protocols most commonly employed in our laboratory.

Denaturing gradient gel electrophoresis (DGGE) allows the rapid screening for single base changes in PCR-amplified genomic DNA[62]. The technique is based on the migration of double-stranded DNA molecules through polyacrylamide gels containing linearly increasing concentrations of a denaturing agent. The mobility of the DNA molecule is strongly retarded at the concentration at which the DNA strands with the lowest melting domain dissociate. This branched structure becomes entangled in the gel matrix and no further movement occurs. Complete strand separation is prevented by the presence of a high melting domain, which is usually artificially created at one end of the molecule by incorporation of a GC clamp. The latter is usually accomplished during PCR amplification using a primer with a 5' tail consisting of a random sequence of approx. 40 GC. Single base substitutions, deletions and insertions are detected with high sensitivity by DGGE analysis[62]. For HNPCC mutation analysis, individual MSH2, MLH1 and MSH6 exons are amplified by PCR, with one of the two primers implemented with a 5' GC-clamp. The PCR product is then loaded on the denaturing gradient gel. Exons with altered patterns of migration on DGGE are subsequently sequenced to determine the nucleotide alteration[335, 336].

Mutation detection techniques such as DGGE detect "point mutations", i.e. single base substitutions and small (1-10 nt) deletions and insertions. However, larger genomic rearrangements such as deletions, duplications, insertions, and inversions also represent a frequent cause of hereditary conditions. To detect this type of genetic defect the implementation of other mutation analysis strategies is required.

Southern blot analysis is a rather old and cumbersome method but, to date, it still represents the most reliable and accurate approach to detect large rearrangements in total genomic DNA. High molecular weight DNA is usually extracted from peripheral blood lymphocytes though many other types of tissues can be employed. DNA is then digested by restriction endonucleases, fractionated according to the molecular weight of the resulting fragments by electrophoresis through agarose gels, and then transferred onto a nylon membrane. The latter are hybridized with (radioactively) labelled DNA probes that recognise specific single-copy restriction fragments among the complexity of genomic DNA[253].

Conform to our previously established protocol[334], Southern analysis of the *MSH2* gene is performed with XbaI, EcoRI, HindIII, NsiI, genomic DNA digests, followed by hybridization with three *MSH2*-specific probes (encompassing exons 1-7, 7-12, and 10-16 respectively). *MLH1* and *MSH6* Southern analysis is performed with XbaI, ApaI, HindIII and NsiI genomic DNA digests followed by hybridization with two *MLH1*- (exons 1-12 and 11-19) and two *MSH6*-specific probes (exons 1-4 and 5-10)[334]. As reported in chapter 2, the implementation of the Southern blotting procedure in the analysis of the MMR genes in HNPCC has resulted in the identification of a significant number of genomic deletions and other less common types of rearrangements[297, 315, 334]. Although Southern analysis is still the method of choice in a "discovery phase", i.e. to define the spectrum of genomic rearrangements at any given disease locus, its protocol is too cumbersome and time-consuming to be efficiently implemented in diagnostic laboratories. PCR-based methods for the detection of genomic deletions and duplications are nowadays available.
Multiplex Ligation-dependent Probe Amplification (MLPA) is based on quantitative multiplex PCR in order to determine the relative copy number of each exon within a gene of interest[77]. Two specific probes are hybridised with the denatured target DNA. If hybridised, the probes are ligated and PCR amplified. The amount of probe amplification product reflects the copy number of the target sequence. MLPA has been successfully applied for the detection of MMR genomic rearrangements in HNPCC patients[35, 36, 77, 189, 220, 232, 307, 315, 321]. However, there are some limitations. For example, rearrangements not encompassing the coding region will not be detected by MLPA unless specific PCR strategies are designed to cover extensive portions of the flanking regions where regulatory elements are likely to be located. Also, Southern analysis, when performed with at least two different restriction enzymes, allows accurate mapping of the extent of the rearrangement and the consequent design of PCR primers to specifically amplify the breakpoints and the rapid detection of the disease-causing deletion/insertion in other affected family members. MLPA does provide indication of the presence of a genomic deletion/duplication but not on the exact location of the breakpoint, especially when dealing with disease genes with very large introns. Moreover, inversions are not detected by MLPA as no quantitative change at the genomic DNA level is observed[113].
Other analytical approaches are available to aid molecular genetic investigations on specific cases, e.g. missense mutations of questionable pathogenicity, or MMR mutation-negative individuals belonging to Amsterdam positive families with MSI-H tumours and/or informative IHC assays.
Mono-Allelic Mutation Analysis (MAMA or conversion technology) represents a useful tool for the analysis of "masked" mutations (hard to detect by conventional techniques),

