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Mismatch Repair Deficiency in Colorectal Cancer

Prognosis and prediction for basic treatment strategies.

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Ένα μεγάλο ΕΥΧΑΡΙΣΤΩ από καρδιάς σε όλους.

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Abstract

Colorectal cancer (CRC) remains a significant healthcare problem worldwide, being the third most common cancer and the fourth most frequent cause of cancer death. Environmental and dietary factors such as alcohol abuse, cigarette smoking, and genetic predisposition seem to constitute the main aetiologies.

Two major distinct molecular genetic pathways have been recognised as models of transition from normal epithelium to adenoma and carcinoma. The first involves chromosomal instability (CIN) and the second involves microsatellite instability (MSI). The MSI pathway constitutes 2-4% of CRCs with a hereditary Mismatch Repair (MMR) defect (dMMR) and approximately 15% of sporadic MMR defects due to epigenetic silencing of the MutL homologue 1 (*MLH1*) promoter. Extracellular factors and spontaneous copy errors necessitate molecular systems to survey and repair human genetic information, and to protect it from chemical disruption. A complicated and entangled network of DNA damage response mechanisms, including multiple DNA repair pathways, damage tolerance processes, and cell cycle checkpoints safeguard genomic integrity. It has recently become apparent that key proteins contributing to cellular survival by taking part in DNA repair become executioners in the face of excess DNA damage. All prokaryotic and eukaryotic organisms have major DNA repair pathways. In each of these DNA repair pathways there are key proteins that have dual functions in DNA damage sensing/repair and apoptosis, taking advantage of the fact that DNA is a double helix with the same information present on both strands. Damages that affect one strand can easily be repaired by excision and replacement with newly synthesised DNA using the complementary strand as a template. MMR plays a critical role in the repair of errors that occur spontaneously during DNA replication, such as single base mismatches. dMMR increases the mutation frequency in an affected cell by approximately 1000 times, leading to MSI through the accumulation of short repetitive DNA sequences called microsatellites. Carcinogenesis in dMMR cases can present as hereditary cases (Lynch syndrome) due to germline mutation in in one of the main MMR genes – *MLH1, MSH2, MSH6,* and *PMS2* or somatic/sporadic cases (epigenetic silencing or somatic inactivation of *MLH1* promoter. dMMR seems to have a favourable prognosis as these CRCs seems to be less prone to metastasising. This phenomenon is much more obvious for tumour stages II and III, while in advanced disease dMMR seems to lose its positive prognostic effect. Even if the underlying mechanism is not fully understood, some studies attribute the positive effect of dMMR tumours to their increased immunogenicity leading to a stronger more effective immune response. On the other hand, the predictive value of the dMMR mechanism is less well understood and has only gained attention in recent years. In general, dMMR seems to predict a poor response to 5-FU, the basis of gastrointestinal chemotherapy.

The aims of this thesis were: 1. To review the latest publications on the role of MSI status as prognostic factor in stage II colon cancer (CC) patients (Study I);

2. To validate MMR status as a prognostic factor in patients with CC Stage II (Study II); 3. To verify MMR status as a predictive factor in relation to the administration of adjuvant chemotherapy in patients with stage II CC (Study III); 4. To investigate the potential role of MMR status as a risk factor for acute CC surgery (Study IV); and finally 5. To investigate the association between CRC with sporadic dMMR and non-colorectal malignancy (Study V).

Study I, a meta-analysis reviewing recently published papers, revealed that MSI status in stage II CC patients does not seem to affect overall survival (OS) and disease-free survival (DFS). This lack of impact could be explained by selection bias and the extremely high proportion of patients receiving adjuvant chemotherapy in the studies included. This was the first meta-analysis specifically evaluating patients with colon cancer stage II. The optimal treatment algorithm for these patients remains unclear, and approximately 20% experience relapse and finally die from disseminated disease.

Study II verified the prognostic role of MMR status in patients with stage II CC. Patients with a dMMR tumour have a significantly lower risk for cancer recurrence, a finding that is particularly important for CC treatment. This relationship does not correlate to a better OS since these patients are older and often die from other causes. Debate on the best postoperative strategy in stage II CC continues. What this study contributes is the idea that determination of MMR status can have prognostic value in these patients.

Study III also verified the predictive role of MMR status in patients with stage II CC, only this time in relation to treatment with adjuvant chemotherapy. Patients with proficient MMR (pMMR) status receiving adjuvant chemotherapy have a significantly better OS than those not receiving adjuvant treatment. This relationship was not seen in patients with a dMMR tumour. Furthermore, patients with a pMMR tumour receiving adjuvant treatment have a significantly longer survival time after the first relapse compared to those not receiving adjuvant treatment.

Study IV revealed the higher probability of dMMR tumours to present as a surgical emergency. Stage III and IV tumours were also associated with acute surgery. This association was significant regardless of the potential bias due to retrospective methodology and possible heterogeneity between the different cohorts. Further research is required before our conclusions can be applied in clinical practice due to the multicomplex relationship and interactions between variables that influence the oncologic outcome of acute CC surgery.

Study V revealed that patients with sporadic, non-hereditary dMMR CRC run a greater risk for having non-colorectal cancer prior to or after the diagnosis of CRC. This implies that patients with a dMMR tumour should be screened for other non-colorectal cancer, more so than in the the general population.

Conclusion

CRC continues to be a significant healthcare problem worldwide, and treatment algorithms for patients with different genomic backgrounds can vary significantly. This thesis supports the idea of using MMR status as a prognostic and predictive factor in everyday clinical practice, especially in stage II CC and acute cases.

Abbreviations

5-FU	5-Fluorouracil
AJCC	American Joint Committee on Cancer
APC	Adenomatous polyposis coli
BRAF	v-raf murine sarcoma viral oncogene homologue B1
CC	Colon cancer
CRC	Colorectal cancer
CI	Confidence interval
CIN	Chromosomal instability
CIMP	CpG island methylator phenotype
CRC	Colorectal cancer
DNA	Deoxyribonucleic acid
DFS	Disease-free survival
dMMR	Deficient mismatch repair
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin-embedded
HNPCC	Hereditary non-polyposis colorectal cancer
HR	Hazard ratio
H&E	Haematoxylin & Eosin
IHC	Immunohistochemistry

IRR	Incidence rate ratio
KRAS	Kirsten rat sarcoma viral oncogene
Ln	Logarithm
LS	Lynch syndrome
LCRT	Long-course chemoradiotherapy
MLH1	MutL homologue 1
MMR	Mismatch Repair
MSH2-6	MutS homologue 2-6
MSI	Microsatellite instability
MSS	Microsatellite stable
MutL/S	Mutator L/S
OR	Odds ratio
OS	Overall survival
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
pMMR	Proficient mismatch repair
PMS2	Post-meiotic segregation
PRS	Post relapse survival
RER	Replicative errors
TNM	Tumour node metastasis
Tp53	Tumour protein 53
TTP	Time to progression

VIP	Västerbotten Intervention Programme
UICC	Union for International Cancer Control

Original Papers

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

I. Gkekas, I., Novotny, J., Pecen, L., Strigård, K., Palmqvist, R., & Gunnarsson, U.: Microsatellite instability as a prognostic factor in stage II colon cancer patients, a meta-analysis of published literature. *Anticancer research* (2017), *37*(12), 6563-6574.

II. Gkekas, I., Novotny, J., Fabian, P., Nemecek, R., Palmqvist, R., Strigård, K., Ladislav, P.Svoboda, T.Gurlich, R., & Gunnarsson, U.: Deficient mismatch repair as a prognostic marker in stage II colon cancer patients. *European Journal of Surgical Oncology* 45.10 (2019): 1854-1861.

III. Gkekas, I., Novotny, J., Fabian, P., Nemecek, R., Palmqvist, R., Strigård, K., John, S.Pecen, L.Reginacova, K., & Gunnarsson, U.: Mismatch Repair status predicts survival after adjuvant treatment in stage II colon cancer patients. *Journal of surgical oncology* 121.2 (2020): 392-401.

IV. Gkekas, I., Novotny, J., Kaprio. T., Beilmann-Lehtonen I., Fabian, P., Edin, S., Strigård, K., Svoboda, T., Hagström, J., Barsova, L., Jirasek, T., Haglund, C., Palmqvist, R., Gunnarsson, U.: Colon cancer patients with mismatch repair deficiency are more likely to present as acute surgical cases. *Submitted*.

V. Gkekas, I., Novotny, J., Kaprio. T., Beilmann-Lehtonen I., Fabian, P., Edin, S., Strigård, K., Svoboda, T., Hagström, J., Barsova, L., Jirasek, T., Haglund, C., Palmqvist, R., Gunnarsson, U.: Higher incidence for sporadic deficient mismatch repair tumours in the colorectum and non-colorectal malignancies. *In manuscript*.

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Aims

This thesis focuses on the MMR status of CC, especially in patients with stage II disease. The overall aim was to assess the impact of MMR status and its potential for prognostic and predictive applications in basic treatment strategies.

