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CLINICAL ASPECTS OF LYNCH SYNDROME

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Clinical aspects of Lynch syndrome

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To Martin, Simon, Amanda and Raoul
ABSTRACT

Colorectal cancer is the second most common cancer in women and the third most common cancer in men worldwide, with 1.8 million new cases and almost 861000 deaths in 2018. Approximately 5–10% of the annual colorectal cancer burden can be attributed to inherited high risk germline mutations. The most common inherited colon cancer syndrome is Lynch syndrome, which accounts for up to 5% of all CRCs. Lynch syndrome displays both genotypic and phenotypic heterogeneity and can be suspected on the basis of a strong family history of colorectal- or endometrial cancer, but also of other tumors. Genetic counselling is recommended for families with Lynch syndrome, to provide the patient and family members with information about cancers risk and options for surveillance and management.

In paper I we compared disease associated haplotypes in families from Sweden, Germany and France, all carrying the MLH1 mutation c.2059C>T, in order to elucidate if this is a founder mutation. When analyzing the haplotype in the families with Swedish descent, a shared region of approximately 0.9–2.8 Mb was identified. The MLH1 c.2059C>T mutation thus act as a founder in the Swedish population, but is also found in Europe, Asia and Australia, indicating that this is a recurring mutation globally albeit with a very low allele frequency.

In paper II and III we investigated if genetic anticipation was part of the clinical picture in a Swedish cohort of Lynch syndrome families, as well as in a larger European cohort of PMS2 mutation carriers. In paper II, a total 1003 proven mutation carriers from 239 families with Lynch syndrome were included. An anticipation effect of 2.55 years and hazard rate of 1.33 between generations was seen in families with MSH2 mutation. In addition, an anticipation effect of 7.33 years and a hazard ration of 1.86 per generation was shown in families with PMS2 mutation. In paper III, 637 individuals from 123 families with PMS2 mutation were recruited from Netherlands, Norway, Germany, Denmark, Spain, including the Swedish PMS2 patients in paper II. Participants were assigned mutation probabilities in cases of unknown carrier status. As opposed to the result in paper II, an anticipation effect initially shown in a crude analysis was no longer statistically significant when corrections were made for gender and birth cohort.

In paper IV we characterized the tumor spectrum, excluding colorectal- and endometrial cancers, in a nationwide cohort of 235 Swedish Lynch syndrome families. Data was stratified for gender, primary cancer, age, and mutated gene. Relative proportions of specific cancer types were compared to corresponding proportions in the reference population from the national Swedish Cancer Registry. Individuals of both sexes in our cohort had a higher proportion of gastric cancer, small bowel cancer and urinary tract cancer compared to the general population. In female mutation carriers, the proportion of ovarian cancer and non-melanoma skin cancer was increased compared to the general population.

Key words: Lynch syndrome, founder mutation, genetic anticipation, MSI, MLH1, MSH2, MSH6, PMS2
LIST OF SCIENTIFIC PAPERS


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AFAP</td>
<td>Attenuated FAP</td>
</tr>
<tr>
<td>BRAF</td>
<td>v-raf murine sarcoma viral oncogene homolog B</td>
</tr>
<tr>
<td>RB1</td>
<td>Retinoblastoma 1</td>
</tr>
<tr>
<td>CIMP</td>
<td>CpG Island Methylator Phenotype</td>
</tr>
<tr>
<td>CIN</td>
<td>Chromosomal Instability Pathway</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal Cancer</td>
</tr>
<tr>
<td>DCC</td>
<td>Deleted in Colorectal Carcinoma</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DPC4</td>
<td>Deleted in Pancreatic Carcinoma 4</td>
</tr>
<tr>
<td>EPCAM</td>
<td>Epithelial Cell Adhesion Molecule</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial Adenomatous Polyposis</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary Non-Polyposis Colorectal Cancer</td>
</tr>
<tr>
<td>KRAS</td>
<td>Kirsten rat Sarcoma gene</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of Heterozygosity</td>
</tr>
<tr>
<td>LS</td>
<td>Lynch syndrome</td>
</tr>
<tr>
<td>MLH1</td>
<td>Mut L Homolog 1</td>
</tr>
<tr>
<td>MLH2</td>
<td>Mut L Homolog 2</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch Repair</td>
</tr>
<tr>
<td>MSH3</td>
<td>Mut S Homolog 3</td>
</tr>
<tr>
<td>MSH6</td>
<td>Mut L Homolog 6</td>
</tr>
<tr>
<td>MSI H/L</td>
<td>Microsatellite Instability- high/low</td>
</tr>
<tr>
<td>MUTYH</td>
<td>Mut Y Homolog</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PMS2</td>
<td>Postmeiotic Segregation 2</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor Protein P53</td>
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The names of genes are written in *italics* and their protein products are written in roman.
1 INTRODUCTION

1.1 CANCER INCIDENCE

Cancer is one of the leading causes of death and morbidity worldwide with an estimated global incidence of over 18 million cases in 2018, leading to over 9.5 million cancer-related deaths (1). In terms of incidence, cancers of the lung, female breast, and colorectum are the top three cancer types worldwide. In countries with rapidly growing economies a shift is seen from cancers related to infections or poverty, for example cancers in cervix, stomach and liver, to cancers that are related to a western lifestyle (2-4).

Since the 1970s, the incidence of cancer has increased by about 40 percent in Sweden, where prostate cancer, breast cancer, and cancer of the colon or rectum are the most common cancer forms (5). Aiming at early detection of tumors, there are national screening programs for breast cancer and cervical cancer in Sweden. From 2019, the Swedish National Board of Health and Welfare recommends a nationwide screening program for colorectal cancer, something that has been introduced in several other developed countries (6).

1.2 CANCER ETIOLOGY

A lot of research is focused on how cancer occur, why some types of tumors are more common than others, or more common in some populations compared to other populations. The emerging picture is that lifestyle and local environmental factors interacts with rare high-risk genetic variants and more common, low-risk genetic variants. Due to an ageing and growing population, as well as social and economic development in parts of the world, the global cancer burden is increasing. Unhealthy diets, obesity, low physical activity and smoking are contributing factors, in addition to improved diagnostics and screening. According to the World cancer research fund, over one third of the most common cancers worldwide could be prevented through a healthy lifestyle (1).

Approximately 80% of all cancer occurs sporadically, as a result of normal cellular processes and environmental factors that constantly alter the DNA in our cells. Most of the alterations are repaired, however a small fraction become permanent changes in the DNA sequence (7). Accumulating alterations are usually randomly distributed throughout the genome, and when somatic mutations (tumor restricted; not present in the germline) occur in genes with potential to initiate or maintain tumor development, cancer might develop (8).

In some families there appears to be more cancer than would be expected to occur by chance, i.e. there is a familial clustering. Shared moderate and low-penetrance genetic variants, together with environmental factors are thought to contribute to familial clustering, which represents about 10-15% of all cancer (9). The cancer risk for unaffected family members can be difficult to determine and various empirical models are available for this purpose (10-12).
In approximately 5-10% of all diagnosed cancer cases, patients have a strong family history of cancer caused by a well-defined hereditary cancer syndrome (9, 13). In most of the cases, the syndrome is caused by germline mutations inherited from parent to offspring in an autosomal dominant manner. Those mutations increase the risk of developing certain tumors, commonly with an early onset. Noteworthy, cancer is a common disease and depending on cancer type predominant in the family, the definition of a strong family history varies. The subject of this thesis is Lynch Syndrome, which is one of the most common hereditary cancer syndromes. Lynch Syndrome predisposes primarily for colorectal cancer (CRC) and endometrial cancer (EC), but also several other types of cancer.

The difference between hereditary and sporadic cancers can be illustrated by Knudson’s two-hit theory of tumorigenesis, a model that relies on the concept that we inherit one allele from each parent (14). An inherited mutation in a gene involved in maintaining genomic integrity, such as cell cycle regulation, suppression of growth factors or regulation of signaling pathways, represents a first “hit”. When a somatic mutation occurs later in life, the second hit, both alleles of the gene become deactivated (Fig.1). This can result in that normal cells acquire capabilities such as resistance to cell-death, sustaining proliferative signaling, inducing the formation of new blood vessels and the ability to invade neighboring tissues (15).

Figure 1. Illustration of Knudson’s two-hit theory of tumorigenesis. Reprinted from S.Nowsheen, A.G. Georgakilas and E.S. Yang. Staying a Step Ahead of Cancer, Cancer Prevention - From Mechanisms to Translational Benefits (16) 2012, under the Creative Commons Attribution license (CC BY-NC-ND 4.0)
2 MOLECULAR GENETICS

The development of a tumor is a multistep process whereby an initial “gatekeeping” mutation enables a normal cell to outgrow the surrounding cells, by providing the cell with selective growth advantages (17). The process of cancer development has been compared with Darwinian evolution, influenced by inherited germline variants and somatic alterations acquired during tumor formation; new genetic changes are acquired as the cancer grows, and each change can contribute to new advantages for tumor formation (17).