missense mutations, splicing errors and genomic alterations[216, 224, 344]. To allow RNA and protein expression analysis of putative disease-causing MMR alleles in a heterologous genetic background, somatic cell hybrids are obtained by fusing patient-derived cells (typically blood lymphocytes) with *Msh2*-deficient mouse cells (GMP Conversion Technologies Inc., Waltham, MA)[224, 344]. Somatic cell hybrids carrying the chromosome of interest can be identified by genotyping with dinucleotide repeat markers and are then selected for mono-allelic expression analysis, e.g. by northern or western blot analyses or, if *MSH2* is the gene of interest, by more functional assays taking advantage of the absence of endogenous *Msh2* expression in the recipient murine cells.
Additional functional assays can be performed in yeast based on interference of recombinant human MLH1 with the yeast MMR machinery[261]. Alternatively, yeast strains expressing mismatch repair genes mutations at codons homologous to 'suspected' substitutions found in HNPCC patients can be analysed by MMR assay that measures stability of mono- or dinucleotide repetitive tracts *in vivo*[55, 234]. Other functional assays make use of the ability of MLH1 and PMS2 or MSH2 and MSH6 to dimerize by co-immunoprecipitation analysis after transfection of mutant or wildtype *MLH1*, *PMS2*, *MSH2* or *MSH6* in *MLH1*- or *MSH2*-deficient colorectal cancer cells or in insect cells(Sf9)[25, 125, 219, 294].
In the Netherlands, diagnostic mutation analysis of the *MLH1*, *MSH2* and *MSH6* genes is provided by university-affiliated DNA laboratories. Each gene analysis usually takes 3 to 6 months. At present, *PMS2* testing is not offered in diagnostic settings. The costs per gene per 2004 amount to approximately ∈650. MSI and IHC analysis of HNPCC related tumours are offered by several pathology departments at variable costs. Costs for MSI, IHC, and mutation analyses are fully covered by health insurance companies. Diagnostic MMR gene testing is also offered by several laboratories in Europe on a non-profit basis (www.EDDNAL.com). Commercial tests are available in the United States (Myriad Genetics) and Europe (Gendia).

### 1.15 Selection for mutation analysis

The Amsterdam criteria, established by the International Collaborative Group on HNPCC (Table 2)[299, 301], are valuable guidelines for the clinical recognition of HNPCC. Families fulfilling these criteria have a high *a priori* risk (45%- 64%) to carry germline mutations in one of the two major MMR genes[73, 333]. Also families not fulfilling the Amsterdam criteria may harbour a MMR gene mutation, though chances are significantly lower (0%-47%)[73].

We showed that failure to detect a MMR gene mutation in Amsterdam criteria positive families could be explained by either (1) marginal fulfilment of the criteria in true-mutation negative families, or (2) incorrect patient selection (phenocopy) in mutation positive families, or (3) failure of mutation detection techniques in mutation positive families. Due to the relatively high incidence of colorectal cancer in the general population, families may fulfil the Amsterdam criteria by chance. Katballe et al.[130] calculated that the Amsterdam criteria I would be met by chance only in 1 out of 77 families with a young colorectal cancer patient (age of diagnosis <50 years). We detected a MMR mutation (mainly in *MLH1* and *MSH2*) in all families tested with 2 additional HNPCC-associated tumours apart from the Amsterdam criteria[315] and in all families fulfilling the Amsterdam criteria by the presence of a relative with a non-colorectal HNPCC-associated cancer, as formulated in the revised Amsterdam criteria. HNPCC-associated cancers become more common at older age. To minimise the risk of testing a phenocopy, the patient tested for MMR mutations should therefore preferentially be the youngest affected individual within the family. Using a comprehensive set of mutation detection techniques, we detected a *MSH2* or *MLH1* mutation in more than 90% of Amsterdam criteria positive families[315].

