MISMATCH REPAIR DEFICIENT CANCER DIAGNOSTIC ASPECTS IN COLORECTAL CANCER AND THE ROLE OF UROLOGICAL CANCER IN LYNCH SYNDROME

Joost, Patrick

2015

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Mismatch Repair Deficient Cancer
Diagnostic Aspects in Colorectal Cancer and
the Role of Urological Cancer in Lynch Syndrome

Patrick Joost, MD

DOCTORAL DISSERTATION
By due permission of the Faculty of Medicine, Lund University, Sweden.
To be publicly defended in the Segerfalk Lecture Hall, Sölvegatan 19, BMC, Lund
on Friday the 22th of January 2016, at 9.00 a.m.

Faculty opponent
Professor Richard Palmqvist
Department of Pathology,
Umeå University, Sweden
Mismatch repair (MMR) deficiency is carcinogenic and can either have somatic/sporadic causes (i.e. epigenetic silencing or somatic inactivation) or hereditary causes (Lynch syndrome due to a germline mutation in one of the MMR genes - *MLH1*, *MSH2*, *MSH6*, *PMS2*). The identification of MMR defective colon cancer is clinically relevant for diagnostic, prognostic and potentially also for treatment-predictive purposes.

The aims of this thesis were to validate the application of the MMR index for the prediction of MMR deficiency in colon cancer (study I), to investigate heterogeneous MMR protein expression in colorectal cancer (study II) and to study urological cancers in Lynch Syndrome (studies III-V). The most common cancers in Lynch syndrome are colorectal cancer and endometrial cancer, but also other tumour types are linked to the syndrome. We investigated the roles of bladder cancer (study III), prostate cancer (study IV) and renal cell cancer (study V) in Lynch syndrome.

Study I confirmed that the MMR index is easy to apply and identifies MMR defective colon cancers with high sensitivity (93%) and specificity (76%). The MMR index evaluates features such as expanding growth pattern, lack of dirty necrosis, mucinous differentiation and presence of tumour-infiltrating lymphocytes. Study II showed that heterogeneous (retained/lost) MMR protein expression occurred in three distinct patterns, i.e. as intraglandular, clonal and compartmental protein expression. These patterns co-existed in 9/14 tumours and correlated to differences in the MMR status. Attention to this phenomenon is recommended to prevent false-positive or false-negative evaluations of MMR protein immunostaining. Studies III-V linked urinary bladder cancer, prostate cancer and renal cell cancer to Lynch syndrome families. The cancers frequently showed MMR defects in line with the underlying disease-predisposing mutation, indicating that these cancer types should be considered part of the Lynch syndrome tumour spectrum. Urothelial cancer development was predominantly linked to *MSH2* mutations. These findings should be considered in risk estimates and surveillance recommendations.

Key words: Mismatch repair, Lynch syndrome, immunohistochemistry, microsatellite instability, heterogeneity, cumulative incidence, colorectal cancer, urothelial cancer, prostate cancer, renal cell cancer

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Signature

Date December 7th, 2015
Mismatch Repair Deficient Cancer

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the Role of Urological Cancer in Lynch Syndrome

Patrick Joost, MD
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Original Studies

This thesis is based on the following studies referred to by their Roman numerals in the text:


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Abstract

Mismatch repair (MMR) deficiency is carcinogenic and can either have somatic/sporadic causes (i.e. epigenetic silencing or somatic inactivation) or hereditary causes (Lynch syndrome due to a germline mutation in one of the MMR genes - MLH1, MSH2, MSH6, PMS2). The identification of MMR defective colon cancer is clinically relevant for diagnostic, prognostic and potentially also for treatment-predictive purposes.

The aims of this thesis were to validate the application of the MMR index for the prediction of MMR deficiency in colon cancer (study I), to investigate heterogeneous MMR protein expression in colorectal cancer (study II) and to study urological cancers in Lynch Syndrome (studies III-V). The most common cancers in Lynch syndrome are colorectal cancer and endometrial cancer, but also other tumour types are linked to the syndrome. We investigated the roles of bladder cancer (study III), prostate cancer (study IV) and renal cell cancer (study V) in Lynch syndrome.

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Studies III-V linked urinary bladder cancer, prostate cancer and renal cell cancer to Lynch syndrome families. The cancers frequently showed MMR defects in line with the underlying disease-predisposing mutation, indicating that these cancer types should be considered part of the Lynch syndrome tumour spectrum. Urothelial cancer development was predominantly linked to MSH2 mutations. These findings should be considered in risk estimates and surveillance recommendations.
# Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>AFAP</td>
<td>Attenuated familial adenomatous polyposis</td>
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<tr>
<td>AJCC/UICC</td>
<td>American Joint Committee on Cancer/Union Internationale Contre le Cancer</td>
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<tr>
<td>AUC</td>
<td>Area under curve</td>
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<tr>
<td>BER</td>
<td>Base excision repair</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CIMP</td>
<td>CpG island methylator phenotype</td>
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<tr>
<td>CIN</td>
<td>Chromosomal instability</td>
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<tr>
<td>CIS</td>
<td>Carcinoma in situ</td>
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<td>CRC</td>
<td>Colorectal cancer</td>
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<tr>
<td>DI</td>
<td>DNA index</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EGD</td>
<td>Esophagogastroduodenoscopy</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
</tr>
<tr>
<td>FCCTX</td>
<td>Familial colorectal cancer type X</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin-fixed paraffin-embedded</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin &amp; Eosin</td>
</tr>
<tr>
<td>HGD</td>
<td>High-grade dysplasia</td>
</tr>
<tr>
<td>HNPPC</td>
<td>Hereditary Non-polyposis Colorectal Cancer</td>
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<tr>
<td>HPF</td>
<td>High-power field</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IDL</td>
<td>Insertion/deletion loop</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IRR</td>
<td>Incidence rate ratio</td>
</tr>
<tr>
<td>ISUP</td>
<td>International Society of Urological Pathology</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
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<tr>
<td>LPF</td>
<td>Low-power field</td>
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<tr>
<td>LS</td>
<td>Lynch syndrome</td>
</tr>
<tr>
<td>MAP</td>
<td>MUTYH-associated polyposis</td>
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<tr>
<td>MLH1/3</td>
<td>MutL homologue 1/3</td>
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<td>MMR</td>
<td>Mismatch repair</td>
</tr>
<tr>
<td>MSH2-6</td>
<td>MutS homologue 2-6</td>
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<td>MSI</td>
<td>Microsatellite instability</td>
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<tr>
<td>MSS</td>
<td>Microsatellite stable</td>
</tr>
<tr>
<td>MutL/S</td>
<td>Mutator L/S</td>
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<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide excision repair</td>
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<tr>
<td>NGS</td>
<td>Next generation sequencing</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
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<tr>
<td>NPV</td>
<td>Negative predictive value</td>
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<tr>
<td>NSAI/NSAD</td>
<td>Nonsteroidal anti-inflammatory drug</td>
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<td>OR</td>
<td>Odds ratio</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PIN</td>
<td>Prostatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>PMSI/2</td>
<td>Post-meiotic segregation 1/2</td>
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<tr>
<td>PPV</td>
<td>Positive predictive value</td>
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<tr>
<td>PSA</td>
<td>Prostate-specific antigen</td>
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<tr>
<td>RCC</td>
<td>Renal cell carcinoma</td>
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<tr>
<td>RER</td>
<td>Replicative errors</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>ROC</td>
<td>Receiver operator curve</td>
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<tr>
<td>RTU</td>
<td>Ready to use</td>
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<tr>
<td>TIL</td>
<td>Tumour-infiltrating lymphocytes</td>
</tr>
<tr>
<td>UC</td>
<td>Urothelial cancer</td>
</tr>
<tr>
<td>UUT</td>
<td>Upper urinary tract</td>
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<tr>
<td>VHL</td>
<td>von Hippel-Lindau</td>
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<td>WHO</td>
<td>World Health Organization</td>
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## Thesis at a glance

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Summary in Swedish

Populärvetenskaplig sammanfattning

Felaktig DNA-reparation av typen mismatch repair (MMR) uppkommer i 15-17% av cancer i tjock- och ändtarm (kolorektalcancer). MMR defekter är av betydelse i tre situationer:

- MMR defekta tumörer har en bättre prognos än övrig tjock- och ändtarmscancer, vilket kan användas för att avgöra huruvida en patient skall erbjudas sk. adjuvant cytostatikabehandling i syfte att minska risken för tumöraterfall.
- Tumörerna har en sk. ”mutationsprofil” med en hög grad av små genetiska skador, vilket har visat sig vara av betydelse för svaret på behandling med immunterapi (sk. PD-1 hämmare).
- Tumörerna är starkt associerade med det ärfliga cancersyndromet Lynch syndrom som beräknas utgöra 2-4% av alla tjock- och ändtarmscancerar. Identifiering av dessa individer och familjer är av stor betydelse för att genom kontrollprogram med hög grad av kostnadseffektivitet kunna minska sjuklighet och dödlighet i cancer.

Avhandlingen studerar tre aspekter av MMR defekt cancer:

- Möjligheten att identifiera MMR defekt tjocktarmscancer genom noggrann analys och klassificering av tumörens histopatologiska egenskaper (studie I).
- Betydelsen av ett variabelt uttryck av MMR proteiner inom samma tumör bedömt med immunhistokemisk färgning (studie II).
- Kopplingen mellan MMR defekta tumörer och Lynch syndrom i urologiska tumörer, inkluderande urotelial cancer i njurbäcken, urinledare och urinblåsa (Studie III), prostatacancer (studie IV) och njurcancer (studie V).
De metoder som har använts innefattar:

- Histopatologisk kartläggning med hjälp av standardfärgning (Hematoxylin & Eosin) samt kartläggning av tumördifilterande lymfocyter, "dirty nekros", tumörens växtsätt (rundat versus infiltrativt) och slembildning
- Immunhistokemisk MMR-proteinfärgning för MLH1, PMS2, MSH2 och MSH6
- Mikrodissektion för analys av MMR status i ett fåtal tumörkryptor
- DNA-extraktion
- Analys av mikrosatellitinstabilitet
- Riskberäkningar i relation till olika mutationsgrupper och i jämförelse med en matchad kontrollbefolkning
- Statistiska metoder

Resultat och diskussion

Studie 1

MMR defekter påvisades i 108/474 (22.8%) av tumörerna och var vanligare hos kvinnor, i högre ålder och i tumörer från högra delen av tjocktarmen. Flera histopatologiska egenskaper var överrepresenterade i MMR defekta tumörer; expanderande växtsätt (73.8% versus 7.6%), avsaknad av "dirty" nekros (80.6% versus 26.1%), mucinös/signet-ring cell differentiering (67.6% versus 26.3%) and tumör-difilterande lymfocyter (66.7% versus 16.9%). Dessa faktorer användes för att skapa ett MMR index för identifiering av MMR defekta tumörer. MMR index användes i 438 patienter och ≥4 faktorer identifierade MMR defekta tumörer med 93% känslighet och 76% specificitet. Reproducerbarhetsanalys visade ett kappa-värde 0.88.

Jämförelse med andra MMR-prediktionsmodeller utfördes i 200 tumörer. MMR index med gräns ≥4 faktorer föll väl ut med en yta under kurvan (AUC) på 0.94 för MMR index jämfört med 0.81 för modellerna PREDICT, 0.80 för REItest6, 0.70 för MsPath and 0.77 för MSI probability score. Sensitiviten varierade från 60-100% och specificiteten från 41-99%. MMR index var likvärdigt med modellerna PREDICT/simplified PREDICT (p=0.38/p=0.27) och med REItest6 (p>0.42) och significant bättre än MsPath (p<0.0001) och MSI probability score >1 (p<0.0001).
Sammanfattningsvis var

- ett expanderande växtsätt, tumörinfiltrerande lymfocyter, avsaknad av "dirty nekros" och muciöns/signet ring-cellsdifferentiering de starkaste prediktorerna för en MMR defekt tjocktarmcancer
- MMR index reproducerbart och användbart med resultat jämförbara med eller bättre än andra modeller
- MMR index ett säkert sätt att identifiera 39/40 MMR defekta tumörer

**Studie II**

Immunhistokemisk färgning används i ökande omfattning för att identifiera MMR defekta tumörer. I vissa tumörer har en heterogen färgning med varierande bild noterats. I studien undersöktes 14 tjock- och ändtarmscancrar med variabel färgning för att kartlägga hur varierande färgningar uttrycks och huruvida dessa kopplas till mikrosatellitinstabilitet (MSI).

Immunohistochemisk variabel färgning definierades i tre mönster; "intraglandulär" (bevarad/förlorad färgning i eller mellan körtlar), "klonal" (bevarad/förlorad färgning i hela körtlar eller körtelgrupper) och "kompartmentell" (bevarad/förlorad färgning i större tumörområden). Dessa olika mönster samvarierade i 9/14 tumörer. MSI identifierades i 13/14 tumörer och var delvis i överensstämmelse med färgningsmönstret. I vissa tumörer kunde den variabla färgningen kopplas till skillnader i differentiering.

Sammanfattningsvis är heterogen immunfärgning för MMR proteiner

- ovanligt, men viktigt att notera då det kan kopplas till skillnader i tumörens MMR status
- ett färgmönster som riskerar att ge såväl falskt positiva som falskt negativa tolkningar av immunhistokemiska färgningar
- ett fynd som kan uppträda i tre definierade mönster

**Studie III**

Cancer i urinvägarna kan uppkomma såväl i de övre urinvägarna (njurbäcken och urinledare) som i de nedre urinvägarna (urinblåsan och urinröret). Vi undersökte urinvägscancer i alla de 288 familjer i Danmark som diagnosticerats med Lynch syndrom. Studien undersökte frekvensen av dessa tumörer och kartlade vilka sjukdomsorsakande gener som låg bakom och hur stor andel av tumörerna som uppvisar MMR defekter. Därutöver bestämdes risken för urinvägscancer i relation till de olika gener som associerats med Lynch syndrom.
Totalt analyserades 48 urêtärcancrar, 34 njurbäckencancrar och 54 urinblåsecancrar från 97 patienter i 75 familjer. Av de 136 cancrrarna uppkom 106 (78%) i familjer som inte tidigare hade drabbats av sjukdomen. Hos 16 av patienterna uppkom flera tumörer samtidigt och hos 12 diagnosticerades fler urinvägscancrar över tiden.

Utveckling av urinvägscancer kunde kopplas till mutationer i genen MSH2, vilket återfanns i 73% av fallen. Cancer i de övre urinvägarna diagnosticerades i medelålder 62 år och 55% av patienterna var kvinnor. Urinbläsecancer diagnostiserades i en medelålder av 61 år och bland patienterna var 58% män och MSH2 mutationer fanns hos 69%.

Förlust av immunhistokemiskt uttryck för MMR proteiner påvisades i 93% av övre urinvägscancer och i 86% av urinbläsecancer. MSI var däremot mindre vanligt med fynd av MSI i 32% av tumörerna i de övre urinvägarna och i 20% av urinbläsecancer.

Risken för urinvägscancer studerades i relation till en ålders- och könsmatchad dansk population med användande av data från de nordiska cancerregistren. Risken för urinvägscancer vid 70 års ålder var 6.7%. De högsta riskerna noterades för individer med mutationer i MSH2.

Sammanfattningsvis visade studien att

- urinvägscancer diagnostiseras i 26% av alla Lynch syndrom familjer i Danmark med 40% av tumörerna i urinblåsan, 35% i urinledare och 25% i njurbäcken
- förlust av immunhistokemiskt uttryck för det muterade MMR proteinet identifieras i 93% av tumörerna i de övre urinvägarna och i 86% av urinbläsecancer
- immunohistokemi är betydligt mer känslig än MSI analys för identifiering av dessa tumörer
- tumörer såväl i de övre som i de nedre urinvägarna kan genom MMR defekter tydligt kopplas till Lynch syndrom med en risk på 6.7%
- urinvägscancer är starkt kopplad till MSH2 mutation, vilket återfanns i 73% av fallen

**Studie IV**

Betydelsen av prostatacancer vid Lynch syndrom är oklar, vilket motiverade analys av prostatacancer i familjer med Lynch syndrom. Bland 1609 män (677 mutatationsbärare och 932 förstgradssläktingar) från 288 Lynch syndrom familjer identifierades 28 prostatacancrar. Medelåldern vid diagnos var 63 år och Gleason graden var 6-10 (med Gleason score ≥8 i 7/10 MMR defekta cancrrar).
Prostatacancer uppkom hos individer med mutationer såväl i MLH1 som MSH2 och MSH6. MMR-analys kunde utföras på 16 tumörer med immunhistokemisk förlust i 69%, medan MSI endast påvisades i 2 tumörer. Risken för prostatacancer vid 70 års ålder beräknades till 3.7%.

Sammanfattningvis visade studien att

- två tredjedelar av prostatacancrar hos mutationsbärare och förstgradssläktingar med Lynch syndrom bär MMR defekter som indikerar att tumörerna utvecklats inom syndromet
- MSH2 var den vanligaste (46%) bakomliggande genetiska avvikelsen
- MSI analys visade svag korrelation med MMR-defekten i prostatacancer
- en stor andel av tumörerna visade hög malignitetsgrad, låg differentiering och tumör-infiltrerande lymfocyter
- prostatacancer ingår i det spektrum av tumörsjukdomar som associeras med Lynch syndrom

Studie V


Sammanfattningvis visar studien att:

- njurcancer är ovanligt vid Lynch syndrom, men en ökad incidens ratio kan påvisas jämfört med normalbefolkningen
- njurcancer kan utvecklas inom ramen för tumörspektrum vid Lynch syndrom
Aims

This thesis focuses on MMR defective tumours of the colorectum, the urinary tract, the prostate and the kidney. The overall aims were to assess histopathological features and MMR deficiency for diagnostic, prognostic and predictive applications.

The detailed aims were to:

- Define whether histopathological characteristics allow recognition of MMR defective colon cancers that develop after age 50 (study I)
- Compare the performance of the MMR index relative to other predictive models for the identification of MMR defective colon cancers (study I)
- Assess patterns of heterogeneous MMR protein expression in colorectal cancer (study II)
- Correlate heterogeneous aberrant MMR protein expression to microsatellite instability (MSI) (study II)
- Define the link between Lynch syndrome and urological cancer through assessment of MMR status, i.e. MMR protein expression analysis and MSI status evaluation, in cancer of the renal pelvis, the ureter, the urinary bladder, the prostate and the kidney (studies III-V)
- Determine the risk of urological cancer in relation to mutations in the different MMR genes (studies III and IV) and to a sex- and age-matched control cohort from the general population (study V).
Mismatch repair function

In 2015 the Nobel Prize in chemistry was awarded to the researchers Lindahl, Modrich and Sancar for the molecular mapping of the DNA repair machinery. Their work has led to fundamental insights into cellular function and provides a basis for development of novel cancer treatments. Molecular systems continuously surveil and repair our genetic information and thereby protect it from a chemical chaos. External causes, spontaneous errors and copy errors necessitate DNA repair mechanisms, which constitute a vital part of the cell’s genetic stability and functionality.

Several DNA repair mechanisms exist and in general take advantage of the fact that DNA is double-stranded with the same information present in both strands. Damages that affect one of these strands can accurately be repaired by excision and replacement with newly synthesized DNA using the complementary strand as template. All prokaryotic and eukaryotic organisms employ at least 3 excision mechanisms: MMR discovered by Modrich, base excision repair (BER) discovered by Lindahl and nucleotide excision repair (NER) discovered by Sancar [1].

The MMR mechanism reduces the error frequency during DNA copying thousand fold, but when constitutionally defective causes hereditary cancer. The MMR system is responsible for correcting base mismatches and insertion/deletion loops (IDLs) generated during DNA replication due to DNA polymerase slippage [2]. This system was first described in Escherichia coli and is highly conserved in prokaryotes and eukaryotes [3, 4]. The human MMR system consists of 2 interacting heterodimerising protein complexes, i.e. MutS (MSH2/MSH6 or MSH2/MSH3) and MutL (MLH1/ PMS2, MLH1/MLH3 or MLH1/PMS1) [5, 6]. The MutSα complex (MSH2 and MSH6) recognizes single-base mismatches and single-base IDLs, whereas the MutSβ (MSH2 and MSH3) complex recognizes larger IDLs consisting of 2 to 8 nucleotides [7]. MutSα binds preferably to MutLα (MLH1 and PMS2), whereas MutSβ predominantly interacts with MutLγ (MLH1 and MLH3), though the exact role of the different MutL heterodimers that bind to MutSβ is not fully understood [8]. The steps of MMR are illustrated in figure 1. The MutS complex recognizes the mismatch and forms a sliding clamp around the
DNA strand. The complex then binds to a MutL heterodimer and moves along the DNA chain until reaching the DNA polymerase complex. The MMR protein sliding clamp interacts with DNA polymerase, exonuclease-1 and proliferating cell nuclear antigen (PCNA). This new large complex excises the daughter strand all the way back to the mismatch. The MMR protein sliding clamp falls off and the mismatch is finally corrected by DNA resynthesis [8].

MLH1 and MSH2 are the 2 obligatory proteins in their respective heterodimer and their mutational or epigenetic inactivation leads to destabilisation of the corresponding binding partners and results in complete loss of MMR activity [6]. Loss of MLH1 leads to destabilisation/secondary loss of expression of PMS2, and loss of MSH2 disrupts expression of MSH6 and MSH3. By contrast, loss of MSH6 or MSH3 has no major impact on the stability of other MutS proteins. In case of mutational loss of MSH6, MSH2 can still bind to unaffected MSH3, preserving some functionality of the MMR system. Likewise, in case of mutational loss of PMS2, MLH1 can still bind to unaffected MutS proteins, i.e. MLH3 or PMS2 [6].

This pattern of primary and secondary losses of protein expression forms the basis for the interpretation of MMR protein immunohistochemistry. The redundancy of the MMR system with partially preserved function of the alternative MutS and MutL complexes may also explain the various degrees of MSI in cancers and the attenuated phenotype in Lynch syndrome caused by mutations in MSH6 and PMS2 [6].
Figure 1. Model of the mismatch repair system in humans – showing an example with a single-base mismatch. Adapted from Boland et al., 2010 [8].
Defective MMR induces MSI

MSI is induced by failure to repair errors that occur during replication of repetitive DNA sequences [9-11]. Mutational or epigenetic inactivation of certain MMR genes, including MLH1, PMS2, MSH2 and MSH6, typically results in MSI [12]. MSI is defined as changes in the length of microsatellites, i.e. small DNA repeat units consisting of 1-6 bases. Microsatellites are unique and uniform in length in different tissues from the same individual, but are polymorphic among different individuals. The deletion or insertion of repeated units in tumour DNA results in novel length alleles compared with non-tumour DNA. Microsatellites can be located in promoter sequences, exons, introns and 3′-untranslated regions or intergenic regions and most of these are considered to be silent. Intragenic alterations may, however be important regulators of gene expression by influencing the rate of transcription, the stability of RNA, the efficiency of splicing and the interaction between RNA and proteins. Microsatellites within intergenic regions may also have functional roles in chromatin organisation and recombination [13]. Several genes, e.g. BAX, CHK1, IGFR2, MLH3, MSH3, MSH6, PMS2, PTEN and TGF-β, contain microsatellites in their coding regions and represent mutation targets in MSI cancers [14-16].