Studies of human breast- and colorectal tumors have indicated that most genetic variation in a tumor is composed of a handful frequently mutated genes, and a much larger number of genes mutated at a lower frequency (18). There is a vast variation in specific genetic pathways and genes that can be altered across different tumor types, as well as between tumor samples (19). The genes that underlie the driving forces of carcinogenesis are commonly divided into two major groups; tumor suppressor genes that are inactivated by mutations, and proto-oncogenes that turn into oncogenes when activated by a mutation.

2.1 PROTO-ONCOGENES AND ONCOGENES

Early studies of RNA tumor viruses revealed genes that were capable of inducing cell transformation, when they were transferred to normal cells (20). This discovery led to the identification of proto-oncogenes, a cellular counterpart that is closely related to retroviral oncogenes (21). Proto-oncogenes encode proteins that are involved in cell proliferating functions such as regulation and progression of the cell cycle, cell division and differentiation. A mutation in a proto-oncogene can turn the gene to an oncogene, and this “gain of function”-mutation might lead to an increase in gene expression or render the gene constitutively active (e.g. producing inappropriate stimulatory signals in response to growth factors). Such an activating mutation act dominantly, meaning that one mutated allele usually is sufficient to increase the selective growth advantage of the cell. The proto-oncogenes HRAS, KRAS and NRAS encode the Ras protein family, which is involved in cell proliferation and cell survival. Those genes are among the earliest genes mutated in a variety of cancers, and act as prototypic oncogenes in about one-third of all human cancers (22).

2.2 TUMOR SUPPRESSOR GENES

The function of tumor suppressor genes is to protect cells from degenerating into cancer cells, by coding for proteins that inhibit cell proliferation. Those genes are generally involved in cell cycle regulation, control of apoptosis, suppression of growth factors and regulation of signaling pathways. When the protein product of a tumor suppressor become functionally inactivated by mutations, this “loss of function” promotes abnormal proliferation of tumor cells. One of the
first tumor suppressor genes discovered, the retinoblastoma gene \((RB1)\) located on chromosome 13, was identified by studies of the rare childhood eye tumor retinoblastoma (23). Based on statistical studies of this tumor in children, Dr. Alfred Knudson proposed that the development of retinoblastoma requires loss of both functional copies of the retinoblastoma gene (14). While gain-of-function mutations are often dominant (as in the case of oncogenes), loss-of-function mutations usually are recessive.

In the inherited form of retinoblastoma, an inherited \(RB1\)-mutation represents the first hit and a somatic tumor-restricted mutation represents the second hit, in accordance with Knudson’s two-hit theory. The second hit results in loss of the remaining wild type allele (loss of heterozygosity; LOH) and is commonly a result of a partial or complete chromosomal deletion or epigenetic silencing through hypermethylation (24) (25). The two-hit hypothesis was later shown to be valid also for other tumor suppressor genes (26). Another well-known tumor suppressor gene is \(TP53\), which is frequently inactivated in several human cancers (27). Interestingly, \(TP53\) exhibit both oncogenic and tumor-suppressor functions and can have mutations conferring selective advantage when only one of the alleles are inactivated (28, 29).

### 2.2.1 Gatekeepers and caretakers

Tumor suppressor genes that are responsible for hereditary cancer syndromes can broadly be divided into two major categories, known as caretakers and gatekeepers (30). Gatekeepers normally inhibit uncontrolled cell growth by regulating cell proliferation and differentiation. The encoded proteins prevent cells from passing through cell cycle checkpoints, through arrested mitosis or induction of apoptosis (31). Several dominant hereditary cancer syndromes involve gatekeepers, such as the \(TP53\) gene or the \(APC\) gene involved in familial adenomatous polyposis coli (FAP) discussed below (32, 33).

While gatekeepers directly regulate tumor growth, caretakers maintain and protect the stability of the genome by increasing the fidelity of DNA replication and repair. Thus, caretakers prevent tumorigenesis more indirectly, as inactivation likely accelerate an accumulation of genetic changes within genes that directly affect cell proliferation and survival. Caretakers may sometimes be referred to as DNA stability genes or genome maintenance genes. One of all important caretaker systems in eukaryotes is the mismatch repair (MMR) machinery, which maintain genomic stability by removing mismatched nucleotides and deletion / insertion loops that arise during replication (34, 35). Inactivation of MMR results in an increase in the overall mutation rate (36). Mutations leading to loss of function of the MMR genes are central in the hereditary colorectal cancer syndrome Lynch syndrome (37).
3 CARCINOGENESIS IN COLON CANCER

In the last century, several more or less overlapping models of carcinogenesis have been proposed in the evolving field of cancer research (15, 38). Tumors of the colon and rectum are highly accessible, and tissue specimens can be obtained at different stages of tumor development for DNA- and histopathological analysis. This enabled early studies of how accumulation of somatic mutations is involved in cancer development, especially in colorectal tumors (39, 40). Studies of families with heritable CRC syndromes further expanded the knowledge of molecular pathways involved in both heritable and sporadic cancer, and how germline mutations accelerate cancer development. The subject of this thesis is the heritable colorectal cancer syndrome Lynch syndrome, and therefore emphasizes the role of DNA repair and genome instability in cancer progression. In this context, instability refers to a range of genetic changes, from point mutations to larger variation such as chromosome number- or structure changes (41).

According to pioneering studies by Vogelstein and coworkers in the 1980s- and 1990s, stepwise genetic alterations in critical genes underlies the initiation and progression of colorectal tumors. This early model, referred to as the adenoma-carcinoma sequence (40), illustrates a simplified process when normal tissue in the lining of the colon, acquires specific mutations and develop from hyperplastic epithelia and early adenomas into intermediate and late adenomas (Fig.2). In parallel with key gene mutations such as activation of oncogenes, or loss and gain of chromosomes, the transformation process continues into carcinoma (41). Two major molecular subtypes of genomic instability in CRCs have been recognized; the chromosomal instability (CIN) and microsatellite instability (MSI) (42, 43). These pathways of tumorigenesis are not mutually exclusive, and an alternative mechanism involved in genomic instability, the serrated/CIMP pathway, has also been presented (44, 45).

3.1 CHROMOSOME INSTABILITY (CIN) PATHWAY

Chromosomal instability (CIN) is detected in up to 85% of sporadic CRCs and represents the most prevalent form of genomic instability in CRC (46). As the name indicates, this pathway refers to a high rate of chromosome mis-segregation and involves genes that regulate chromosome stability at mitosis (41). The CIN pathway is characterized by changes in chromosome number (aneuploidy) or unbalanced structural rearrangements of chromosomes. A common early key mutation in the conventional adenoma–carcinoma pathway is inactivation of the tumor suppressor gene APC. This leads to activation of the WNT-signaling pathway, which is involved in e.g. proliferative and self-renewal signaling. APC is involved both in the hereditary CRC syndrome Familial Adenomatous Polyposis (FAP) and sporadic cancers (47). CIN tumors often have inactivating mutations in additional tumor suppressor genes such as TP53 and DCC/DPC4, and activating mutations oncogenes such as COX2 and KRAS (48). Other prominent genes that are involved in later stages of the adenoma–carcinoma progression
are SMAD2 and SMAD4. Both genes encode molecules in the transforming growth factor β (TGF-β) signaling pathway, which is involved in cell growth and differentiation among other cellular processes (Fig.2).

### 3.2 MICROSATELITE INSTABILITY (MSI) PATHWAY

About 15% of the sporadic CRCs and most patients with the colorectal cancer syndrome Lynch syndrome can be identified with a biomarker in tumor tissue called microsatellite instability (MSI) (49, 50). Microsatellite DNA is short repetitive DNA stretches of 1–10 nucleotides, present ~100,000 times throughout the human genome (51). Microsatellite DNA is evolutionary relevant due to its instability, and in order not to pass damaged DNA on to progeny cells, several cellular repair mechanisms have evolved. One DNA proof reading system, the mismatch repair system (MMR), act to increase the fidelity of DNA polymerase by repairing slippage mistakes and nucleotide base errors at DNA replication. Deficient MMR results in a high rate of mutations particularly in microsatellite DNA, leading to microsatellites of varying lengths. Microsatellite instability have been observed in CRC, gastric cancer, and EC, among other cancer types (52). Mutation in the MMR genes MLH1, MSH2, MSH6 and PMS2 leads to MSI, which is a characteristic phenotype of most tumors associated Lynch syndrome, discussed below (Fig.2). MSI can also be a result from epigenetic inactivation of the MLH1 promotor (53).