Detailed aims were to:

- To ascertain from recently published studies whether MMR status has prognostic value, focusing on patients with stage II CC (Study I)
- Investigate whether MMR status has prognostic value in patients with stage II CC (Study II)
- Investigate whether MMR status has predictive value in patients with adjuvant chemotherapy for stage II CC (Study III)
- Investigate the risk of presentation of CC at acute surgery in relation to MMR status (Study IV)
- Investigate the relationship between sporadic dMMR CRC and risk for non-CRC prior to or after the diagnosis of CRC (Study V)

Sammanfattning på svenska

Kolorektalcancer (CRC) utgör, som den tredje vanligaste cancerformen och den fjärde vanligaste orsaken till cancerdöd, fortfarande ett viktigt hälsoproblem över hela världen. Defekt Mismatch repair system (dMMR) är genom felaktig DNA-reparation en av de viktigaste orsakerna till utveckling av CRC. Mismatch repair (MMR) spelar en avgörande roll vid reparation av fel som uppträder spontant under DNA-replikation såsom enkla basmatchningar, korta insertioner eller deletioner. dMMR ökar mutationsfrekvensen i en drabbad cell cirka 1000 gånger, vilket leder till mikrosatellit-instabilitet (MSI) genom ackumulering av korta repetitiva DNA-sekvenser som kallas mikrosatelliter. Tumörer med dMMR karakteriseras av hög mutationshastighet vilket leder till förekomst av neoantigener på cellmembranet. Detta möjliggör för kroppens immunsystem att utveckla ett effektivt immunologiskt svar. Upptäckten av dMMR är grunden för nya behandlingar vilka kan påverka immunologiska kontrollpunkter. dMMR förekommer i cirka 20 % av all CRC och utgörs dels av en ärftlig typ, som finns hos familjer med Lynch syndrom eller Hereditary Non-polyposis Colorectal Cancer (HNPCC), och en sporadisk typ utan ärftlighet. För närvarande finns huvudsakligen två metoder för att fastställa MMR-status. Den första och mest använda bygger på Polymerase Chain Reaction (PCR) som kan utföras på färsk, fryst eller paraffinbäddad tumörvävnad. Analysen bygger på en granskning av fem definierade mikrosatellite markörer. Dessa markörer har valts ut vid en konsensuskonferens i Bethesda (1997). MSI graderas som MSI high (MSI-H) där två eller flera av markörerna identifieras, MSI low (MSI-L) om en markör identifieras och stabil (MSS) om ingen av dessa markörer återfinns. Graderingen MSI-L och MSS slås ofta samman till en grupp, det vill säga stabila tumörer. Den andra metoden utgörs av immunohistokemi (IHC) där man använder monoklonala antikroppar mot fyra riktade proteiner (MLH1, MSH2, PMS2, MSH6). Idag används IHC oftare då denna teknik är enklare och billigare. Flera publicerade studier med syfte att jämföra dessa båda metoder har påvisat sammstämighet i mer än 94 % av tumörerna.

Avhandlingen undersöker följande aspekter av MMR status vid kolorektal cancer:

- Betydelsen i de senast publicerade studierna av MSI-status som prognostisk faktor för patienter med CC stadium II (studie I).
- Möjligheten att MMR-status utgör en signifikant prognostisk faktor för patienter med CC stadium II (studie II).
- Möjligheten att MMR-status utgör en signifikant prediktiv faktor för patienter med CC stadium II som behandlas med adjuvant kemoterapi (studie III).
- Möjligheten att dMMR-status utgör en riskfaktor för behov av akut kirurgi vid CC (studie IV).

- Möjligheten att sporadiska tumörer med dMMR-status löper en högre risk för utvecklingen av andra cancertyper före eller efter CRC (studie V).

Resultat och diskussion

Delarbete 1: I denna meta-analys utgjordes 20.8% av CC i stadium II av MSI/dMMR. Det fanns ingen signifikant skillnad i total överlevnad (OS) mellan MSI/dMMR och MSS/pMMR grupperna. Hazard ratio (HR) för OS var 0.73 (95% konfidenceintervall (CI); 0.33-1.65). Sjukdomsfri överlevnad (DFS) för ovanstående grupper uppvisade inte heller någon signifikant skillnad. HR för DFS var 0.60 (95% CI; 0.27-1.32). Ingen signifikant skillnad identifierades heller när studier som använde genotypning (MG) eller IHC testades separat (MG vs IHC: HR OS 0.45,95% CI; 0.10-2.05 vs 0.95, 95%CI; 0.57-1.58. HR DFS 0.51, 95% CI: 0.14-1.85 vs. 0.67, 95% CI 0.26-1.70). I 8 av 19 studier ingick även fall av rektalcancer och i 3 av dessa var andelen rektalcancer inte angiven. Vid analys av studier inkluderande endast koloncancerpatienter var HR för OS 0.72 (95% CI: 0.31-1.71); och HR för DFS 0.60 (95% CI: 0.27-1.31).

Vår metaanalys, den första som endast utvärderar patienter med CC stadium II, påvisades inget signifikant samband mellan MSI/dMMR och OS eller DFS. Huvudorsaken till resultatet är de motstridiga resultat som studierna presenterar. Dessutom har vi i metaanalysen upptäckt att 47.3% av patienterna fått adjuvant kemoterapi, jämfört med cirka 25% som numera behandlas i modern onkologisk kontext. Detta resulterar i en dubbelt så lång överlevnad i gruppen med MSS/pMMR jämfört med MSI/dMMR. Den senare gruppen har betydlig sämre respons på klassisk kemoterapi.

Delarbete 2: I denna retrospektiva studie fanns 93 dMMR fall bland 452 koloncancerfall (20.6%). Det förelåg inget signifikant samband mellan dMMR och OS (Log-Rank, p=0·583, 95% CI 0.76 – 1.67). Däremot konstaterades en höggradigt signifikant skillnad för tid till progress (TTP) med halverad hazard ratio för dMMR (TTP: HR 0·50, 95% CI 0.28 – 0.87, p = 0·012). Således har dessa patienter en betydligt mindre risk för återfall och cancerrelaterad död. Detta fynd korrelerar inte med bättre OS. En trolig förklaring är att koloncancer i detta stadium har en generellt god prognos efter kirurgi. I och med att dMMR-patienter är äldre än de med pMMR drabbas de oftare av andra dödsorsaker än cancerrelaterade. TTP-analys visar att dMMR-status utgör en prognostisk faktor avseende återfall och koloncancerrelaterade dödsfall för patienter med koloncancer stadium II. Som en följd av detta bör MMR-status användas i klinisk

praxis som grund för beslut om adjuvant terapi, även om de flesta studier visar att vikten av MMR-status påverkas även av andra faktorer.

Delarbete 3: I denna retrospektiva multicenterstudie som inkluderar 451 patienter med CC stadium II förelåg dMMR i 20.4%. Denna patientgrupp fick ingen överlevnadsvinst av adjuvant kemoterapi (HR 1.05; 95% CI 0.47-2.38, p=0.897). Å andra sidan visade sig patienter med pMMR-status som fått adjuvant kemoterapi ha en signifikant bättre OS jämfört med de som endast behandlades med kirurgi (HR 0.54; 95% CI 0.35-0.82, p=0.004). Detta förhållande bibehölls vid multivariabel analys (HR 0.42; 95% CI 0.22-0.78, p=0.007). Patienter tillhörande pMMR gruppen som drabbades av återfall efter adjuvant kemoterapi hade en signifikant bättre överlevnad jämfört med de som inte fick adjuvant postoperativ behandling (HR 0.55; 95% CI 0.32-0.96, p=0.033). Detta var signifikant även i den multivariabla modellen (HR 0.49; 95% CI 0.26-0.93, p=0.030). Fynden är mycket intressanta gällande beslut om behandlingsstrategi för CC stadium II, då de visar en tydlig relation mellan MMR-status och respons på adjuvant kemoterapi.

Delarbete 4: I denna retrospektiva multicenterstudie som inkluderar 870 koloncancer-patienter från tre olika länder (Sverige, Finland och Tjeckien) var 399 patienter kvinnor (46%), medianålder vid kirurgi var 69 år och dMMR status förelåg hos 190 patienter (22%). Akut kirurgi, dvs kirurgi vid samma vårdtillfälle som när CC diagnosen ställdes, genomfördes hos 179 patienter (21%). dMMR status förelåg i 57 fall (32%) (p=0.001). Patienterna delades in i en skandinavisk undersökningsgrupp (Sverige och Finland) som inkluderade 412 patienter och en tjeckisk valideringsgrupp som utgjordes av 458 patienter. I den skandinaviska gruppen förelåg ett signifikant samband mellan dMMR-status och akut kirurgi i såväl uni- (Odds Ratio (OR) 1.82, 95% CI 1.11-3.02, p=0.017) som multivariabel (OR=2.21, 95% CI 1.28-3.95, p=0.005) analys. Detta samband bekräftades i den tjeckiska valideringsgruppen vid såväl uni- (OR=1.94, 95% CI 1.09-3.26, p=0.022) som multivariabel (OR=1.77, 95% CI 1.15-3.18, p=0.021) analys. Dessa fynd är intressanta då det rör sig om variabler som påverkar utfallet vid akut insjuknande i CC.

Delarbete 5: I denna retrospektiva multicenterstudie som inkluderar 1753 CRC-patienter från tre olika länder (Sverige, Finland och Tjeckien) var 838 patienter kvinnor (48%), medianåldern vid kirurgi var 67 år och sporadisk dMMR status förelåg hos 236 patienter (13%). Av dessa hade 327 patienter (19%) diagnostiserats med minst en annan icke-kolorektal malignitet innan eller efter diagnosen av CRC. Det förelåg ett signifikant högre incidence rate ratio (IRR) för förekomst av icke kolorektal malignitet 20 år innan och efter (fram till stoppdatum för uppföljning) debuten av CRC, såväl i den uni- (IRR=1.45, 95% CI 1.10-1.92, p=0.009) som i den multivariabla (IRR=1.46, 95% CI 1.09-1.95,

p=0.011) modellen för patienter med sporadiska dMMR jämfört med pMMR fall. Detta fenomen var ännu mer uppenbart för icke kolorektal malignitet som uppträdde efter den primära diagnosen för CRC både i den uni- (IRR=1.64, 95% CI 1.15-2.36, p=0.007) och multivariabla (IRR=1.61, 95% CI 1.10-2.35, p=0.014) modellen. Dessa fynd behöver verifieras men skulle kunna vara betydelsefulla vid utformning av framtida uppföljningsprogram vid CRC riktad mot patienter med icke ärftlig dMMR tumör.

Introduction

CRC incidence and mortality rates vary widely worldwide, with distinct gradients across human development levels. Trends point towards widening disparities and an increasing burden in countries in transition. CRC is the third most commonly diagnosed malignancy and the fourth leading cause of cancer death worldwide[1]. This is especially so in Sweden, where CRC constitutes the third most common cancer among both men and women[2].

Environmental and dietary factors such as alcohol abuse, cigarette smoking, and genetic predisposition seem to be the main aetiologies[3]. Although tumourigenesis is thought to be a complex multistep process in which heterogenous genetic alterations accumulate, two major pathway concepts have been recognised as models of transition from normal epithelium to adenoma and carcinoma[4,5]. These are the chromosomal instability (CIN) pathway, which accounts for 85% of CRC, and the microsatellite instability (MSI) pathway which accounts for 2-4% of CRC with a hereditary MMR defect and approximately 15% of sporadic MMR defects mainly due to epigenetic silencing of the *MLH1* promoter[6,7]. Recently, efforts have been made to provide a more complete picture of this complex topic, and an up-to-date consensus molecular subtype classification based on gene expression has been proposed (Fig 1)[8,9].