Families with a MMR gene mutation may fail to meet the Amsterdam criteria for several reasons, namely reduced penetrance of specific MMR mutant alleles (e.g. *MSH6* and *PMS2*), early death of relatives due to cancer unrelated causes, non-paternity, lack of family history data, and small family size. However, most of these families will eventually qualify as Amsterdam positive, after efforts have been made to expand the pedigree structure and to obtain additional medical information. Anamnestic data on family history should be verified whenever possible[203].

In addition to family history, MSI and IHC analysis have been proven useful tools for the selection for mutation analysis[18, 47, 148, 175, 282]. More than 90% of HNPCC-associated colorectal cancers display microsatellite instability[3, 67], in contrast to 12-18% of the sporadic colorectal cancers[114]. Reversely, Liu et al.[168] detected a MMR gene germline mutation in 73% of families with familial colorectal cancer and a MSI-High tumour in one of the patients. MSI as the only pre-screening tool for MMR gene mutation analysis in unselected colorectal cancer will result in a mutation detection rate of only 5-10%. Since MSI is an expensive and laborious technique, and in view of the many sporadic MSI-High tumours that would be detected, screening of all colorectal cancer by MSI analysis is unattractive as a pre-screen tool.

The Bethesda guidelines for selection of tumours for MSI analysis were formulated in 1997 and revised in 2004 (Table 3)[248, 296]. When compared to the Amsterdam criteria as a

clinical prescreen tool, the Bethesda guidelines improve the MMR gene mutation analysis sensitivity, but lowers its specificity as a considerable number of families with more atypical phenotypes are included [277]. However, these atypical families may harbour *MSH6* or *PMS2* mutations[42]. This pleads for a liberal selection of cases for MSI analysis as appreciated in the revised Bethesda criteria.

MSI analysis is preferentially performed on colorectal cancer of the youngest patient in the family. If no colorectal cancer is available, endometrial cancer, or other HNPCC-associated cancers, or adenomas, are often tested. However, the interpretation of normal MSI results (MSS) in these latter settings is more difficult and has to be scored as non-conclusive.

Immunohistochemical (IHC) analysis of MMR proteins has earned a place as a pre-screening tool for MMR gene mutation analysis[42, 43, 100, 160]. Of note, positive IHC staining does not exclude the presence of a MMR gene mutation, as certain mutation types do not interfere with the translation of a protein detectable by IHC[317]. Also, many sporadic colorectal cancers show a decreased MLH1 expression due to promotor hypermethylation. MSI and IHC patterns generally show a high degree of concordance[38, 39, 47, 160, 240, 317]. Berends et al. indicate that IHC analysis might be the best prescreen tool in colorectal cancers diagnosed under the age of 50 years[20].

Few studies have been carried out to evaluate the concordance between the presence of a *MLH1*, *MSH2* or *MSH6* germline mutation, and the MSI and/or IHC status of the corresponding tumours[20, 39, 47, 100, 317, 323]. The results of these studies are summarised in Table 8. In total, 98 out of 106 tumours from mutation carriers (92%) tested as MSI-High, 89 (84%) revealed aberrant MLH1, MSH2 or MSH6 staining patterns, whereas 87 (82%) had both. Eleven mutation carriers (10%) only displayed MSI with no IHC staining aberrations. Two patients (2%) only displayed aberrant IHC and no MSI. Therefore, a total of 13 mutation carriers (12% of all tumours) displayed either only MSI, or aberrant IHC staining in their tumours. Three of the mutations in these 13 carriers were of unknown significance. By combining MSI and IHC analysis, 100 out of 106 (94%) mutation carriers were detected; the 6 mutations unnoticed by MSI or IHC analysis were of unknown significance. Sixteen mutation negative index patients (33%) displayed both MSI and IHC aberrations, suggesting failure of mutation detection techniques. In 82 tumours from MMR gene mutation carriers in which at least MLH1 and MSH2 IHC-staining results were available, a strong correlation was found between aberrant staining and the presence of a germline mutation: 29 tumours from 30 *MLH1* mutation carriers were MLH1-negative (no nuclear staining) but positive for MSH2; 39 out of 42 tumours of *MSH2* mutation carriers showed no MSH2 staining and were positive for MLH1. Also, all the 10 tumours available

from *MSH6* mutation carriers displayed no MSH6 staining but were positive for MLH1 and MSH2. Among those cases tested for all three MMR proteins, MSH6 staining was often negative in tumours from *MSH2* mutation carriers and occasionally in *MLH-* related tumours.