**Figure 2.** Conventional adenoma-to-carcinoma sequence. Reprinted with permission from De Palma FDE et al., Cancers, 2019 (44).
3.3 THE CpG ISLAND METHYLATION (CIMP) PATHWAY

The CpG island methylation (CIMP) pathway is responsible for the CpG island methylator phenotype, which is due to epigenetic instability. This pathway exhibits transcriptional silencing of tumor suppressor genes, including certain DNA repair genes, as a result of hypermethylated promoter CpG islands (54). The CIMP phenotype can be divided into CIMP-high and CIMP-low, based on certain thresholds of methylated markers (55). CIMP-low tumors have been associated with a high rate of KRAS mutations, while CIMP negative tumors generally have a high rate of p53 mutations (56). Many CIMP-high CRC tumors display MSI and mutations in the BRAF gene (p.V600E), thus involving both genetic and epigenetics in tumorigenesis (57). The majority of those tumors are thought to arise through methylation-associated inactivation of MLH1 in CIMP-high sessile serrated adenomas. (58).

The development of sporadic MSI CRC from sessile or traditional serrated adenomas is described by the serrated neoplasia pathway, as opposed to the classical adenoma-carcinoma sequence pathway in hereditary CRC displaying MSI (59).

Definitions of the CIN, MSI and CIMP pathways not mutually exclusive, and tumors can occasionally exhibit features of several pathways. Both CIMP-positive tumors and MSI CRCs can exhibit a high degree of chromosomal aberrations, while there are CIN-positive tumors with MSI (60-62). In addition, the CIMP phenotype accounts for most CIN-negative tumors displaying MSI.
CRC is the second most common cancer in women and the third most common cancer in men worldwide, with expected cumulative life time risks of 1.2% for men and 0.65% for women in 2018. It is 3 to 4 times more common in developed countries than in developing countries (63). Geographic differences in incidence display the highest rates in New Zealand, Australia, Europe and North America, while South-Central Asia and Africa have the lowest incidences (64). Cancer of the colon or rectum is the third most common form of cancer in Sweden, constituting up to 11% of newly diagnosed malignancies (65). The lifetime risk of colorectal cancer is approximately 5% in Sweden (66). In 2016, the median age for coloncancer was 74 years in Sweden, and approximately 90% of all patients were above 60 years old (67). Between 2007–2011, 4% of all colon cancers and 5% of all rectal cancers were diagnosed in patients younger than 50 years (65). The risk of developing cancer increases with age for most types of cancer. However, recent international reports have shown a decrease in incidence among older age groups, while the incidence has risen in the group with a younger age at onset (68, 69).

Prognosis is highly dependent upon stage of disease at diagnosis, and the 5-year survival rate ranges from 90% for tumors detected at the localized stage, to 10% for patients diagnosed with distant metastatic cancer. For patients diagnosed between 2005–2009 the survival rates from colon cancer were 61% respective 65% for men and women (65). For rectal cancer equivalent numbers were 61% and 64% respectively. The relative 5-year survival in Sweden have increased in recent decades. The increase can in part be attributed to screening or surveillance and improved primary and adjuvant treatments. However, there are global disparities and the increased survival is mostly seen in countries with access to modern specialized health care and high life expectancy.

The vast majority of tumors of the colon and rectum are carcinomas, of which approximately 90% are adenocarcinomas. Examples of adenocarcinomas are the mucinous carcinomas that produce mucin (which may be secreted or remain within the cells), non-gland-forming adenocarcinomas named signet ring cell carcinoma, and the medullary carcinoma subtype which is heavily infiltrated by small lymphocytes. Different morphologic variants come with different prognosis. For instance, the subtype signet ring cell carcinomas which has a poor prognosis overall, while the medullary adenocarcinoma subtype (often associated with MMR protein deficiency and Lynch syndrome) mostly is associated with a relatively favorable prognosis (70). There are standardized protocols when classifying tumors. Tumor grading describes the grade of differentiation of a tumor; a well differentiated tumor is described as low-grade, while a poorly differentiated tumor is described as high grades. The recommended staging system for CRC is the TNM system by the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC (71) This system considers the stage of the local tumor (T), spread to regional lymph nodes (N) and the occurrence of distant metastasis (M).
Approximately one third of all CRCs is thought to be related to heritable factors (72). Between 5%–10% are caused by high-risk mutations associated with known CRC syndromes, while the remaining supposedly are related to less penetrant, more common genetic variants (72, 73). The heritable CRC syndromes can broadly be subclassified into polyposis-associated syndromes and non-polyposis syndromes.

Among the major polyposis-associated syndromes are familial adenomatous polyposis (FAP), attenuated FAP (AFAP) and MUTYH-associated polyposis (MAP). In approximately 80% of those families, the syndrome is due to a germline mutation in the tumor suppressor gene APC or biallelic mutations in the MUTYH (74, 75). The FAP syndrome is characterized by the development of hundreds to thousands colorectal adenomas. While approximately 25% of FAP is caused by de novo mutations in APC, somatic mosaicism accounts for about 20% of unexplained adenomatous polyposis cases (76, 77). In the attenuated form of FAP (AFAP), patients have an inherited mutation that only involves the 5' or 3' region of the APC gene and typically have less than 100 adenomas. In MUTYH-associated polyposis (MAP), patients have bi-allelic inactivation of the MUTYH based-excision repair that result in a similar phenotype as in AFAP, displaying less than 100 polyps (78). The rarer polyposis-associated syndromes include hyperplastic polyposis and the hamartomatous polyposis conditions, including Peutz-Jeghers syndrome, Cowden syndrome and juvenile polyposis.

The non-polyposis syndrome Lynch Syndrome, described below, is caused by mutations in MMR genes. Noteworthy, adenomatous polyps are also a part of the LS phenotype, albeit with much fewer polyps than in the classical polyposis-associated syndromes.

5.1 LYNCH SYNDROME

Lynch syndrome (LS) is the most common hereditary CRC susceptibility syndrome and arises via the MSI pathway. LS was formerly known as hereditary non-polyposis colorectal cancer (HNPCC), highlighting the lesser amount of colon polyps in contrast to the polyposis syndromes. LS is inherited in an autosomal dominant manner and is defined in terms of having a pathogenic germline mutation in any of the DNA MMR genes MLH1 on chromosome 3p21, MSH2 on chromosome 2p16, MSH6 on chromosome 2p16 or PMS2 on chromosome 7p22 (79-86). A minority of LS cases are due to germline deletions in the 3' end of the EPCAM gene located directly upstream of MSH2, resulting in methylation-induced transcriptional silencing of MSH2 (87, 88).
5.1.1 History of Lynch syndrome

In the late nineteenth century, pathologist Aldred Warthin at the University of Michigan identified several families with colorectal- gastric- and uterine cancer, one of which was the family of his seamstress. Dr. Warthin published her pedigree in 1913 and labeled it “Family G” (Fig.3) because the family had immigrated from Germany to America (89). Family G displayed similarities with two large Midwestern families named N and M, reported in 1966 by Dr. Henry Lynch (90, 91). Families G, N, M, and other similar families were documented and categorized in the 1960s and 1970s, to help define the cardinal features of LS. In 1971 the term Cancer Family Syndrome was coined to describe the clustering of cancers in families, and after initial skepticism, the hereditary link finally became widely accepted (92). From 1984 the terms HNPCC and LS was used interchangeably (93, 94).

![Figure 3. The pedigree of Family G drawn by dr A.Warthin. Reprinted with permission from A. I Wolf et al., J. Coloproctol. (Rio J.) 33:2, 2013, under the Creative Commons Attribution license (CC BY-NC-ND 4.0)(95).](image)

The molecular genetics era of LS diagnostics began in 1993, when the LS genes MSH2 and MLH1 was mapped to chromosome 2p respective 3p by Peltomäki et al. and Lindblom et al. (79, 80). During the same time MSI was identified in LS-associated tumors, which was followed by the discovery of other MMR genes including MSH6 and PMS2 (96-98). The association between PMS2 and LS was described in 1994 but reliable genetic testing
for Lynch-associated $PMS2$ mutations was not offered until later. This was partly due to pseudogenes that are highly homologous to the 5' region of $PMS2$ complicating the analysis, but also to difficulties in identifying mutation carriers because of low penetrance (99-101).

Today, over 4000 unique germline MMR sequence variants have been deposited to date in the InSiGHT (International Society for Gastrointestinal Hereditary Tumors) DNA Variant Database, which a databases for LS-associated mutations maintained by The International Society for Gastrointestinal Hereditary Tumors (102).

5.1.2 Molecular basis MMR

The DNA MMR system is involved in the correction of base substitution- and small insertion-deletion mismatches that arise during DNA replication. It is a well conserved DNA proof reading system from bacteria to humans. In prokaryotes, the main MMR proteins shown in prokaryotes are MutS and MutL, which are working together in homodimers. In humans, five protein homologues to MutS (MSH2, MSH3, MSH4, MSH5 and MSH6) and four protein homologues to MutL (MLH1, MLH2, MLH3, PMS1 and PMS2) have been identified (103-106).