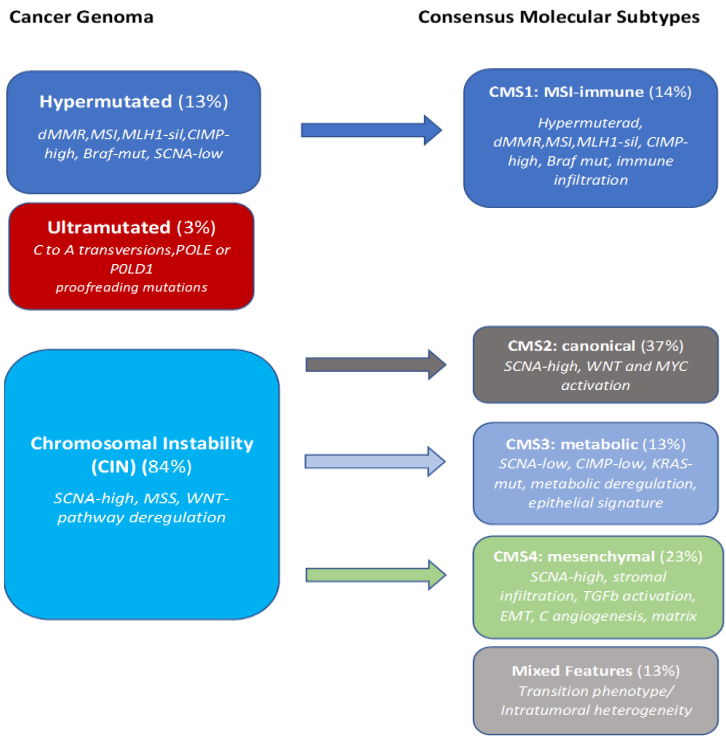

Figure 1. *The consensus molecular subtypes of colorectal cancer*[8,10].

Extracellular factors and spontaneous copy errors necessitate molecular systems to survey and repair human genetic information, and to protect it from chemical agents. A complicated and entangled network of DNA damage response mechanisms, including multiple DNA repair pathways, damage tolerance processes, and cell-cycle checkpoints safeguard genomic integrity[11]. It has recently become apparent that key proteins that contribute to cellular survival by acting in DNA repair become executioners in the face of excessive DNA damage. All prokaryotic and eukaryotic organisms have major DNA repair pathways. In each of these DNA repair pathways, key proteins occur with dual functions in DNA damage sensing/repair and apoptosis, taking advantage of the fact that DNA is a double helix with the same information present on both strands. Damage that affects one strand can be easily repaired by excision and replacement with newly synthesised DNA using the complementary strand as a template[12,13].

Mismatch repair status and its function in colorectal cancer patients

In the human MMR system, cluster proteins with dual roles (to identify and correct errors during DNA replication) consist of 2 interacting heterodimers: MutS (MSH2/ MSH6) and MutL (MLH1/PMS2). The MMR system is responsible for correcting DNA base mismatch and insertion/deletion loops generated during DNA replication. The MutS complex recognises the mismatch and forms a sliding clamp around the DNA strand. This complex then binds to the MutL heterodimer and moves along the DNA chain until reaching the DNA polymerase complex. This new complex excises the daughter strand all the way back to the mismatch. Finally, the MMR complex clamp falls off and the mismatch is corrected by DNA resynthesis (Fig 2)[12,13].

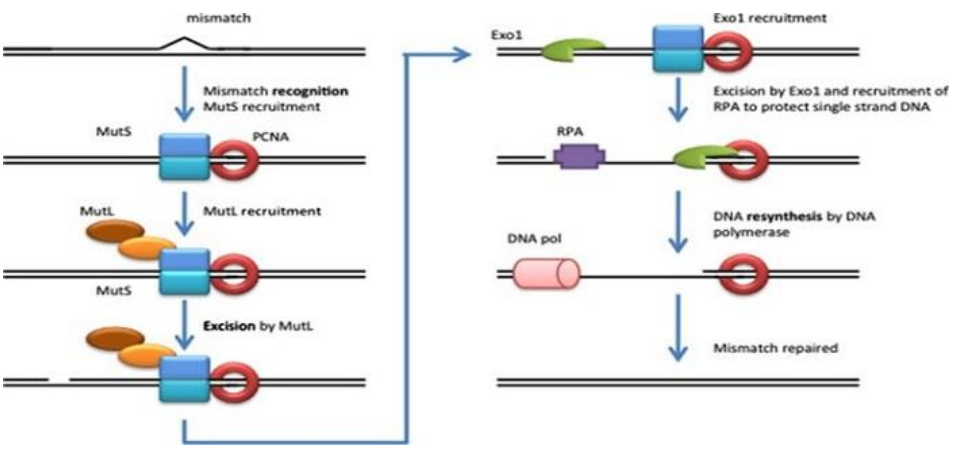

Figure 2. *Model of the mismatch repair system in single-base excision. Adapted from Guilottin et al, 2014[13].*

MLH1 and MSH2 are the 2 obligatory proteins in their respective heterodimer. Any mutational or epigenetic inactivation in these proteins leads to destabilisation of the corresponding binding partners (PMS2 and MSH6 respectively), resulting in complete loss of MMR activity. Loss of MLH1 leads to destabilisation/secondary loss of expression of PMS2 and loss of MSH2 disrupts expression of MSH6. This pattern of primary and secondary loss of protein expression forms the basis of MMR protein immunohistochemistry interpretation (IHC)[14,15]. Mutational or epigenetic inactivation of the certain MMR genes, including *MLH1, MSH2, MSH6* and *PMS2* typically results in MSI due to failure to repair errors that occur during replication of repetitive DNA sequences. MSI is defined as changes in the length of microsatellites, small DNA

repeat areas consisting of 1-6 bases. MSI status is usually evaluated with multiplex polymerase chain reaction (PCR) assays using a panel of defined microsatellite markers[12,16]. Mutations arising within microsatellites associated with critical target genes are believed to play a causative role in the evolution of dMMR tumours[15]. The development of antibodies against the basic MMR proteins allows the immunohistochemical identification of MSI tumour tissue. Since the MMR proteins functionally interact in heterodimers, mutations in *MLH1* genes cause downregulation of the expression of both MLH1 and PMS2 proteins, in the same way that mutations in *MSH2* lead to loss of expression of both MSH2 and MSH6. *MSH6 and PMS2* mutations lead to loss of expression of respective genes that may be accompanied by downregulation of MSH2 and MLH1 respectively (Table 1)[17,18].

Table 1. *Immunohistochemical patterns in dMMR tumours[18].*

Affected genes	**Immunohistochemical expression**			
	MLH1	**PMS2**	**MSH2**	**MSH6**
MLH1	Loss	Loss	Non-affected	Non-affected
PMS2	Loss or Non-affected	Loss	Non-affected	Non-affected
MSH2	Non-affected	Non-affected	Loss	Loss
MSH6	Non-affected	Non-affected	Loss or Non-affected	Loss

The combined loss of expression of the MLH1/PMS2 protein complex indicates an underlying defect in *MLH1 or PMS2*, whereas an isolated loss of PMS2 protein expression indicates a mutation affecting this gene. In the same way, the combined loss of MSH2/MSH6 complex indicates an underlying defect in *MSH2* or *MSH6*, whereas isolated loss of MSH6 expression indicates a mutation affecting that gene only. Immunostaining of these basic MMR proteins is generally recommended for the identification of deficient MMR (d-MMR) tumours having approximately 92% sensitivity and 100% specificity[15,17].

Mismatch repair status as a prognostic and predictive factor in CRC.

Several studies have already investigated the potential prognostic and predictive value of dMMR in CRC. CRCs with dMMR seem to have a more favourable prognosis as these tumours appear to be less prone to metastasise[19]. This phenomenon is much more obvious in stage II and III tumours, while in advanced

disease, dMMR seems to lose its positive prognostic value[20-22]. Even if the underlying mechanism is not fully understood, some studies attribute this positive effect of dMMR to the tumour having a high level of immunogenicity and leading to a strong immune response (Fig 3)[23,24].

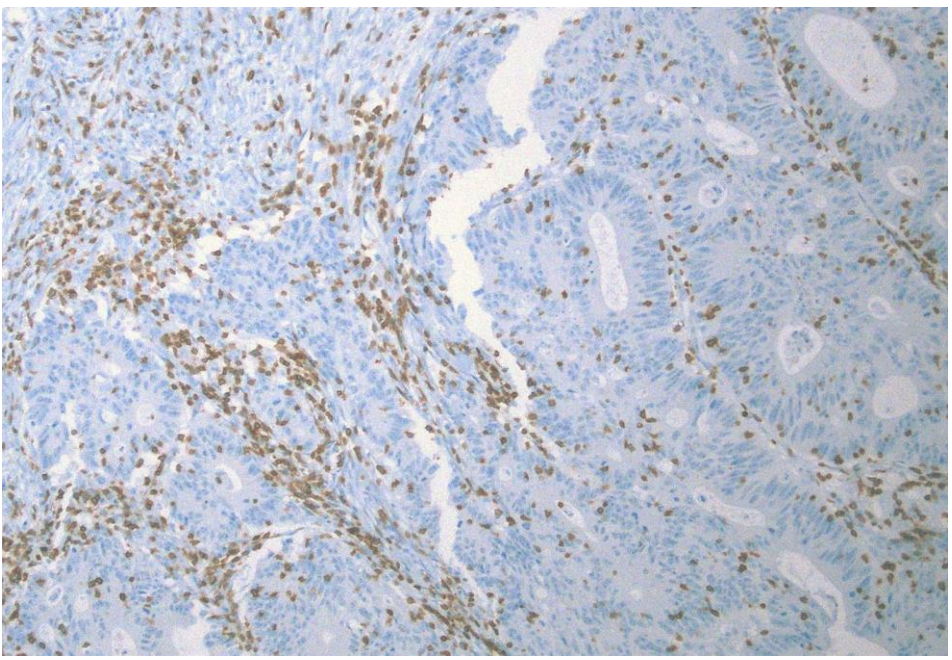

Figure 3. *High T-lymphocyte infiltration in a dMMR tumour.*

On the other hand, the predictive effect of dMMR is less well understood and only in recent years has this gained our attention. In generally, dMMR tumours seem to predict a poor response to 5-Fluorouracil (5-FU), the basis of gastrointestinal chemotherapy[25,26]. Briefly, 5-FU seems to incorporate into DNA and this incorporation results in DNA damage that is in turn detected by the MMR system responsible for detecting errors in DNA replication. pMMR recognition of DNA damage gives rise to cell arrest and apoptosis. Cells with dMMR cannot recognise such DNA damages even if 5-FU has been incorporated into DNA structures and cell death programmes are not activated. This may be the reason for impaired response to 5-FU chemotherapy as seen in Table 2. Secondly, the inherited DNA errors in dMMR cells make them less sensitive to other defense mechanisms [27,28]. This is why some authors only propose adjuvant treatment, especially 5-FU monotherapy, for stage II dMMR tumours with risk factors (perforations, obstruction, T4 tumours). On the other hand, a combination of adjuvant chemotherapies are recommended for the majority of stage III tumours because of their significantly higher risk for relapse[25,29]. Some recent studies have