To date, a combined approach of family history, MSI and IHC analysis is generally recognised as the most evidence-based and cost-effective way to select patients for MMR gene mutation analysis[42, 47, 175, 240, 296]. A practical flowchart of the steps involved in the selection of individuals with multiple or early onset HNPCC-associated tumours for MMR gene mutation analysis, and of families with some clustering of HNPCC-associated tumours, is shown in Figure 10.

Wijnen et al.[336] proposed a statistical approach for the selection of families for MMR gene mutation detection. They devised a logistic model for estimating the likelihood of a *MLH1* or *MSH2* mutation based on clinical data (Table 9). They recommended mutation analysis for any individual with a likelihood over 20% of carrying an MMR gene mutation. Loukola et al.[175] proposed to perform MSI analysis on tumours of individuals with a likelihood of more than 5% according to the algoritm developed by Wijnen et al.

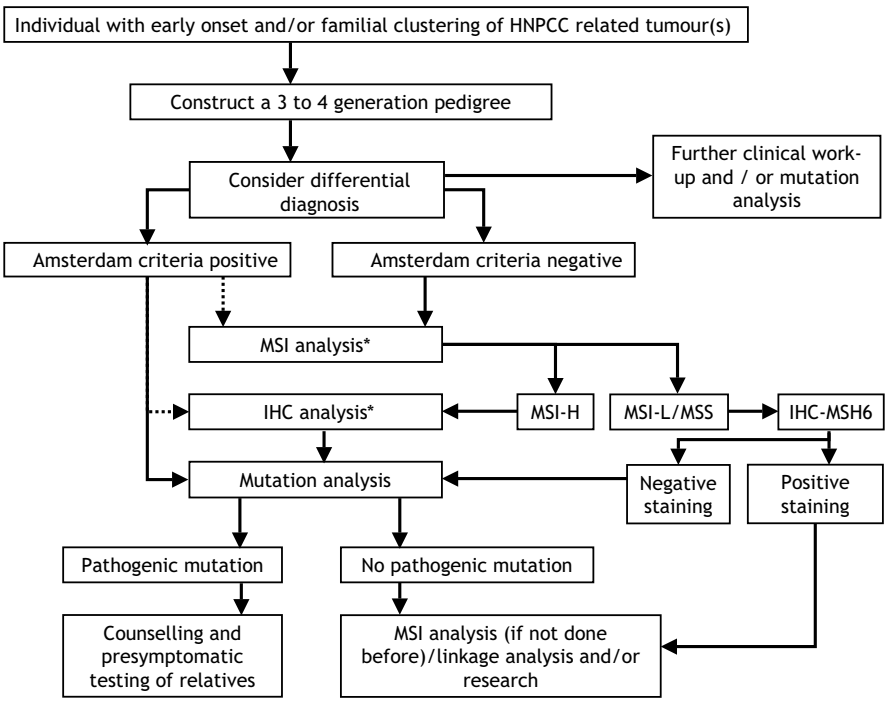


**Figure 10.**
A practical flowchart for the management of individuals with multiple or early onset HNPCC-associated tumours, and/of families with clustering of HNPCC-associated tumours. * The more "classic" the family, the less additional value has MSI and/or IHC, however IHC can be used to direct mutation analysis.

**Table 8.**
Comparison of MSI and IHC analysis in 154 tumours of 119 families analysed for *MLH1*, and *MSH2* mutations[20,39,47,100,317,323]. In all studies except in that of Debniak et al. the international markers for MSI analysis[24] (sometimes enhanced with BAT40) were used. Debniak et al. used 10 markers including 3 international markers and BAT40. Hendriks et al. and Berends et al. also performed IHC for MSh6 and mutation analysis of *MSH6*.