![Figure 4. Human DNA MMR protein complexes. Reprinted from P. Peltohämäki, Human Molecular Genetics, 2001, with permission from Oxford University Press (107).](image)

The MSH2 protein forms a heterodimer with MSH6 or MSH3, forming the MutSα respective the MutSβ complex. Both complexes can recognize mismatched base-pairs such as insertion/deletion loops of more than two bases. However, MutSα preferentially recognizes one or two-base insertion-deletion loops and single base mispairs while MutSβ recognizes
slippages at dinucleotide or longer repeats (Fig. 4). Upon mismatch binding, the MutSα and MutSβ complex recruits a protein heterodimer formed by MLH1 coupled with either PMS2, PMS1 or MLH3, named MutLa, MutLβ or MutLγ respectively. An excision machinery consisting of several other proteins (e.g. DNA Helicase, DNA polymerase and DNA ligase) is then recruited to the MutS-MutL complex, to remove and synthesize new DNA (107, 108).

The *MSH2* and *MLH1* genes are essential for the MutS- and MutL complex to function, and have therefore been defined as the major MMR genes, while *MSH6*, *PMS2*, *MLH3* and *MSH3* are known as the minor MMR genes (109).

### 5.1.3 Clinical manifestation and risk reduction

LS is characterized by genetic heterogeneity and a great variability in phenotypic manifestations. A combination of environmental factors and genetic factors may contribute to the heterogeneity, which complicates both recognition and management of patients. LS is characterized by a high lifetime risk for primarily CRC and ECs, accounting for 3%–5% of all CRCs and 2%–3% of all ECs (110-113). In addition, LS is associated with cancers of the ovary, stomach, urinary tract (kidney, renal pelvis, ureter and bladder) and small bowel, and less frequently cancers of the brain, biliary tract and pancreas (114). Rare skin lesions, such as sebaceous lesions and keratoacanthomas, can be indicative of a rare variant of LS named Muir Torres (115, 116). Biallelic MMR mutations (BMMRD), with homozygous or compound heterozygosity, manifests in a high risk of childhood cancer including hematological, cerebral and gastrointestinal tumors (117).

Cancer risks are commonly stratified according to tumor type, mutated MMR gene and gender. As our knowledge increase and diagnostic capabilities improve, estimated risk numbers for patients with LS are being adjusted from having up to ~80% risk of cancer by the age of 80, to sometimes lower figures depending on mutated gene (118).

#### 5.1.3.1 CRC in Lynch Syndrome

LS associated CRC often has a proximal colon location and an early age of diagnosis (~45 years) compared with sporadic CRC (109). Approximately 7% of the patients have more than one cancer by the time of diagnosis, in addition to an increased risk for metachronous CRCs (119). The lifetime risk of CRC depends on gender and mutated MMR gene. In a recent report based on data from the Prospective Lynch Syndrome Database, the cumulative CRC incidence for *MLH1* and *MSH2* mutation carriers at 75 years of age was estimated to 48% for females and 57% for males (120). Equivalent numbers for *MSH6* mutation carriers was estimated to 20% for females and 18% for males, while the risk for *PMS2* mutation carriers (male and females) was 10%.
The incidence of adenomas in LS families is reported to be similar to the incidence of adenomas in late-onset colon cancer families without MMR mutation (121, 122). However, it is thought that most LS-associated CRC arise from adenomatous polyps, but with an accelerated development to carcinoma (~2-3 years vs. ~8-10 years in sporadic CRC) (123, 124). LS-associated adenomas have a high degree of dysplasia and villous architecture, which together with the accelerated progression to carcinoma would be related to a relatively pronounced risk of malignancy (125). Similarly, colorectal tumors associated with LS commonly have histologic features like tumor-infiltrating lymphocytes, poorly differentiation with mucinous features, or a Crohn's-like lymphoid reaction (126). However, MSI-high tumors generally have a better prognosis compared to MSS sporadic tumors and the overall 10-year survival from LS-associated CRC is high (91%)(127).

5.1.3.1.1 Surveillance and risk reduction

Colonoscopy screening has proven to significantly reduce CRC incidence in individuals with LS, and according to several guidelines the recommended interval of colonoscopy with polypectomy is every 1 to 2 years (128-133). Due to the efficacy of control programs, prophylactic surgery such as colectomy is not a standard intervention. However, recent data suggest that some LS–associated colorectal tumors might develop directly as invasive cancer, as opposed to the classical adenoma-to-carcinoma pathway (134). In line with this, it has been shown that some patients develop tumors regardless of short surveillance intervals, which has started a discussion about optimal intervals and the preventive effect of intense control programs (135, 136). Most guidelines agree that screening programs with polypectomy should begin at between 20-25 years of age regardless of mutated MMR gene. Noteworthy, since lower rates of CRC are reported for MSH6 and PMS2 mutation carriers later initiation of screening for those individuals have been discussed (137, 138).

Data from observational studies indicate a potential role for aspirin in CRC prevention. In a multinational prospective study of chemoprevention, the CAPP2 study, MLH1 and MSH2 mutation carriers were randomly assigned to aspirin or aspirin placebo. The result showed that 600 mg aspirin per day for a mean of 25 months reduced cancer incidence (139). Further studies are dedicated to find the optimal dose and duration for chemoprevention in LS. Thus, for the CAPP3 study (http://www.capp3.org/) 3000 MMR mutation carriers will be invited to compare 100, 300 or 600 mg daily dose of aspirin (140).

5.1.3.2 Gynecological cancer in Lynch Syndrome

The most common extracolonic tumor in women with LS is EC followed by ovarian cancer, with different risks depending on mutated MMR gene. In a recent report from the Prospective Lynch Syndrome Database, the cumulative incidence of EC up to 75 years of age was similar for MLH1 and MSH2 mutation carriers; 37- 49%, while the risk was 41% respective 13% for
PMS2 and MSH6 mutation carriers (141). Equivalent numbers for ovarian cancer was 11% for MLH1 mutation carriers, 17% for MSH2 mutation carriers, and 3% respective 11% for PMS2 and MSH6 mutation carriers. Previous studies have reported the CRC risk associated with an EPCAM mutation to be similar to risks associated with MSH2 mutations, but a lower risk for endometrial- or other extracolonic cancer given that the deletion in EPCAM does not extend into MSH2 (142, 143).

The majority of LS-associated ECs are characterized by endometroid histology, similar to sporadic EC. However, also non-endometrioid subtypes have been reported, including serous carcinomas and clear cell carcinomas (144). MMR mutations increase the risk of different types of ovarian cancer of varying proportions between studies, including mucous, endometrioid and mixed or clear cell tumors (145).

5.1.3.2.1 Surveillance and risk reduction
Several studies have investigated the benefit of screening for endometrial and ovarian cancer in LS with techniques that include transvaginal ultrasonography, endometrial biopsies or tumor marker testing with cancer antigen 125 (CA-125). However, there are no consistent data that show an effect on cancer incidence or mortality (146). Screening for EC have shown modest sensitivity for detection of endometrial hyperplasia or carcinoma (147) and most guidelines recommend patients annual transvaginal ultrasound and endometrial biopsy at age 30 to 35, until risk reducing surgery at the completion of childbearing has been done (129, 131, 132).

Hysterectomy and salpingo-oophorectomy have shown to be efficient in preventing prevent endometrial- and ovarian cancer, but the survival benefit remains unclear (148). Noteworthy, data from the CAPP2 study indicated that aspirin may also have a preventive effect for endometrial and ovarian cancer in LS, but further studies are needed to evaluate this finding (140).

5.1.3.3 Other extracolonic cancers in Lynch Syndrome
LS predisposes to an increased risk of various extracolonic cancer, apart from endometrial and ovarian cancer. Sites includes the urothelium-, gastric-, pancreas-, hepatobiliary system, small intestine- sebaceous gland and brain (149). According to a recent report from the Prospective Lynch Syndrome Database, MSH2 carriers displayed the highest risk among the four MMR-genes, with approximately 18 % cumulative risk (up to 75 years of age) for ureter and kidney cancer in both sexes. Male carriers of a mutation in MLH1 or MSH2 were reported to have about 20% risk for cancer in the stomach, small bowel, bile duct, gallbladder and pancreas, while female carriers had half of that risk (141). Several studies have analyzed an association between LS and prostate cancer or female breast cancer, with different outcome. For instance, a previously proposed increase of breast cancer risk in MSH6 and PMS2 mutation carriers has
been questioned in a recent study showing cumulative risks across all four MMR genes, comparable with general population risks (141, 149-151). Prostate cancer is emerging as part of the LS tumor spectrum with lifetime risks reported to be up to 24% for MSH2 carriers, while risks are lower for other MMR genes (141, 152, 153).

A few patient cases have suggested an association of MLH3 variants and brain tumors, and a heterozygous MSH3 variant (in combination with a MSH2 variant) with the classical LS-phenotype (154, 155). In addition, it has been suggested that PMS2 and MSH6 mutation carriers might present with a phenotype similar to hereditary breast-ovarian cancer (156). These patients are likely to be missed by LS screening, which mainly focuses on the occurrences of CRC and EC.

Currently there is no convincing data to support surveillance for cancer in the urinary tract, pancreaticobiliary-, gastric-, small intestinal-, or brain tumors (157). Nevertheless, in the presence of a strong family history of a specific cancer, tailored surveillance might be considered according to some guidelines (128).