indicated that dMMR tumours show a remarkable response to treatment with anti-programmed cell death protein 1 (PD-1) immune check point inhibitors[30]. PD-1 is a cell surface receptor cooperating with a negative feedback system that inhibits autoimmunity by suppressing the T-cell-mediated cytotoxic immune response[31]. The use of PD-1 inhibitors seems to activate the antitumour immune response which is already highly activated in dMMR tumours *per se*[32]. This advanced immunogenic histological phenotype which is usually observed in dMMR tumours, has been used as a prospective surrogate for PD-1 inhibitor responsiveness. In fact, dMMR status has been shown to be such an effective biomarker that the United States Food and Drug Administration (FDA) granted unprecedented accelerated approval of PD-1 inhibitors for metastatic dMMR solid tumours regardless of site of origin[33].

Table 2. *DNA damage in normal cells induced by 5-FU in pMMR and dMMR tumours.*

Normal cell	Normal DNA damage -> DNA adducts -> Recognised by MMR -> Cell cycle arrest -> Cell death
Chemotherapy pMMR tumours	5-FU -> Severe DNA damage -> Recognised by MMR -> Cell cycle arrest -> Cell death
Chemotherapy dMMR tumours	5-FU DNA incorporation and damage -> No recognition from MMR -> No cell cycle arrest -> No cell death

On the other hand, some studies suggest that dMMR status is not the only mechanism resulting in an immunogenic phenotype and suggest a significant discordance between MMR status and histological appearance[34]. It is well-known that the characteristic histological appearance of dMMR CRC includes mucinous features, lymphocytic tumour infiltration, and high-grade histology[35,36]. This is the reason why further investigation should be conducted when encountering a tumour that is histologically suggestive of MMR deficiency, but exhibits retained MMR expression in polymerase chain reaction (PCR) or IHC testing. This discordance between MMR status and histology may be explained by dysregulation mechanisms other than dMMR.

Mismatch repair status in tumours presenting as an emergency.

Many studies have tried to clarify the issue of acute presentation of CRC. Around 15 to 30% of CRCs present as a surgical emergency, the most common causes being obstruction, perforation , and bleeding[37]. The majority of these studies have a case-mix of colon and rectal cancers despite the fact that rectal cancers seldom present as acute surgery[38]. Left colon and sigmoid are the most common sites for obstructing tumours , though the risk for obstruction seems to be highest for tumours appearing at the splenic flexure[38]. The caecum and sigmoid colon are reported to be the most common sites of perforation, and perforation can occur either at the site of the tumour or proximal to it due to distension[39]. Previous reports have shown a higher proportion of patients with a dMMR tumour presenting as emergency cases compared to those with a pMMR tumour, though the relationship between the genetic background and the acute clinical presentation is not fully understood[40,41]. On the other hand, there are reports presenting the opposite, making theories behind acute presentation of CRC even more uncertain[42,43]. Generally, the relationship between the biological effect of different genetic aberrations and acute presentation remains unclear and more knowledge must be gained before results can be applied in routine clinical practice.

Mismatch repair status in sporadic and familial tumours.

The aetiology of dMMR tumours can be either hereditary/genetic or somatic/sporadic. The first, known as Lynch syndrome, represents an autosomal dominant cancer predisposition, caused by a germline mutation in one of the basic MMR genes (*MLH1, PMS2, MSH2, and MSH6*). The main genes *MLH1* and *MSH2* are the most commonly mutated accounting for about 75% of these cases (42% and 33% respectively), whereas mutations in *MSH6 and PMS2* account only for 18% and 7% respectively of the cases. Somatic mutation, known as the second hit in the wild-type allele of the target tissue, needs to inactivate both MMR genes resulting in accumulation of somatic mutations and in the hypermutable phenotype observed in tumour tissues from mutation carriers [44,45]. An overview of the aetiology of CRC is illustrated in Fig 4.

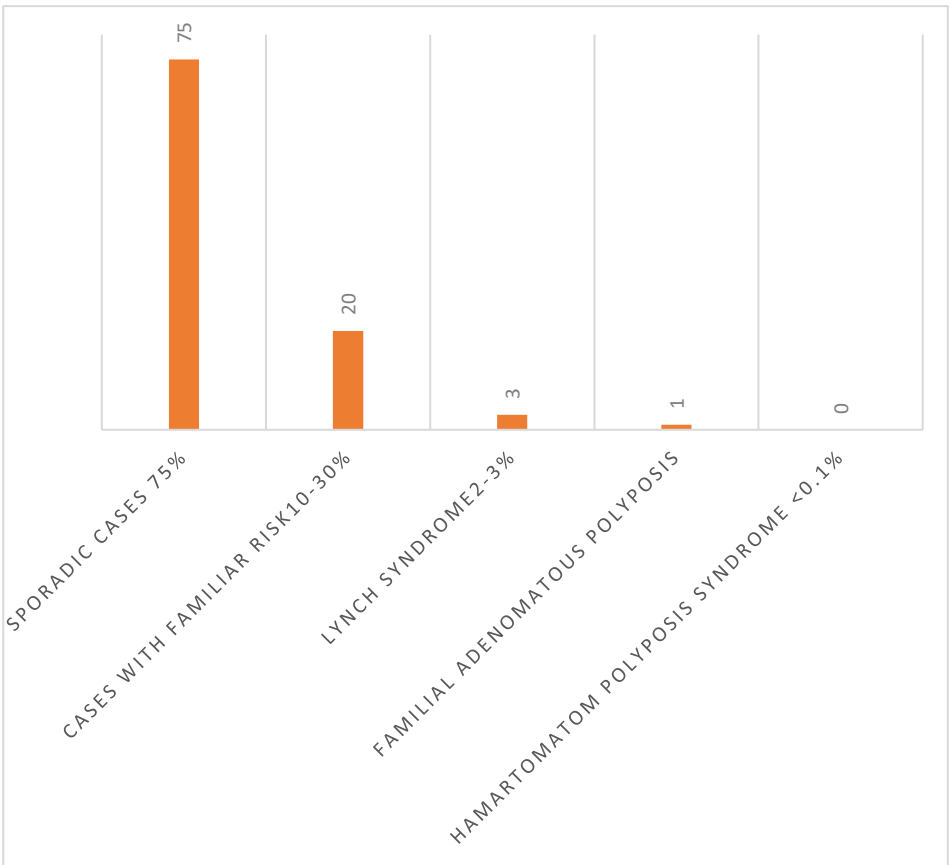

Figure 4. *Overview of CRC aetiology*[46].

Somatic inactivation of MMR function is observed in 15-20% of all CRCs due to bi-allelic methylation of the *MLH1* promoter. The majority of these tumours are CC cases and it is rare in rectal cancer. Analysis of *BRAF* ^{V600} mutation firstly distinguishes sporadic from hereditary cases. Further analysis of methylation of the *MLH1* promoter can supply more information, since *BRAF* mutations are generally not observed in hereditary cases as shown in Fig 5[46].

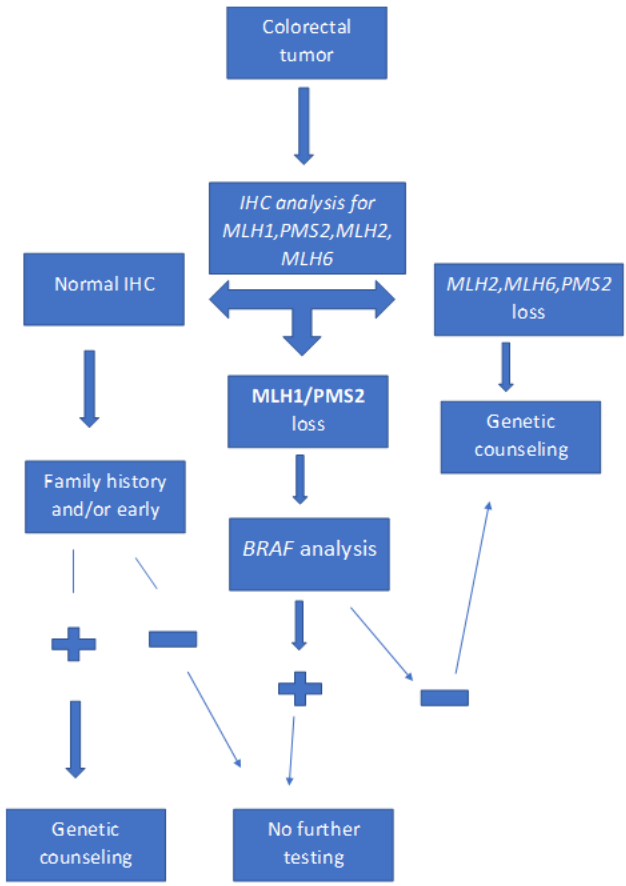

Figure 5. *Universal screening algorithm for the identification of patients with Lynch syndrome.*

It is well known that hereditary cases (Lynch Syndrome) are associated with the occurrence of specific tumours and represent specific germ-line mutations. Typical characteristics of this syndrome are autosomal dominant inheritance, early onset of cancer, increased incidence of CRC and endometrial cancer, and increased risk for synchronous and metachronous cancer[47].