There are abundant microsatellite loci that could potentially be used for MSI analysis distributed throughout the genome [17]. Initially, many different markers were used, but this led to variable MSI frequencies in the same type of cancer. This can most likely be explained by variations in the sensitivity of individual markers [18, 19].

Therefore, a National Cancer Institute (NCI) workshop in 1997 recommended the use of a reference panel, known as the Bethesda panel, that consisted of only 5 markers and developed guidelines for MSI classification [20]. The Bethesda panel consists of 2 mononucleotide markers (BAT-25 and BAT-26) and 3 dinucleotide markers (D2S123, D5S346, and D17S250).

Instability in 2 or more of the Bethesda markers was defined as MSI-high, no instability in any of the 5 markers as microsatellite stable (MSS), and instability in only one of the 5 markers as MSI-low. However, the significance of MSI-low is unclear [20]. Laiho et al. hypothesised that if a high number of different markers are used, most colorectal cancers would show some degree of MSI. Indeed, in an analysis of 90 tumours with 377 microsatellite markers 79% of the tumours displayed MSI in 1 to 11 markers [21].

A follow-up NCI workshop [22] recognised that the dinucleotide markers of the Bethesda panel are less sensitive and specific than mononucleotide markers for the identification of MSI-high tumours. Suraweera et al. proposed that a panel of 5 mononucleotide markers may be more sensitive in detecting MMR deficiency
A further advantage of mononucleotide markers is that they are often monomorphic or quasi-monomorphic, which allows for MSI analysis without corresponding normal tissue [23-26].

Bacher et al. [18] evaluated a set of 266 mono-, di-, tetra-, and penta-nucleotide microsatellite loci and confirmed that mononucleotide markers are more sensitive and specific. The group developed a fluorescent multiplex assay that uses 5 mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27). This assay, the MSI Analysis System (Promega Corp., Madison, WI), was used for studies II-V in this thesis. Other studies have subsequently confirmed a higher sensitivity and specificity of the MSI Analysis System compared with the Bethesda panel [27, 28]. The MSI Analysis System detects MMR deficiency with approximately 96% sensitivity and 99% specificity [27]. An example of a MSI analysis is shown in figure 2.

The frequency and degree of MSI in MMR defective cancers may also depend on the type of inactivation (mutational or epigenetic) and on the specific MMR gene affected. Whereas biallelic methylation of the MLH1 promoter results in epigenetic silencing of the MLH1 gene and widespread MSI [29], the mutations found in Lynch syndrome may be associated with various occurrences and degrees of MSI [30, 31]. Studies have shown that a variable fraction of colorectal cancers associated with MSH6 mutations were MSS or MSI-low [32-35].

The widespread instability throughout the genome of MSI cancers, with a high frequency of frameshift mutations within coding regions, also leads to production of a range of neoantigens and may explain the stronger immunogenicity in MSI cancers [36]. Frameshift neopeptides have been identified in MSI-high cancers and research has begun to develop vaccines based on these neopeptides. The humoral response may also provide the basis for serological testing in the diagnostics/monitoring of patients with MSI cancers [37, 38].
Figure 2. Example of a MSI analysis showing instability in all 5 mononucleotide markers of the MSI Analysis System. Each marker shows the presence of 2 major peaks, in which the blue shaded peak represents shorter microsatellite lengths in tumour cells and the non-shaded peak normal microsatellite lengths in non-neoplastic cells. In this case, a right-sided colon cancer, the tumour was designated MSI-high.
Defective MMR protein expression

Development of antibodies against the MMR proteins allows for immunohistochemical analysis of MMR protein expression. Since the MMR proteins functionally interact in heterodimers, mutations in \textit{MLH1} lead to loss of expression for MLH1 and PMS2 and mutations in \textit{MSH2} lead to loss of expression for MSH2 and MSH6 (table 1). Mutations in \textit{MSH6} and \textit{PMS2} may lead to loss of expression of the mutated gene only or be accompanied by loss of MSH2 and MLH1, respectively [39]. Hence the combined loss of expression of MLH1/PMS2 indicates an underlying defect in \textit{MLH1} or \textit{PMS2}, whereas the loss of PMS2 only suggests a mutation affecting this gene. Similarly, the combined loss of MSH2/MSH6 indicates a mutation in either of these 2 genes, whereas loss of only MSH6 points to a mutation in \textit{MSH6}. Certain mutations, particularly missense mutations (especially in \textit{MLH1}), may be associated with retained staining or partially reduced staining compared with the surrounding non-neoplastic cells [40, 41].

\textbf{Table 1.} Immunohistochemical patterns in MMR defective cancer

<table>
<thead>
<tr>
<th>Affected gene</th>
<th>Immunohistochemical expression</th>
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<tr>
<td></td>
<td>MHL1</td>
</tr>
<tr>
<td>\textit{MLH1}</td>
<td>Loss</td>
</tr>
<tr>
<td>\textit{MSH2}</td>
<td>Preserved</td>
</tr>
<tr>
<td>\textit{MSH6}</td>
<td>Preserved</td>
</tr>
<tr>
<td>\textit{PMS2}</td>
<td>Preserved or loss</td>
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</table>

MMR protein immunostaining identifies MMR defective tumours with approximately 92\% sensitivity and 95-100\% specificity (figure 3) [39]. Use of all 4 MMR proteins is generally recommended since the functional interaction described above can be used as a control and safety check. However, studies have also indicated that a first-line screening using only PMS2 and MSH6 achieves favourable sensitivity for the identification of MMR defective tumours [42-44].
Figure 3. Colon cancer demonstrating combined loss of MLH1 (A) and PMS2 (B) with retained staining for MSH2 (C) and MSH6 (D). This staining pattern represents the most common abnormal staining pattern in routine diagnostics and may be caused by \textit{MLH1} promoter hypermethylation or \textit{MLH1} mutation. Colon cancer with isolated loss of PMS2 (F) with retained staining for MLH1 (E), MSH2 (G) and MSH6 (H). This pattern is rare and suggests an underlying mutation in \textit{PMS2}. 
Germline and somatic causes of defective MMR

A defective MMR system can have either hereditary or somatic/sporadic causes.

Germline mutations

Lynch syndrome is an autosomal dominant cancer predisposition syndrome caused by a germline mutation in one of the MMR genes (MLH1, MSH2, MSH6, PMS2) or within the epithelial cell adhesion molecule (EPCAM) gene adjacent to the MSH2 gene [45-51]. MLH1 and MSH2 are the most commonly mutated genes in patients with Lynch syndrome accounting for ~75% of the mutations identified (~42% in MLH1 and ~33% in MSH2), whereas mutations in MSH6 and PMS2 account for ~18% and ~7%, respectively [52]. An additional somatic event, called “second hit”, in the wild-type allele of the target tissue is necessary to inactivate both MMR gene copies. This in turn predisposes to the accumulation of somatic mutations and finally results in the hypermutable phenotype observed in tumour tissues from mutation carriers [53].

The MLH1 gene is located on chromosome 3p21.3, has 19 exons and encodes a 756-amino acid protein. Over 200 different mutations in MLH1 have been reported in patients with Lynch syndrome [52, 54].

The MSH2 gene, located on chromosome 2p22–p21, consists of 15 exons and encodes a 934-amino acid protein. More than 150 different pathogenic mutations have been described [52, 55]. Individuals with mutations in the MSH2 gene have a high risk for developing extracolonic cancer and a slightly lower risk for colorectal cancer when compared with individuals with MLH1 mutations [56, 57]. Germline 3’ deletions of the EPCAM (TACSTD1) gene have been demonstrated to cause Lynch syndrome through secondary epigenetic silencing of the neighbouring MSH2 gene by promoter methylation [50, 51].

The MSH6 gene is located on chromosome 2p16, has 11 exons and encodes a 1360-amino acid protein. MSH6 mutation carriers are characterised by a lower risk for colorectal cancer, nearly equal risk for endometrial cancer and later age at diagnosis [35, 58].

The PMS2 gene on chromosome 7p22 has 14 exons and encodes a 862-amino acid protein [59]. Of note, a non-expressed pseudogene exists in the region of PMS2 and polymorphisms in this pseudogene may falsely be interpreted as PMS2 mutations [60]. PMS2 mutation carriers are characterised by a lower penetrance and elevated risk for colorectal cancer and endometrial cancer [59].

MMR gene mutations include missense mutations, nonsense mutations, splice site alterations, small insertions/deletions and large intragenic deletions [52, 54, 55,
A small subset of patients with loss of MLH1 expression and a Lynch syndrome phenotype, but without a germline mutation, harbours a constitutional MLH1 epimutation. These germline epimutations are characterised by soma-wide hypermethylation and silencing of a single allele of the MLH1 promoter in the absence of any DNA sequence changes [61, 62].

**Somatic inactivation**

Biallelic methylation of the MLH1 promoter is the most common cause of MLH1 inactivation and is present in 15-20% of all colon cancer, but is rare in rectal cancer [16, 63]. In tumours with MSI and/or loss of MLH1/PMS2 protein expression further analysis can help explain the underlying cause. Methylation analysis of the MLH1 promoter may distinguish a sporadic from a hereditary origin. Alternatively, BRAF V600E mutation analysis can be applied, since BRAF mutations are generally not observed in Lynch syndrome cases, but are present in approximately half of the somatically mutated colon cancers [64, 65]. MLH1 promoter methylation is not restricted to colon cancer and has also been described in several extracolonic cancers, i.e. gastric cancer and endometrial cancer [66, 67]. Recently, somatic mutations in MLH1 and MSH2 have been demonstrated in tumours with loss of MLH1 or MSH2 [68]. This implies that somatic mutation analysis of tumour tissue could be considered in tumours with unexplained MMR defects.

**Prognostic and predictive impact**

The prognostic and predictive value of MMR deficiency has mostly been investigated in patients with colorectal cancer. Although considerable frequencies of defective MMR have been described in different extracolorectal cancers, e.g. endometrial and urothelial cancer, little is known about the prognostic and predictive value of MMR deficiency in these tumours.

MMR deficient colorectal cancers have been found to have a favourable prognosis and to be less prone to metastases [69]. Several studies and large meta-analyses have shown that MMR deficiency is a positive prognostic factor in stage II-III tumours [70-75]. In advanced stage tumours with distant metastasis (stage IV), a recent meta-analysis indicates that MMR deficiency is a negative prognostic factor, likely driven by the presence of BRAF mutations [76].

The underlying mechanism is unknown, but MSI tumours have a stronger immunogenicity and commonly show an increased immune response with e.g. TIL and Crohn-like reactions, which may contribute to a favourable outcome [36, 77]. Mutations in B2M (β2-microglobulin), frequently found in MSI tumours, have
also been suggested to represent a possible mechanism since these mutations lead to an inability of antigen-presentation through HLA class I molecules, which in turn triggers tumour cell death mediated by natural killer (NK) cells [78]. The increased immune response may also be the reason for the higher yield of lymph nodes in resection specimens of stage I and II MMR defective cancers [79].

MMR has lately gained increased attention related to its predictive role. MMR defective tumours have been shown to show poor response to 5-FU, which constitutes the base in most gastrointestinal cancer chemotherapy regimens [80, 81]. Hence, MMR defective colorectal cancer stage II can be spared adjuvant treatment, particularly 5-FU monotherapy, whereas stage II tumours with risk factors or stage III tumours should be discussed for combination treatment because of their significantly higher risk for relapse [75, 80].

Recently, MMR deficient tumours have been demonstrated to respond to treatment with the anti-PD-1 immune checkpoint inhibitor pembrolizumab [82]. The results of the study suggest that the MMR status represents a treatment-predictive marker and that patients with MMR defective tumours may benefit from treatment with PD-1 inhibitors. The programmed cell death protein 1 (PD-1) is a cell surface receptor and part of a negative feedback system that inhibits autoimmunity by suppressing the T-cell mediated cytotoxic immune response [83]. It has been shown that the PD-1 pathway is up-regulated in different tumour types and that treatment with PD-1 inhibitors activate the anti-tumour immune response [84].
Lynch syndrome

Historical perspective

The first known documentation of a family with Lynch syndrome - “cancer family G” was made 1913 by Dr. Aldred S. Warthin, who was professor and chair of Pathology at the University of Michigan [85]. Inspired by the family history of his seamstress he conducted a study of hereditary cancer by analysing her pedigree and the corresponding pathological documentation of cancers in her family. He was the first to discover a link between gastrointestinal cancer, endometrial cancer and family history. However, despite an extended follow-up study by Warthin [86], “cancer family G” did not receive further attention from the scientific community.

Progress in the understanding of Lynch syndrome would come nearly half a century later when Dr. Henry T. Lynch, oncologist and professor of Medicine at the Creighton University in Omaha, and colleagues published their study “Hereditary factors in cancer. Study of 2 large midwestern kindreds” in 1966 [87]. Dr. Lynch was consulted by a gastroenterologist on a patient with a strong family history of colorectal cancer without apparent polyposis [88, 89]. The detailed pedigree analysis of this family formed the basis for the study and Lynch found a strong predilection for colorectal cancer and also noted the presence of other tumour types.

The data reminded Dr. Majorie W. Shaw, a medical geneticist at the University of Michigan at Ann Arbor, of a family with similar features, resulting in the collaborative study of “family N” and “family M”. For lack of a better term, the syndrome was initially referred to as “cancer family syndrome” [87, 89]. Lynch revisited Warthins’s “cancer family G” and published an update of the family in 1971, containing over 650 individuals [90]. Typical characteristics of the syndrome, such as (1) autosomal dominant inheritance, (2) early onset of cancer, (3) increased incidence of colorectal cancer endometrial cancer, and (4) increased risk for synchronous/metachronous tumours, were noted.

Lynch’s findings were in the beginning met with substantial scepticism, but gradually gained international recognition. In 1984 the term “Lynch syndrome” was suggested and the syndrome was initially divided into type I when only colorectal cancer occurred in a family and type II when extracolonic cancer (e.g. in the endometrium, ovaries, gastrointestinal tract and the urinary tract) also occurred [91]. In the following years several studies recognised and further characterised the syndrome [92-96].

In order to emphasize the hereditary nature and to distinguish Lynch syndrome from the polyposis syndromes such as familial adenomatous polyposis (FAP), it
was later renamed to “hereditary non-polyposis colorectal cancer” (HNPCC). In 1989 an international collaborative group on hereditary non-polyposis colorectal cancer (ICG-HNPCC) was founded and established criteria known as the “Amsterdam criteria I” for the diagnosis of the syndrome [97].

The molecular basis of Lynch syndrome was discovered in 1993 to 1994. The first loci linked to Lynch syndrome were identified on chromosomes 2p and 3p in 1993 [48, 98]. Thereafter, 3 studies independently identified a specific molecular phenotype in colorectal cancer with widespread alterations in the sequence length of repetitive sequences. The observation was variably described as “replicative errors” (RERs), “microsatellite instability” and “ubiquitous somatic mutations in simple repetitive sequences” [9-11]. During the same time period MSH2 on chromosome 2p and MLH1 on chromosome 3p were cloned and corresponding germline mutations were found in Lynch syndrome families [45-47, 49]. The identification of germline mutations in PMS2 and MSH6 followed [99, 100]. In 1999, the Amsterdam criteria were further expanded to include extracolonic tumours and were referred to the “Amsterdam criteria II” [101]. A standardised panel of microsatellite markers was defined and the Bethesda guidelines for testing of colorectal tumours for MSI were established by the NCI in 1997 [20, 102]. These guidelines were revised in 2004 [22] to incorporate family history and specific pathological features of colorectal cancer (table 3).

In 2009, the Jerusalem Workshop suggested a broader screening program in order to improve the identification of patients with Lynch syndrome. This is especially relevant for MSH6 or PMS2 mutation carriers, who typically present at a later age, and would not be selected for testing under the revised Bethesda guidelines. The workshop therefore recommended that either MMR protein immunohistochemistry (IHC) or MSI analysis should be performed on all colorectal cancers diagnosed before age 70 [103].

The term hereditary non-polyposis colorectal cancer (HNPCC) was typically used for families that either fulfilled the various clinical criteria to suspect an underlying MMR syndrome or had proven MMR gene mutations. To distinguish between these different family types, families with mutations were again redefined as Lynch syndrome families [104]. Families suspected of Lynch syndrome with MMR defective tumours, but without any identified MMR gene mutation, were defined as Lynch-like families. These families likely consist of a mixture of families with missed MMR gene mutations and cases with somatic MMR gene inactivation. Families that fulfil the Amsterdam criteria and show MMR proficient tumours have an unidentified genetic defect and are referred to as familial colorectal cancer type X (FCCTX) [105].
Clinical manifestation

Lynch syndrome has several unique clinical and pathological features. The syndrome is characterised by a high penetrance with 70-90% lifetime risk for cancer in mutation carriers [106-108]. Cancers related to Lynch syndrome typically develop at an earlier age (mean ~45 years) compared with corresponding cancers the general population and individuals with Lynch syndrome show high rates of multiple (synchronous and metachronous) primary tumours [106-108]. Colorectal cancer and endometrial cancer are 2 of the most common cancer types caused by Lynch syndrome [107, 108]. A wide cancer spectrum may be found in individuals/families with Lynch syndrome and cancers at several extracolorectal sites, e.g. the stomach, small bowel, biliary tract, urinary tract, ovaries and the brain can occur within the syndrome [107, 108]. The tumour spectrum, the cancer risk and the age at onset vary substantially depending on the sex and the MMR gene mutated. The tumour spectrum and cumulative risks in Lynch syndrome are illustrated in figure 4.

The lifetime risk of colorectal cancer for MLH1 and MSH2 gene mutation carriers is in the range of 30-74%, whereas lower risks (10-22% and 15-20%, respectively) have been found in patients with MSH6 and PMS2 gene mutations [35, 58, 109-112]. Male mutation carriers exhibit a higher lifetime risk for colorectal cancer than female mutation carriers [106, 109]. The mean age at diagnosis is 27-46 years for MLH1 and MSH2 gene mutation carriers, whereas MSH6 and PMS2 gene mutation carriers show a later onset for colorectal cancer with a mean age of 54-63 years and 47-66 years, respectively [58, 59, 109]. In Lynch syndrome, colorectal cancers are in 60-80% of the cases located in the proximal colon (proximal to the splenic flexure) [113]. Lynch syndrome patients are at a high risk (16-19% at 10 years; 41-47% at 20 years) of metachronous colorectal cancer after segmental surgical resection of the initial tumour [114, 115]. Adenomas of limited size can transform into adenocarcinoma within 35 months compared with 10-15 years in sporadic cancer [116].

The second most common malignancy in Lynch syndrome is endometrial cancer, which occurs in up to 71% of women with MSH6 gene mutations and in 54% with MLH1 or MSH2 gene mutations [35, 109]. There is a lower risk (15%) for PMS2 gene mutation carriers [59]. MSH6 mutation carriers are characterised by a later onset of endometrial cancer [35]. Lynch syndrome-associated endometrial cancers are predominantly of the endometroid subtype [117, 118]. Female MLH1 and MSH2 mutation carrier also have an increased risk for ovarian cancer with a cumulative lifetime risk of 4-20% [107, 108].

Several studies have reported an increased lifetime risk for urothelial cancers of the upper urinary tract in Lynch syndrome. The risk estimates range from 0.4% to 20.3%, with the highest risk in male carriers and those with an MSH2 mutation.
The role of bladder cancer in Lynch syndrome is uncertain though an increased risk has been suggested in some studies and MMR defective bladder cancers have been identified in individuals with Lynch syndrome. The risk for non-urothelial tumours was not increased. Estimates of the lifetime risk of bladder cancer in Lynch syndrome range from 0 to 16.4%, with the highest risk for MSH2 gene mutation carrier [119-123].

Lynch syndrome is a multi-tumour syndrome and as such a number of other tumour types may develop, though at low absolute frequencies. The role of prostate cancer is unresolved though a few studies suggest that there is an increased risk is ranging from 9 to 30%, with the highest risk for MSH2 gene mutation carriers [124-126]. An increased risk for adenocarcinomas of the kidney has been observed in one study of Lynch syndrome families, compared with the general population [106, 127]. MMR defects are rarely observed in kidney cancer and the age at onset of this cancer does not seem to be lower than that in sporadic tumours. Therefore, it has been proposed that kidney cancer may not be part of the tumour spectrum of Lynch syndrome [128, 129]. Increased lifetime risks of tumours in the stomach, small bowel, biliary tract, pancreas, breast and brain and of cutaneous sebaceous neoplasms in Lynch syndrome families have also been described [109, 130-134]. A high risk of gastric cancer was commonly observed in Lynch syndrome families in earlier studies, but the risk decreased over the years, possibly due to the decreasing incidence in Western countries [135]. The association of breast cancer with Lynch syndrome is unclear [136]; an increased lifetime risk has been reported in some studies [137], but not in others [130, 138]. However, early-onset breast cancer with an MSI phenotype has been described in Lynch syndrome [139, 140]. There is probably an association between sarcoma and Lynch syndrome, but the risk estimates are uncertain [141]. Turcot syndrome is a rare variant of Lynch syndrome with mutations in the APC gene or MMR genes; it is characterised by glioblastomas, medulloblastomas and colorectal tumours [142]. Another rare variant is the Muir-Torre syndrome linked to MLH1 and MSH2 mutations, which is characterised by typical skin lesions in the form of sebaceous gland tumours, keratoacantomas and additional colorectal cancer or other internal cancer [143].
Figure 4. Cumulative risks of tumours by age 70 years in Lynch syndrome. The corresponding risks in the general population are provided in parentheses. Based on data from Vasen et al., 2013 and Giardiello et al., 2014 [107, 108].
Identification of Lynch syndrome

Lynch syndrome is one of the most common cancer susceptibility syndromes with an estimated carrier frequency of 1/2000-1/660 in the general population [144]. A number of criteria, guidelines and detection strategies exist, but none are optimal and identification is challenged by extensive phenotypic variation and a broad spectrum of associated tumours.

The Amsterdam criteria

The Amsterdam criteria were developed in 1991 [97] and modified in 1999 [101] to identify and classify families with Lynch syndrome. They require clinical evaluation of the patient and the patient's pedigree for colorectal and other Lynch syndrome-associated cancers. The Amsterdam criteria were originally intended for use as research criteria, but became instrumental in identifying Lynch syndrome and are still widely used clinically (table 2). Amsterdam criteria II identify Lynch syndrome with 22% sensitivity and 98% specificity [117, 145-147]; the low sensitivity may mainly be due to small families, skipped generations and lack of the central triad of the required first-degree relatives.