5.1.3.4 Genetic anticipation

There is a large variation in age of onset between and within families with LS, and genetic anticipation (progressively earlier age at onset in successive generations) have been suggested. Anticipation was described already in the case report on Family G, where dr. Warthin had noticed an earlier age at cancer occurrence in younger generations. Anticipation has also been suggested in hereditary cancer syndromes such as familial melanoma, ovarian- pancreatic- and breast cancer (158-162). Genetic anticipation is well known neurological and neuromuscular diseases, where repetitive trinucleotide DNA sequences that expands during meiosis is a proven underlying mechanism (163). However, an alternative mechanism leading to anticipation in hereditary cancer is not known at present. In paper II and III we analyze anticipation in Swedish respective European families with LS, trying to elucidate whether anticipation in part of the clinical picture in those cohorts. Several other reports have been published providing evidence for and against anticipation in LS, and the issue remains controversial (164, 165).

5.1.4 Clinical criteria

In the 1980's when the genetic background to LS was still unknown, the diagnose of LS was solely based on family history. In 1990 a collaborative effort was initiated by a group of researchers at the International Collaborative Group meeting in Amsterdam, in order to standardize clinical criteria for LS. The guidelines referred to as the Amsterdam I Criteria were published in 1991 and focused on a strong family history of CRC with a young age of onset (166). These criteria were later revised to Amsterdam II (167), to include extracolonic manifestations such as cancers of the endometrium, small bowel or pelvic-ureter system. The
guidelines evolved further as the knowledge on molecular and clinical characteristics of LS emerged (Table 1). The discovery of microsatellite instability as a hallmark of LS tumors led to the understanding that LS is a result of germline MMR gene mutations (50, 98, 168). This prompted the development of the Bethesda Guidelines, a list of guidelines to identify patients who should be tested for microsatellite instability (169, 170). Later, the Revised Bethesda Guidelines was developed to include panels for microsatellite instability testing, directions for molecular evaluation of tumors and germline DNA, and a clinical selection of cases (171).

However, neither the Amsterdam criteria nor Bethesda guidelines are solid screening tests for LS. When only clinical criteria is used to identify LS, there is a likelihood of chance clustering of cancer within a family as CRC is a relatively common malignancy. While the Bethesda guidelines are reported have high true positive rate (sensitivity) but too low true negative rate (specificity), computer-based empirical models have been suggested to detect high risk individuals, but with a low sensitivity (172, 173).
**Table 1. Clinical guidelines for identifying Lynch Syndrome.**

**Amsterdam I criteria (166)** requires at least three relatives with histologically verified CRC, and the following:

- Familial Adenomatous Polyposis has been ruled out
- One should be first-degree relative to the other two
- At least two successive generations are affected
- At least one of the affected is diagnosed <50 years of age

**Amsterdam II criteria (167)** requires at least three relatives with a Lynch associated cancer (colorectal, endometrial, small intestine, ureter, renal pelvis) verified by pathologic examination, and the following:

- Familial Adenomatous Polyposis has been ruled out in the CRC cases
- One should be first-degree relative to the other two
- At least two successive generations are affected
- At least one of the affected is diagnosed <50 years of age

**Revised Bethesda guidelines (171)** for testing of colorectal tumors for microsatellite instability (MSI) in families that meet the Amsterdam criteria:

- At least one CRC is diagnosed <50 years of age
- Presence of synchronous or metachronous LS- associated tumors* regardless of age
- CRC with the MSI-H histology** diagnosed in a patient <60 years of age
- CRC or LS-associated tumor* diagnosed under 50 years of age, in at least one first-degree relative
- CRC or LS-associated tumor* in two first- or second-degree relatives, regardless of age

* Endometrial, ovarian, gastric, hepatobiliary, or small-bowel cancer or transitional cell carcinoma of the renal pelvis or ureter LS-related tumors including colorectal, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract, and brain (usually glioblastoma as seen in Turcot syndrome) tumors, sebaceous gland adenomas and keratoacanthoma in Muir–Torre syndrome, and carcinoma of the small bowel

** Presence of tumor-infiltrating lymphocytes, Crohn disease-like lymphocytic reaction, mucinous or signet-ring differentiation, or medullary growth pattern.
5.1.5 Screening for Lynch syndrome

The two standard tests for screening of colorectal, endometrial or ovarian tumor tissue are the MSI and IHC, described below. According to the National Comprehensive Cancer Network (https://www.nccn.org/) IHC or MSI-testing is recommended for all patients diagnosed with CRC younger than 70 years, and in patients above 70 years if the family meets the Bethesda guidelines. MSI and/or MMR IHC detect over 90% of LS patients in sharp contrast to the clinical Amsterdam criteria (174) and if either MSI or MMR IHC is positive, the recommendation is to proceed with germline DNA testing (175, 176).

Universal CRC tumor screening have shown to be a cost-effective way to increase in the detection rate of LS, and is also recommended for EC (128, 177). In addition, there is data indicating that universal tumor screening of ovarian cancer and upper tract urothelial cancer might identify Lynch-associated cancers that would be missed using only clinical criteria (178, 179).

5.1.5.1 MSI assay

Cells with defective MMR display an inconsistent number of microsatellite nucleotide repeats, compared to normal tissue from the same individual. This can be assessed by PCR-based amplification of a panel with specific microsatellite markers, to compare the number of repeats at each locus, between tumor tissue and normal tissue.

In order to standardize MSI analysis, the National Cancer Institute (NCI) created a reference panel called the Bethesda panel in 1997. The Bethesda panel consists of five standardized loci with three dinucleotide repeats and two mononucleotide repeats (180). However, panels containing more mononucleotide markers are reported to have higher sensitivity and specificity in CRC diagnostics (181, 182).

Based on the extent of the genetic instability, MSI can be divided into MSI-High (MSI-H) or MSI-Low (MSI-L) (169, 183). Tumors with with ≥30–40 % marker instability are defined as MSI-high, while tumors with <30–40 % are MSI-low (169, 184). If the same number of repeats is present in each marker in both the tumor and the normal tissue, the tumor is microsatellite stable (MSS).

5.1.5.2 Immunohistochemistry

Loss of MMR protein expression can be detected by immunohistochemistry (IHC), which also is an indirect way to detect microsatellite stability (although not specific for LS). The principle of IHC is to use antibodies to bind antigens within tumor tissue sections and visualize the antibody-antigen interaction by any of various techniques for tissue staining.
IHC is a well-accepted initial screening test for LS, where antibodies to the MMR proteins \textit{MLH1}, \textit{MS2}, \textit{MSH6}, and \textit{PMS2} are used to determine expression of their corresponding genes in CRC and EC. The protein expression of the minor MMR genes depend the expression of \textit{MLH1} or \textit{MSH2} (185). Thus, if there is a mutation in any of the minor MMR genes, the major MMR genes are still expressed.

IHC relies on evaluation of staining patterns and intensity, and therefore requires experience on the part of the pathologist to interpret. For a clearly positive or negative result, the interpretation of IHC is straightforward. However, when there is ambiguous staining of the tissues and weak protein expression, results may be inconclusive (186).

5.1.5.3 Founder mutations

Founder mutations originates from a single ancestor (or are introduced into a population by a single individual) who passes it on to succeeding generations. While some founder mutations are detected in only a few families with a single origin, others are frequently found in specific geographic regions or countries (187). Founder mutations may affect the prevalence of LS in certain populations and influence the relative proportion of specific mutations. Therefore, founder mutations represent a useful tool in genetic screening, as testing for them as a first step in relevant populations may lower the cost of molecular diagnosis.

For instance, an exon 16 deletion in \textit{MLH1} in the Finnish population probably dates back over 1000 years. Together with another Finnish founder in \textit{MLH1}, c.454-1G>A, those mutations accounts for more than 50\% of all LS in Finland (188, 189). In the Ashkenazi Jewish population, the majority of LS cases appear to be caused by the mutations c.3959_3962delCAAG and c.3984_3987dupGTCA in \textit{MSH6} and the mutation c.1906G>C in \textit{MSH2} (190, 191). Another \textit{MSH2} mutation (c.942+3A>T) has been detected in 27\% of LS cases in the province of Newfoundland in Canada. This mutation is a common MMR mutation and repeatedly arises \textit{de novo} because of 26 consecutive adenines in the DNA strand, leading to DNA misalignment (192). Nevertheless, mutation carriers in Newfoundland share a common haplotype that is not present in carriers from England, Japan Hong Kong or Italy. Thus, the \textit{MSH2} c.942+3A>T presents a founder effect in this population (193, 194). At least three Swedish MMR founder mutations have previously been identified the MMR genes (195, 196). In paper I, we analyze the presumptive Swedish founder mutation c.2059C>T in \textit{MLH1}, identified in ten families of Swedish origin.

5.1.5.4 Next-Generation Sequencing

Identification of germline MMR mutations is critical for confirmation of LS and possibly the clinical management due to varying risks associated with different MMR mutations (197). In addition, it has been suggested that patients with MMR deficiency might benefit from immunotherapy (Fig. 5). Fortunately, the detection of LS has evolved from only using clinical
criteria to tumor-based screening with germline confirmation, and strategies may evolve further when costs for mutation screening drop further.