A plethora of criteria, guideline strategies and detection techniques have been proposed but no gold standard rules exist. The Amsterdam criteria (developed 1991 and modified 1999, Table 3) were developed to identify and classify families with the Lynch syndrome. They reveal a sensitivity of about 22% and specificity of 98%[48,49].

Table 3. *The revised Amsterdam criteria for Lynch Syndrome.*

All criteria must be fulfilled to identify families with Lynch Syndrome
Three or more relatives with a Lynch associated cancer (CRC, endometrial, small bowel, ureter, or renal cancer).
One relative should be first degree relative of the other two
Probably affected two or more generations
One or more patients diagnosed before 50 years of age
Familial adenomatosis polyposis should be excluded from the CRC cases
Cancer disease should be verified by pathological examination

After the implementation of molecular examination, the Bethesda guidelines (developed in 1997 and revised 2004, see Table 4) were used to select patients with Lynch syndrome-associated tumours[50,51].

Table 4. *The revised Bethesda criteria for Lynch Syndrome.*

The Revised Bethesda Guidelines. One criterion fulfilled necessary to warrant clinical testing
CRC diagnosed in a patient younger than 50 years
Presence of synchronous or metachronous Lynch-associated tumour (CRC, endometrial, stomach, ovaria, pancreas, ureter, or renal cancer).
CRC diagnosed with MSI histology in a patient younger than 60 years
CRC diagnosed in one or more first degree relatives with a Lynch-associated tumour, with one cancer diagnosed in a patient younger than 50 years
CRC diagnosed in two or more first or second-degree relatives with Lynch-associated tumours, regardless age

Though both have limitations, the Amsterdam and Bethesda criteria are the most widely applied tools to identify patients with Lynch syndrome. Usually, an initial IHC evaluation of MLH1, PMS2, MSH2 and MSH6 is performed followed by genetic counselling for tumours to reveal loss of PMS2, MLH2 and MSH6. For the 15-20% of cases with IHC verified loss of MLH1/PMS2, further analysis for *BRAF* mutation and/or subsequent analysis for *MLH1* promoter methylation provide information to distinguish somatic alterations from germline defects (Fig 4)[52].

The clinical management of Lynch syndrome consists primarily of surveillance programmes and secondly of prophylactic surgery or chemopreventive options. Current guidelines recommend colonoscopy at 1-2 years intervals from the age of 20-25 years in persons with Lynch syndrome. In patients with a diagnosis of CRC, subtotal colectomy should be an option because of the risk for synchronous or metachronous colorectal tumours[53,54]. Endometrial surveillance with pelvic examination and endometrial biopsy each year, starting from the age of 30-35 years is recommended for females with Lynch syndrome. Women who have finished childbearing or have reached the age of 40 should be advised hysterectomy and bilateral salpingo-oophorectomy[54,55]. Screening for gastric cancer should include gastroscopy control at 1-2-year intervals from the age of 30, while urine cytology and ultrasound examination from the age of 25 at intervals of 1-2 years should be recommended due to risk for urinary tract cancer[56]. Risk estimates for cancer in 70 year-old patients with known Lynch syndrome undergoing the recommended surveillance programme are seen in Table 5**.**

Table 5*. Risk estimates for cancer in different organ systems in 70-year-old patients with Lynch syndrome and recommendation for surveillance programs[56-58].*

Tumour localisation	**Risk %**	**Mean age at diagnosis**		
Colon and rectum	30-80	44-61		
Endometrium	20-70	48-62		
Ovary	7-12	42		
Stomach	2-13	56		
Urine tract	1-12	55		
Small bowel	1-7	49		
Target organ	**Surveillance**		**Beginning**	**Interval**
Colon and rectum	Colonoscopy		20-25	1-2
Endometrium and ovary	Endometrial biopsy/ultrasound		30-35	1-2
Urinary tract	Urine cytology/ultrasound		25	1-2
Stomach	Oesophago-gastro-duodenoscopy		30	1-2

In contrast to the well-established screening, surveillance, therapy and/or prevention programmes for hereditary dMMR cases, little is known about the risk for cancer other than CRC for sporadic cases and no surveillance or screening programmes have been recommended for these patients. The main reason is that most studies have blended series of sporadic tumours with Lynch associated tumours[59,60]. A small series of patients comparing sporadic and Lynch-associated CRC tumours revealed that classical MSI characteristics were significantly less frequent in the Lynch group. On the other hand, lymphocytic reactions, peritumoural infiltration, and serrated adenomas occurred more often in Lynch patients but these differences were not significant[61]. Different MSI prediction models have been developed and used as pre-screening tools to identify cases that should undergo MMR testing, but these models focus on the identification of Lynch-associated tumours[36,59,60,62-65].

Materials and Methods

Ethics and approval

All studies presented in this thesis were conducted in accordance with the regulatory norms and standards of the Helsinki Declaration. Ethics approval was granted prior to each study.
Data extraction from the the Swedish National Board of Health and Welfare Cancer Register was authorised by the board of the register.

The studies were approved by the Regional Ethics Review Board of Umeå University, the Masaryk Memorial Cancer Institute, and the Finnish National Institute for Health and Welfare (registration number 2014/371-31, 2015/838/MOU and Dnro THL/2137/5.05/00/2017 respectively).

Study population

All patient and tumour materials used for this thesis were obtained from the departments of Pathology at the University Hospital of Umeå and Sunderby Hospital, Sweden, Helsinki University Hospital, Finland, Masaryk Memorial Cancer Institute, Regional Hospital of Liberec and the University Hospital of Pilsen, Czech Republic.

Study I was a meta-analysis based on previously published data and no further tumour material was used in this study.

Studies II and III were based on a series of stage II CC patients diagnosed between January 1995 and December 2012. Included were a consecutive series of 128 colon cancer patients operated at the University Hospital of Umeå (Sweden) between January 1995 and December 2003, along with 324 patients operated at Sunderby Hospital (Sweden), Masaryk Memorial Cancer Institute, Pilsen University Hospital and Kralovske University Hospital in the Czech Republic between January 2002 and December 2012.

Inclusion criteria were: i) histologically confirmed CC; ii) curative R0 resection of the primary tumour and lymphadenectomy; iii) pathologically confirmed stage II disease according to the American Joint on Committee on Cancer (AJCC) TNM classification; iv) having necessary demographic data and data regarding the course of the disease; and v) follow-up over a minimum of 5 years[66,67]. Adjuvant treatment with chemotherapy was allowed but was not mandatory. Patients younger than 18 years and patients with a tumour in the rectosigmoid junction or rectum were excluded. Unscheduled resection performed during the same admission as diagnosis of the primary tumour was classified as acute surgery. Data collected included demographic information, stage at diagnosis, histological

type, differentiation grade, number of lymph nodes examined, vascular and lymphatic invasion, perineural involvement, localisation of the primary tumour, type and duration of adjuvant chemotherapy.

Study IV included CC patients diagnosed between January 1995 and December 2019. A total of 412 patients formed the Scandinavian study cohort. Of these, 299 were CC patients diagnosed and operated at Umeå University Hospital between January 1st, 1995 and December 31st, 2003, the rest were 113 CC patients diagnosed and operated at Helsinki University Hospital between January 1st, 2004 and December 31st, 2005. The Czech validation cohort comprised a total of 458 patients; 124 CC patients diagnosed and treated between January 1st, 2018 and December 31st, 2019 at the Regional Hospital of Liberec, and 334 patients diagnosed and treated at the University Hospital of Pilsen during the same period. Acute surgery was defined as a patient operated during the same hospital admission as when the diagnosis of colon cancer was made. Through retrospective examination of hospital records, the following data were gathered: age and sex; stage at diagnosis; histological type; differentiation grade; and right or left tumour location. All tumour tissue materials were formalin-fixed and paraffin-embedded (FFPE).

Study V was based on a total of 2107 CRC patients diagnosed between March 1986 and December 2019. Of these, 527 patients were included from the Västerbotten Intervention Programme, and diagnosed and treated at a hospital in Västerbotten County, Sweden, between October 17th, 1986 and March 31st, 2009. Another 589 were patients who had been diagnosed and treated at Umeå University Hospital between January 1st, 1995 and December 31st, 2003 as well as 577 patients diagnosed and operated at Helsinki University Hospital between September 1st, 1998 and December 31st, 2005. Finally, 414 colon cancer (CC) patients treated at the University Hospital of Pilsen in the Czech Republic between January 1st, 2018 and December 31st, 2019.

The algorithm for determination of sporadic dMMR cases is shown in Fig 6.

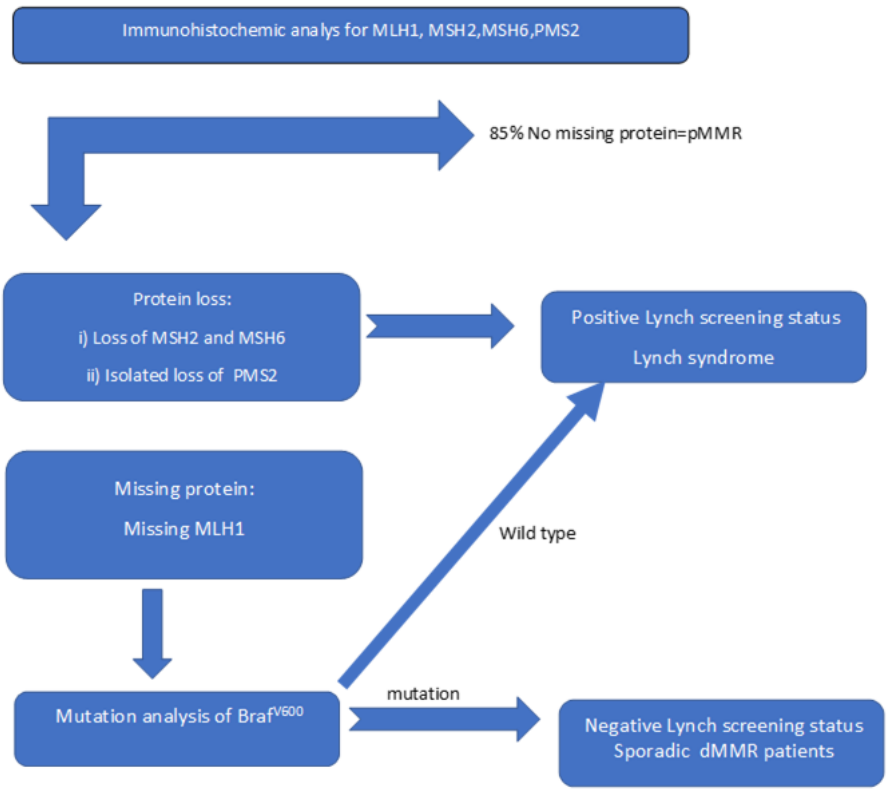

Figure 6. *Algorithm for determination of sporadic dMMR cases.*

Cases with conclusive immunohistochemistry showing protein loss of MSH2 or MSH6, or isolated loss of PMS2, were considered to be positive for Lynch syndrome. Cases with loss of MLH1 were further analysed for *BRAF*-mutation status. MLH1 loss cases with wild-type *BRAF*^{V600} were also considered Lynch syndrome. Patients with loss of MLH1 but with *BRAF*^{V600} mutation were considered negative for Lynch syndrome (*i.e.*, sporadic dMMR).