Some 40% of the families that meet the Amsterdam criteria have MMR proficient tumours and no MMR gene mutation. Families with non-Lynch syndrome colorectal cancer have been termed “familial colorectal cancer type X” [105]. The families are characterised by MSS tumours, a lower relative risk for colorectal cancer, later onset and no evidence of excess of extracolorectal tumours. The genetic basis of this group is unknown.

Table 2. The Amsterdam criteria II [101]

<table>
<thead>
<tr>
<th>Amsterdam criteria II: Clinical criteria for the identification of families with Lynch syndrome*</th>
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<tbody>
<tr>
<td>≥3 relatives with an Lynch syndrome-associated cancer (Colorectal cancer, cancer of the endometrium, small bowel, ureter or renal pelvis)</td>
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<tr>
<td>1 should be a first-degree relative of the other 2</td>
</tr>
<tr>
<td>≥2 successive generations should be affected</td>
</tr>
<tr>
<td>≥1 diagnosed before the age of 50 years</td>
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<tr>
<td>Familial adenomatous polyposis should be excluded in the colorectal cancer case(s)</td>
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<tr>
<td>Tumours should be verified by pathological examination</td>
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</table>

*All criteria must be fulfilled.

The Bethesda guidelines

With the implementation of molecular tumour testing, the Bethesda guidelines were developed in 1997 and revised in 2004 to select patients with colorectal...
cancer who should undergo MSI testing [22, 102]. The guidelines are considerably broader, include the expanded spectrum of Lynch syndrome-associated tumours and incorporate the MSI morphology of colorectal cancer (table 3). The selection of patients who fulfil any one of criterion in the revised Bethesda guidelines has an 82% sensitivity and 77% specificity for the diagnosis of Lynch syndrome [117, 148].

**Table 3.** The revised Bethesda guidelines [22]

<table>
<thead>
<tr>
<th>The Revised Bethesda Guidelines: Clinical guide for the testing of colorectal tumours for MSI*</th>
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<tbody>
<tr>
<td>Colorectal cancer diagnosed in a patient who is less than 50 years of age</td>
</tr>
<tr>
<td>Presence of synchronous or metachronous colorectal or other Lynch syndrome-associated tumours, regardless of age (colorectal, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract and brain [usually glioblastoma as seen in Turcot syndrome] tumours, sebaceous gland adenomas and keratoacanthomas in Muir-Torre syndrome, and carcinoma of the small bowel)</td>
</tr>
<tr>
<td>Colorectal cancer with the MSI-H histology diagnosed in a patient who is less than 60 years of age (presence of tumour-infiltrating lymphocytes, Crohn’s-like lymphocytic reaction, mucinous/signet-ring differentiation or medullary growth pattern)</td>
</tr>
<tr>
<td>Colorectal cancer diagnosed in one or more first-degree relatives with a Lynch syndrome-related tumour, with one of the cancers being diagnosed under age 50 years</td>
</tr>
<tr>
<td>Colorectal cancer diagnosed in 2 or more first- or second-degree relatives with HNPCC-related tumours, regardless of age</td>
</tr>
</tbody>
</table>

*Fulfilment of only 1 criterion is necessary to warrant clinical testing.

The Amsterdam criteria II and the revised Bethesda guidelines are the most broadly applied strategies to identify Lynch syndrome tumours, though both have limitations. Both set of criteria fail to identify up to one-third of Lynch syndrome cases and suffer from the requirement of a comprehensive family history [149-152]. Although the Bethesda guidelines recommend MSI analysis in patients with colorectal cancer below age 50, but more than 50% of Lynch syndrome cases would escape detection by limiting MMR testing to this subset of patients [153].

With the development of MMR protein immunohistochemistry and an increased availability of molecular tests, the guidelines are primarily relevant for the homogenous classification of families. Broad-scale MMR assessment is introduced to improve the identification of patients with Lynch syndrome. The need for a broader screening program was also recognised by the Jerusalem Workshop (see chapter “Historical perspective”) and screening for MMR deficiency in colorectal cancers in patients below age 70 was suggested [103].

Population-based universal screening/reflex MMR testing of all newly diagnosed colorectal cancers at ≤70 years of age has been shown to be cost effective with substantial clinical benefit with different possible screening strategies [149, 154-159]. Regardless of the strategy, however, an ethics platform needs to be
developed that establishes whether informed patient consent is required before screening, given the potential impact when germline MMR gene alterations are identified [160, 161].

A common approach is to perform an initial immunohistochemical evaluation of MLH1, PMS2, MSH2 and MSH6 with referral to genetic counselling for patients whose tumours reveal the loss of PMS2, MSH2 and/or MSH6. In the 15-20% of cases with immunohistochemical loss of MLH1/PMS2 further analysis, by BRAF mutation analysis and/or MLH1 promoter methylation analysis may provide information to distinguish somatic alterations from germline defects (figure 5).

Figure 5. Example of universal screening algorithm for the identification of patients with Lynch syndrome. Adapted and modified from Bellcross et al., [162].

Germline genetic testing for Lynch syndrome is performed on blood samples and available mutation analyses include full-length sequencing of MLH1, PMS2,
MSH2 and MSH6, essays for large rearrangements, and additional analysis for EPCAM deletions. Genetic testing is recommended for at-risk patients when the test will influence their own medical management or that of their at-risk relatives [107]. The presence of a pathogenic germline mutation in 1 of the 4 MMR genes or in the EPCAM gene, confirms the diagnosis of Lynch syndrome and other at-risk relatives of the family can be tested for the same mutation through single-site, gene-specific DNA mutation analysis [107, 163].

Although germline mutation analysis is the gold standard in Lynch syndrome diagnostics, its application as a universal screening method is not feasible. Genetic testing is still too expensive, time-consuming and difficult to perform in such a setting. With new emerging DNA sequencing techniques, e.g. next generation sequencing (NGS) and lower costs of genetic tests, the current strategies in Lynch syndrome diagnostics may considerably change in the future.

Clinical management

The main rationale for genetic testing and counselling is to decrease the morbidity and mortality from cancer. Clinical management in Lynch syndrome consists primarily of surveillance (table 4), but may also include prophylactic surgery and chemoprevention [107, 108].

Colonoscopy has been found to effectively reduce the mortality from colorectal cancer by 65-71% [164-166]. Studies have shown that shorter screening intervals (≤2 years) were associated with an earlier stage at diagnosis and a lower risk of cancer development than longer intervals [167-169]. Current guidelines recommend that high-risk individuals should undergo colonoscopy with (1-2) year intervals from age 20-25 years [107, 108]. Because of the high risk of metachronous cancer, subtotal colectomy can be recommended following a diagnosis of colorectal cancer. The decision of extended surgery should consider patient’s age, preferences and ability to undergo future colonoscopy [170].

Endometrial cancer is the second most common cancer type in Lynch syndrome and is the first malignant diagnosis in half of female mutation carriers. Approximately 75% of women who develop endometrial cancer as part of Lynch syndrome present with stage I disease. The 5-year survival rate has been estimated as 88% [171, 172]. Due to the favourable prognosis, it is difficult to prove whether surveillance improves survival. Current guidelines recommend endometrial surveillance with pelvic examination and endometrial biopsy annually starting from age 30-35 years. Evidence for ovarian cancer surveillance is lacking (table 4), but hysterectomy and bilateral salpingo-oophorectomy is recommended for women who have finished childbearing or reached age 40 years [107, 108].
In the past gastric cancer was frequently observed in Lynch syndrome families, but the risk has decreased over time. A Dutch study found a 5.3-8% lifetime risk in mutation carriers, but no evidence for clustering in Lynch syndrome families [173]. In Sweden, there are no general surveillance recommendations, though esophagogastroduodenoscopy is occasionally recommended for such cases.

Screening for urinary tract cancer with urine cytology has in one retrospective study shown a poor sensitivity (29%) [174]. Annual analysis for haematuria using urinary dipsticks from age 45-50 years has been suggested [175]. No studies are available for the application of ultrasound. The current US guidelines recommend annually urine analysis for microscopic haematuria starting from age 30-35 years, though with a low strength of recommendation (table 4). The revised European guidelines do not recommend any surveillance outside the setting of a research project [108]. Surveillance for other tumour types is not recommended and has been left to depend on the individual expert decisions and the occurrence of these cancer types in the families.

Table 4. US and European Guidelines for surveillance of at-risk or affected persons with Lynch syndrome [107, 108]  

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Intervention</th>
<th>Recommendation</th>
<th>Strength of recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal cancer</td>
<td>Colonoscopy</td>
<td>Every 1-2 y beginning at age 20-25 or 2-5 y before the youngest age at diagnosis of CRC in the family if diagnosis before age 25</td>
<td>Strong</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Considerations:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Start at age 30 in MSH6 and at age 35 in PMS2 families</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Annual colonoscopy in MMR mutation carriers</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Moderate</td>
</tr>
<tr>
<td>Endometrial cancer¹</td>
<td>Pelvic examination with endometrial sampling</td>
<td>Annually beginning at age 30-35</td>
<td>Low</td>
</tr>
<tr>
<td>Ovarian cancer¹</td>
<td>Transvaginal ultrasound</td>
<td>Annually beginning at age 30-35</td>
<td>Low</td>
</tr>
<tr>
<td>Gastric cancer²</td>
<td>EGD with biopsy of the gastric antrum</td>
<td>Beginning at age 30-35 and subsequent surveillance every 2-3 y can be considered based on patient risk factors</td>
<td>Low</td>
</tr>
<tr>
<td>Urinary tract cancer³</td>
<td>Urine analysis</td>
<td>Annually beginning at age 30-35</td>
<td>Low</td>
</tr>
</tbody>
</table>

Abbreviations: CRC, colorectal cancer; EGD, esophagogastroduodenoscopy; y, years
1 revised European guideline: every 1-2 years beginning at age 35-40
2 revised European guideline: only in families from countries with a high incidence of gastric cancer, preferably in a research setting
3 revised European guideline: no surveillance (outside of research setting)
Chemoprevention with resistant starch and aspirin has been studied in individuals with Lynch syndrome. The CAPP2 trial evaluated the long-term effect (up to 4 years) of aspirin, resistant starch or both on adenoma and colorectal cancer development and found no effect [176]. However, longer follow-up revealed a decline of colorectal adenomas by 45% with a daily dose of 100-300 mg aspirin [177]. The CAPP3 trial started in 2014 and evaluates the optimum dose and duration of aspirin treatment over a period of 5-10 years. The US and European guidelines recommend that Lynch syndrome patients should consider daily medication with aspirin starting with their regular surveillance [107, 108]. Recently, a link has been suggested between benefit from NSAIDs and presence of PIK3CA mutations in the tumour tissue [178]. The role of this association in Lynch syndrome remains to be demonstrated, but there is potentially a link to the MSI pathway.

Tumour types in focus

**Colorectal cancer**

*Epidemiology and aetiology*

Colorectal cancer is one of the major causes of morbidity and mortality; worldwide it is the second most common cancer among females and the third most common among males [135]. The incidence is highly variable with the highest incidence rates in Europe, Northern America, and Australia/New Zealand and lower rates in Africa and Asia [135]. In Sweden colorectal cancer is the third most common cancer in both females and males with about 6000 new cases each year. Approximately 4% of all colon cancers and 5% of the rectal cancers are diagnosed in patients younger than 50 years, whereas 29% of the colon cancers and 23% of the rectal cancers are diagnosed in patients with age ≥80 years [179].

The aetiology of colorectal cancer is diverse and influenced by multiple factors, which include environmental and dietary factors, longstanding inflammatory bowel diseases (IBD) and genetic predispositions [180, 181]. Environmental and dietary risk factors that have been associated with colorectal cancer are for example alcohol abuse, cigarette smoking, a diet high in animal fat and red meat and low in fibre, physical inactivity and obesity [182]. While physical activity, a Mediterranean diet and long-term therapy with low-dose aspirin exerts possible preventive effects [183-185].

Genetic factors have been estimated to contribute to up to one-third of colorectal cancer [186] and this group can broadly be divided into familial and hereditary
colorectal cancer. Hereditary colorectal cancer is characterised by high-penetrance germline mutations; it accounts for less than 6% of all colorectal cancers and include Lynch syndrome and the different polyposis syndromes [187, 188]. Familial colorectal cancer is likely to be a consequence of the co-inheritance of multiple low-penetrance variants [189].

**Histopathology, grading and differentiation**

Over 90% of colorectal cancers are adenocarcinomas, the majority of which develop from a precursor adenoma. To be defined as a carcinoma tumour invasion through the lamina muscularis mucosae is required [190]. The prognosis of colorectal cancer is strongly correlated to tumour stage, which is based on the depth of infiltration through the bowel wall and the presence of lymph node metastases or distant metastases. The 5-year survival rate is >90% in stage I, 75-85% in stage II, 45-60% in stage III and 0-5% in stage IV [179].

MMR defective tumours, whether sporadic or hereditary, are characterised by a number of features that include:

- predilection for right-sided location (proximal to the splenic flexure)
- poor differentiation (including medullary type)
- mucinous differentiation (including signet-ring cell differentiation)
- histologic heterogeneity (i.e. at least 2 distinct growth patterns)
- increased number of TIL, peritumoural lymphocytic reaction/banding, Crohn-like reaction
- absence of dirty necrosis
- a circumscribed tumour margin/pushing border [191, 192].

Colorectal cancer has traditionally been graded as well, moderately, or poorly differentiated. In 2010 the WHO proposed a refined grading system aimed at better reproducibility and based on the similar clinical behaviour of well-differentiated and moderately differentiated carcinomas. The WHO proposed a two-tiered grading system with low-grade defined as ≥50% gland formation and high-grade defined as <50% gland formation. In tumours with heterogeneous patterns of differentiation the highest-grade component should be used for grading, with the important restriction that small areas of poor differentiation at the tumour’s advancing edge (i.e. epithelial-mesenchymal transition, tumour budding) should not be used to classify a tumour as high-grade [190].

33
Mucinous adenocarcinoma is composed of >50% of extracellular mucin containing single tumour cells, acinar structures or sheets/strips of tumour cells. Carcinomas with <50% mucinous areas are classified as having a mucinous component. Signet-ring cell carcinoma is defined by >50% tumours cells with prominent intracytoplasmatic mucin, typically pushing the nucleus to one side of the cell. Signet-ring cells can infiltrate in a diffuse manner within a fibrous stroma or may occur floating in pools of free mucin. Signet-ring cell carcinoma comprises only 0.7-2.6% of all colorectal cancers [190]. Mucinous adenocarcinoma and signet-ring cell carcinoma were by definition classified as poorly differentiated according to the previous WHO classification (2000). However, the current WHO classification (2010) considers these tumours as low-grade if they have MSI and as high-grade if they are MSS [190].

Medullary carcinomas are rare (<1% of all colorectal cancers) and are characterised by sheets of undifferentiated, large epithelial cells with vesicular chromatin, prominent nucleoli, abundant cytoplasm and prominent TIL (figure 6A). Medullary carcinomas are poorly differentiated with a distinct clinical behaviour. They occur more often in older females and are common in the right-sided colon, are less prone to lymph node metastases and generally have a more favourable prognosis. Medullary carcinomas are MSI-H in most cases [190, 193].

Morphologic heterogeneity is common in sporadic and hereditary MMR tumours and is defined as the presence of 2 or more distinct growth patterns within a tumour. Both mucinous and signet-ring cell differentiation may appear as heterogeneous components in 10-50% of the tumour area [194, 195] (figure 6D).

Lymphocytic reactions

Lymphocytic reactions can be categorised into 3 major forms: as TIL, peritumoural lymphocytic reactions/banding and Crohn-like reactions.

TIL are intraepithelial are CD3/CD8 co-expressing cytotoxic T-cells within tumour tissue (figure 6B). The underlying mechanism for increased TIL is unknown and it has been hypothesised that the presence of TIL reflects a response to the formation of tumour neoantigens that occurs in MSI tumours. TIL also constitute a possible explanation for the favourable prognosis of MSI tumours [196]. Several studies have suggested that TIL are the most sensitive morphological biomarker for MMR deficiency [191, 197]. Many different cut-off values have been used to define TIL, though most studies have applied a cut-off of between 5 and 10 TIL per 10 high-power fields (HPF) in haematoxylin & eosin (H&E) stained sections. A higher sensitivity for the detection of MMR defective tumours may be achieved with immunohistochemical staining for CD3 [191, 193-195, 197, 198]. In order to maximize the sensitivity, at least 7 TIL/10 HPF should
be identified in H&E-stained slides. TIL should be counted in hot spot areas and only in invasive parts of the tumour [198].

Peritumoural lymphocytes are defined as a lymphoid cuff/banding or cap at the leading edge of the tumour [195].

A Crohn-like reaction is characterised by prominent nodular lymphoid aggregates that are located in front of the advancing edge of the tumour, characteristically at the junction of the muscularis propria and mesocolonic/mesorectal fat (figure 6C). Different cut-offs have been used: 2 or more large lymphoid aggregates in one section, a minimum of 3 lymphoid aggregates per section, a single 4x field of at least 3 nodular aggregates of lymphocytes, and at least 4 nodular aggregates in one low-power field [191, 193, 195, 199].

**Growth pattern and necrosis**

The growth pattern is best evaluated at the leading front of a tumour and at low power and is typically classified as pushing or expanding [200] (figure xE). By contrast, MMR proficient tumours often exhibit an infiltrating growth pattern with irregular bordering and tumour budding with single cells or cluster of less than 5 cells at the leading edge of the tumour [201].

Dirty necrosis is characterised by inflammatory cells and cell detritus within glandular lumina and its absence correlates with MMR deficiency [191] (figure 6F).

**Sporadic MMR deficient versus Lynch syndrome-associated colorectal cancer**

Most studies that assess histopathological features linked to MSI in colorectal cancer have been conducted on MSI-high tumours in general and are therefore largely based on a mixed series of sporadic tumours with a small fraction of Lynch syndrome tumours [191-193, 199, 202]. Few studies have compared the frequencies of pathological features between sporadic and Lynch syndrome-associated tumours. Young et al. reported in a series of 112 Lynch syndrome and 57 sporadic tumours that proximal location, poor differentiation, mucinous differentiation and tumour heterogeneity occurred often but were significantly less frequent in Lynch syndrome tumours than in sporadic tumours. Also, a contiguous serrated adenoma was more often observed in sporadic tumours (29% versus <1%). By contrast, lymphocytic reactions such as TIL, peritumoural lymphocytic banding and Crohn-like reaction were more frequent in Lynch syndrome tumours though the difference was not statistically significant. No differences were found for expanding growth pattern [195]. Another study identified only mucinous differentiation and a higher TIL count at significantly higher frequencies in Lynch
syndrome tumours compared with sporadic tumours [194]. Tumour budding occurs rarely in sporadic MSI tumours, but is identified in approximately 20% in Lynch syndrome tumours and has been suggested to signify a worse prognosis [201].

Figure 6. MSI morphology in colorectal cancer: (A) Poor medullary differentiation, (B) Tumour-infiltrating lymphocytes (TIL) marked with white rings, (C) Crohn-like reaction marked with white arrows, (D) Tumour heterogeneity in an adenocarcinoma with mucinous component, (E) Mucinous adenocarcinoma with “pushing border”, (F) Dirty necrosis in an MMR proficient tumour.
**MSI prediction models**

The clinicopathological features associated with MSI-high colorectal cancer can be used as a pre-screening tool to identify cases that should undergo MMR testing. The features have been integrated into the revised Bethesda guidelines [22] and have also - in different constellations - been combined into several prediction models (table 5).

Five different MSI prediction models have been developed: the MMR index [203], MsPath [199, 204], PREDICT [205], the MSI probability score [206] and RERtest6 [202, 207]. These models have predominantly focused on the identification of Lynch syndrome-related tumours, but the MMR index and the RERtest6 model were developed in series that had a substantial contribution from sporadic MMR deficient tumours. The models are based on 6-7 clinicopathological variables and most often integrate proximal tumour location, TIL and mucinous/signet-ring cell differentiation. However, large differences exist in the cut-offs and also in composition of the remaining variables (table 5). Study I focused on the validation of the MMR index and on its comparison with the other existing models.
Table 5. Overview of the different MMR prediction models in colorectal cancer

<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of variables</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Sex</td>
<td>Female</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)</td>
<td>≥60</td>
<td>&lt;50</td>
<td>&lt;50</td>
<td>&lt;50</td>
<td>-</td>
</tr>
<tr>
<td>Tumour location</td>
<td>Proximal</td>
<td>Proximal</td>
<td>Proximal</td>
<td>Proximal</td>
<td>Proximal</td>
</tr>
<tr>
<td>Growth pattern</td>
<td>Expanding</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Expanding</td>
</tr>
<tr>
<td>Dirty necrosis</td>
<td>Lack of</td>
<td>-</td>
<td>Lack of</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mucinous/signet-ring components</td>
<td>≥10%</td>
<td>≥50% (incl. medullary)</td>
<td>Any component</td>
<td>Any component</td>
<td>Amount in %</td>
</tr>
<tr>
<td>Solid component</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TIL</td>
<td>≥7 TIL/10 HPF</td>
<td>≥5 TIL/HPF (10 HPF searched)</td>
<td>≥5 TIL/HPF (10 HPF searched)</td>
<td>≥2 TIL/mean of 5 HPF</td>
<td>≥4TIL/HPF</td>
</tr>
<tr>
<td>Differentiation</td>
<td>-</td>
<td>Poorly differentiated</td>
<td>-</td>
<td>Well or poorly differentiated</td>
<td>-</td>
</tr>
<tr>
<td>Crohn-like reaction</td>
<td>-</td>
<td>≥4 nodules/LPF</td>
<td>-</td>
<td>≥3 nodules/section</td>
<td>≥3 nodules/LPF</td>
</tr>
<tr>
<td>Peritumoural lymphocytic reaction</td>
<td>-</td>
<td>-</td>
<td>Banding of lymphocytes beyond tumour edge</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Increased stromal plasma cells</td>
<td>-</td>
<td>-</td>
<td>&gt;25% plasma cells/stromal immune cells</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Scoring system</td>
<td>No score, 7-factor index, cut-off ≥4 features</td>
<td>Score: cut-off ≥1</td>
<td>Score: cut-off ≥2.5; “Simplified PREDICT”: No score, ≥2 features</td>
<td>Score: cut-off ≥1 or ≥1.5</td>
<td>Score: cut-off &lt;0.8</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>92.3%</td>
<td>93%</td>
<td>96.9%</td>
<td>92%</td>
<td>78.0%</td>
</tr>
<tr>
<td>Specificity</td>
<td>75.3%</td>
<td>55%</td>
<td>76.6%</td>
<td>46%</td>
<td>93.4%</td>
</tr>
</tbody>
</table>

Abbreviations: HPF, high-power field; IHC, immunohistochemistry; LPF, low-power field; MMR, mismatch repair; MSI, microsatellite instability; TIL, tumour-infiltrating lymphocytes.
Molecular alterations in colorectal cancer

Colorectal cancer constitutes a prime example of how genetic changes accumulate during the progression from an adenoma to a carcinoma. The adenoma-carcinoma sequence was initially proposed in 1990 by Fearon and Vogelstein [209] and suggests that the accumulation of certain mutations results in the stepwise activation of oncogenes and inactivation of tumour suppressor genes. The proposed model is still valid but has since, in light of extensive epidemiological, histopathological and genetic data, been further refined. Three major pathway concepts - chromosomal instability (CIN), microsatellite instability (MSI) and the CpG island methylator phenotype (CIMP)/serrated pathway - have been recognised [53, 209-211]. The adenoma-carcinoma sequence with its underlying genetic changes is illustrated in figure 7.