During the last decade next-generation sequencing of multigene panels for germline testing has emerged, as an alternative to traditional syndrome-specific gene testing. This has, though, raised the issue of management of unexpected findings (198). In addition, a number of the reported mutations are missense, silent or intronic variants with uncertain pathogenicity, and are therefore classified as Variants of Uncertain Significance (VUS) (199). The InSiGHT Variant Interpretation Committee (VIC) provides interpretation of variants, with classification criteria available at https://www.insight-group.org/criteria/. To facilitate the management of families with suspected LS, a collaborative effort was undertaken in 2014, to reclassify the MMR variants deposited in the InSiGHT database (102).

![Figure 5](image_url) Management of patients with LS. Reprinted with permission from Durato et al. Oncol Lett. 2019 Mar; 17(3), under the Creative Commons Attribution license (CC BY-NC-ND 4.0) (200).

### 5.1.6 Epidemiology

The heterogeneity in phenotype and penetrance of LS poses challenges in establishing population-based prevalences, however it appears that LS is relatively common across a diversity of ethnicities. Results from a recent study suggests that the prevalence of LS is higher in the European population compared to non-European ancestry populations (201). Minority populations are underrepresented in LS studies and prevalence data in those populations are therefore limited and may be biased. Moreover, despite similar rates of colorectal tumor testing,
minority patients are reported to be less likely to be referred to genetic counselling and testing (202).

Prevalence numbers of LS and MMR germline mutations varies depending on if patients are ascertainment based on family history of CRC/EC, or if measurements are made in the general population. For the most part, calculations have been made in the context of a family history with cancer, and LS-associated CRC and EC then accounts for 2% and 3% of the total cases respectively (198, 203-208). The majority of germline mutations have been detected in MLH1 and MSH2, while germline mutations in MSH6, PMS2 and EPCAM are represented in fewer LS-cases (198, 203, 205, 209-211). However, recent epidemiologic data indicates that LS-associated mutations are less penetrant and more common in the overall population than previously estimated. By analyzing clinical data from families of 5744 CRC cases (probands) in the Colon Cancer Family Registry (https://www.coloncfr.org/), Win et al. estimated the population prevalence to be 1/1946 for MLH1 mutations, 1/2841 for MSH2 mutations, 1/758 for MSH6 mutations, and 1/714 for PMS2 mutations (212). Altogether, the estimated the population prevalence for carrying any MMR mutation was estimated to be 1/279, making LS one of the most common heritable cancer syndromes. Noteworthy, mutations in PMS2 and MSH6 was reported to be the most common among MMR-mutations, in spite of being relatively uncommon among patients ascertained with LS based on personal history of cancer. This is in line with studies that suggest that carriers of PMS2 and MSH6 mutations have lower cancer risk or later age at onset, compared to MLH1 and MSH2 mutation carriers (101, 213-217). Further, this has led some authors to suggest modified guidelines for genetic counseling and surveillance for those genotypes (141).

5.1.7 Genetic counselling

Identification of patients with LS has greatly improved over the last two decades, still the syndrome is underdiagnosed, not only in minority populations (218). It is of utmost importance that clinicians recognize affected families, to offer genetic counselling and genetic screening, as well as surgeries and therapy to reduce mortality and morbidity.

Individuals with LS have no distinguishing traits other than an increased incidence of cancer. Due to a lack of specific phenotypic features, LS can be difficult to diagnose. Therefore, a comprehensive family history of all cancer among relatives, with attention paid to the cardinal features of LS, is important in facilitating the process. This allows for the identification of patterns of disease or possible differential diagnosis, for both patient and family. Ideally, a 3- to 4-generation family pedigree can be obtained including relatives with their current age or age at death, to identify patterns of inheritance. Additionally, both maternal and paternal ethnic backgrounds are of interest with particular attention to e.g Ashkenazi Jewish ancestry because of founder mutations in this population.

One cornerstone in genetic counselling is to establish an effective process for education regarding the inheritance and should focus on unbiased information and non-directive support
in the patient’s decision-making process (219). Individuals of reproductive age might benefit from information regarding prenatal testing options, including preimplantation genetic diagnosis (220).

The amount of information that reaches at-risk relatives depends upon how effective the intrafamilial communication is. Most patients notify first-degree family members (children, parents, siblings) but are less likely to inform family members that are more distant, because of lack of closeness and concerns that the information will not be understood (221). Thus, one challenge is to optimize the process of notification of at-risk relatives and subsequent genetic testing. Due to this, resources to assist patients in the process of notifying at-risk relatives, seeking appointment with genetic counselors and possibly give informed consent is under development in several countries.

One challenge to patient uptake is the significant heterogeneity among patients with LS, some of which can be attributed to which of the MMR genes is mutated. According to recent studies, aging patients display distinct patterns of cancer risk and survival depending on MMR mutation (137), information that should be taken into consideration during counselling. Adding to the complexity of LS, there is heterogeneity among family members carrying the same mutation. This possibly illustrates that environmental or polygenic factors and possibly genetic anticipation may influence phenotypic expression, something that we address in our study of anticipation in paper II and III.

Clearly, it takes a concerted effort from both patients and clinicians to improve current approaches to the diagnosis and prevention of LS but in the long term, lessons learned can be applied to other hereditary conditions.
6 AIM OF THE THESIS

The overall aim was to study the clinical picture in Swedish LS families, specifically the tumor spectrum, if the age at cancer onset is decreasing in successive generations, and a Swedish founder mutation. We hope that this work will contribute to further research, that in the long run improve the clinical management of individuals with LS. Future improvements that would be of high value is increased effectiveness of surveillance, by individually adjusted control programs and personalized risk estimations.

Specific aims in this thesis;

- Compare disease associated haplotypes in families from Sweden, Germany and France, all carrying the frequent MLH1 mutation c.2059C>T, in order to elucidate if this mutation act as a founder mutation in Sweden.

- Investigate if genetic anticipation is part of the clinical picture in a Swedish cohort of LS families, as well as in a larger international cohort of PMS2-mutation carriers.

- Characterize the tumor spectrum, excluding CRC- and ECs, in Swedish LS families.
7 MATERIALS AND METHODS

7.1 PAPER I

Patients in the haplotype analysis: Eight Swedish families that were identified at the Karolinska University Hospital in Stockholm, carrying the \( MLH1 \) c.2059C>T mutation, were enrolled in the study. In addition, one Finnish family with Swedish descent, three families from Germany and one family from France were included. In the Swedish families, 11 patients participated altogether. From the other families, one individual from each family participated, thus a total of 20 patients were analyzed.

Patients in prevalence study: The case cohort was composed of 2982 CRC patients that underwent surgery in Stockholm or Uppsala county between years 2004-2009. The control cohort was composed of 1610 anonymous blood donors from the same geographic region as the CRC patients, including 448 healthy spouses to CRC patients.

TaqMan analysis in prevalence study: Screening of the c.2059C>T mutation in the case cohort and control cohort was performed using TaqMan SNP Genotyping Assay.

Haplotype analysis: For genotyping, 19 polymorphic microsatellite markers surrounding the \( MLH1 \) gene. Microsatellites were amplified using PCR and analyzed using electrophoresis and a manually determined.

7.2 PAPER II

Patients: Participants were recruited from the regional department of Clinical genetics in Umeå, Uppsala, Stockholm, Linköping, Göteborg or Lund, where they had received genetic counselling and genetic testing between 1990-2013. In this population-based cohort, 1003 mutation carriers from 239 families with pathogenic MMR variants were identified with sufficient pathological and medical information to enroll in the study. Altogether, 96 \( MLH1 \) families, 89 \( MSH2 \) families plus one \( EPCAM \)-deletion family, 39 \( MSH6 \) families, 12 \( PMS2 \) families, and 2 families with mutation in both \( MLH1 \) and \( PMS2 \) were included.

Statistic methods: The patient follow-up period was defined as the time from birth until the time of first cancer diagnosis or censoring. Two models were used; a normal random effects model (NREM), and an extension of the Cox proportional hazards model with a family-level random effect (COX-R). NREM calculates the anticipation effect in mean change in age at diagnosis between consecutive generations while COX-R calculates the effect of anticipation between generations as a log-hazard ratio. Both models take generation (coded with respect to oldest observed generation in each family), sex and mutational status into account. All analysis were done in the R software package (222).
7.3 PAPER III

**Patients:** Data from 157 families with a total of 637 family members divided over three generations was collected from clinical genetic departments in the Netherlands, Norway, Germany, Sweden, Denmark, and Spain between the years 2009-2017. Clinical and pathologic diagnoses as well as polypectomy, and hysterectomy were confirmed using patient records. Not all family members were genetically tested but mutation probabilities were calculated from kinship coefficients, resulting in an estimated number of 360 mutation carriers in the sample.