Methods

Study design and methods are summarised in Table 6.

Table 6. *Summary of methods used in the different studies.*

Study	Design	Methods/statistical analyses
I	Meta-analysis	Fixed-effect meta-analysis of hazard ratio according to the method of Peto.
II	Retrospective Multicentre Study	IHC, BRAF mutation test, statistical analyses (Kaplan-Meier method/log rank test, Cox proportional hazards regression model for uni- and multivariable analyses to estimate Hazard Ratio)
III	Retrospective Multicentre Study	IHC, statistical analyses (Kaplan-Meier method, Cox proportional hazards regression model for uni- and multivariable analyses to estimate Hazard Ratio)
IV	Retrospective Multicentre Study	IHC, statistical analyses (Pearson x^2 test, logistic regression model for uni- and multivariable analyses to estimate Odds Ratio)
V	Retrospective Multicentre Study	IHC, statistical analysis (Pearson x^2 test, Poisson regression model for uni- and multivariable analyses to estimate Incidence Rate Ratio)

Meta-analysis

All studies matching the inclusion criteria were individually analysed. Data extraction from selected publications was performed by two authors. Review articles, case reports, abstracts, letters, and former meta-analyses were not included but were screened for additional publications in their reference lists. Finally, one author independently evaluated the information collected. Due to non-standardised microsatellite (in)stability terminology, simplification was employed as seen in Table 7 where the inclusion criteria are listed.

Table 7*. Combinations of scrutinised terms, inclusion criteria and simplifications.*

Terms and combination	Colon cancer, rectal cancer, colorectal cancer and outcome, prognosis, survival, recurrence, relapse, and MMR, mismatch repair, replication error, microsatellite instability and hazard ratio, relative risk, log-rank test and localised disease, stage II, stage III, Dukes B, Dukes C.
Inclusion criteria	Studies were included if at least one hazard ratio for overall survival or/and disease-free survival for stage II disease was presented or could be easily derived from published results.
Simplifications	MSI-low, MSS and RER-negative tumours considered as MSS. Only MSI-high and RER-positive tumours classified as microsatellite-instable tumours (MSI).

MMR protein IHC: Studies II-V

All available cases were evaluated by IHC analysis of the protein products of genes involved in DNA MMR. Antibodies against all four key MMR proteins (MLH1, MSH2, PMS2 and MSH6) were used to explore the proficiency of the MMR system. Deficient MMR was indicated if one or more of these proteins were absent (Figure 7)[68]. The dependent expression of specific MMR protein heterodimers MSH2/MSH6 and MLH1/PMS2 was taken into account when interpretating IHC results by two independent investigators according to a standardised protocol (R.Palmqvist and P.Fabian)[69,70]. For patients included from the three university hospitals in the Czech Republic and the regional hospital in Sweden, the IHC assay was performed as follows: Formalin-fixed, paraffin-embedded tissue was cut into 4-6 micrometre-thick slices and placed on glass slides. After deparaffination and rehydration in xylene and ethanol, antigen retrieval in Dako target retrieval solution pH 9 was performed for 40 minutes at 96°C. After incubation with the primary antibodies, the Dako visualisation system (DAKO EnVision™+ System, HRP) was applied according to the manufacturer's instructions. For patients from the University Hospital of Umeå, immunohistochemistry was performed in a similar way but using another semiautomatic immunostaining machine (Ventana (Roche)) and reagents used were according to the manufacturer's recommendations[71]. In all cases, internal positive control cells (normal crypt epithelium, lymphocytes or stromal fibroblasts) were evaluated for comparison prior to tumour tissue assessment. Specimens with no positive internal control cells were excluded from further evaluation. Only nuclear staining was considered positive. Tumours that yielded at least one negative stain were considered to be MMR protein-deficient.

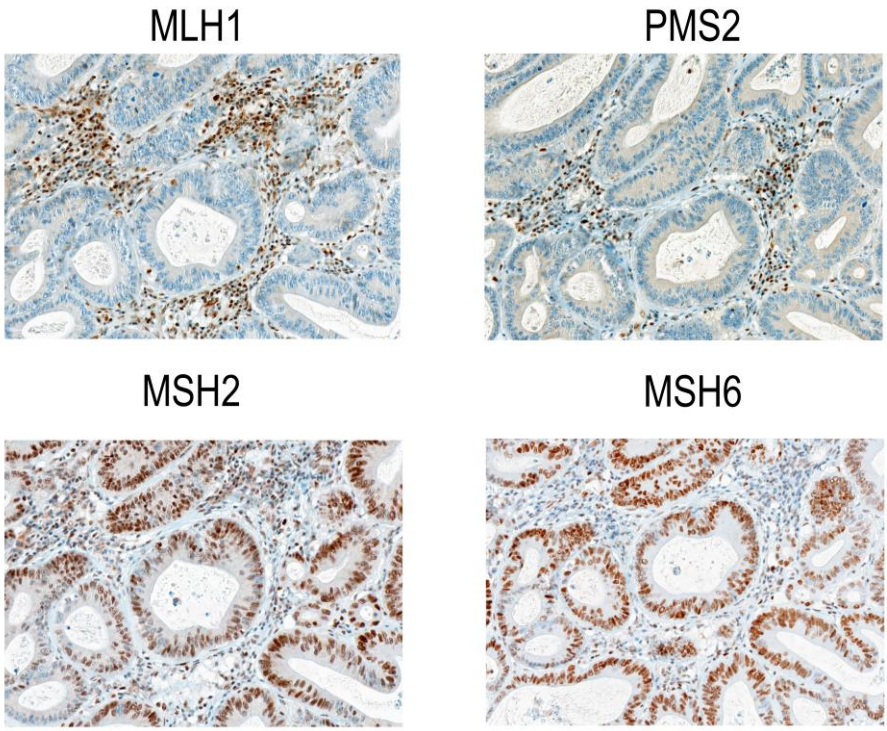

Figure 7. *IHC analysis: Preserved and lost protein expression.*

BRAF mutation test: Studies II - V

In all cases, DNA for analysis was extracted from formalin-fixed, paraffin-embedded (FFPE) specimens either by the DNA Sample Preparation Kit (Roche) or QIAamp® DNA FFPE Tissue Kit (Qiagen), with macrodissection when necessary[72,73]. For patients from the Czech Republic and the regional hospital in Sweden, *BRAF* mutation testing was carried out using Cobas® 4800 *BRAF*^{V600} Mutation Test (Roche). Besides the most common mutation V600E (c.1799T>A) the test can also detect mutations V600K (c.1798_1799delGTinsAA) and V600D (c.1799_1800delTGinsAT). Analytical sensitivity of the assay is approximately 5%. For cases from the University Hospital of Umeå, *BRAF* was instead detected by a *BRAF*^{V600E} mutation-specific Taqman allelic discrimination assay (reagents from Applied Biosystems)[73]. For the Finnish patients, *BRAF* mutation status was analysed with immunhistochemistry using a *BRAF*^{V600E} mutation-specific antibody.

Histopathology: Studies II-IV

All available H&E-stained tumour sections were reviewed for correct classification of the tumours according to a standardised protocol by two independent investigators (R.Palmqvist and P.Fabian) who were blinded to the IHC results as well as to the results from the other pathologist. The tumour stage was determined according to the available AJCC/UICC TNM classification at the time of primary staging. Unscheduled resection performed during the same admission as diagnosis of the primary tumour was classified as acute surgery.

Statistical analyses

Study I

A fixed-effect meta-analysis of hazard ratio according to the method of Peto was applied in the analysis of the papers included. Firstly, the natural logarithm (ln) of the hazard ratio (HR) from each study was calculated. Standard errors for the ln (HR) were calculated from CIs if available. Weighted mean of ln (HR) together with weighted standard error of mean HR estimations between different studies were calculated as well as the mean of the standard errors from each study. Weights were equated to the number of patients where MSI was accessed. Because standard errors were not available from all studies included, the formula weight = 1/(standard error) could be used. Standard errors between studies and mean standard errors from studies involved were appropriately combined according to Peto's method[74].

Study II

SAS software (IBM) version 25.0 was used for all analyses. All continuous variables were simplified by categorisation. Pearson χ^2 test was used to compare categorical variables. Available variables included age, gender, tumour site, un-/scheduled operations, vascular, perineural or lymphatic invasion, tumour staging, the number of lymph nodes examined, differentiation grade, adjuvant chemotherapy administration, relapse, and cause of death. Survival curves were estimated using the Kaplan-Meier method and compared by mean of the log rank test. The Cox proportional hazards regression model was used for uni- and multivariable analyses. Covariates that were significantly associated with overall survival and those with a p value < 0.2 in the univariable test were included in the multivariable model. All statistical tests were performed as two-sided at a significance level of 0.05 (5%) with 95% confidence intervals. Overall Survival (OS) was defined as the time from the date of surgery to death from any cause. For patients still alive at the end of follow-up, the date of last contact was used as censoring date. Time to relapse (TTP) was defined as the time from the date of

surgery to the date of first relapse. Thus, patients without relapse and those with non-cancer-related death were censored from further analysis.