**Figure 7.** The adenoma-carcinoma sequence in colorectal cancer. Three major pathway concepts have been recognised: the chromosomal instability (CIN) pathway, the microsatellite instability (MSI) pathway, and CpG island methylator phenotype (CIMP)/serrated pathway. Adapted and modified from Sepulveda and Aisner, 2009 [212].

The CIN pathway

The CIN pathway plays a role in 80-85% of colorectal cancers [213]. The pathway is associated with an accelerated rate of gains or losses of genetic material. Carcinomas are usually highly aneuploid, and show frequent loss of
heterozygosity (LOH) and a striking variation in the number of gene copies [214]. Genomic changes include either mutational inactivation of the tumour suppressor gene APC or loss of chromosome 5q, harbouring the APC gene; additional genomic changes include the activation of proto-oncogenes such as KRAS, loss of chromosome 18q and deletion of chromosome 17p, harbouring the suppressor gene TP53 [215].

Loss of the APC gene is an early key event in the CIN pathway. Genetic disruption of the APC gene or epigenetic silencing of the APC promoter leads to unsuppressed activation of the Wnt/β-catenin signalling cascade. This process is suggested to be crucial for the development of adenoma and mutations can be found in up to 80% of sporadic colorectal cancer and adenomas [216, 217]. Loss of APC protein function increases the stabilisation of β-catenin; as a result, β-catenin accumulates in the cytoplasm and is translocated into the nucleus. The β-catenin translocation will in turn deregulate several genes that are involved in cellular growth, apoptosis and differentiation [218]. The relevance of Wnt/β-catenin signalling in the tumourigenesis is also demonstrated by the high frequency of activating mutations in the gene encoding β-catenin (50%) in colorectal cancer with intact APC genes [219].

Another important gene in the CIN pathway is the proto-oncogene KRAS. Mutations in KRAS are early events in the pathway and can be found already in adenomas. Activating mutations in KRAS lock the protein in a constitutively active form leading to disruption of the Ras/Raf/MEK/MAPK pathway, which plays an important role in cell division, cell differentiation, and apoptosis. Activating mutations in KRAS are found in approximately 40% of all colorectal cancers [220-222]. Loss of TP53 and 18q LOH are also important contributors to the CIN phenotype [209]. The long arm of chromosome 18 harbours the important tumour suppressor gene SMAD4, which is lost by 18q deletion, resulting in tumourigenesis via the TGF-β signalling pathway [223]. LOH of chromosome 18q has been proposed to represent an adverse prognostic marker for survival in patients with colorectal cancer [224].

*The MSI pathway*

The MSI pathway characterises 2-4% of colorectal cancers with a hereditary MMR defect and approximately 15% of sporadic colon cancers with epigenetic silencing of the MLH1 promoter [16]. Involvement of the MSI pathway is rare in sporadic rectal cancers [63]. In contrast to the CIN pathway tumours that develop along the MSI pathway are typically diploid or near-diploid with high mutation rates [225].

Defective MMR leads to an accumulation of mutations, in coding and non-coding microsatellites and may cause somatic frameshift mutations in cancer-associated genes that promote tumourigenesis [16]. Somatic frameshift mutations in
microsatellites of genes that are part the immune surveillance mechanism, also allow tumour cells to escape from the physiological immune response. Most MSI tumours have lost expression of either β2-microglobulin (B2M) or HLA class I, resulting in an impaired presentation of processed abnormal antigens on the cell surface [226, 227].

Mutations in the TGFβR2 gene are found in 80% of the MSI tumours [228]. TGFβR2 mutations are found in advanced adenomas with high-grade dysplasia and in adenocarcinomas. These mutations correlate with further neoplastic progression and the development of metastatic disease in the MSI pathway [229]. Additionally, inactivating mutations in the tumour suppressor genes SMAD2 and SMAD4 in the TGF-β signalling pathway are commonly found [230]. Another frequent mutational target is the ACVR2 gene, which encodes a transmembrane receptor that promotes cell differentiation and inhibits proliferation by phosphorylation of the SMAD2 and SMAD3 proteins. The mutation occurs in up to 83% of MSI tumours, frequently together with TGFβR2 mutations [231]. Mutations in the pro-apoptotic tumour suppressor gene BAX can be found in 50% of the MSI tumours, promoting the tumour cell’s escape from intrinsic apoptosis mechanisms [232, 233]. In addition to the genes mentioned above, several other genes are less frequently mutated, e.g. MSH3 (36%), CCND1 (28%), IGFR2 (22%), MSH6 (17%), BLM (16%), PIK3CA (15%) [234-238].

It is notable that hereditary tumours and sporadic tumours have somewhat different mutational spectra. Although the frequency of mutations is similar in some genes, e.g. BLM, FLT3LG and TEAD2, considerable differences are observed for other genes, e.g. Axin2, B2M, BRAF and CHK1 [16]. Sporadic MSI tumours harbour V600E mutations in the BRAF gene in 40-70% of the tumours. The proto-oncogene BRAF is a member of the Raf family involved in the Ras/Raf/MEK/MAPK pathway. Mutations in BRAF are almost never seen in Lynch syndrome-associated tumours [65, 239-241] and the presence of a BRAF mutation strongly indicates a somatic MMR defect. This demonstrates that Lynch syndrome-associated and sporadic MSI tumours, despite having similarities with regard to the MSI pathway, have different underlying molecular pathways of tumour development. There is therefore support for separate analysis of Lynch syndrome-associated and sporadic MSI tumours [16].

The CIMP/serrated pathway

A third pathway concept is the CIMP/serrated pathway [210, 211]; the name of the pathway is derived from the serrated morphology of the tumour precursors. This pathway is characterised by the epigenetic silencing of genes involved in cell cycle regulation, adhesion, apoptosis, DNA repair, invasion and angiogenesis, e.g. APC, CDH13, CDKN2A, MCC, MGMT, MLH1, THBS1, TIMP3 and several others [242]. Hypermethylation of CpG dinucleotides in gene promoters results in the loss of gene expression, and increased methylation has been shown to correlate
with advanced age, smoking and other environmental factors [211, 243, 244]. The CIMP pathway overlaps with the MSI pathway and the epigenetic silencing of the MLH1 gene in sporadic MSI tumours is considered to be part of CIMP pathway by some investigators [245, 246]. In light of this view the CIMP pathway plays a role in approximately 15%–20% of colorectal cancers and shows similar clinical characteristics to sporadic MSI tumours [245, 246].

CIMP is defined by the presence of hypermethylation of multiple genes and a test panel of 5 markers, i.e. CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1, has been chosen to define CIMP positivity. Methylation of at least 3 markers is classified as CIMP-positive or CIMP-high and methylation of 0 to 2 markers is classified as CIMP-negative or CIMP-low [247]. An early event in the CIMP pathway is the silencing of CDKN2A, which promotes uncontrolled cell proliferation and neoplastic transformation [248]. Also BRAF mutation, i.e. V600E, is an early event and can already be detected in sessile serrated polyp/adenoma (SSP/SSA) but rarely in ordinary adenomas [249].

Cancer of the urinary tract

Epidemiology and aetiology

Urothelial cancers can develop within the entire urinary tract from the renal pelvis to the urethra. While urothelial carcinomas of the bladder and the upper urinary tract share morphological characteristics, they also have significant clinical, anatomical, biological and molecular differences and are therefore considered to represent 2 different diseases.

Bladder cancer is the most common malignancy involving the urinary system, representing the 9th most common malignancy worldwide [250] and the 6th most common malignancy in Sweden. Approximately 2400 new cases of bladder cancer are diagnosed each year in Sweden, representing an annual incidence of about 25 new cases per 100,000 individuals. Around 600 individuals die each year from bladder cancer, while the prevalence is about 23,000 individuals. There is a male predominance, with a male:female ratio of about 3:1. The median age at onset is ~70 years and urothelial cancers are rarely diagnosed in individuals younger than 40 years [179]. At diagnosis 20-30% of the tumours are muscle-invasive [251].

Urothelial carcinoma in the upper urinary tract (the ureter and the renal pelvis) is rare and accounts for 5–10% of all urothelial carcinomas. The estimated annual incidence in Western countries is approximately 2 new cases/100,000 inhabitants [252, 253]. In Sweden the incidence of cancer in the renal pelvis is approximately 100 cases per year and for cancer in the ureter it is 60-70 cases per year [179]. At diagnosis, 60% of the tumours are muscle-invasive [254, 255]. In 17% of the
cases, synchronous bladder cancer is present [256] and recurrences in the bladder occur in 22.47% after urothelial cancer in the upper urinary tract [257-259], compared with 2.6% after cancer in the contralateral upper urinary tract [260, 261].

Tobacco smoking is the most important risk factor for urothelial cancer. Other risk factors include occupational exposure to carcinogenic aromatic amines and for bladder cancer also radiotherapy and infections with Schistosoma species [262].

A positive family history is a risk factor for bladder cancer. The risk for first-degree relatives of patients with bladder cancer is about 2-fold higher than that for the general population [262]. In upper urinary tract cancer approximately 21% of the cases are associated with Lynch syndrome [120, 263].

Urothelial cancers can develop in a synchronous or metachronous way at different sites in the urinary tract. Two main hypotheses have been generated to explain this phenomenon, the “intraluminal seeding and implantation hypothesis” and the “field change hypothesis” [264, 265]. The first hypothesis proposes the clonal development of a multifocal cancer through intraluminal tumour cell migration and implantation on the urinary tract wall or through the intraepithelial expansion of cells from the primary tumour. The second hypothesis suggests that mutagenic factors in the urine transform urothelial cells at multiple sites inducing the development of multiple tumour clones.

Histopathology, grading and differentiation

Urothelial carcinoma (previously referred to as transitional cell carcinoma) is the predominant histological type and accounts for over 90% of all bladder cancers and upper urinary tract cancers. Less frequent histological types include squamous cell carcinoma, small cell carcinoma and adenocarcinoma. It is important to differentiate these types from urothelial carcinoma, because treatment and prognosis differ [251].

The classification and grading systems for papillary lesions have changed over time. Although the WHO 1999 classification system with 3 grades is still used in Sweden, the WHO changed the grading system for papillary lesions into a 2-tiered system in 2004 [251]. Urothelial cancer is, based on the degree of nuclear atypia and architectural changes, graded as low-grade or high-grade. All muscle-invasive tumours are usually high-grade (earlier grade 2-3 according to WHO 1999).

Urothelial cancers are clinically divided into non-muscle-invasive (superficial) and muscle-invasive tumours. Non-muscle-invasive tumours include tumours that are either non-invasive or invasive but stay superficial to the muscularis propria. At initial diagnosis, 70-80% of patients with bladder cancer and 40% of patients
with upper urinary tract cancer have superficial disease. Non-muscle-invasive carcinomas can be separated into 3 distinct types [251]:

- non-invasive papillary urothelial carcinoma
- flat urothelial carcinoma in situ (CIS) and
- invasive urothelial carcinoma

Most non-muscle-invasive urothelial carcinomas present as non-invasive, exophytic tumours with finger-like papillae, whereas some tumours exhibit an endophytic/inverted growth pattern. Approximately 70% of these tumours will recur and 10-15% will eventually progress to invasive carcinomas [266]. Non-invasive papillary carcinomas (stage pTa) can either be of low grade or of high grade, with high-grade tumours exhibiting a higher risk of progression [251].

Flat urothelial lesions with strong atypia are classified as urothelial carcinoma in situ (CIS) (stage pTis); these lesions are highly aggressive and progress more rapidly than papillary tumours.

Non-muscle-invasive urothelial carcinomas comprise a minority of tumours that invade only superficially into the connective tissue (lamina propria) of the bladder (stage pT1); these tumours recidivate in 80%, and high-grade tumours progress in 60% of cases [251].

Approximately 30% of all urothelial carcinomas are muscle-invasive at diagnosis (stages pT2-pT4). These cancers are highly aggressive and may metastasise via lymph and blood vessels to bone, liver and lungs [251].

The pathological diagnosis includes the histological type, grade, pTNM stage and other prognostic factors, such as vessel infiltration, presence of urothelial dysplasia or carcinoma in situ in the surrounding urothelium, and margins in cystectomy specimens. In non-muscle-invasive tumours (pTa or pT1) the risk of recurrence correlates with a high-grade, multifocal growth and the presence of urothelial dysplasia or carcinoma in situ in the surrounding urothelium [251].

**MSI morphology**

No morphological differences have been discerned between MMR deficient and MMR proficient urothelial cancer in the bladder [123]. For urothelial cancer in the upper urinary tract one study has suggested that the occurrence of an inverted growth pattern of papillary tumours is predictive of an MSI-high tumour [267] (figure 8).
Molecular alterations in urothelial cancer

Clinical behaviour and mutually exclusive genetic changes suggest that urothelial cancer of the bladder derives and progresses along at least 2 different pathways. Superficial/low-grade non-invasive papillary urothelial cancer originates from benign urothelium through urothelial hyperplasia, whereas muscle-invasive urothelial cancer derives through progression from dysplasia/carcinoma in situ or high-grade non-invasive papillary urothelial cancer or develops de novo from normal urothelium. Progression from superficial to muscle-invasive urothelial cancer occurs within a minor fraction (10-15%) of the tumours [268-270].

Low-grade, superficial urothelial cancers frequently harbour mutations in RAS, FGFR3 or PIK3CA. Studies have shown that activating FGFR3 mutations and RAS mutations are mutually exclusive in urothelial cancer, given that both signal through the same MAPK pathway. FGFR3 and PIK3CA mutations occur together, suggesting a synergistic oncogenic effect [271-273].

High-grade, muscle-invasive urothelial cancers carry mutations in the TP53 and/or RB genes in >50% of the tumours. During progression from dysplasia/cancer in situ the lesions accumulate multiple chromosomal abnormalities in addition to LOH of chromosome 9, which is the earliest alteration in both pathways of urothelial cancer development [270, 274, 275]. The divergent oncogenic pathways...
in urothelial cancer and their genetic alterations during progression are illustrated in figure 9.

Urothelial cancers of the upper urinary tract carry many of the same mutations and chromosomal abnormalities observed in urothelial cancers of the bladder, but the progression model for urothelial cancer of the upper urinary tract is less well characterised. *FGFR3* mutations occur with similar frequencies in urothelial cancer of the upper urinary tract and in urothelial cancer of the bladder. Likewise, *FGFR3* mutations confer a favourable prognosis in both tumour types [276]. However, large differences exist between urothelial cancer of the upper urinary tract and urothelial cancer of the bladder regarding the contribution of MMR defects/the MSI pathway. MMR defects are rarely (~3%) encountered in urothelial cancers of the bladder, whereas 15-45% of the urothelial cancers in the upper urinary tract show MMR defects [277-279]. Also within in the upper urinary tract the frequency of MSI seems to depend on the tumour location; studies have demonstrated MSI in 33% of the urothelial cancers of the ureter and in only 10% of the urothelial cancers of the renal pelvis [278, 279].

**Figure 9.** The progression model of urothelial cancer in the bladder with 2 divergent pathways: The main genetic changes in the development of non-invasive low-grade urothelial carcinoma involve *HRAS, FGFR3* and members of the mTOR pathway. By contrast, progression to muscle-invasive and non-invasive high-grade urothelial carcinoma involves the tumour suppressor genes *TP53* and *RB*, and several alterations of the tumour microenvironment. Based on Netto, 2013 [266].
Prostate cancer

Epidemiology and aetiology

Prostate cancer is the second most common malignancy in men worldwide and (after lung cancer) the second leading cause of death in men in industrialised countries. The incidence varies, partly due to differences in the coverage of prostate-specific antigen (PSA) testing [280, 281]. Sweden has one of the highest prostate cancer incidences in the world with more than 9600 new cases annually. Prostate cancer accounts for 5.4% of all cancer-related deaths in men in Sweden [179]. While the incidence is increasing, the mortality is decreasing [281].

The aetiology of prostate cancer is not well understood, although several risk factors have been identified. Increasing age, ethnic origin and hereditary factors are well-established risk factors, though prostate cancer development is regarded to be multifactorial [282]. Environmental factors and lifestyle factors seem to be relevant, based on observations of an increased incidence in populations migrating from low- to high-prevalence regions [283].

A family history of prostate cancer is an established risk factor for the disease. The risk for first-degree relatives of men with prostate cancer is about 2-fold higher than that for men in the general population [284] and it is more than 4-fold higher if prostate cancer was diagnosed before age 60 [285]. The risk increases to 7- to 8-fold for those with 2 or more affected first-degree relatives [284].

Histopathology, grading and differentiation

More than 95% of the malignant tumours in the prostate are adenocarcinomas and the most common subtype (>90%) is acinar adenocarcinoma [251]. Morphological adenocarcinoma variants like ductal, mucinous, signet-ring, pseudohyperplastic, foamy gland and small cell cancers contribute to less than 5% of the tumours. The prognosis of several of these variants is unclear because they are rare and usually occur in combination with the acinar type. Some variants, e.g. ductal, signet-ring and small cell cancers have a particularly aggressive clinical behaviour and should therefore be distinguished from conventional acinar adenocarcinoma. Over 75% of the tumours develop in the peripheral zone [251]. The diagnosis is often made on transrectal ultrasound-guided core biopsies, but coincidental identification occurs in 8-10% of the specimens from transurethral resections (TUR-P) [251].

The morphology of acinar adenocarcinomas ranges from well-differentiated gland forming to poorly differentiated with diffuse and/or solid growth. Well-differentiated carcinomas in particular are often difficult to distinguish from benign prostatic glands. Histological changes include:
• glandular architecture (invasive/dissecting pattern, perineural infiltration, microglands and cribriform glands),
• intraluminal features (dense eosinophilic and irregular crystal-like structures),
• cellular atypia (often enlarged nuclei with prominent nucleoli) and
• lack of basal cells

Immunohistochemistry with basal cell markers CK5 (membrane stain), 34BE12 (membrane stain) and p63 (nuclear stain) is most often used to prove the lack of basal cells in suspected carcinomas. Tumour cells can be stained with α-methylacyl-CoA racemase (AMACR/p504s, cytoplasmatic stain), which is commonly up-regulated in prostate carcinoma.

The standard grading of adenocarcinoma in the prostate is the Gleason grading system, which was first introduced in 1966 [286, 287]. The current version, proposed by the International Society of Urological Pathology (ISUP) and recommended by the WHO, is the 2014 ISUP modified Gleason score [288]. Gleason grading is based on the glandular architecture, evaluated at low-power magnification in standard H&E-stained tissue sections. Overall, 5 grades are distinguished, based on the increasing architectural disorganisation/glandular pattern, but Gleason grade 1 is no longer used [288]. In core biopsies and TUR-P specimens only grades 3 to 5 are used, which means the lowest possible score is 6. The most abundant pattern and the most aggressive pattern/highest grade are added to give the Gleason score, e.g. 4+3 or 3+5. In radical prostatectomy specimens, grades 2 to 5 are used. The most common pattern and the second most common pattern (>5%) are added to give the Gleason score, e.g. 4+3 or 4+4. If smaller foci with a tertiary higher grade are present, this should be mentioned separately to the Gleason score with an estimated percentage of the extent [288].

The pathological diagnosis includes the histologic type, Gleason grades and score, presence of high-grade PIN, perineural growth, and presence of extraprostatic growth and infiltration of the seminal vesicles. In core biopsies the total length of the tumour and the length of the biopsy are reported in mm. In radical prostatectomy specimens the diagnosis also contains the pTNM stage, vessel infiltration and the surgical margin status. Prognostic factors include the serum PSA level, the Gleason score, TNM stage and the surgical margin status [251].

**MSI morphology**

Data on the association between MSI/MMR status and histopathological phenotype are scarce. Only one study has reported a high frequency of TIL (cut-off ≥4 TIL/HPF) in 12 of 16 MMR deficient compared to 3 of 10 MMR proficient...
prostate carcinomas; all carcinomas were of the acinar type [125]. This study and an additional study have found a higher frequency of Gleason score ≥8 in MMR deficient tumours compared to MMR proficient tumours [125, 289]. An example of a prostate carcinoma with TIL and Gleason score ≥8 is shown in figure 10.

Figure 10. MMR deficient prostate carcinoma with abundant TIL (examples marked with white rings) from an MSH2 mutation carrier. Radical prostatectomy specimen with Gleason score 5+4=9.

Molecular alterations in prostate cancer
The pathogenesis of prostate cancer and its underlying molecular changes are still not well defined. It is thought that there is a continuous progression from normal epithelium to prostatic intraepithelial neoplasia (PIN), followed by progression to localised invasive adenocarcinoma, which may metastasise and develop into a hormone refractory disease; the progression is thought to be reflected by an increasing Gleason grade [290, 291]. Multiple molecular and cytogenetic alterations have been described, but the demonstration of associations with specific steps of cancer progression has been hampered by the multifocal and heterogeneous nature of prostate cancer [292].

Common chromosomal changes include the loss of 8p and 13q [293, 294] and less frequently the loss of 6q, 5q, 16q, 18q, 2q, 4q, 10q and Y [295]. Loss of 8p, harbouring the tumour suppressor gene NXX3.1, commonly occurs in PIN and represents an early event in cancer development [296, 297]. Frequent gains are found on chromosomes 7, 8q, 17q, 3q, 9q, 1q and X [295].
Loss of gene expression due to promoter methylation of the \textit{GSTP1} gene, which codes for glutathione-S-transferase P1 (an enzyme that protects DNA from free radicals), is the most common epigenetic alteration and is found in more than 70\% of PIN and in more than 90\% of invasive adenocarcinomas [298]. Another epigenetic alteration is promoter methylation of the \textit{CD44} gene, which correlates with progression to metastasising disease [299].