**Method:** Within the families there were a total number of 123 CRCs. Genetic anticipation was estimated as the effect of generation on a person's hazard for age at first cancer diagnosis using a cox-type random effects model in the R package survival. The effect of sex and year of birth was included in a second adjusted analysis. Ascertainment bias was controlled by including individuals who had been at risk for at least 65 years and excluding the probands. As not all individuals were genetically tested, mutation probabilities based on kinship were used to avoid possible testing bias.

7.4 PAPER IV

**Patients:** Families were recruited from five of six regional department of Clinical genetics in Uppsala, Stockholm, Linköping, Göteborg or Lund where they had received genetic counselling and genetic testing. In total, 235 LS families participated, of which 445 individuals had a pathogenic variant, 343 were obligate carriers and 265 individuals were assigned a 50% carrier probability ($MLH1 n = 97$, $MSH2 n = 87$, $MSH6 n = 37$ and $PMS2 n = 14$).

**Statistical method:** The data was stratified for gender, primary cancer, age and mutated gene. The relative proportions of specific cancer types in relatives to the index patients were compared to the relative proportion in the general population, using data from the Swedish Cancer registry as reference. Comparison was done at two different time points (1970 and 2010) to compensate for difference in incidence rates over time. The population distribution of cancer was weighted by the age and sex of cases in the data (relatives to index cases). Using binominal distributions, confidence intervals (95%) were calculated for each cancer site and transformed to proportions (number of cancers at each site/ total number of cancers). The confidence intervals were then compared with corresponding confidence intervals for the general population.

Categorical data was tested for heterogeneity with chi-square tests and p-values were calculated using Monte Carlo simulations. All calculations were performed in the R software package (R core team, 2012).
8 RESULTS AND DISCUSSION

8.1 PAPER I

In the prevalence study, one individual in the cohort of 2982 CRC cases was carrying the mutation, a patient who subsequently was included in the haplotype study. There were no carriers of the MLH1 c.2059C>T mutation among the 1610 normal controls, which was expected as the global carrier status in the normal population is approximately 1/25,100 according to gnomAD database (https://gnomad.broadinstitute.org/).

When analyzing the haplotype in the eight Swedish families and the Finnish family with Swedish ancestry, a shared region of about 0.9–2.8 Mb was identified. Crossovers were seen in two different families at the 3´ or the 5´ end respectively. The downstream and upstream haplotypes in both families were consistent with a common haplotype, which might imply that the shared haplotype is larger than 2.9 Mb, and that there have been mutation events in the markers rather than recombination events in the two differing families. Mutation carriers in the French and German families did not share this haplotype, indicating that variant is a founder mutation in Sweden. The probable origin is in the north of Sweden since the earliest verified case in the Swedish families was in a geographical area in the middle-north of Sweden in the nineteenth century. One Swedish family and the Finnish family had ancestors from the northern part of Sweden. In addition, there are two additional families in the northern part of Sweden previously identified with this mutation, but samples from these families were not available when we did the haplotype analysis.

The MLH1 c.2059C>T mutation thus act as a founder in the Swedish population, but is also found in Europe, Asia and Australia, indicating that this is a recurring mutation globally albeit with a very low allele frequency. The effect of genetic drift is more pronounced in isolated populations than in heterogeneous populations. Still, mutations occurring in hotspots in the DNA sequence can be prevalent in diverse populations, and at the same time display a founder effect in populations, as heterogenous as the Swedish. Other examples of recurring founder mutations are a MSH2 mutation found in Portugal, and one MSH2 mutation in Newfoundland (223, 224). Both mutations displayed a founder effect in respective country but were also found in other populations in Europe or Asia on different haplotypes. Another founder mutation that occur in a heterogenous populations is a MLH1 mutation detected in America and Italy, but on different haplotypes (225).

Founder mutations and recurrent mutations could provide a tool in trying to identify the cancer risk attributable to specific mutations. In addition, single nucleotide polymorphisms present in shared haplotypes might provide valuable information when striving to predict risk in specific LS families. In line with this, a MLH1 intron 14 founder mutation identified in the Danish population has been suggested to be associated with a reduced risk of extracolonic tumors (226), while a nontruncating MLH1 missense mutation identified in Italy, was reported to causes a particularly aggressive phenotype and being frequently associated with extracolonic
cancers(227). Moreover, a recurrent MSH2 mutation at an intron 5 splice site is reported to be associated with a higher risk for CRC in male carriers, while female carriers seemed to have a relatively higher risk for ovarian cancer (228, 229). Optimally, both mutated MMR gene and phenotype observed in each family is taken into account when designing surveillance programs, and evaluation of the specific cancer risk attributable to specific mutations may be helpful in this work.

8.2 PAPER II AND III

In paper II, a total of 719 confirmed mutation carriers were diagnosed with at least one Lynch-associated cancer during the follow-up period, of which the first diagnosis was used in the analysis. The median age of first diagnosis in the whole cohort was 51 years (95% CI: 50–53). Stratified by mutated gene, it was 49 years in both MLH1 and MSH2 patients and 58 and 67 years, respectively, for MSH6 and PMS2 patients.

Both NREM and COX-R models suggest the presence of anticipation; a 2.1 year decrease in the age of diagnosis per generation and a hazard ratio of 1.19 between consecutive generations respectively, across the whole cohort. When the patients were stratified accorded to mutated gene, the confidence intervals for an anticipation effects of MSH2 and PMS2 lie far from their null values, while the effect of anticipation in MLH1 and MSH6 is less evident (Table 2).

<table>
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<th>Gene</th>
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<th>COX-R</th>
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<tbody>
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<td>MSH2</td>
<td>Estimate</td>
<td>-2.55 (0.003)</td>
</tr>
<tr>
<td></td>
<td>Wald p-value</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hazard ratio</td>
<td>1.33</td>
</tr>
<tr>
<td>MSH6</td>
<td>Estimate</td>
<td>-1.10 (0.366)</td>
</tr>
<tr>
<td></td>
<td>Wald p-value</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hazard ratio</td>
<td>0.99</td>
</tr>
<tr>
<td>PMS2</td>
<td>Estimate</td>
<td>-7.33 (0.014)</td>
</tr>
<tr>
<td></td>
<td>Wald p-value</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hazard ratio</td>
<td>1.86</td>
</tr>
</tbody>
</table>

In paper III, the analysis included 637 individuals from 152 families, with 123 CRCs in total. The median age of first CRC diagnosis was 70 years with a standard deviation of 12.94 (other Lynch-associated tumors were not part of the analysis). Not all individuals were genetically
tested, therefore mutation probabilities based on kinship were used to avoid possible testing bias. After weighing, the estimated number of mutation carriers in the sample was 360.

Genetic anticipation was estimated as the effect of generation on a person’s hazard for age at first cancer diagnosis, using a similar hazard model as in paper II, the cox-type random effects model. The effect of sex and year of birth was included in a second adjusted analysis. Hazard ratios were calculated by the Cox-type random effects model and shows an increase in the crude analysis for the second and third generation (Table 3). When corrected for gender and birth cohort, hazard ratios decreased and was no longer statistically significant. The adjusted analysis indicated that year of birth affected the result, equaling a roughly 5% increase of risk for every year (HR = 1.05; 95% CI = 1.02–1.07).

### Table 3. Anticipation effects of per generation in paper III.

<table>
<thead>
<tr>
<th>COX-model</th>
<th>Crude analysis</th>
<th>Analysis adjusted for gender and birth cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMS2-families</td>
<td>HR (95% CI)</td>
<td>(95% CI)</td>
</tr>
<tr>
<td>Generation 1</td>
<td>reference</td>
<td>reference</td>
</tr>
<tr>
<td>Generation 1</td>
<td>2.24 (1.16–4.33)</td>
<td>1.30 (0.65–2.62)</td>
</tr>
<tr>
<td>Generation 1</td>
<td>2.64 (1.08–6.46)</td>
<td>1.07 (0.41–2.84)</td>
</tr>
</tbody>
</table>

When studying genetic anticipation, various biases might cause a false anticipation effect. In paper I, an anticipation effect was seen in Swedish families with *MSH2* and *PMS2* mutation, with the most prominent effect in families with *PMS2*-mutation. When a cohort with European families with *PMS2*-mutation were analyzed in paper III, the anticipation effect was not significant when corrected for birth cohort. A cohort effect has been a proposed bias in other studies of anticipation, and the phenomenon might be difficult to differentiate from anticipation. However, Boonstra and colleagues published a review of statistical methods used in studying anticipation, and confirmed the presence of anticipation in LS families in the Danish HNPCC-register using the most common methods (230). Further, a germline founder mutation in *MSH2* was reported to be clearly associated with anticipation in 4 LS families, in the absence of a birth cohort effect (231). In addition, a study of a large Danish LS cohort reports a progressive decrease in age at onset excluding birth cohort effects (232).