Study III

All analyses were performed as in Study II. OS was defined as the time from the date of surgery to death from any cause. For patients still alive at the end of follow-up, the date of last contact was used as a censoring date. Post-relapse survival (PRS) was calculated as the time from the date when relapse was diagnosed, using the same procedure of censoring as for OS.

Study IV

SPSS (IBM) version 26 was used in this study. Primarily, all continuous variables were simplified by categorisation and proportions were compared with the Pearson x^2 test (Fisher exact test if expected numbers in the contingency table were below five). Secondly, uni- and multivariable logistic regression was used to estimate odds ratios (OR) for acute surgery as dependent variable with the independent predictor variables age, sex, MMR status, tumour site (right vs. left colon), and stage. In this study, all statistical tests were performed as two-sided at a significance level of 0.05 with 95% CI.

Study V

In this study SPSS (IBM) version 26 was used. Primarily, all continuous variables were simplified by categorisation and proportions were compared with the Pearson x^2 test (Fisher exact test if expected numbers in the contingency table were below five). Secondly, uni- and multivariable Poisson regression was used to estimate incidence rate ratio (IRR) for non-CRC as the dependent variable with the independent predictor variables age, sex, MMR status, tumour site (colon vs. rectal), and stage. In this study, all statistical tests were performed as two-sided at a significance level of 0.05 with 95% CI.

Results

Study I

From a total of 19 studies, 16 were eligible for analysis of OS and 14 for analysis of DFS. Determination of MSI status was performed retrospectively. Genotyping was used in 12 studies, IHC in 6 studies, and no information on methodology was given in one study. The time from diagnosis until the first recurrence or relapse, second cancer, or death was defined as DFS, and OS was defined as the time from diagnosis to the date of death from any cause. Flow diagram of studies included and those excluded is shown in Fig 8.

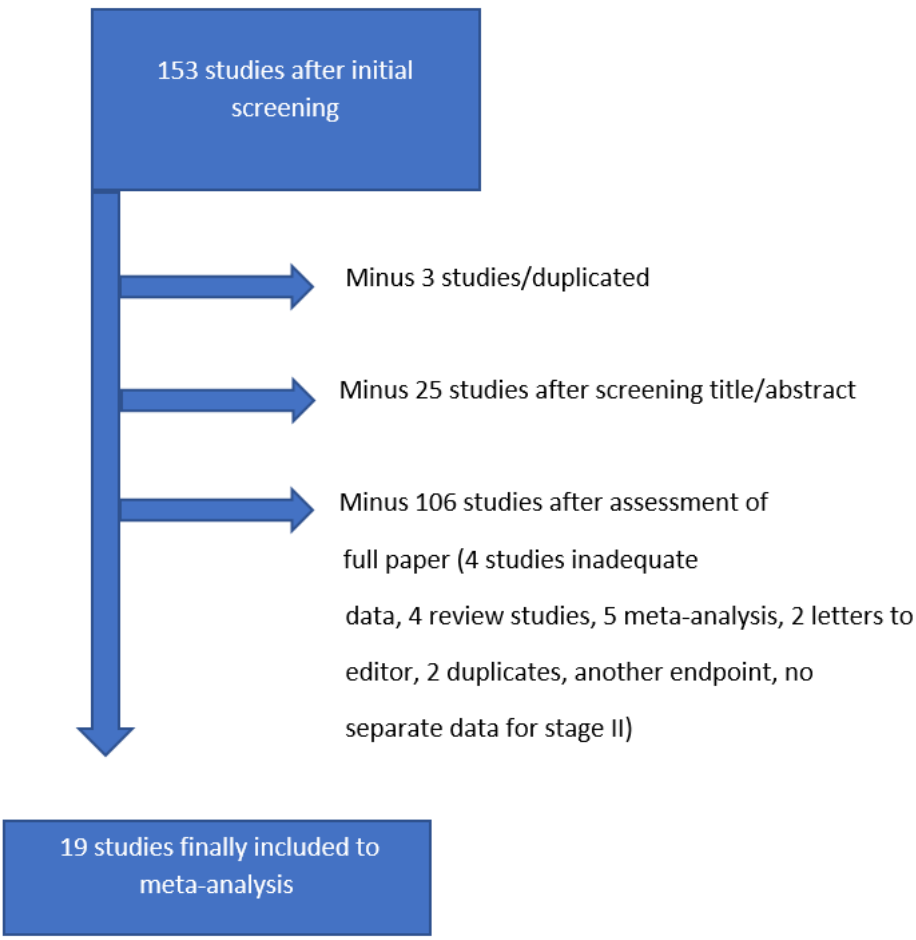

Figure 8. *Flow diagram of included and excluded studies.*

Overall survival data

HR was > 1.0 in six studies available for analysis of OS, 10 studies gave HR < 1.0, and p-values were given in 13 studies. Only three studies revealed statistical significance, but these evaluated only 575 patients. OS for the entire group revealed no significant difference between MSI and MSS patients (HR 0.73 (95%CI 0.33-1.65)). Identical results were observed for the subgroup of studies evaluating colon cancer patients only. No significant difference was noticed when studies using immunohistochemistry or genotyping were analysed separately.

Disease-free survival data
Four studies available for analysis of DFS revealed HR > 1.0 and 10 studies HR < 1.0. Six of 13 studies with available p-values showed statistical significance (five of them with HR < 1, one with HR > 1). No significant difference between patients with MSI and MSS tumours was noticed in the analysis of all studies (HR 0.60 (95% CI=0.27-1.32)). Separate analysis of studies that included colon cancer only did not reach the threshold for statistical significance either (HR 0.60 (95% CI 0.27-1.31)). No difference was noticed between studies using immunohistochemistry (HR 0.67, 95%CI 0.26-1.70) or genotyping (HR 0.51, 95%CI 0.14-1.85).

Study II

From a cohort of 463 curatively operated patients, 452 were available for statistical analysis. Median follow-time up was 77 months. dMMR was detected in 93 of 452 specimens (20.6%). Analysis for *BRAF*^{V600} was available for 166 of the cases from the subgroup of patients from Umeå university hospital. Sixty-two operations were unscheduled (14%). Other causes of death apart from colon cancer-specific death were recorded as: surgical complication, oncological complication, non-cancer-related, and other cancer-related death.

Overall survival data

One hundred and sixty-two patients died during the study period. Twelve dMMR patients died due to relapse. Another 14 dMMR patients died of other causes during the follow–up period. No significant association was detected in the univariable model between dMMR and OS (Log-Rank, p = 0.583, 95% CI 0.76 – 1.67; Wilcoxon p = 0.263, 95% CI 0.55 – 1.16; HR 1.28, 95% CI 0.82 – 1.08, p = 0.323) (Fig 9).

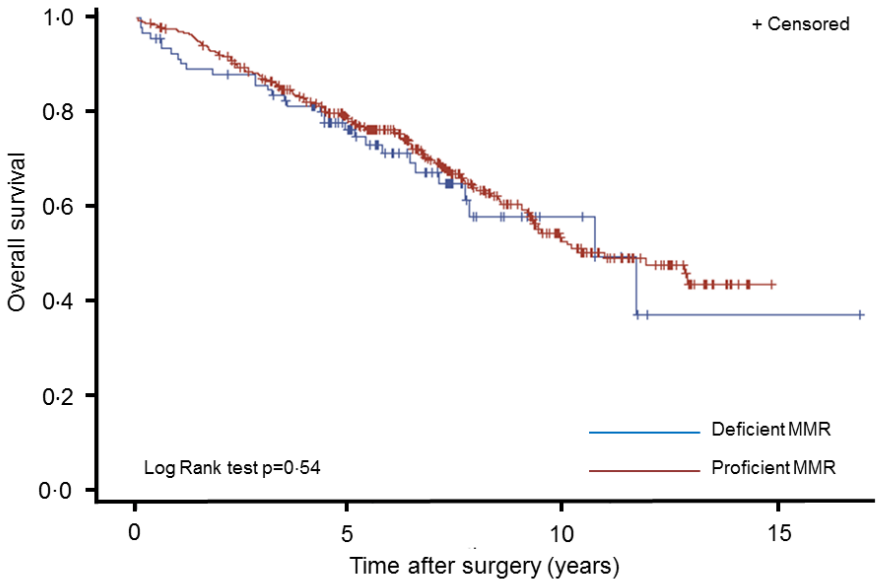

Figure 9. *OS comparing dMMR and pMMR patients.*

The multivariable model adjusted for all variables with significant association or a p value <0.2, revealed a significant impact of dMMR. Acute surgery, age over 70 years, less than 12 lymph nodes found in the surgical specimen and the administration of adjuvant treatment were the covariates showing a statistically significant association with OS as seen in Table 8. Furthermore, only stepwise removal of acute surgery and number of lymph nodes in the multivariable model led to loss of dMMR significance.

Table 8. *Univariable and multivariable analyses of Overall Survival in 452 patients with colon cancer stage II.*

Variables	**Univariable**			**Multivariable**		
	HR	**95 % CI**	**p value**	**HR**	**95 % CI**	**p value**
MMR						
Deficient	1.00			1.00		
Proficient	1.28	0.82-1.80	0.323	1.75	1.05–2.93	0.033
Acute operation						
Yes	1.00			1.00		
No	0.50	0.34-0.74	0.001	0.35	0.21–0.59	<0.001
Age						
<70	1.00			1.00		
≥70	2.15	1.57-2.95	<0.001	1.87	1.23–2.82	<0.001
Lymph Nodes examinated						
≥12	1.00			1.00		
<12	1.39	0.93-2.06	0.106	1.45	0.97-2.20	0.073
Adjuvant chemotherapy						
No	1.00			1.00		
Yes	0.58	0.40-0.86	0.007	1.47	0.92-2.35	0.108

Time to relapse

dMMR patients revealed a highly signifiantly lower risk for relapse or colon cancer-specific death (TTP: HR 0.50, 95% CI 0.28 – 0.87, p = 0.012) (Fig 10).