Various gene expression changes have also been identified, which partly reflect specific chromosomal losses, e.g. \textit{NKX3.1} (loss of 8p), \textit{PTEN}, \textit{MXII} and \textit{ANXI} (loss of 10q), or are due to gene mutations, e.g. \textit{TP53}, \textit{RB}, \textit{CDKN2A} and \textit{AR} (figure 11) [291]. Mutations in \textit{AR} are a late event in prostate cancer and lead to hormone refractory disease [300].

Several gene fusions have also been identified in prostate cancer, the most common being the fusion between the androgen-regulated gene \textit{TMPRSS2} (21q22.3) and the ETS-related gene \textit{ERG} (21q22.2). The \textit{TMPRSS2-ERG} fusion is found in up to 50\% of localised prostate cancers and leads to the androgen-regulated overexpression of the oncogene \textit{ERG} [301, 302].

\textbf{Figure 11.} Progression model of prostate cancer. Normal prostatic epithelium transforms into prostatic intraepithelial neoplasia (PIN), which in turn progresses to localised invasive cancer, which may become metastasising and finally turn into hormone refractory disease, with the increasing severity reflected in an increasing Gleason grade. This figure illustrates the variable molecular alterations observed in the progression of prostate cancer. Model adapted and modified from Garnis et al., 2004 [303].
The molecular basis of hereditary prostate cancer is complex with several loci suggested, but no distinct high-risk gene has been identified. In 2012, a germline mutation in the *HOXB13* gene was identified that was specifically associated with familial prostate cancer [304-309]. The *HOXB13* G84E mutation is believed to represent a founder mutation of Scandinavian origin [307-309]. Further *HOXB13* mutation variants have subsequently been identified in individuals of Southern European, African and Asian origin [304, 305, 310, 311].

Before the linkage of *BRCA1* and *BRCA2* to the hereditary breast/ovarian cancer (HBOC) syndrome [312, 313], studies had already found an association between prostate cancer and breast cancer [314, 315]. Male *BRCA2* mutation carriers have a 2.5 to 8.6-fold increased risk of developing prostate cancer [316, 317]. The association with *BRCA1* mutations is uncertain with conflicting risk estimates [318-320].

An increased risk of prostate cancer has also been reported in Lynch syndrome [125, 132, 137, 289]. MMR gene mutation carriers are reported to be at 2- to 3-fold higher risk, which is possibly even higher for *MSH2* carriers [125, 132, 137]. The association with Lynch syndrome is still controversial, as other studies have not identified an increased risk for prostate cancer [106, 321, 322]. The role of MMR in prostate carcinogenesis is unclear. MSI has been reported at different frequencies (4-12%) in prostate cancer [323, 324]. Somatic mutations in the *MSH2* and *MSH6* genes have also been described [324, 325].

**Renal cell cancer**

*Epidemiology and aetiology*

Renal cell carcinoma (RCC) is the 7th most common cancer worldwide and represents approximately 2-3% of all cancers [135]. The incidence is highest in Western countries. Worldwide the incidence of RCC has increased by approximately 2% over the last 2 decades, with the exception of Denmark and Sweden, where the incidence has declined [326]. In Sweden the incidence of RCC is approximately 14.5/100,000 for males and 7.8/10,000 for females, corresponding to 1000 new cases in Sweden each year [179]. There is a 1.5-2:1 male/female ratio, with the highest incidence between 60 and 70 years. RCC mortality in Scandinavia has fallen in the last 3 decades to reach a 5-year survival rate of up to 90% for localised disease [327].

The main aetiological factors include obesity, hypertension and smoking [328]. Individuals with a first-degree relative with RCC are at increased risk for RCC [329]. Heredity has been estimated to cause 5-8% of the tumours; histological type, age at onset and extra-renal manifestations differ between the syndromes,
which include von Hippel-Lindau syndrome, hereditary papillary renal carcinoma, hereditary leiomyomatosis, Birt-Hogg-Dubé syndrome and tuberous sclerosis [330]. Due to the widespread use of ultrasound and computed tomography, RCC is increasingly diagnosed incidentally, at a lower stage [331].

Histopathology, grading and differentiation
RCC encompasses a wide spectrum of histological types described in the WHO classification 2004; the classification was modified by ISUP in 2013, resulting in the ISUP Vancouver classification [332]. The classification has been confirmed by genetic analyses [333-335]. The most frequent histological subtypes are clear cell RCC (75%), papillary RCC type I and II (10%), and chromophobe RCC (5%) [336]. Less common types include uncommon sporadic and hereditary carcinomas, e.g. collecting duct carcinoma, multilocular cystic cell carcinoma, medullary RCC, mucinous tubular and spindle cell carcinoma, Xp11.2 translocation RCC and carcinoma associated with neuroblastoma. “RCC of unclassified type” is a diagnostic category of exclusion and constitutes about 4-6% of the cases.

Clear cell RCC is the most common variant and originates from the epithelium of the proximal convoluted tubules (renal cortex). Macroscopically, RCC commonly presents as a unilateral, rounded and well-circumscribed tumour. The cut surface is typically golden-yellow with a variable extent of haemorrhage, necrosis, cystic degeneration or calcifications. Bilateral and/or multicentric growth is rare (<5%) in sporadic cases, whereas it is more common in hereditary cases. Microscopically, the tumour cells form nests, sheets, alveolar structures or acinar structures separated by small blood vessels. The tumour cells are characterised by clear cytoplasm due to loss of their lipid- and glycogen-rich content during tissue processing and staining. In high-grade lesions, the tumour cells frequently lose their clear cell morphology and become more eosinophilic.

Papillary RCC typically presents as a well-circumscribed tumour with a pseudocapsule. Some tumours appear entirely necrotic. Papillary RCC is more often multifocal than other RCC subtypes. Microscopically, the tumour is characterised by variable papillary, tubulopapillary and tubular growth. The papillae commonly contain fibrovascular cores infiltrated by foamy histiocytes. Acute and chronic inflammation, necrosis, haemorrhage, hemosiderin deposition and psammoma bodies are frequent findings. Two histological subtypes are recognised [337]. Papillary RCC type I is the most common subtype and accounts for about two-thirds of the cases. Type I tumours typically show short papillae with a single layer of basophilic cells surrounding the basal membrane. The tumour cells have scarce clear cytoplasm and small hyperchromatic nuclei. Papillary RCC type II is
characterised by large papillae lined with eosinophilic cells that have large pseudostratified nuclei with prominent nucleoli. The prognosis is better with type I than with type II tumours [337].

Chromophobe RCC typically presents as a solitary, well-circumscribed and non-encapsulated tumour: the cut surface is light brown and solid, usually without haemorrhage and necrosis. Large tumours characteristically show a central stellate scar. Microscopically, the tumour cells commonly show a solid growth pattern, sometimes with minor areas with tubulocystic architecture. The tumour cells are large and polygonal with a “plant cell like” cell membrane. The cytoplasm is finely reticulated due to numerous microvesicles. The nuclei are irregular and hyperchromatic and often show a typical perinuclear halo.

For grading the four-tiered Fuhrman nuclear grading system is commonly applied, which was first described in 1982 (table 6) [338]. Several studies have confirmed that the Fuhrman grade is an independent prognostic factor for clear cell RCC [339]. The grading system is applied to clear cell and papillary RCC, but not to chromophobe RCC [340].

Table 6. Fuhrman nuclear grading system for clear cell RCC and papillary RCC [338]

<table>
<thead>
<tr>
<th>Fuhrman grade</th>
<th>Nuclear size</th>
<th>Nuclear shape</th>
<th>Chromatin</th>
<th>Nucleoli</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;10 µm</td>
<td>Round</td>
<td>Dense</td>
<td>Inconspicuous</td>
</tr>
<tr>
<td>2</td>
<td>15 µm</td>
<td>Round</td>
<td>Finely granular</td>
<td>Small, not visible at 10x magnification</td>
</tr>
<tr>
<td>3</td>
<td>20 µm</td>
<td>Round/oval</td>
<td>Coarsely granular</td>
<td>Prominent, visible at 10x magnification</td>
</tr>
<tr>
<td>4</td>
<td>&gt;20 µm</td>
<td>Pleomorphic, multilobulated</td>
<td>Open, hyperchromatic</td>
<td>Macronucleoli</td>
</tr>
</tbody>
</table>

The pathological diagnosis includes the RCC type, the nuclear grade, the pTNM stage and prognostic factors such as vessel infiltration, sarcomatoid features, necrosis and infiltration of the perirenal fat. The prognosis is worse for clear cell RCC than for papillary and chromophobe RCC [341], even after stratification for stage and grade [342].

**MSI morphology**

No morphological differences have been described between MMR deficient and MMR proficient RCCs. An example of clear cell RCC from an MSH2 mutation carrier is shown in figure 12.
Molecular alterations in renal cell cancer

Much of the current knowledge of the molecular biology and the genetics of RCC is based on studies of hereditary kidney cancer, in particular related to von Hippel-Lindau syndrome [334]. The underlying genetic changes of the different histological subtypes of RCC are diverse, but subtype-specific.

Clear cell RCCs do in 70-90% of the tumours harbour alterations of the short arm of chromosome 3, including deletions, mutations or methylation of the VHL (3p25-26), RASSF1A (3p21) and FHIT genes (3p14.2). The second most common finding is the duplication of 5q22. Other genetic alterations include the loss of chromosomes 6q, 8p12, 9p21, 9q22, 10q, 17p or 14q [343-345]. The majority (98%) of sporadic clear cell RCCs show LOH of the VHL gene and 18-82% of the tumours have somatic mutations in VHL. Epigenetic silencing of the VHL gene through methylation of the VHL promoter has been described in 5-20% of the cases [346-348]. The VHL gene is a tumour suppressor gene that encodes the VHL protein, which is crucial for the regulation of the cellular response to hypoxia. VHL regulates the hypoxia-inducible factor (HIF), which is a transcriptional factor that is hydroxylated under normoxic conditions. The wild-type VHL protein binds hydroxylated HIF leading to HIF degradation [349]. As a result, normal cells with intact VHL have low HIF levels under normoxic conditions. Under hypoxic
conditions, HIF is not hydroxylated and not bound by VHL. HIF therefore accumulates, which in turn leads to the activation of downstream hypoxia-driven genes, including factors involved in angiogenesis (VEGF and PDGF-β), cell proliferation or survival (TGFα), anaerobic metabolism (Glut-1), acid base homeostasis (CA IX), and erythropoiesis (erythropoietin) [349]. Several signal transduction pathways are activated, including the PI3K-Akt-mTOR pathway and the Ras-Raf-Erk-Mek pathway, which are involved in cell proliferation, differentiation and survival [348, 350]. Loss of VHL, through mutation or epigenetic silencing of the VHL gene, leads to uncontrolled activation of this VHL/HIF oxygen-sensing pathway and promotes tumourigenesis in clear cell RCC. The VHL/HIF oxygen-sensing pathway and other involved pathways are illustrated in figure 13. Several critical components of the involved pathways have become targets in clinical trials for the treatment of RCC, e.g. the neutralising antibody bevacizumab targets VEGF; sorafenib and sunitinib (small molecule inhibitors of tyrosine kinase) target PDGFR and VEGFR; erlotinib targets EGFR, and temsirolimus targets mTOR [351, 352].

Figure 13. Key pathways involved in clear cell RCC biology and tumourigenesis. Reprinted from Klatte and Pantuck [353].
The underlying genetic changes in papillary RCC are different. Papillary RCC is in 75% of the cases associated with trisomy 7 [354]. Other common changes include trisomy 17 and in men loss of the Y chromosome [355]. Papillary RCC type I and type II have distinct genetic features. Type I is more commonly associated with the gain of 7p and 17p, whereas deletion of 9p and loss of heterozygosity at 9p13 are associated with type II tumours and a shorter survival [356]. In hereditary papillary RCC the responsible proto-oncogene MET (mesenchymal-epithelial transition factor) has been identified through linkage analysis [357]. The MET proto-oncogene encodes a tyrosine kinase membrane receptor (the MET receptor). The gene for the MET receptor and the gene of its ligand, HGF (hepatocyte growth factor), are both located on chromosome 7. On binding of HGF to the MET receptor, a signalling cascade is initiated, resulting in several events such as activation of the Ras-MAPK and PI3K-Akt pathways [358]. Gain-of-function mutations constitutively activate the MET pathway and promote tumourigenesis in papillary RCC. Several tyrosine kinase inhibitors targeting the MET pathway as well as monoclonal antibodies against HGF are in clinical trials [349, 358].

The genetic changes in chromophobe RCC are characterised by chromosomal loss, which most commonly affect chromosomes 1, 2, 6, 10, 13, 17, 21 and Y [359].

The contribution of deficient MMR in renal carcinogenesis is largely undefined. Low frequencies (2-6%) of MSI-high RCC have been reported in unselected series using the Bethesda panel or the MSI Analysis System [360, 361]. A study from Rubio-Del-Campo et al. analysed 89 RCCs (66% clear cell RCC) and found no mutational changes in MLH1 and MSH2, no methylation of the MLH1 promoter and no MSI. They concluded that defective MMR related to MLH1 or MSH2 is not involved in sporadic RCC and found that chromosomal instability was the main genetic alteration [362]. Variable MLH1 and MSH2 protein expression levels have also been described in the different histological subtypes of RCC, but their functional relevance is unknown [360, 363, 364].
Materials and Methods

Patients and materials

The patient and tumour materials used for the studies in this thesis have been collected from the Department of Pathology, Helsingborg Hospital and from various Pathology Departments in Denmark identified through the national Danish HNPCC register, Hvidovre University Hospital, Copenhagen.

Study I is based on a consecutive series of colon cancer patients diagnosed between 2002-2006 at the Department of Pathology, Helsingborg Hospital. The patients in study II were identified during evaluations between 2007 and 2011 at the Departments of Pathology, Helsingborg Hospital, Sweden and Hvidovre Hospital, Denmark.

The patients in studies III-V were recruited by register searches in the Danish HNPCC register performed in May 2014 (study III-IV) and in May 2015 (study V). The HNPCC register was founded in 1991 and is continuously updated. All departments of genetics, pathology, surgery and biochemistry in Denmark report clinical data, genetic test results, surgical procedures, and family data to the register. The register covers a population of 5.6 million and contains >5000 families with suspected or verified hereditary colorectal cancer. One-third of the families are estimated to have a moderate risk of colorectal cancer based on 2 or fewer colorectal cancers occurring at varying age in these families. An equal proportion of families are categorised as high-risk families including about 300 Lynch syndrome families, with the remaining third consisting of grey-zone families, families under evaluation and families with other genetic syndromes disposing to colorectal cancer, e.g. attenuated familial adenomatous polyposis (AFAP), MUTYH-associated polyposis (MAP) etc.

Families with a high risk:

- Lynch syndrome families with a proven mutation (n=323) and FCCTX families fulfilling the Amsterdam criteria I/II (n=249)
- Lynch syndrome-likely families, which fulfil all but 1 of the Amsterdam criteria (all HNPCC-like n=408)
• Families with late onset (age >50 years), which fulfil the Amsterdam criteria except for age at diagnosis

Families with a moderate risk:

• Families with 1 family member with colorectal cancer diagnosed before age 50 years (n=554)
• Families with 2 family members (first-degree relatives) with colorectal cancer diagnosed after age 50 years (n=717)
• Grey-zone category, for families who are difficult to classify (n=484, under evaluation n=618)

In May 2015 the Danish HNPCC register contained 1494 mutation carriers from 313 families with Lynch syndrome, based on proven disease-predisposing MMR gene mutations.

Tumour material

Studies I-IV are based on paraffin-embedded tumour tissue. All diagnoses were confirmed by review of the original histopathological reports and if necessary additional data was obtained from clinical files. All available sections from routine diagnostics were re-evaluated. If only paraffin blocks were available, fresh H&E stained sections were evaluated. One or more representative tumour blocks were selected for MMR protein immunohistochemistry (studies I-IV) and representative tumour blocks and/or tumour areas were subjected to DNA-based analyses (studies II-IV).

Study I

A consecutive series of all colon cancers from patients ≥ age 50 operated for colon cancer at Helsingborg Hospital between 2002 and 2006 was studied. The series contained 474 colon cancers from 462 patients with a mean age of 76 (range 50-100) years and a male:female ratio of 1:1.2. Synchronous colon cancers were identified in 12 patients. None of the patients had metachronous colorectal cancer. 58% of the tumours developed within the proximal colon. The tumour stage was pT1 in 2%, pT2 in 10%, pT3 in 69% and pT4 in 19%.

Study II

During routine immunohistochemical evaluations at the Departments of Pathology, Helsingborg Hospital and Hvidovre Hospital, Denmark, colorectal 58
cancers with heterogeneous MMR protein expression were identified. Following the first observation of heterogeneous MMR protein staining in 2007, all such cases identified at these 2 institutions were collected during 5 years. These cases were re-evaluated and 12 colon cancers as well as 2 rectal cancers with a verified heterogeneous MMR protein expression were included in the study. None of the patients received neoadjuvant treatment. The mean age was 66 (range 33-85) years, the male:female ratio was 1:1.3 and the tumour stage was pT2 in 3 cases, pT3 in 9 cases and pT4 in 2 cases.

**Study III**

All Lynch syndrome mutation carriers and their first-degree relatives in the Danish cohort who had developed primary malignant tumours of the renal pelvis, the ureter or the urinary bladder were identified. In total, 48 ureter cancers, 34 renal pelvic cancers and 54 urinary bladder cancers developed in 97 patients from 75 families. The mean age at diagnosis was 61 (range 24-89) years, the male:female ratio was 1:1.3. The tumour stage was pTa in 46%, pT1 in 19%, pT2 in 18%, pT3 in 13%, pT4 in 1% and pTx in 3%. From 49/136 tumours formalin-fixed, paraffin-embedded tissue was available for histologic re-evaluation and further analysis.

**Study IV**

Study IV is based on the same cohort from the Danish HNPCC register as in study III. The register was used to identify all adenocarcinomas of the prostate that had developed in carriers of a disease-predisposing MMR gene mutation in *MLH1*, *MSH2*, *MSH6* or *PMS2* and in their first-degree relatives. In this cohort of 1609 males (677 mutation carriers and 932 first-degree relatives), prostate cancers developed in 15 mutation carriers and in 13 first-degree relatives. The median age at diagnosis was 63 (range 52-81) years. From 16/28 tumours formalin-fixed, paraffin-embedded tissue was available for histologic re-evaluation and further analysis. The Gleason score was 6 in 12.5%, 7 in 31.3%, 8 in 25%, 9 in 18.7% and 10 in 12.5%.

**Study V**

Study V is also based on the cohort from the Danish HNPCC register. The register was accessed in May 2015 and used to identify all primary malignant tumours of the kidney that had developed in carriers of a disease-predisposing MMR gene mutation.
1494 mutation carriers from 313 families with Lynch syndrome, based on proven disease-predisposing MMR gene mutations, were eligible for the study. In total, 13 RCCs were diagnosed in 8 men and 5 women with mutations linked to Lynch syndrome. The disease-predisposing genes were $MSH2$ in 6 cases, $MLH1$ in 6 cases and $MSH6$ in 1 case. The median age at diagnosis was 62 (47-82) years. In 2 families 2 RCCs had developed. The tumour stage was pT1 in 3 cases, pT2 in 4 cases, pT3 in 2 cases and pTx in 4 cases. From 6/13 tumours formalin-fixed, paraffin-embedded tissue was available for histologic re-evaluation and further analysis. Clinical data and the MMR status for the different studies are summarised in table 7.

Table 7. Summary of patients and materials for the different studies

<table>
<thead>
<tr>
<th>Study</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>462</td>
<td>14</td>
<td>97</td>
<td>28</td>
<td>13</td>
</tr>
<tr>
<td>No. of tumours</td>
<td>474</td>
<td>14</td>
<td>136 (49 with available tissue)</td>
<td>28 (16 with available tissue)</td>
<td>13 (6 with available tissue)</td>
</tr>
<tr>
<td>Male:female ratio</td>
<td>1:1.2</td>
<td>1:1.3</td>
<td>1:1.3</td>
<td>-</td>
<td>1.6:1</td>
</tr>
<tr>
<td>Mean age (range)</td>
<td>76 (50-100)</td>
<td>66 (33-85)</td>
<td>61 (24-89)</td>
<td>63 (52-81)</td>
<td>62 (47-82)</td>
</tr>
<tr>
<td>MSS</td>
<td>-</td>
<td>1</td>
<td>26/48</td>
<td>8/16</td>
<td>4/5</td>
</tr>
<tr>
<td>MSI-low</td>
<td>-</td>
<td>0</td>
<td>10/48</td>
<td>6/16</td>
<td>1/5</td>
</tr>
<tr>
<td>MSI-high</td>
<td>-</td>
<td>13</td>
<td>12/48</td>
<td>2/16</td>
<td>0/5</td>
</tr>
<tr>
<td>Intact MMR protein expression</td>
<td>366/474</td>
<td>0/14</td>
<td>5/49</td>
<td>5/16</td>
<td>2/6</td>
</tr>
</tbody>
</table>

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Methods

Study design and methods used for the different studies are summarised in table 8.

Table 8. Summary of methods used in the different studies

<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Cohort study</td>
<td>Histopathological evaluation, IHC, Statistical analysis (multiple logistic regression model, ROC, Inter-observer variability, Comparison of different prediction models with PPV, NPV and ROC)</td>
</tr>
<tr>
<td>II</td>
<td>Descriptive</td>
<td>Histopathological evaluation, IHC, Laser capture microdissection, DNA extraction, MSI analysis, Methylation analysis, Flow cytometry</td>
</tr>
<tr>
<td>III</td>
<td>National cohort study</td>
<td>Histopathological evaluation, IHC, DNA extraction, MSI analysis, Statistical analysis (Cumulative risk assessment, IRR)</td>
</tr>
<tr>
<td>IV</td>
<td>National cohort study</td>
<td>Histopathological evaluation, IHC, DNA extraction, MSI analysis, Statistical analysis (Cumulative risk assessment)</td>
</tr>
<tr>
<td>V</td>
<td>National cohort study</td>
<td>Histopathological evaluation, IHC, DNA extraction, MSI analysis, Statistical analysis (age-specific incidence/Poisson regression, IRR)</td>
</tr>
</tbody>
</table>

Histopathology (studies I-V)

In study I all available H&E-stained sections were morphologically reviewed for correct classification of the tumours. All cases were evaluated according to a standardised protocol by 2 independent investigators (B. Halvarsson and P. Joost) who were blinded to the IHC results as well as to the results from the other reviewer. The tumour location was classified as proximal/distal in relation to the splenic flexure [365]. The tumour stage was determined according to the American Joint Committee on Cancer /Union Internationale Contre le Cancer (AJCC/UICC) staging system and the grade according to the WHO system [366]. Mucinous/signet-ring cell cancers were considered as poorly differentiated. For the following variables only invasive tumour components were considered and intramucosal/early invasive tumour components were not taken into account [197, 198]. The growth pattern was classified as expanding if a continuous, rounded infiltration margin (with a pushing, well-circumscribed border) was found and as infiltrating if invading foci (dissecting tumour infiltrates) were identified [200]. Dirty necrosis was defined as the presence of cell detritus and inflammatory cells within the glandular lumina and was scored as present or absent [191]. A tumour was classified as mucinous or signet-ring cell cancer if more than 50% of the tumour area showed such differentiation [366]. Tumours with mucinous/signet-
ring cell components that encompassed 10-50% of the area but did not fulfil the criteria for mucinous/signet-ring cell tumours were considered to have a mucinous/signet-ring cell component [203]. The presence or absence of TIL was evaluated on H&E-stained sections and defined as the presence/absence of intraepithelial lymphocytes between tumour cells; hot spot areas were primarily analysed. TIL were scored as present if there were ≥7 TIL per 10 high-power fields (40x, field diameter 0.53 mm) [197, 198]. For comparison of the MMR index [203] with other prediction models of MMR deficiency additional variables, including variants of the variables above, were evaluated. They are summarised in the “Background” chapter, table 5.