| | **Mutation** | | **Normal IHC (%)** | **Aberrant IHC (%)** | **Total (%)** |
|---|---|---|---|---|---|
| MSI-H/L (%) | positive | *MLH1* | 8 (25) | 24 (75) | 32 |
| | | *MSH2* | 1 (4) | 26 (96) | 27 |
| | | *MSH6* | 2 (5) | 37 (95) | 39 |
| | | Subtotal | 11 (11) | 87 (89) | 98 (92) |
| | Negative | | 8 (33) | 16 (67) | 24 |
| | *Subtotal* | | *19* | *103* | *122 (79)* |
| MSS (%) | positive | *MLH1* | 3 (75) | 1 (25) | 4 |
| | | *MSH2* | 3 (100) | 0 | 3 |
| | | *MSH6* | 0 | 1 (100) | 1 |
| | | Subtotal | 6 (75) | 2 (25) | 8 (8) |
| | Negative | | 23 (96) | 1 (4) | 24 |
| | *Subtotal* | | *29* | *3* | *32 (21)* |
| **Total** | | | **48** | **106** | **154** |
| All (%) | positive | *MLH1* | 11 (31) | 25 (69) | 36 |
| | | *MSH2* | 4 (13) | 26 (87) | 30 |
| | | *MSH6* | 2 (5) | 38 (95) | 40 |
| | | Subtotal | 17 (16) | 89 (84) | 106 (69) |
| | Negative | | 31 (65) | 17 (35) | 48 (31) |
| **Total** | | | **48 (31)** | **106 (69)** | **154** |

**Table 9.**
Logistic regression model for selection of families for *MSH2* and *MLH1* mutation analysis[336].

| **Including Amsterdam criteria I** | |
|---|---|
| Log Odds = L<br>$L = 1,4 + (-0.1)V_1 + 1,7V_2 + 2,4V_3$ | $V_1$ = mean age at diagnosis of all crc patients in the family |
| | $V_2$ = equal 1 if at least 1 relative has ec; otherwise it equals 0 |
| | $V_3$ = equals1 if family meets ACI; otherwise it equals 0 |
| **Including Amsterdam criteria II** | |
| Log Odds = L<br>$L = 2,34 + (-0,1)V_1 + 0,25V_2 + 1,6V_3 + 0,96V_4$ | $V_1$ = mean age at diagnosis of all crc patients in the family |
| | $V_2$ = number of patients with crc |
| | $V_3$ = number of patients with both crc/ec |
| | $V_4$ = equals1 if family meets ACII; otherwise it equals 0 |
| **Excluding Amsterdam criteria I and II** | |
| Log Odds = L<br>$L = 2,79 + (-0,1)V_1 + 0,33V_2 + 1,45V_3$ | $V_1$ = mean age at diagnosis of all crc patients in the family |
| | $V_2$ = number of patients with crc |
| | $V_3$ = number of patients with both crc and ec |
| **Mutation analysis of *MSH2* and *MLH1* is performed if $e^L/(1-e)^L \geq 0.2$ (probability of finding *MLH1* or *MSH2* mutation ≥20%)** | |

crc = colorectal cancer ec = endometrial cancer ACI/II = (Revised) Amsterdam criteria

### 1.16. Genetic counselling

Appropriate counselling is a prerequisite before genetic testing for HNPCC takes place, in view of the medical, psychological and social consequences for mutation carriers and their relatives. With respect to genetic diseases, the World Health Organization defines counselling as the 'provision of accurate, full and unbiased information in a caring, professional relationship that offers guidance, but allows individuals and families to come to their own decisions'[332]. General guidelines for cancer susceptibility testing were formulated by the American Society of Clinical Oncology in 1996 and updated in 2003[1, 2].

The counselee who initially seeks genetic advice generally fulfils a central role in the counselling of the family. He/she is asked to contact affected relatives for their participation in MSI/IHC and/or MMR gene analysis. Screening advises for relatives are initially communicated through the initial counselee. It is the task of the genetic counsellor to assist and advice the counselee in this process. Also, the implications of not finding a MMR gene mutation in a family must be understood by the counselee prior to testing.

In families with a known mutation in *MLH1*, *MSH2* or *MSH6*, genetic testing is offered to all affected and healthy adult risk carriers. Since the cancer risks in HNPCC start rising from about 20-25 years of age, genetic testing is not offered before adulthood. The use of genetic testing in families with a known MMR gene mutation has been studied by us and others[12, 154, 316]. Though the studies were performed in different settings, the test rate in individuals with *a priori* risk of 50% of carrying a mutation varied from 57 to 75%. If the counselee is affected by an HNPCC-related tumour, the risk of carrying the mutation is high though not 100%, and is therefore worth confirming by DNA analysis.