Other biases to take into account when analyzing anticipation is be the rising incidence of CRC, specifically in younger patients, that might be reflected also in the group of LS patients. Together with possibly underreported diagnosis in older generations, today’s improved screening with cascade testing of family members, those factors likely affect age at diagnosis in successive generations. The research design and statistical methods applied in paper II at the time was superior to many previous studies, however it has shortcomings compared to paper III, and vice versa. First of all, the strong anticipation effect found for *PMS2* in paper II might be questioned since there were only 12 *PMS2* families in the total sample, compared with 152 *PMS2* families in paper III. Still, the result in paper II reached statistical
significance, but results from studies with smaller sample sizes may in general be interpreted with caution. In addition, the larger sample size in paper III did allow for the exclusion of probands reducing bias of including index patients.

Discrepancies in result between different studies may be due to the different study designs and different inclusion criteria. Both study II and III calculated the effect of generation on an individual’s hazard of cancer diagnosis, in addition to a normal random effects model used in paper II. Paper II included confirmed mutation carriers in \( MLH1, MSH2, MSH6 \) and \( PMS2 \), while paper III assigned participants probabilities of carrying a mutation in \( PMS2 \) (the estimated number of carriers were 360). Thus, the cohort in paper III consisted exclusively of families with the \( PMS2 \) mutation, as opposed to paper II that included families with different mutations. The anticipation effect for \( MSH2 \) in paper II is therefore not in conflict with the results in paper III. Notably, 90 families with \( MSH2 \) were included in the study thus the findings do not suffer from the same risk of biases as the \( PMS2 \) cohort.

In contrast to triplet expansions, which is a well-known underlying mechanism to anticipation in neurological and neuromuscular diseases, the mechanism behind anticipation in hereditary cancer is largely unknown. In summary, a clinical observation of anticipation and statistical analyses in different studies cannot indicate the underlying mechanism in hereditary cancer. Until a possible molecular mechanism for genetic anticipation is identified, it will be difficult to elucidate whether the anticipation is due to genetic effects or sampling errors. Today, screening protocols for LS patients recommend starting endoscopic surveillance between 20-25 years of age, or 10 years before the earliest cancer diagnosis in the family, which may be sufficient if one would take anticipation into consideration.

8.3 PAPER IV

Several previous reports have analyzed the tumor spectrum in LS and in this retrospective study, we define the relative tumor frequencies in a nationwide Swedish cohort of LS families, excluding the most common cancers CRC and EC. Compared to the reference population from the Swedish Cancer Registry, individuals of both sexes in our cohort had a higher proportion of gastric cancer, small bowel cancer and urinary tract cancer. In female mutation carriers, the proportion of ovarian cancer and non-melanoma skin cancer was increased compared to the reference population.

When stratified by mutated gene, the number of PMS2 carriers with non-colorectal/non-ECs was too low for further analysis. However, \( MLH1 \) carriers had an elevated frequency of gastric, pancreas and small bowel cancer, and \( MSH2 \) carriers had an elevated proportion of gastric-, small bowel, urinary tract, non-melanoma skin (only in female carriers) and ovarian cancer. The proportion of gastric cancer in \( MSH6 \) carriers was higher compared to the general population but the difference was not statistically significant.
When dr. Warthin initially described LS kindreds he noted predominantly gastric and ECs, and to a much lesser extent CRC (only two patients had CRC). However, the distribution of LS-associated malignancies seems different today and an alternative picture also about extracolonic tumors is evolving. According to the Swedish cancer registry, the annual incidence for gastric cancer in the general population has decreased between 1970 and 2010, something that is reflected also in LS tumor spectrum. Of note, there are differences between Eastern and Western populations with regard to gastric cancer in LS patients. It is reported to be the most common or second most common extracolonic malignancy in Chinese and Brazilian LS populations, compared to western population where it presents as the third or fourth most common malignancy (233, 234).

MLH1 and MSH2 carriers in both sexes had an increased frequency of small bowel cancer in our cohort, compared to the reference population. The incidence of small bowel cancer varies in different studies, with numbers between 0.6-7% (235, 236). Despite a relatively low risk, it is clearly elevated in association with LS compared to the normal population. In a recent prospective study, cancers of the upper gastrointestinal cancers (including stomach, duodenum, bile duct, gall bladder or pancreas) were altogether diagnosed predominantly at old age with highest risks in MLH1 and MHS2 carriers (21 % risk and 10% risk respectively)(141). In MSH6 carriers the risk for upper gastrointestinal cancers was 7%, i.e still higher than the normal population, which was not confirmed in our Swedish cohort.

Ovarian cancer is one of the most common gynecologic cancers globally with a cumulative risk up to 75 yrs of age of 1.06% (5), after cervical and uterine cancer(1). Ovarian cancer has a much lower prevalence compared to breast cancer, but it three times more lethal and has the worst prognosis among gynecological cancers in (237). In the Swedish female LS cohort, ovarian cancer was elevated in the MSH6 and MSH2 group, but not in the MLH1.

The same trend was also seen in a large prospective study where MSH6 and MSH2 have higher frequency of ovarian cancer compared to MLH1(137). Incidences for ovarian cancer were 17% and 13% in MSH2 and MSH6 respectively while 10% in MLH1 carriers and occurred mainly premenopausally, similar to our study. Female carriers overall also had elevated frequencies of urinary tract cancer, as did MSH2 mutation carriers when the cohort was stratified by gene.

Cancer of the upper urinary (urinary bladder, ureter or kidney) tract is a well described malignancy of LS with recently reported risks of 8.0%, 24.9% and 11.0% in MLH1, MSH2 and MSH6 carriers respectively (137). However, numbers vary between different studies and several reports indicate that MSH2 mutations account for a majority of UC (238-240). Non-melanoma skin cancer also had an increased proportion in the female group of LS patients, and in the group of MSH2 carriers.

Among skin cancers described in LS cohorts the most notable is that of the Muir-Torre which often comes with at least one sebaceous neoplasms and typically at least one gastrointestinal or urological malignancy(241). Regarding pancreatic cancer, most cases are sporadic, but up to 10% are estimated to be related to hereditary factors(242). In the Swedish cohort we could
see a relative increase only in \textit{MLH1} carriers. A similar trend was shown in a recent prospective study, with a lifetime risk of 6.2 associated with \textit{MLH1}, while risks associated with \textit{MSH2} and \textit{MSH6} mutations amount to 0.5\% and 1.4\% respectively\cite{137}. Conversely, other reports suggest that mutations in \textit{MSH2} are responsible for the majority of Lynch associated pancreatic cancer, underscoring that there are potential variable phenotypes for different germ-line MMR mutations\cite{243}.

In summary, the tumor spectrum apparently varies depending on mutated gene, gender and age, in addition to the influence of individual and environmental factors. Also, the incidence of LS was thought to be relatively rare, a belief that is slowly changing. The number of tumor types included in LS is growing, allowing diagnostic criteria and surveillance to be a subject of discussion. This, in addition to variable ages at onset, is of high relevance during genetic counseling and in the strive for precision surveillance programs.
9 FUTURE PERSPECTIVES

Even with intense surveillance, colorectal cancer is reported to develop in between controls, in some patients. Recent data showed that the stage of CRC and interval since last colonoscopy were not correlated in patients, and despite surveillance with colonoscopy and polypectomy the lifetime risk of CRC (in MLH1 and MSH2 carriers) was approximately 50%. This raises questions regarding the molecular mechanisms behind the development of carcinomas. Important future research would aim at the different carcinogenetic mechanisms as opposed to the traditional adenoma-carcinoma pathway. In addition, case reports of spontaneous regression of sporadic CRC displaying MSI, which encourages future research on immune responses that might act in removing developing precancerous cells.

The Swedish MLH1 founder c.2059C>T identified in paper I did not segregate perfectly with disease in the investigated families, and other sequence variants (genetic modifiers) are likely influencing the penetrance of the known mutation. In the aim of improving the clinical surveillance of patients, it would be interesting to extend the mutation screening from the known pathological founder mutation to include other genetic modifiers in the screening of those families. Newer techniques, such as whole-genome sequencing (WGS) or whole-exome sequencing (WES) should preferably be used for this purpose.

It would also be interesting to extend the analysis in other MMR mutations displaying very different penetrance in different patients. For instance, PMS2 mutations are mostly detected in families not fulfilling Amsterdam criteria and were recently suggested to have no increased risk for CRC, endometrial, or ovarian cancer before the age of 50. Nevertheless, there are a number of PMS2-carriers reported with early-onset cancers. This highlights the variability of PMS2 penetrance and possible interaction effects with low penetrance mutations. If possible, identifying families with shared haplotypes might facilitate the search for modifying variants.

Identification of patients with LS has greatly improved over the last two decades, still the syndrome is underdiagnosed. One underlying factor is the phenotypic heterogeneity in LS, which is proposed to be influenced of both environmental and genetic factors. The suggested genetic anticipation in Lynch syndrome is debated, with some studies reporting anticipation while the effects have been ambiguous or absent in others. Recent studies adopting advanced research design and robust statistical methods still show conflicting results, which also is illustrated in the different results found in paper II and III. Possible future studies should include subjects with proven mutations in all the four MMR genes, with biases such as cohort bias taken into account. Future studies also need to include larger representative samples, preferably pooled from several centers and countries.
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