In study II all cases were histologically re-evaluated by one pathologist (P. Joost). The tumour stage was determined according to the AJCC/UICC staging system and the grade according to the WHO system. A tumour was classified as mucinous cancer if more than 50% of the tumour area showed such differentiation [366]. Intraprotumoural differences in tumour differentiation were compared with MMR protein expression.

In study III all available urinary tract cancers were histologically re-evaluated by one pathologist (P. Joost). Tumour stage was determined according to the AJCC/UICC staging system and grade according to the WHO 2004 grading system [367].

In study IV all available core biopsies, as well as all prostatectomy specimens, were pathologically reviewed regarding their Gleason scores [287] and the presence of TIL (cut-off ≥4 per high-power field) [125, 195] by one pathologist (P. Joost), who was blinded to the MMR status.

In study V all available core biopsies, as well as all nephroureterectomy specimens were pathologically reviewed regarding their Fuhrman nuclear grade [338] and the presence of TIL (cut-off ≥5 per high-power field) was determined [197]. Tumour stage was determined according to the AJCC/UICC staging system and tumour type according to the WHO classification 2004 [367].

**MMR protein immunohistochemistry (studies I-V)**

Fresh 4-μm sections from formalin-fixed, paraffin-embedded tumours were mounted on Dako REAL™ capillary gap microscope slides (Dako, Glostrup, Denmark). The slides were dried overnight at room temperature and thereafter at 60°C for 1–2 hours. The tissue was deparaffinised in xylene for 2 times 5 min, followed by 5 min each in 99.5% and 95% ethanol and 5 min in distilled water. Heat-induced epitope retrieval was achieved by pressure boiler treatment in
ethylene diamine tetraacetic acid (EDTA)-Tris buffer (1:10 mM, pH 9.0) for 20 min. Hereafter the slides were cooled for 20 min and rinsed in distilled water.

Immunostaining was performed using the Dako Autostainer and the EnVision™ visualization method (Dako, Glostrup, Denmark). Endogenous peroxidase activity was blocked for 5 min and primary mouse monoclonal IgG antibodies were used. Following primary antibody incubation, the slides were incubated with EnVision™/horseradish peroxidase (HRP) rabbit/mouse (Dako) and stained using the EnVision™ detection system peroxidase/DAB rabbit/mouse (Dako). In study II sections from all tumour blocks were additionally subjected to independent MMR protein staining using alternative MMR protein antibodies from other manufacturers and partly using Ventana BenchMark Ultra (Ventana Medical Systems, Tucson, AZ) as an alternative platform. All specimens were stained at least 3 times. Antibodies, clones, suppliers and staining protocols are given in table 9.

### Table 9. Data on the MMR protein antibodies used

<table>
<thead>
<tr>
<th>MMR protein</th>
<th>Study</th>
<th>Lab</th>
<th>Supplier</th>
<th>Clone</th>
<th>Dilution</th>
<th>Immunogen/ epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLH1</td>
<td>I-V</td>
<td>L</td>
<td>BD Pharmingen</td>
<td>G168-15</td>
<td>1:100</td>
<td>Full-length</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>C</td>
<td>Dako</td>
<td>ES05</td>
<td>RTU</td>
<td>Recombinant protein, 210 aa</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>L</td>
<td>Dako</td>
<td>ES05</td>
<td>1:100</td>
<td>Recombinant protein, 210 aa</td>
</tr>
<tr>
<td>PMS2</td>
<td>I-V</td>
<td>L</td>
<td>BD Pharmingen</td>
<td>A16-4</td>
<td>1:300</td>
<td>431-862 a, C-terminal</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>H</td>
<td>Ventana (Cell Marque)</td>
<td>EPR3947</td>
<td>RTU</td>
<td>100 aa, C-terminal</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>C</td>
<td>Epitomics</td>
<td>EPR3947</td>
<td>1:50</td>
<td>100 aa, C-terminal</td>
</tr>
<tr>
<td>MSH2</td>
<td>I-V</td>
<td>L</td>
<td>Calbiochem</td>
<td>FE11</td>
<td>1:100</td>
<td>C-terminal</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>H</td>
<td>Ventana (Cell Marque)</td>
<td>G219-1129</td>
<td>RTU</td>
<td>Full-length</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>C</td>
<td>Novocastra</td>
<td>25D12</td>
<td>1:50</td>
<td>Full-length</td>
</tr>
<tr>
<td>MSH6</td>
<td>II</td>
<td>C</td>
<td>Epitomics</td>
<td>EP49</td>
<td>1:100</td>
<td>Synthetic peptide, N-terminal</td>
</tr>
<tr>
<td></td>
<td>I-V</td>
<td>L</td>
<td>Epitomics</td>
<td>EP3945</td>
<td>1:100</td>
<td>Synthetic peptide, N-terminal</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>L</td>
<td>BD Transduction Lab</td>
<td>44</td>
<td>1:500</td>
<td>Synthetic peptide, 225-333 aa</td>
</tr>
</tbody>
</table>

Abbreviations: aa, amino-acids; C, Copenhagen; H, Helsingborg; L, Lund; RTU, ready to use.
Immunohistochemical stainings were classified as retained, lost or reduced. Reduced immunostaining implies weaker than expected nuclear staining in tumour cells compared to non-neoplastic cells, i.e. stromal cells, lymphocytes and normal epithelium (figure 14). In study II the areas of the respective expression patterns were estimated in each section and expressed as percentages.

**Figure 14.** Examples of MMR protein expression patterns: (A) Normal/retained expression in tumour nuclei, (B) Lost expression in tumour nuclei and retained expression in non-neoplastic cells, (C) Reduced/weak expression in tumour nuclei compared to non-neoplastic cells.
Several technical artefacts may hamper the interpretation of immunostainings and these were considered carefully in the evaluations. Failure of the antibody to penetrate the tissue and the presence of air bubbles lead to general absence of staining and lack of an internal control (non-neoplastic cells). Poor fixation, which often occurs in the central parts of the specimen, may lead to a general absence of staining or a staining gradient in these areas. Generally weak staining can lead to false-negative interpretation or to non-evaluable samples, due to the lack of positivity in the non-neoplastic cells serving as internal controls. Excessive staining/overstaining may lead to a false-positive interpretation and may disguise weak/reduced expression and heterogeneous expression patterns. Necrotic tumour areas can exhibit both false-negative and non-specific positive staining [368].

Pitfalls in the evaluation of MMR stains also arise from biological and technical factors. Heterogeneous staining patterns due to biological tumour heterogeneity may lead to a false-positive interpretation. Also, neoadjuvant chemotherapy and radiotherapy may influence the results with a particular effect on MSH2/MSH6 staining [369, 370]. None of the patients in studies I-V, where tissue was evaluated with IHC, had received neoadjuvant chemotherapy and/or radiotherapy.

Examples of artefacts and pitfalls in MMR protein immunohistochemistry are shown in figure 15.
Figure 15. Artefacts and pitfalls in MMR protein immunohistochemistry: (A) Artefact created from an air bubble, (B) Overstaining with extensive background staining that hampers evaluation, (C)-(D) Lack of internal control staining in stromal components, (E) Staining gradients often observed in areas with poor fixation (F). Heterogeneous staining patterns for MSH6 with intraglandular mixture of nuclear positivity and negativity.
Laser capture microdissection (study II)

To assess the implications of heterogeneous MMR protein staining for MMR protein function, the tumours were subjected to MSI analysis. Depending on the extent of the area involved, laser capture microdissection was used to obtain material from areas with retained/lost expression patterns. Paraffin blocks from tumours with heterogeneous staining were prepared for laser capture microdissection. Polyethylene Teraphthalate (PET)-membrane FrameSlides (Carl Zeiss MicroImaging, Germany) were first pre-treated with UV-light for 30 min in order to make them less hydrophobic and then mounted with fresh 10-µm formalin-fixed, paraffin-embedded tissue sections. The tissue was deparaffinised and IHC was performed in the same way as described above. However, xylene and a cover slip were not applied after immunostaining. Tissue of heterogeneous areas was isolated using Carl Zeiss non-contact Laser Capture Microdissection (LCM) (Carl Zeiss MicroImaging GmbH, Munich, Germany). The 20x magnification was used, and for each analysis at least 2.5 mm² tissue from 2-6 tumour areas with retained as well as lost expression was harvested into separate adhesive caps of 500-µl microtubes (figure 16) [371].

![Laser capture microdissection](image)

**Figure 16.** Laser capture microdissection. The specimen is microdissected by a focused laser beam. The cut piece is then catapulted out of the object plane into a collection device by a defined laser pulse.
**DNA extraction (studies II-V)**

DNA was extracted from 1-3 fresh 10-µm sections from formalin-fixed and paraffin-embedded tissue blocks. Macrodissected tissue or whole tumour sections were deparaffinised by using xylene and ethanol, followed by tissue digestion adding 180 µl Buffer ATL (ready to use, Qiagen) and 20 µl Proteinase K (20 mg/ml, Qiagen). The tubes were incubated overnight at 56°C and at 90°C for 1 hour. Thereafter, DNA was extracted either using the QIAcube machine (Qiagen) or the QIAamp® DNA FFPE tissue kit (Qiagen) following the manufacturer’s protocol for FFPE (formalin-fixed paraffin-embedded) tissue sections, finally eluting with 60 µl Buffer ATE (ready to use, Qiagen). Lysis of laser microdissected tissue was obtained by adding 15 µl Buffer ATL (ready to use, Qiagen) and 10 µl Proteinase K (20 mg/ml, Qiagen) into each cap. The tubes were put upside down for digestion for at least 16 hours at 56°C. DNA was extracted using the QIAamp® DNA micro kit (Qiagen) following the manufacturer’s protocol for laser microdissected tissues, finally eluting in 20 µl Buffer AE (ready to use, Qiagen). After extraction, the DNA concentration was measured using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific Inc).

**MSI analysis (studies II-V)**

MSI status was determined using the MSI Analysis System Version 1.2 (Promega, Madison, WI). The system consists of 5 nearly monomorphic mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) for MSI determination and 2 polymorphic pentanucleotide markers (Penta-C and Penta-D) for sample identification (table 10).

**Table 10. Data on markers used for MSI analysis**

<table>
<thead>
<tr>
<th>Locus name</th>
<th>Gene</th>
<th>Repeat motif</th>
<th>Size range (bp)</th>
<th>Fluorescent label</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAT-25</td>
<td>c-kit</td>
<td>(A)25</td>
<td>113-124</td>
<td>JOE</td>
</tr>
<tr>
<td>BAT-26</td>
<td>MSH2</td>
<td>(A)26</td>
<td>103-115</td>
<td>FL</td>
</tr>
<tr>
<td>NR-21</td>
<td>SLC7A8</td>
<td>(A)21</td>
<td>94-101</td>
<td>JOE</td>
</tr>
<tr>
<td>NR-24</td>
<td>ZNF-2</td>
<td>(A)24</td>
<td>130-133</td>
<td>TMR</td>
</tr>
<tr>
<td>MONO-27</td>
<td>MAP4K3</td>
<td>(A)27</td>
<td>142-154</td>
<td>JOE</td>
</tr>
<tr>
<td>Penta-C</td>
<td>-</td>
<td>(AAAAG)3-15</td>
<td>143-194</td>
<td>TMR</td>
</tr>
<tr>
<td>Penta-D</td>
<td>-</td>
<td>(AAAAG)2-17</td>
<td>135-201</td>
<td>FL</td>
</tr>
</tbody>
</table>
The markers were amplified in 10-μl multiplex PCR reactions using 1 μl Gold ST
★R 10X Buffer, 1 μl MSI 10X Primer Pair mix, 0.15 μl AmpliTaq Gold® DNA
Polymerase (5 units/μl; Applied Biosystems, CA, USA), 2 μl template and
supplemented with nuclease-free water. As a positive amplification control, K562
Genomic DNA was used (diluted 1:10 to 1ng/μl; Promega) and as a negative
control nuclease-free water. The PCR was performed on a C1000 Thermal Cycler
(Bio-Rad Laboratories) and the program consisted of the following steps: 1
denaturation cycle at 95°C for 11 min and 1 cycle at 96°C for 1 min; 10 cycles of
30 sec at 94°C, 30 sec at 58°C, ramp 0.5°C/sec, 1 sec at 70°C and ramp 0.3°C/sec;
30 cycles of 30 sec at 90°C, 30 sec at 58°C, ramp 0.6°C/sec, 1 sec at 70°C and
ramp 0.3°C/sec; final extension for 30 min at 60°C; unlimited at 4°C. By the
fluorescently labelled markers the PCR products were size separated on a 3130xl
Genetic Analyzer (Applied Biosystems) after mixing the PCR products with 9 μl
deionised formamide (Hi-Di formamide, Applied Biosystems) and 1 μl ILS 600
(Promega) as the internal size marker and denaturing at 95°C for 3 min. The
GeneMapper® software was used for data analysis (figure 17). Tumours with
instability for 1 marker were classified as MSI-low, tumours with instability for ≥2
markers were classified as MSI-high, and tumours with stability for all markers
were classified as microsatellite stable (MSS).
Figure 17. MSI analysis from paraffin-embedded tissue. After DNA extraction the different markers are amplified by PCR with fluorescence labelled primers. The curves represent the fragment length analysis, where instability is defined as altered length of repetitive sequences. Blue shading indicates additional shorter peaks in microsatellite instable samples (figure reproduced and modified with permission from Kajsa Ericson-Lindquist).

**Methylation analysis (study II)**

*MLH1* promoter methylation analysis was performed at the Department of Clinical Genetics, Vejle Hospital, Vejle, Denmark according to the following protocol. Extracted DNA was treated with bisulfite using the EZ DNA Methylation-Lightning™ Kit (Zymo Research, CA, USA) according to the manufacturer's instructions. The *MLH1* promoter methylation status was analysed by a fluorescence-based, real-time methylation-specific PCR assay, as described previously [372]. Two sets of primers and probes, designed specifically for bisulfite-converted DNA, were used: MLH1-M2B for the methylation-specific
reaction [373] and ALU-C4 for the methylation-independent control reaction used to measure the amount of bisulfite-converted input DNA [374]. Amplification was performed on the QuantStudio™ 12 K Flex Real-Time PCR System (Life Technologies). The samples were run in doublets, with 2 reactions for each of the 2 primer sets with positive and negative controls included in each run.

**Flow cytometry (study II)**

In 1 tumour in study II heterogeneous tumour areas were analysed for DNA content by flow cytometric DNA analysis as previously described [375, 376]. Briefly, the 2 formalin-fixed samples were processed to form cell suspensions. Separated cells were treated with ribonuclease (Sigma-Aldrich, Stockholm, Sweden), incubated with trypsin for 48 h (Merck, Darmstadt, Germany) and stained with propidium iodide (Sigma-Aldrich, Stockholm). Flow cytometric DNA analysis was performed using the FACSCalibur platform (BD Biosciences, USA). Up to 20,000 nuclei were analysed from each sample. The DNA histograms obtained were automatically processed using Modfit LT™ 3.3 software. The DNA index (DI) was calculated as the ratio of the respective modal channel values of the non-diploid and the diploid G0/G1 peaks. The S-phase fraction (Spf) was estimated assuming that the S-phase compartment constituted a rectangular distribution between the modal values of the G0/G1 and G2 peaks.

**Statistical analysis (studies I, III-V)**

*Study I*

The MMR index includes the factors female sex, age ≥60 years, proximal tumour location, expanding growth pattern, lack of dirty necrosis, any mucinous/signet-ring cell differentiation (mucinous/signet-ring cell tumour or mucinous/signet-ring cell component) in ≥10% of the tumour area and presence of TIL. The index was applied to all tumours in the series. For statistical calculations, the software package Stata 12.1 (StataCorp. 2012, College Station, TX, USA) was used. The histopathologic variables were dichotomised and assigned equal weights. The association between the MMR status and the other histopathologic factors was analysed by means of contingency tables and the Fisher’s exact test.Patients with any missing value were excluded from the analysis (n=24). A multiple logistic regression model that contained the 7 dichotomised clinicopathologic factors as covariates was fitted to determine the independent contribution of each factor at predicting MMR deficiency. These effects were summarised as odds ratios (OR) with 95% confidence intervals (CI). The sensitivity and specificity of the MMR index were calculated by means of a receiver operating characteristic (ROC) curve.
In order to evaluate inter-observer agreement, 200 randomly selected tumours evaluated by B. Halvarsson were independently re-evaluated by P. Joost. Complete data were obtained for 189 tumours, which were included in the final calculation. Inter-observer variability was expressed using the chance-corrected measure of agreement kappa.

The MMR index results were compared with those from 5 other predictive models (“Background” chapter, table 5), i.e. MsPath [199, 204], PREDICT/simplified PREDICT [205], MSI probability score [206] and RERtest6 [202, 207] in 200 randomly selected tumours, 20% (n=40) of which were MMR deficient. The performance of the different models was evaluated by calculating the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) and area under the ROC curve (AUC).

**Studies III and IV**

The analyses in study III included all urinary tract cancers from mutation carriers and first-degree relatives. For patients who developed synchronous or metachronous tumours in the same organ, the first urothelial cancer in each patient was included in the analyses, whereas synchronous or metachronous cancers in different organs were allowed in their respective groups. In study IV the analyses included all prostate cancers from mutation carriers and first-degree relatives, no metachronous tumours occurred and the calculations excluded females. Mutation carriers were weighted by 1, while first-degree relatives were weighted by 0.5, assuming 50% mutation carriers among the latter. One family with an EPCAM mutation that also affected MSH2 was pooled with the MSH2 groups. In total, 7 families with PMS2 mutations were excluded, since no urothelial cancers were observed in this cohort. The event times used were age at diagnosis or age at death in unaffected individuals. Individuals who remained free from cancer were censored in May 2014 based on data on vital status from the Danish civil registry. Patients without a civil number were censored at the time of death based on the death certificate or the family record, or at the last follow-up date. Genotypic and phenotypic pedigree data on mutation carriers and first-degree relatives of these were transferred into R 3.1.0 (R: A Language and Environment for Statistical Computing, Vienna, Austria). Cumulative incidences were calculated with death as a competing risk (cmprsk: Subdistribution Analysis of Competing Risks, 2011, Bob Gray, R package version 2.2-2). Confidence intervals were calculated at age 70 using a non-parametric boot-strap, with re-sampling and repeated calculations of the cumulative incidences. Group p-values were calculated using permutation tests with 10,000 replicates with significance between the groups set at p<0.05. Incidence rate ratios (IRR) were calculated for urinary tract cancers in MSH2 mutation carriers compared to MLH1/MSH6 mutation carriers, again with boot-strapping as
described above. The spectrum of MSH2 mutations was mapped to investigate potential clustering to certain genetic regions and cumulative incidences and confidence intervals of urothelial cancer were determined for each specific MSH2 mutation. The risk of urinary cancer was compared between families with MSH2 mutations with more than 5 events and all families with other MSH2 mutations using group p-values as described above.

Study V

Determination of the incidence ratios of RCC in the general Danish population and in the Lynch syndrome cohort was possible - due to data availability and completeness - for all cases diagnosed between January 1, 1978 and December 31, 2012. Person years at risk were defined as the period from entry date (January 1, 1978) or date of birth, whichever occurred last, to exit date (December 31, 2012) or date of death, whichever occurred first. Cancer events were stratified by age, sex, mutated gene and time period. Data from the Danish background population were collected from the Nordcan database (available from http://www.ancr.nu, accessed on May 1, 2015) [377]. Person years at risk were calculated in the Pyrsstep SAS-macro (http://sourceforge.net/projects/pyrsstep/) using SAS software, version 9.4 (SAS Institute Inc., Cary, NC, USA) with incidence rates from the Danish background population as reference [378]. Lynch syndrome-associated RCCs and their respective person years at risk were subtracted from the background population-based data in the Nordcan database to eliminate double registrations. Cancer events were pooled into 4 age groups: 0-29 years, 30-49 years, 50-69 years, and 70+ years. The Lynch syndrome cohort and the Nordcan data set were linked using personal identifiers such as age, sex and period, and the expected RCC events were calculated for all age groups. The stratified and aggregated data were transferred into R i386 3.1.0 (R: A Language and Environment for Statistical Computing, 2011, R Foundation for Statistical Computing, Vienna, Austria). Incidence rate ratios, 95% confidence intervals and p-values were calculated using Poisson regression analyses. The significance level was set at p < 0.05.
Results and Discussion

Study I

A pre-screening procedure that identifies tumours with a high likelihood of MMR deficiency could be clinically valuable for institutions that have not implemented universal assessment of the MMR status. Several MMR prediction models have been established (see table 5 in the Background chapter) [199, 202, 204-207]. For such models to be implemented in the routine histopathological work-up, the assessment should be reproducible and easy to apply, i.e. it should preferentially be based on factors that can be evaluated on standard sections. We assessed the performance of the MMR index and validated the index in an independent series of 474 colon cancers and provide data on reproducibility and performance in comparison with other MMR/MSI predictive models.

MMR deficiency, defined as the immunohistochemical loss of at least one MMR protein, was identified in 108/474 (22.8%) tumours. No tumour showed weak or reduced MMR protein staining. The MMR deficient tumours predominantly developed in women (74.8%), in the proximal colon (91.7%), and at a mean age of 76 (range 50-100) years (table 11). The MMR defects involved MLH1/PMS2 in 93 tumours, PMS2 in 1, MSH2/MSH6 in 5, MSH6 in 4, and MLH1/PMS2 and MSH6 in 5. This means that defects highly suggestive of Lynch syndrome (mutations in $MSH2$ and $MSH6$) were identified in 14/474 (3%) cases.