The recommended protocol for genetic testing includes three counselling sessions, a pre-test session, a session during which blood sampling is performed, and the test disclosure session [13, 154, 179]. Generally, this counselling procedure is judged as sufficient (see chapter 3.4)[13]. Of note, Dutch mutation carriers indicated that they would appreciate a regular update on new developments or scientific progress in the field of HNPCC, in addition to this protocol (see chapter 3.4).

Clearly, the absence or presence of a mutation is of considerable medical significance. Healthy individuals not carrying the mutation can be dismissed from medical surveillance, sparing them from invasive screening and reducing health-care costs[272]. Importantly, mutation carriers can benefit from medical surveillance programs. In 42 healthy Dutch HNPCC carriers, the compliance for colon screening increased from 31% before testing to 88% after testing (see chapter 3.4).

Genetic testing may also have a considerable psychological impact. Knowledge of having a cancer predisposition increases cancer worry. Several studies showed that most tested individuals are able to cope with a positive test result on the short-term, particularly in HNPCC[12, 13, 171]. After a short period of increased distress after the test result disclosure, the distress generally declines to levels comparable with those of non-carriers at one year follow-up[14]. Like in non-carriers, the ability to cope with a positive test result, is closely related to the ability to cope with problems overall, and to pre-test distress[171]. Also, on the long term the majority of Dutch mutation carrier was able to cope with being a gene carrier (chapter 3.4). In our study, about 10% of the mutation carriers experienced cancer worry on the long term. This was significantly associated with a high perceived risk of colorectal cancer. Regret of being tested occurred in 6 of 70 mutation carriers (9%) tested at our department (chapter 3.4). Notably, in 4 of these 6 mutation carriers, serious cancer events had happened in the family since their personal test disclosure. Events in the family are also known to influence perceived cancer risk[196].

Since the majority of the unaffected at risk relatives will receive a negative test result[329], most test applicants are relieved from fear. However, also non-carriers might experience adverse effects of testing. Especially if non-carriers show high pre-test distress and less effective coping strategies, they are at increased risk of post-test psychological distress and complex emotions summarised as "survivors guilt"[14, 171, 178, 289]. Non-carriers might feel guilty because they do not have the predisposition while close relatives have. This can have a large impact on family relationships; people even may become alienated from their family[178]. The relief of personal risk may also activate unresolved grief, and a few non-carriers will continue to have cancer fear[172].

Genetic testing may have considerable social consequences. First of all at the family-level, where several conflicts may arise. To search for the familial germline MMR mutation, co-operation of an affected relative often needs to be sought. Relatives may be unwilling to co-operate, depending on prior relationships and also on personal views. Sometimes unaffected relatives put too much pressure on their affected relatives to obtain their co-operation. Different coping strategies of relatives may disturb relationships. Informing children and family members about a genetic cancer risk is generally experienced as a burden, especially if more distant family members are concerned. There is no legal duty to inform relatives about a MMR gene mutation in the family, but most family members and professionals feel a moral duty to do so[57]. Peterson et al.[229] interviewed 39 members of 5 mutation positive HNPCC families. All participants had suspected a hereditary predisposition for cancer in their family prior to the identification of the HNPCC gene mutation. The pattern of communicating the news was

the same as the pattern used to communicate other non-urgent family news. The information was experienced as private but not secret and discussed first with the nuclear family members. Individuals who are encouraged by their relatives to seek genetic counselling seem to opt for genetic testing more frequently[85, 229].
Another important issue is the (fear of) employment and insurance discrimination. There is large international variation in legislation concerning individuals with a genetic predisposition to cancer. In the Netherlands, employers or insurers are forbidden by law to exclude individuals with a genetic predisposition for cancer from jobs or health insurance. For life or disability insurances, no questions about genetic predispositions may be asked by insurance employees for insurances below a certain limit (∈160.00 for life insurance and ∈32.000 in the first year of a disability insurance and ∈22.000 the following years). To our experience, possible future employment and insurance discrimination is an increasingly important reason to postpone or refrain from testing. Of 10 Dutch healthy HNPCC carriers opting for life or disability insurance or mortgage, 4 mentioned some trouble (see chapter 3.4). This is confirmed by Hadley et al.[85] who found potential effect on health insurance as the most important reason to refrain from testing in 50% risk carriers in 15 American families.