Several morphologic features were overrepresented in MMR deficient tumours in comparison with MMR proficient tumours (table 11). This applied to an expanding growth pattern (73.8% versus 7.6%), lack of dirty necrosis (80.6% versus 26.1%), mucinous/signet-ring cell differentiation (67.6% versus 26.3%) and presence of TIL (66.7% versus 16.9%).
Table 11: Distribution of clinicopathological factors in relation to the MMR status (n=474).

<table>
<thead>
<tr>
<th>Factor</th>
<th>Frequency (%)</th>
<th>MMR deficient</th>
<th>MMR proficient</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patients (n=462)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number</td>
<td>103 (22.3)</td>
<td>359 (77.7)</td>
<td></td>
</tr>
<tr>
<td><strong>Sex (n=462)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>26 (25.2)</td>
<td>184 (51.3)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>77 (74.8)</td>
<td>175 (48.7)</td>
<td></td>
</tr>
<tr>
<td><strong>Age (n=462)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>76</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>Age ≥60</td>
<td>102 (99.0)</td>
<td>328 (91.4)</td>
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<tr>
<td><strong>Tumours (n=474)</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Total number</td>
<td>108 (22.8)</td>
<td>366 (77.2)</td>
<td></td>
</tr>
<tr>
<td><strong>pT stage (n=450)</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>pT1</td>
<td>2 (1.9)</td>
<td>6 (1.7)</td>
<td></td>
</tr>
<tr>
<td>pT2</td>
<td>10 (9.6)</td>
<td>37 (10.7)</td>
<td></td>
</tr>
<tr>
<td>pT3</td>
<td>87 (83.7)</td>
<td>224 (64.8)</td>
<td></td>
</tr>
<tr>
<td>pT4</td>
<td>5 (4.8)</td>
<td>79 (22.8)</td>
<td></td>
</tr>
<tr>
<td><strong>pN stage (n=461)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pN0</td>
<td>76 (73.1)</td>
<td>183 (51.2)</td>
<td></td>
</tr>
<tr>
<td>pN1</td>
<td>23 (22.1)</td>
<td>107 (30.0)</td>
<td></td>
</tr>
<tr>
<td>pN2</td>
<td>5 (4.8)</td>
<td>67 (18.8)</td>
<td></td>
</tr>
<tr>
<td><strong>pM stage (n=474)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM1</td>
<td>1 (0.9)</td>
<td>10 (2.7)</td>
<td></td>
</tr>
<tr>
<td><strong>Differentiation (n=474)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good/moderate</td>
<td>42 (38.9)</td>
<td>334 (91.3)</td>
<td></td>
</tr>
<tr>
<td>Poor/no</td>
<td>66 (61.1)</td>
<td>32 (8.7)</td>
<td></td>
</tr>
<tr>
<td><strong>Location (n=473)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>99 (91.7)</td>
<td>173 (47.4)</td>
<td></td>
</tr>
<tr>
<td>Distal</td>
<td>9 (8.3)</td>
<td>192 (52.6)</td>
<td></td>
</tr>
<tr>
<td><strong>Growth pattern (n=459)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expanding</td>
<td>76 (73.8)</td>
<td>27 (7.6)</td>
<td></td>
</tr>
<tr>
<td>Infiltrating</td>
<td>27 (26.2)</td>
<td>329 (92.4)</td>
<td></td>
</tr>
<tr>
<td><strong>Dirty necrosis (n=467)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>20 (19.4)</td>
<td>269 (73.9)</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>83 (80.6)</td>
<td>95 (26.1)</td>
<td></td>
</tr>
<tr>
<td><strong>Mucin/signet-ring differentiation (n=473)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present (&gt;50%)</td>
<td>23 (21.3)</td>
<td>15 (4.1)</td>
<td></td>
</tr>
<tr>
<td>Present (10-50%)</td>
<td>50 (46.3)</td>
<td>81 (22.2)</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>35 (32.4)</td>
<td>269 (73.7)</td>
<td></td>
</tr>
<tr>
<td><strong>TIL (n=470)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>72 (66.7)</td>
<td>61 (16.9)</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>36 (33.3)</td>
<td>301 (83.1)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: MMR, mismatch repair; TIL, tumour-infiltrating lymphocytes.
The strongest predictive indicators of MMR deficiency were an expanding growth pattern (OR 11.6; 95% CI 5.5-24.5), presence of TIL (OR 5.6; 95% CI 2.6-12.1), mucinous/signet-ring cell differentiation (OR 3.0; 95% CI 1.3-6.0) and lack of dirty necrosis (OR 3.0; 95% CI 1.3-7.0).

The MMR index was applied in 438 patients from whom complete data were available. In these patients, the presence of ≥4 factors identified MMR deficient colon cancers with a sensitivity of 92.6% and a specificity of 75.5%, corresponding to an ROC curve with an AUC of 0.94 (95% CI, 0.91-0.96) (figure 18).

Inter-observer agreement was 90%, which corresponds to a kappa value of 0.88. The kappa values for the individual histopathological markers were 0.78 for TIL, 0.94 for mucinous/signet-ring cell components, 0.96 for lack of dirty necrosis and 0.97 for an expanding growth pattern.

The comparison with other predictive models was performed in 200 randomly selected tumours, in which the MMR index - applied with a cut-off of ≥4 factors - resulted in an AUC of 0.83 (95% CI, 0.79-0.87) and identified MMR deficient tumours with a sensitivity of 97.5% and a specificity of 69%. The factors expanding growth pattern, TIL, mucinous/signet-ring cell differentiation and lack of dirty necrosis identified MMR deficient tumours with almost identical performance as that described in the original report of the MMR index [203].
The corresponding AUC values were 0.81 for PREDICT, 0.80 for RERtest6, 0.70 for MsPath and 0.77 for the MSI probability score. The sensitivity values for these models varied from 60% to 100% and the specificity was 41% to 99% (table 12).

The performance of the MMR index was similar to that of the PREDICT/simplified PREDICT models (p=0.38/p=0.27) and the RERtest6 model (p=0.42), but it was significantly better than that of the MsPath model (p<0.0001) and the MSI probability score model (p<0.0001 for a cut-off >1 and p<0.01 for a cut-off >1.5).

Table 12. Performance of the different prediction models for MMR deficiency (n=200).

<table>
<thead>
<tr>
<th>Model</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMR index</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 features present</td>
<td>97.5</td>
<td>68.8</td>
<td>44.3</td>
<td>99.1</td>
<td>0.83</td>
</tr>
<tr>
<td>PREDICT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Score ≥2.5</td>
<td>95.0</td>
<td>66.3</td>
<td>41.3</td>
<td>98.2</td>
<td>0.81</td>
</tr>
<tr>
<td>Simplified PREDICT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 features present</td>
<td>95.0</td>
<td>65.0</td>
<td>40.4</td>
<td>98.1</td>
<td>0.80</td>
</tr>
<tr>
<td>MsPath</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Score ≥1</td>
<td>100.0</td>
<td>40.6</td>
<td>29.6</td>
<td>100.0</td>
<td>0.70</td>
</tr>
<tr>
<td>MSI probability score</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Score ≥1</td>
<td>100.0</td>
<td>45.6</td>
<td>31.5</td>
<td>100.0</td>
<td>0.73</td>
</tr>
<tr>
<td>Score ≥1.5</td>
<td>97.5</td>
<td>56.3</td>
<td>35.8</td>
<td>98.9</td>
<td>0.77</td>
</tr>
<tr>
<td>RERtest6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Score &lt;0.8</td>
<td>60.0</td>
<td>99.4</td>
<td>96.0</td>
<td>90.8</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Abbreviations: AUC, area under curve; MMR, mismatch repair; MSI, microsatellite instability; NPV, negative predictive value; PPV, positive predictive value.

Discussion

The strongest predictive indicators of MMR deficiency were an expanding growth pattern (OR 11.6), the presence of TIL (OR 5.6), mucinous/signet-ring cell differentiation (OR 3.0) and lack of dirty necrosis (OR 3.0) (table 11). In comparison to the series in which the MMR index was developed, the age groups studied differed somewhat (mean age 76 versus 72 in the previous series), but the above 4 histopathological characteristics were observed at similar frequencies, i.e. 59-89% in the previous study and 67-81% in the present series [203]. There were some differences in the predictive values of the individual factors, but when combined into the MMR index, the presence of ≥4 factors identified MMR
defective tumours with similar sensitivity (93%) and specificity (75%) in the 2 sample sets.

Inter-observer agreement was demonstrated through independent and blinded evaluation by 2 reviewers, who identified MMR deficient tumours with a kappa value of 0.88. Hence, the model demonstrates stable performance with good predictive accuracy and it is quick and easy to apply.

When compared with 5 other predictive models, the MMR index demonstrated better performance than the RERtest6 [202, 207], the MsPath [199, 204] and the MSI probability score [206] models and comparable performance to the PREDICT/simplified PREDICT models [205] (table 12). The MsPath, PREDICT and MSI probability score models identified MMR deficient tumours with an equally favourable sensitivity compared with the MMR index, whereas the sensitivity of the RERtest6 model was clearly inferior (60%) in our cohort.

The MMR index identified 39/40 MMR deficient tumours, but also indicated MMR deficiency in 50 MMR proficient tumours, which implies unnecessary MMR testing in 50/160 MMR proficient tumours. The corresponding number of MMR proficient tumours that would be included in MMR status testing was 54 for the PREDICT model, 70 for the MSI probability score model and 95 for the MsPath model. By contrast, the RERtest6 model showed a high specificity. Only 1 tumour identified as MMR deficient was a false-positive. However, as mentioned above, the sensitivity was low: 16/40 MMR deficient tumours escaped detection (see “supplementary table” in original article study I).

With the exception of the RERtest6 model, the comparator algorithms were primarily developed for the identification of Lynch syndrome tumours [191, 199, 205]. The MsPath, PREDICT and MSI probability score models therefore include age <50 years as one of the predictive variables, which means that the comparison with the MMR index in our study was suboptimal given that the study only included tumours diagnosed after age 50. The RERtest6 model was, however, developed without age restriction and has been validated in independent tumour samples [202, 207].

MMR testing is commonly requested without prior selection in the 5% of colon cancers that develop before age 50 [158, 379, 380]. Our study thus focused on patients aged ≥50 years, 23% of whom had MMR deficient colon cancers, with loss of MLH1/PMS2 in 86% of the cases. Most of these cases will be of sporadic origin, involving the consistent silencing of MLH1 through promoter methylation. This epigenetic mechanism has been suggested to lead to more pronounced histopathological features than the variable MMR gene defects observed in Lynch syndrome tumours [70, 73, 195, 381]. However, despite our focus on a cohort aged ≥50, we identified immunohistochemical loss of MSH2 and/or MSH6 suggestive of Lynch syndrome in 3% of the tumours. In particular, the contribution from MSH6 was substantial. MSH6 mutations have been linked to an
overall lower risk of colon cancer, a less striking family history, a higher age at onset and less pronounced tumour morphology, which implies that many cases may escape detection [382, 383]. Indeed, the 4 cases with an isolated loss of MSH6 and the 5 cases with the combined loss of MLH1/PMS2 and MSH6 in our series developed after age 70. Although these cases did most likely not influence the performance of the MMR index, the observation indicates that Lynch syndrome should also be considered also among somewhat older patients.

The MMR index is not intended for the identification of Lynch syndrome cases and has not been validated in younger individuals. However, 11 of 14 MSH2/MSH6 deficient tumours in our series and all 7 MSH2/MSH6 deficient tumours reported by Halvarsson et al. [203] would have been detected by the MMR index.

The study limitations include assessment of the MMR status solely based on immunohistochemical staining and inclusion of a small number of presumed Lynch syndrome tumours, which were not genetically characterised in more detail. The impact from these shortcomings is judged to be minor and the limitations reflect use of the MMR index in clinical routine.

Study II

*Universal assessment of immunohistochemical MMR status is increasingly applied in colorectal cancer diagnostics in order to identify cases suspected of Lynch syndrome for further molecular diagnostics and to obtain prognostic and treatment-predictive information linked to the somatic methylation of MLH1 [154]. MMR protein immunostaining is generally stable and relatively easy to interpret, although challenges and pitfalls have been reported with false-positive as well as false-negative interpretations [384-386]. Most commonly, these observations relate to technical artefacts caused by suboptimal fixation or paraffin-embedding, necrotic areas, sample storage, antibody specificity, clone selection or staining conditions [368, 387]. Also, neoadjuvant chemotherapy and radiotherapy may influence the results with a particular effect on MSH2/MSH6 staining [369, 370]. Heterogeneous expression patterns have been reported, e.g. retained staining in the adenomatous part and loss of staining in an invasive tumour area, though their relevance is uncertain [371]. We systematically collected colorectal cancers with heterogeneous MMR protein staining patterns for the detailed analysis of correlations with the MSI status and MLH1 promoter methylation.*
Immunohistochemical staining using alternative MMR protein antibodies confirmed heterogeneous MMR protein expression in all 14 tumours. The heterogeneity affected MLH1/PMS2 in 3 tumours, PMS2 in 2 tumours, MSH2/MSH6 in 10 tumours (of which 2 also showed heterogeneity for MLH1/PMS2 expression) and MSH6 in only 1 tumour (in which one block also showed heterogeneity for PMS2 expression). Areas with alternative expression patterns were well demarcated and appeared in 3 distinct patterns: “intraglandular” (retained/lost staining within or in between glandular formations), “clonal” (retained/lost staining in whole glands or groups of glands) and “compartmental” (retained/lost staining in larger tumour areas/compartments leading to retained/lost staining in between different tumour blocks) (figure 19). Various heterogeneous expression patterns co-existed in 9/14 tumours, most commonly as intraglandular and clonal heterogeneity (figure 19C). The heterogeneous staining patterns were present in 3-100% of the respective examined tumour area. In 4/14 cases, all tumour blocks showed heterogeneity, whereas the remaining tumours showed heterogeneity in a variable fraction of the tumour blocks (see table 3 “Summary of MMR heterogeneity” in original article study II).

Figure 19. Examples of the different MMR protein staining patterns. (A) Clonal loss, (B) intraglandular loss, (C) co-existence of clonal and intraglandular loss and (D) compartmental loss with different patterns in 2 separate tumour blocks.
In some tumours, a variable MMR status corresponded to variable differentiation, e.g. mucinous areas, poor differentiation or adenomatous components (figure 20).

**Figure 20.** Variable MMR protein expression in relation to tumour differentiation. (A) Retained expression of PMS2 in a mucinous tumour component and loss of PMS2 expression in a non-mucinous component. (B) Clonal and intraglandular heterogeneity for MLH1 in the adenomatous component of a tumour, whereas the remaining tumour areas showed retained expression of MLH1. (C) Clonal heterogeneity of MSH6 expression in a poorly differentiated tumour component and (D) homogenous expression in a well-differentiated tumour component.

MSI was demonstrated in 13/14 tumours. Intratumoural differences in MMR status, i.e. MSI versus MSS, in line with MMR protein expression was observed in 3 tumours (table 3 “Summary of MMR heterogeneity” in original article study II; cases 1, 2 and 9). Non-consistent MSI status in tumours with heterogeneous MMR protein expression was observed in 2 cases (cases 4 and 6). MLH1 promoter methylation was demonstrated in all 7 cases with complete (non-heterogeneous) loss of MLH1/PMS2. In 2 cases (cases 1 and 9) heterogeneous MMR protein staining for MLH1/PMS2 correlated with heterogeneous MLH1 promoter methylation, i.e. tumour areas with retained MLH1 expression did not show MLH1 methylation, whereas areas with loss of MLH1 expression showed MLH1 methylation. DNA flow cytometric analysis was performed in one tumour (case 1) and demonstrated differences in DNA content within the heterogeneous areas, which had DNA indices of 1.13 and 1.57, respectively (figure 21).
Figure 21. Adenocarcinoma (case 1) with 4 different expression patterns and various combinations of heterogeneity, loss of MLH1/PMS2 and heterogeneity/retained expression of MSH2/MSH6. (A) clonal loss of MLH1 staining. (B) MSI corresponding to loss of MMR protein staining, (C) MSS corresponding to retained MMR protein staining. Methylation analysis revealed the (D) presence and (E) absence, respectively, of MLH1 promoter methylation, in line with the clonal MLH1 methylation status. Flow cytometric analysis showed different DNA indices, i.e. (F) 1.13 in the MSI area and (G) 1.57 in the MSS area.
Discussion

Heterogeneous MMR protein expression is a rare phenomenon that corresponds to differences in the MMR status within a given tumour. It is therefore important to recognize its occurrence to prevent false-positive or false-negative evaluations. We identified 3 distinct patterns of heterogeneous MMR protein expression, i.e. intraglandular, clonal and compartmental heterogeneous MMR protein expression. The different patterns co-existed within the same tumour and the extent of the tumour area involved varied.

In-depth analysis suggests that multiple causes may be responsible, e.g. variable epitope expression, variable differentiation, second hit mutations or methylation in selected tumour clones and possibly influence from factors linked to the tumour microenvironment such as hypoxia and oxidative stress [39].

Intraglandular and/or clonal heterogeneity throughout the tumour, which may be caused by variable epitope expression, was identified in 4 tumours (cases 4, 5, 15 and 16; see table 3 “Summary of MMR heterogeneity” in original article study II).

Homogenous loss of MLH1/PMS2 and heterogeneous expression of MSH2/MSH6 was identified in 7 tumours, all of which were MSI-high and showed MLH1 promoter methylation. This expression pattern has been observed previously and may relate either to a germline MSH2/MSH6 mutation that allows for partial epitope binding in the presence of somatic MLH1 methylation or to secondary MSH2/MSH6 inactivation [39, 388, 389]. For example, Shia et al. reported secondary mutations in coding region microsatellites of the MSH6 gene as a possible mechanism for aberrant staining of MSH6 in tumours with loss of MLH1/PMS2 [390].

Heterogeneous MLH1 and/or PMS2 expression, suggestive of variable MLH1 methylation/second hit mutations, was observed in 2 tumours (cases 2 and 6).

Case 1 showed a more complex pattern of MMR protein expression and intra-tumour differences in MSI, MLH1 promoter methylation and DNA content, suggestive of a tumour composed of 2 distinct clones (case 1, figure 21).

Different, though homogenous, MMR protein expression patterns in distinct tumour compartments were observed in a mucinous adenocarcinoma (case 9) with loss of MLH1/PMS2 expression, MSI and MLH1 methylation in 1/7 tumour blocks that corresponded to an adenomatous tumour component (figure 19D). A sample mix was excluded through histologic review and penta-D marker fragment analysis (data not shown).

The homogenous loss/reduced staining of MSH2/MSH6 throughout the tumour and the additional loss of PMS2 were observed in 7/10 tumour blocks from a mucinous adenocarcinoma (case 5). This case most likely reflects a situation where the mucinous tumour component progressed in another line than the non-
mucinous tumour component. Though compartmental loss of MMR protein expression is rare, this observation motivates the thorough evaluation of different tumour compartments, particularly when areas with variable expression are observed.

Limitations to our study include the use of surgical specimens, since biopsy material may produce stainings of better technical quality [391-394]. At the same time, the use of biopsy material implies that restricted tumour areas were analysed and that areas with alternative expression might not have been captured.

Moreover, information on the MMR gene mutation status was not available in all cases. The 4 tumours from Lynch syndrome mutation carriers displayed heterogeneity in different tumour blocks, with homogenous as well as heterogeneous loss in clonal and intraglandular patterns.

So-called “patchy” MMR protein staining has also been reported. This phenomenon differs from the heterogeneous staining patterns in that it primarily relates to MSH6 stainings in neoadjuvantly treated tumour specimens [369, 370] or represents weak or cytoplasmatic staining rather than a pattern of distinct and well-demarcated areas with retained staining on the one hand and loss of staining on the other.

Study III

Lynch syndrome is a multi-tumour syndrome. The highest risks apply to colorectal cancer and endometrial cancer, but the tumour spectrum also includes other tumour types such as cancer of the upper urinary tract. We aimed to assess the frequency or urinary tract cancer in the Danish Lynch syndrome cohort, to provide evidence for the link between urinary bladder cancer and Lynch syndrome, to assess the sensitivity of MMR protein immunostaining and microsatellite instability, and to determine the cumulative risks of urinary tract cancer in relation to the different disease-predisposing MMR genes.

In total, 48 ureter cancers, 34 renal pelvic cancers and 54 urinary bladder cancers developed in 97 patients from 75 Lynch syndrome families. Of the 136 urinary tract cancers, 106 (78%) developed in a family without a prior history of the disease. Synchronous urinary tract cancer was diagnosed in 16 (16.5%) patients, 9 of whom had synchronous upper urinary tract cancer and bladder cancer. Metachronous urinary tract cancer developed in 12/97 (12.4%) patients, 4 of whom developed bladder cancer following a diagnosis of upper urinary tract cancer. The majority of the patients (78.4%) had a previous cancer outside of the urinary tract with urinary tract cancer as the first diagnosis in 15 patients.
The development of urothelial cancer was strongly associated with *MSH2* mutations with 99/136 (73%) of the tumours diagnosed in *MSH2* mutation carriers or their first-degree relatives. In total, urinary tract cancer was diagnosed in 49/135 *MSH2* families, in 10/76 *MLH1* families and in 16/70 *MSH6* families.

**Cancers of the renal pelvis and the ureter**

We identified 82 upper urinary tract cancers in 64 patients (46 mutation carriers and 18 first-degree relatives) from 55 families. The tumours included 48 transitional cell cancers of the ureter and 34 transitional cell cancers of the renal pelvis. The mean age at diagnosis was 62 (36-89) years with a somewhat higher mean age, 69 years, at diagnosis for cases associated with *MSH6* mutations compared with 59 and 61 years, respectively, for cases linked to *MLH1* and *MSH2* mutations. Of the patients, 55% were female. The tumour stage was pTa in 43% with an even stage distribution in the remaining cases. The tumours were predominantly (76%) of high grade and showed papillary differentiation with the exception of 3 cases with non-papillary histology. *MSH2* mutations were present in 76% of the cases.

**Urinary bladder cancer**

In total, we identified 54 cancers of the urinary bladder from 50 patients (35 mutation carriers and 15 first-degree relatives) from 45 families. The tumours were predominantly (67%) of high grade and all tumours showed papillary differentiation. The tumour stage was pTa in 52%; in the remaining cases, the predominant stages were pT1 and pT2. The mean age at diagnosis was 61 (24-82) years with a higher mean age, 71 years, in *MSH6*-associated cases compared with 59 years in cases associated with *MLH1* and *MSH2* gene mutations. Of the patients, 58% were male. *MSH2* mutations were present in 69% of the cases.

**MMR defects in upper urinary tract cancer and bladder cancer**

Loss of immunohistochemical expression of the affected MMR protein was identified in 26/28 (93%) upper urinary tract cancers and in 18/21 (86%) urinary bladder cancers (figure 23). Of the upper urinary tract cancers, 8/25 (32%) showed an MSI-high genotype; among the bladder cancers, the corresponding figure was 4/20 (20%).

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Figure 23. Immunohistochemical staining for the 4 MMR proteins in an urothelial cell carcinoma of the bladder in a patient with a germline MSH2 mutation. (A) normal staining for MLH1, (B) normal staining for PMS2, (C) loss of nuclear staining for MSH2 and (D) loss of staining for MSH6.

Risk of urothelial cancer development

The cumulative risk of urinary tract cancer (including cancer of the renal pelvis and the ureter as well as urinary bladder cancer) at age 70 was 6.7% (95% CI: 5.5-7.8). The cumulative risk estimates for cancers of the renal pelvis and the ureter were 5.1% in women and 4.6% in men and the cumulative risk for urinary bladder cancer was 3.3% in men and 2.6% in women (table 13).

Table 13. Cumulative risk estimates (%) in relation to subgroups

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Cumulative risk (%)</th>
<th>Upper urinary tract</th>
<th>Urinary bladder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men</td>
<td>3.96 (95% CI: 2.57-5.29)</td>
<td>4.11 (95% CI: 2.64-5.45)</td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>5.13 (95% CI: 3.64-6.55)</td>
<td>2.56 (95% CI: 1.54-3.52)</td>
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</tr>
<tr>
<td>MSH2</td>
<td>6.91 (95% CI: 5.11-8.60)</td>
<td>4.4 (95% CI: 2.95-5.77)</td>
<td></td>
</tr>
<tr>
<td>MLH1</td>
<td>2.17% (95% CI: 0.73-3.42)</td>
<td>2.85 (95% CI: 1.32-4.24)</td>
<td></td>
</tr>
<tr>
<td>MSH6</td>
<td>2.89 (95% CI 1.23-4.37)</td>
<td>1.74 (95% CI: 0.40-2.85)</td>
<td></td>
</tr>
</tbody>
</table>
Individuals with *MSH2* mutations had a significantly higher risk for upper urinary tract cancer (6.9%) compared to individuals with mutations in *MLH1* (2.2%) or *MSH6* (2.9%) (p=0.0006) (table 13 and figure 24A). The corresponding IRR (*MSH2*=1) for *MLH1* and *MSH6* was 0.23 and 0.40, respectively.

Also, the risk for urinary bladder cancer was significantly higher in *MSH2* mutation carriers (4.4%) than in individuals with *MLH1* (2.9%) or *MSH6* (1.7%) mutations (p=0.0081) (figure 24B). The corresponding IRR (*MSH2*=1) for *MLH1* and *MSH6* was 0.46 and 0.42, respectively.

![Figure 24](image)

**Figure 24.** Cumulative lifetime risk of (A) upper urinary tract and (B) urinary bladder cancer in relation to the disease-predisposing MMR gene.
The subgroup with the highest risk were men with *MSH2* mutations, who had a cumulative risk of urinary tract cancer of 6.9% (95% CI: 5.08-8.60). The spectrum of *MSH2* mutations in individuals with urothelial cancer is delineated in figure 25. No obvious urothelial cancer cluster region was found and the identified recurrent mutations corresponded to hotspot mutations in the Danish Lynch syndrome cohort. The cumulative risk estimates for urothelial tract cancer did not differ between the different mutations (data not shown).

Figure 25. Distribution of *MSH2* mutations in individuals who developed upper urinary tract cancer (green) and bladder cancer (red). In total, 62/82 upper urinary tract cancers and 37/54 urinary bladder cancers developed in *MSH2* mutation carriers.

**Discussion**

Urinary tract cancer developed in 26% of all Danish Lynch syndrome families and was particularly frequent (36%) in families with *MSH2* mutations. The tumours were located in the urinary bladder (40%), the ureter (35%) and the renal pelvis (25%).

The development of urothelial cancer in MMR mutation carriers, the loss of immunohistochemical expression concordant with the mutated gene in 93% of the upper urinary tract cancers and in 86% of the bladder cancers, and the demonstration of MSI suggest that cancers of the upper urinary tract as well as those of the urinary bladder represent markers of Lynch syndrome.
Immunohistochemical analysis proved considerably more sensitive (90%) than MSI analysis (23%) for the identification of MMR defective tumours.

Urothelial cancers developed at a mean age of 61 years with a predominance of high-grade, papillary tumours.

Cancer of the upper urinary tract is well recognised as part of the Lynch syndrome tumour spectrum with cumulative risk estimates of 1.9-12% [57, 106, 109, 121, 123, 137, 174, 395]. Our data confirm these observations and underscore the strong link to MSH2 mutations, which were present in 76% of the cases of the upper urinary tract.

Whereas sporadic urothelial cancers show a male to female ratio of 2.7:1, we identified a male to female ratio of 1:1.3 in our cohort. The cumulative risks of any urinary tract cancer at age 70 in the Lynch syndrome cohort were 5.1% in women and 4.6% in men, which serves as a reminder to include both sexes in surveillance programs.

Though not directly comparable, e.g. due to differences in the time period studied and the method of ascertainment, the cumulative risk of urinary tract cancer at age 70 in the general Danish population (estimated between 1978 and 2013) was 2.15% in men and 0.7% in women [377]. A direct comparison with a matched control cohort was not included in the present study.

An increased risk of bladder cancer in Lynch syndrome has been suggested in a limited number of studies with risk estimates of 1-7.5%, which fits well with our risk estimates of 3.3% for men and 2.6% for women [121-123, 137, 396-398].

Mutations in MSH2 have been suggested to confer an increased risk for several types of extracolonic tumour types [397]. Urinary tract cancer was strongly associated with MSH2 mutations with 77% of the ureter cancers, 74% of the renal pelvic cancers and 69% of the urinary bladder cancers linked to MSH2 mutations, which fits well with estimates that suggest that 81-82% of the urinary tract cancers develop in MSH2 families [122, 123].

Three studies have also linked the development of bladder cancer to MSH2 mutations, which were identified in 71-76% of the cases [122, 123, 397]. The cumulative risks for upper urinary tract cancer (6.9%) and urinary bladder cancer tract cancer (4.4%) were significantly higher in patients with MSH2 mutations compared to individuals with mutations in MLH1 or MSH6 (p=<0.005) (figure 24).

Within the MSH2 gene, we could, however, not identify any specific mutation cluster region linked to urinary tract cancer.

The revised European guidelines for Lynch syndrome families do not recommend surveillance for urinary tract cancer, whereas the US guidelines call for surveillance for microscopic haematuria from age 30-35 in mutation carriers [107, 89].
Urine cytology is not considered appropriate because it has a poor sensitivity (29%), whereas annual analysis for haematuria using urinary dipsticks from age 45-50 has been suggested as the preferred method [174, 175]. Our data do not support screening limited to families with a history or urinary tract tumours since 78% of the tumours developed in patients with a negative family history of the disease and the increased risk for urothelial cancer in MSH2 mutation carriers suggest that these individuals should be prioritised in the potential surveillance for cancer of the upper urinary tract and the urinary bladder [122, 123, 130, 399].

Study IV

*The role of prostate cancer in Lynch syndrome is unresolved though molecular investigations and epidemiological studies suggest that prostate cancer may be linked to the syndrome [124-126]. Against this background, we assessed the risk of prostate cancer, characterised the clinicopathological features and determined the MMR status in the national Danish Lynch syndrome cohort.*

In total, 288 Lynch syndrome families with disease-predisposing germline mutations in MLH1, MSH2, MSH6 or PMS2 were identified in the Danish HNPPCC register. In this cohort of 1609 males (677 mutation carriers and 932 first-degree relatives), prostate cancer developed in 15 mutation carriers and in 13 first-degree relatives. The median age at diagnosis was 63 (range 52-81) years. All tumours were adenocarcinomas with Gleason scores between 6 and 10. The tumours were linked to disease-predisposing mutations in MLH1 (n=8), MSH2 (n=14) and MSH6 (n=6). Among the 28 men diagnosed with prostate cancer, 16 had a previous cancer diagnosis, which included colon cancer in 15 cases.

Tumour tissue for MMR analysis could be retrieved from 16 tumours, with loss of expression of the respective MMR proteins in 69% of the tumours, including 2/6 MLH1-associated tumours, 7/7 MSH2-associated tumours and 2/2 MSH6-associated tumours (table 14, figure 26). Tumours from proven mutation carriers showed loss in 7/10 cases. MSI analysis with standard diagnostic markers revealed an MSI-high phenotype in 2 tumours, an MSI-low phenotype in 6 tumours and an MSS phenotype in 8 tumours. None of the MLH1-associated tumours showed an MSI-high phenotype (table 14). Pathologic review showed the presence of TIL in all MMR defective tumours and in 75% of all prostate cancers. Among the 11 MMR defective prostate cancers, 7 tumours had Gleason scores of 8-10, indicative of an aggressive tumour phenotype (table 14).
**Table 14.** Prostate cancers analysed for MMR function

<table>
<thead>
<tr>
<th>ID</th>
<th>Status</th>
<th>Age</th>
<th>Gleason score</th>
<th>TILs</th>
<th>MMR gene</th>
<th>IHC</th>
<th>MSI</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
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<tr>
<td>P11</td>
<td>C</td>
<td>63</td>
<td>7 (4+3)</td>
<td>n</td>
<td>MLH1</td>
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<td>+</td>
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<tr>
<td>P22</td>
<td>F</td>
<td>56</td>
<td>7 (3+4)</td>
<td>y</td>
<td>MLH1</td>
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<td>-</td>
</tr>
<tr>
<td>P2</td>
<td>C</td>
<td>60</td>
<td>8 (4+4)</td>
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<td>MLH1</td>
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<td>+</td>
</tr>
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<td>P13</td>
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<td>63</td>
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<td>-</td>
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<td>MSH6</td>
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<td>+</td>
</tr>
</tbody>
</table>

**Abbreviations:** C, carrier; F, first-degree relative; HPF, high-power field; IHC, immunohistochemistry; MMR, mismatch repair; MSI, microsatellite instability high/low; MSS, microsatellite stability; n, no; TIL, tumour-infiltrating lymphocytes; y, yes.
Figure 26. Prostate cancer with normal expression of (A) MLH1 and (B) PMS1 and loss of (C) MSH2 and (D) MSH6 from an individual with a mutation in the $MSH2$ gene.

The risk analysis was performed based on 1488/1609 males for whom complete data were available. The cumulative risk of developing prostate cancer at age 70 was 3.7% (95% CI: 2.32-4.92) with no significant differences depending on the disease-predisposing gene: $MLH1$ 4.4% (95% CI: 1.44-7.04), $MSH2$ 3.9% (95% CI: 1.96-5.70) and $MSH6$ 2.5% (95% CI: 0.56-4.12) (figure 27).

Figure 27. Non-parametric risk estimates in the Danish male Lynch syndrome cohort. Age-specific cumulative risks for prostate cancer in relation to MMR genes.
Discussion

In the Danish Lynch syndrome cohort, two-thirds of the prostate cancers that developed in mutation carriers and in first-degree relatives showed MMR defects concordant with the underlying MMR gene mutation and could thus be linked to the syndrome. Development of prostate cancer was most frequently (46%) linked to MSH2 mutations, which is in agreement with other studies [289, 400-402]. An expanded spectrum of extracolonic tumours, e.g. urothelial cancers, brain tumours and skin tumours, has been demonstrated in MSH2 families [57, 123, 125, 132, 400, 401, 403]. Loss of MMR expression was accurately detected in tumours from MSH2 and MSH6 families, whereas most MLH1-associated tumours showed retained immunohistochemical expression. MSI analysis with the diagnostic markers currently used in routine clinical practice did not detect MMR defective tumours with sufficient sensitivity, which supports an earlier report that MSI defects were detected in only 4-12% of prostate cancers in Lynch syndrome [323, 324].

The median age at onset of prostate cancer in the Danish cohort was 63 years, which fits well with reports of a median age at onset of 59-65 years [132, 137, 289, 400, 401, 404].

The cumulative risk of prostate cancer at age 70 was 3.7% in Lynch syndrome mutation carriers with no significant differences depending on the disease-predisposing gene, but this analysis was hampered by the small size of the study cohort and should be repeated in larger multi-national cohorts. Based on the assumption that MMR defective prostate cancer is part of Lynch syndrome, mutation carriers are estimated to be at a 2- to 3-fold increased risk of prostate cancer compared to the general population [124, 126].

Colorectal cancers that develop as part of Lynch syndrome are often characterised by poor differentiation and the frequent presence of TIL. TIL were also present in all MMR defective prostate cancers (table 14). However, the role of TIL in MMR defective prostate cancer is not known. Studies in prostate cancer in general have indicated an association with a poor prognosis [405-407]. Of the 10 MMR defective prostate cancers in our study, 7 had a Gleason score of ≥8 (table 14).

Growing data support the hypothesis that hereditary prostate cancers, those linked to Lynch syndrome as well as those linked to the BRCA2 syndrome, present as aggressive tumours with an overrepresentation of tumours with Gleason score ≥8, tumour stage pT3/T4, nodal involvement and metastases at diagnosis compared to sporadic cases [408]. Hence, there is a need to develop strategies for genetic counselling related to prostate cancer, recommendations for surveillance and guidelines for the clinical management of prostate cancers that develop as part of hereditary cancer syndromes.
Study V

RCC has been linked to a number of rare hereditary syndromes, but its potential role in Lynch syndrome is uncertain. We used the national Danish HNPCC register and the Danish Cancer Registry to identify all RCCs that had developed in Lynch syndrome mutation carriers and defined the clinical, histopathological and molecular features and incidence rates of these tumours to investigate the development of RCC in Lynch syndrome.

In the national Danish Lynch syndrome cohort, we identified 13 RCCs diagnosed in 8 men and 5 women with mutations linked to Lynch syndrome (table 15). The disease-predisposing genes were MSH2 in 6 cases, MLH1 in 6 cases and MSH6 in 1 case. The median age at diagnosis was 62 (47-82) years. In 2 families 2 RCCs had developed (figure 28).
<table>
<thead>
<tr>
<th>Sex</th>
<th>Age</th>
<th>Tumour stage</th>
<th>Histopathology</th>
<th>TIL (≥5/HPF)</th>
<th>MMR gene</th>
<th>MSI</th>
<th>MLH1</th>
<th>PMS2</th>
<th>MSH2</th>
<th>MSH6</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>82</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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</tr>
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<td>NA</td>
<td>NA</td>
<td>MLH1</td>
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</tbody>
</table>

Abbreviations: F, female; HPF, high-power fields; M, male; MMR, mismatch repair; MSI, microsatellite instability; MSS, microsatellite stable; MSI-L, low microsatellite instability; NA, not available; TIL, tumour-infiltrating lymphocytes.
A comparison of the incidence rates for RCC in MMR mutation carriers with those in a matched cohort from the general population (Nordcan database) revealed increased incidences in the Lynch syndrome cohort for the age groups 30-49, 50-69 and >70 years. The highest incidence rate ratio of 7.7 was observed for the age group of 50-69 years (p=1.08E-10) (figure 29). Due to the limited sample size, differences related to sex and the disease-predisposing genes were uncertain (data not shown).
Figure 29. Age-specific incidence rates for RCC in Lynch syndrome (solid line) compared to the general population (dotted line) with 95% confidence intervals marked in grey.

The histological analysis revealed 7 clear cell cancers, 1 papillary cancer, and 5 unspecified renal cell carcinomas. Histological re-evaluation and MSI analysis were possible in 5 cases, which showed TIL in 3 tumours (figure 30) and an MSI-low genotype in 1 tumour with the remaining tumours being microsatellite stable (MSS). Immunohistochemical results were available in 6 cases and showed loss of MMR protein expression concordant with the mutated MMR gene in 4 tumours (figure 31).

Figure 30. A clear cell RCC with abundant TIL (examples marked with red circles).
Figure 31. IHC for the MMR proteins in a clear cell RCC with the MLHI c.1667+2delTCAinsATTT mutation showing loss of (A) MLH1 and (B) PMS2 staining in tumour cells and positive staining for (C) MSH2 and (D) MSH6.

Discussion

We provide evidence that RCC is linked to Lynch syndrome based on an increased incidence rate compared to the general population and the demonstration of MMR deficient RCCs in mutation carriers.

The 13 RCCs identified in the Danish Lynch syndrome cohort developed at a median age of 62 years. The highest incidence rate of 7.7 applied to the age group of 50-69 years (figure 29). The mean age at cancer diagnosis in Lynch syndrome varies by tumour type with a mean age of 40-45 for ovarian cancer and colorectal cancer, 50-55 for endometrial cancer and around 60 for urinary tract tumours [107, 118, 133]. RCCs thus seem to develop at an age similar to that reported for other urological tumours in Lynch syndrome.

The tumours were predominantly of the clear cell subtype with abundant TIL in 3/5 tumours (figure 30). Lymphocytic reactions, expressed as TIL or Crohn-like reactions, also characterise colorectal cancer and endometrial cancer linked to Lynch syndrome [191, 197, 409, 410].
Loss of MMR protein expression in accordance with the mutated gene was identified in 4/6 RCCs available for analysis, whereas MSI analysis was considerably less sensitive, with only one of 5 RCCs showing an MSI-low phenotype, which is in accordance with previous findings in RCC [128]. This also corresponds to observations in urothelial cancer, where MMR protein immunostaining has a high (90%) sensitivity for the identification of MMR defective tumours, whereas MSI analysis has a low (20%) sensitivity [128, 411-413].

Individuals with Lynch syndrome are estimated to be at a 2- to 22-fold increased risk of cancer of the upper urinary tract and the urinary bladder, which translates to an estimated cumulative risk for urinary tract cancer of 0.4-20% [130, 395, 412]. The majority (70-80%) of the urothelial cancers associated with Lynch syndrome have been linked to mutations in MSH2, whereas the RCCs identified in the present study were associated with any one of the 3 most commonly affected MMR genes, namely MLH1, MSH2 and MSH6. In a previous study in 160 Finnish Lynch syndrome families, 12 RCCs were identified. These tumours developed at a mean age of 64 years and were linked to disease-predisposing mutations in MLH1. This finding may reflect strong founder effects in the Finnish population [127].

Other hereditary RCC-predisposing syndromes might have affected the cancer incidences reported in our cohort. In fact, one of the MSH2 gene mutation families in the cohort also carried a verified pathogenic Birt-Hogg-Dubé-associated mutation in the FLCN gene. The Birt-Hogg-Dubé syndrome predisposes to spontaneous pneumothoraces and skin fibrofolliculomas and confers a 10-30% increased risk of RCC. Though the FLCN gene mutation has not yet been identified in the affected patient, it is possible that the RCC was linked to Birt-Hogg-Dubé syndrome and not to Lynch syndrome. Exclusion of this case resulted in a slightly lower but still significantly increased incidence rate of 7.0 (p=6.32E-09) in the age group of 50-69 years.
Conclusions

Study I
The MMR index provides a validated tool to identify the MMR deficient subset of colon cancers. In addition to factors evaluated as part of the routine diagnostic work-up (i.e. sex, age, tumour location and occurrence of mucinous differentiation), the MMR index only requires evaluation of growth pattern, dirty necrosis and TIL. Its simple application and high reproducibility represent key advantages for clinical implementation to identify individuals with colon cancer who have a good prognosis and may be spared adjuvant chemotherapy.

Study II
Our study confirms that a subset of colorectal cancers has a heterogeneous MMR status. Heterogeneous MMR protein expression appears in 3 major forms (intraglandular, clonal and compartmental), which frequently co-exist and correlate to differences in the MMR status. Attention to this phenomenon is recommended to prevent false-positive or false-negative evaluations.

Study III
We demonstrate that cancers of the upper urinary tract as well as urinary bladder cancers are included in the Lynch syndrome tumour spectrum. These tumours predominantly develop in individuals with mutations in MSH2, which suggests that surveillance programs for urinary tract cancer should be targeted at individuals with MSH2 mutations.

Study IV
In the national Danish Lynch syndrome cohort, we identified 28 prostate cancers that developed at a median age of 63 years, displayed high Gleason scores and frequent TIL, and were predominantly linked to MSH2 mutations. MMR staining adequately identified these tumours and demonstrated that more than half of the prostate cancers were MMR deficient and thus presumably linked to the syndrome. By contrast, MSI analysis had a poor sensitivity for these tumours.
Study V

Though a rare presentation, we suggest that RCC should be considered to be part of the Lynch syndrome tumour spectrum, based on our findings that RCC developed at increased incidence rates in individuals with Lynch syndrome and that the tumours in these patients were MMR defective. This implies that RCC should be considered in risk estimates and surveillance recommendations for families evaluated for or diagnosed with Lynch syndrome.
Future Aspects

A major challenge in Sweden is the widespread introduction of reflex testing for MMR defects in colorectal cancer diagnostics and correct clinical management of patients with MMR aberrancies as regards patient information, considerations related to adjuvant therapy, access to genetic counselling and - for patients with advanced-stage disease - potential treatment with PD-1 inhibitors.

There are now several studies that document the efficient identification of MMR gene mutation carriers using reflex testing with IHC; these studies underscore that the identification of individuals with Lynch syndrome represents an exceptionally cost-effective measure that has not yet been applied in Swedish health care. The decision whether or not reflex testing will be introduced will determine the further application of the MMR index, assessed and validated in study I, though application of this index is highly relevant until broad-scale MMR testing has been introduced.

Our demonstration of heterogeneous MMR status warrants attention to this phenomenon and analysis of larger tumour areas and, if possible, multiple tumour blocks. Recently, somatic causes of MMR gene inactivation have been demonstrated and these mechanisms should be further investigated as potential causes of the MMR status heterogeneity identified in study II.

The link between Lynch syndrome and urological cancer demonstrated in studies III-V warrants further caution. The highest risks apply to urothelial cancer, notably cancer of the renal pelvis, the ureter and the urinary bladder. No general surveillance for these tumour types is recommended in Europe. Since urothelial cancer has a clear alarm symptom, i.e. haematuria, and since the disease is linked to MSH2 and rarely appears before age 50, we believe that a targeted surveillance program should be developed and validated.

The observation that Lynch syndrome is associated with prostate cancer and kidney cancer warrants consideration of these tumour types in the diagnostic work-up of families with suspected Lynch syndrome. To determine the risk of these tumours and identify of potential genotype-phenotype correlations, multinational studies are required to correct for ascertainment bias, to perform prospective analyses and to collect larger numbers of these tumours.
The identification of MMR defective urological cancers may also be highly relevant for prognostic and predictive decisions. The prognosis of Lynch syndrome-associated urological cancer remains to be determined. The observation of frequent TIL and common MMR defects in these tumours may be highly relevant for future therapeutic decisions. Preliminary data indicate that MMR defective tumours outside the colorectum may also respond to PD-1 inhibition - an observation that opens new possibilities for the treatment of Lynch syndrome patients affected by cancer.
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