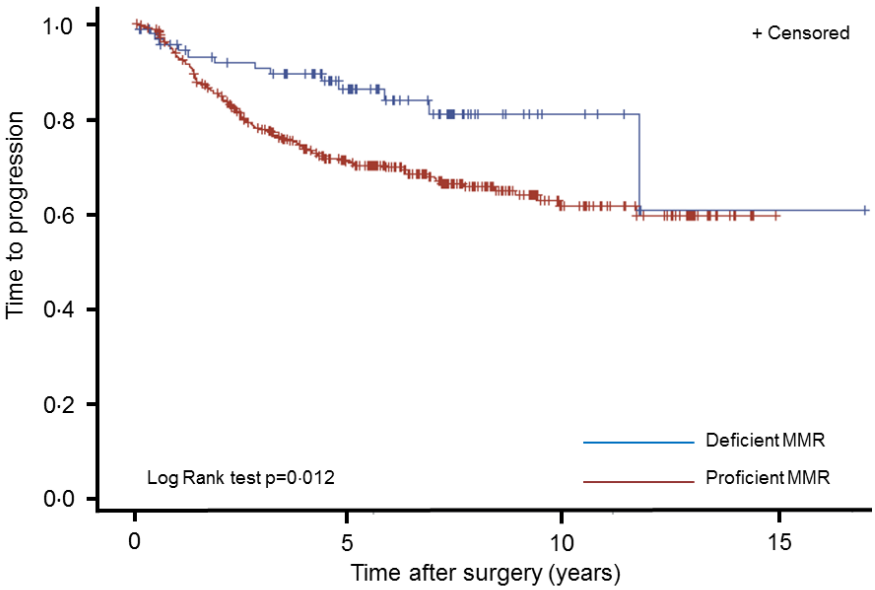

Figure 10. *TTP comparing dMMR and pMMR patients.*

Tumour localisation (HR 0.62, 95% CI 0.43 – 0.91, p = 0.013) and vascular invasion (HR 3.18, 95% CI 1.74 – 5.81, p = 0.001) were the only two other covariates having statistical significance. The last one was the only covariate to retain significance in the multivariable model and its significant association was independent of covariable stepwise removal, as seen in Table 9. No effect on the association between dMMR and TTP was revealed through stepwise removal of non-significant covariables in the multivariable model.

Table 9. *Univariable and multivariable analyses for TTP in 452 patients with stage II colon cancer.*

Variables	Univariable			Multivariable		
	HR	**95 % CI**	**p value**	**HR**	**95 % CI**	**p value**
MMR status						
Proficient	1.00			1.00		
Deficient	0.50	0.28-0.89	0.019	0.60	0.32–1.11	0.104
Tumour location						
Left	1.00			1.00		
Right	0.62	0.43-0.91	0.013	0.71	0.47–1.10	0.125
Age						
<70	1.00			1.00		
≥70	0.78	0.53-1.10	0.141	0.83	0.56-1.27	0.404
Vascular Invasion						
No	1.00			1.00		
Yes	3.18	1.74-5.81	<0.001	3.18	1.77-5.84	<0.001

Study III

MMR status was successfully determined in 451 patients and thus eligible for this study with a median follow-up of 73 months. dMMR status was detected in 92 of these (20.4%). Continuous variables have been simplified by categorisation in all analyses. The mean age at diagnosis was 69.1 years and patients were dichotomised at 70 years-of-age. Sixty-two operations were unscheduled.

Overall survival data

Survival data were available for 92 dMMR and 359 pMMR patients elegible for OS analysis. No OS benefit from adjuvant treatment was detected for the dMMR cohort (HR 1.16; 95% CI 0.63-2.13, p=0.616) (Table 10). No significant difference in median overall survival (MOS) was seen between patients receiving or not receiving adjuvant chemotherapy (10.6 vs 11.57 years).

Table 10. *Univariable and multivariable analyses for Overall Survival in 92 dMMR patients with stage II colon cancer.*

Variables	**Univariable**			**Multivariable**		
	HR	**95 % CI**	**p value**	**HR**	**95 % CI**	**p value**
Gender						
Male	1.00			1.00		
Female	0.34	0.27-1.71	0.194	0.59	0.21-1.63	0.309
Acute surgery						
Yes	1.00			1.00		
No	0.59	0.37-0.92	0.022	0.38	0.21-0.70	0.012
Tumour location	1.00			1.00		
Right				0.80		
Left	0.69	0.42-1.11	0.142		0.52-1.24	0.337
Age						
≥70	1.00			1.00		
<70	0.47	0.27-0.83	0.010	0.77	0.46-0.89	0.023
Tumour stage						
T3	1.00			1.00		
T4	1.05	0.64-2.03	0.599	1.05	0.28-1.76	0.455
Tumour Type						
Adenocarcinoma	1.00			1.00		
Mucinous	1.90	1.26-3.12	0.010	1.27	0.37-4.37	0.187
Tumour Grade						
Low	1.00			1.00		
High	2·17	1.21-3.56	0.009	4.00	1.74-9.34	0.021
Adjuvant Chemotherapy						
No	1.00			1.00		
Yes	1.16	0.63-2.13	0.616	1.10	0.58-1.08	0.058

In contrast, patients with pMMR tumours receiving adjuvant treatment had significantly better OS compared to those who did not (HR 0.52; 95% CI 0.31-0.85, p=0.010). In non-treated pMMR patients, MOS reached 9.19 years. Analysis in the multivariable model revealed that pMMR patients treated with adjuvant chemotherapy had a significantly better OS compared to the group without treatment, as seen in Table 11.

Table 11. *Univariable and multivariable analyses for Overall Survival in 359 pMMR patients with stage II colon cancer.*

Variables	**Univariable**			**Multivariable**		
	HR	**95 % CI**	**p value**	**HR**	**95 % CI**	**p value**
Gender						
Male	1.00			1.00		
Female	1.40	0.94-2.12	0.102	1.09	0.70-1.69	0.693
Acute surgery						
Yes	1.00			1.00		
No	0.65	0.39-1.07	0.096	0.38	0.21-0.70	0.002
Tumour location						
Right	1.00			1.00		
Left	1.13	0.75-1.71	0.540	0.80	0.52-1.24	0.337
Age						
≥70	1.00			1.00		
<70	0.70	0.45-1.06	0.096	0.77	0.46-1.29	0.323
Tumour stage						
T3	1.00			1.00		
T4	0.68	0.30-1.57	0.377	0.70	0.28-1.76	0.455
Tumour Type						
Adenocarcinoma	1.00			1.00		
Mucinous	1.10	0.34-3.49	0.870	1.27	0.37-4.37	0.702
Tumour Grade						
Low	1.00			1.00		
High	1.71	0.99-2.97	0.053	2.54	1.37-4.69	0.003
Adjuvant Chemotherapy						
No	1.00			1.00		
Yes	0.52	0.31-0.85	0.010	0.42	0.22-0.78	0.007

For the pMMR group, the significant impact of adjuvant chemotherapy remained even in the multivariable model (HR 0.42; 95% CI 0.22-0.78, p=0.007), as seen above in Table 11.

Post-Relapse survival

Fourteen dMMR and 112 pMMR patients who relapsed were analysed separately and no significant survival differences due to MMR status were observed (Log-Rank, HR 1.72, 95% CI 0.93-3.19, p=0.083) (Fig 11).

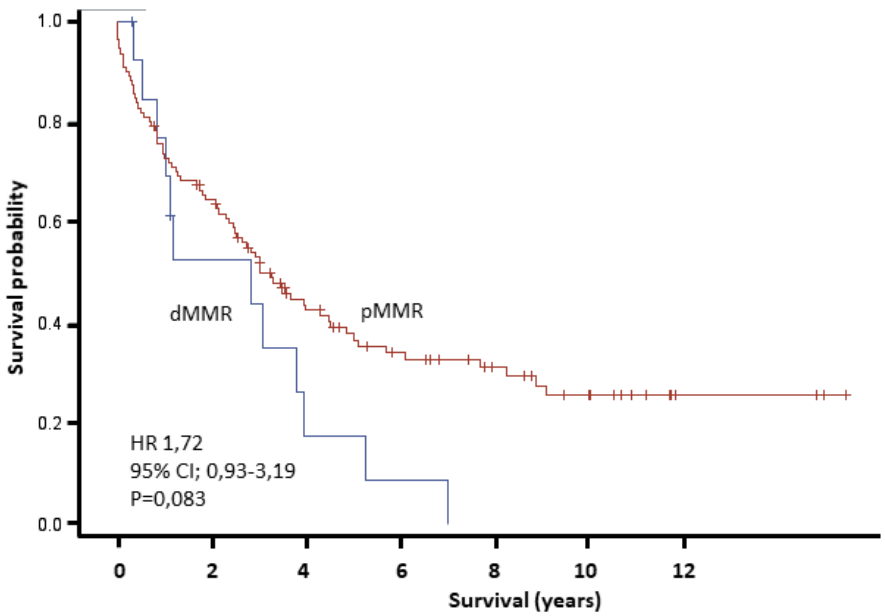

Figure 11. *Post-relapse survival according to MMR status***.**

Subsequent analysis considering postoperative treatment separately for these groups revealed no significant survival differences in the dMMR group. In the pMMR group, survival after relapse was significantly longer for patients postoperatively treated with adjuvant chemotherapy compared to non-treated patients both in the uni- (HR 0.51; 95% CI 0.29-0.90, p=0.021) and multivariable model (HR 0.49; 95% CI 0.26-0.93, p=0.030) (Table 12).

Table 12. *Univariable and multivariable analyses for Post-Relapse Survival in 359 pMMR patients with stage II colon cancer.*

Variables	Univariable			Multivariable		
	HR	**95% CI**	**p value**	**HR**	**95% CI**	**p value**
Gender						
Male	1.00			1.00		
Female	1.12	0.69-1.83	0.623	1.01	0.61-1.69	0.964
Acute surgery						
Yes	1.00			1.00		
No	0.55	0.32-0.93	0.028	0.52	0·29-0·92	0·027
Tumour location						
Right	1.00			1.00		
Left	0.87	0.75-1.71	0.567	0.92	0.55-1.53	0.763
Age						
≥70	1.00			1.00		
<70	0.59	0.37-0.95	0.030	0.72	0.43-1.19	0.202
Tumour stage						
T3	1.00			1.00		
T4	0.57	0.25-1.33	0.199	0.73	0.30-1.72	0.472
Tumour Type						
Adenocarcinoma	1.00			1.00		
Mucinous	4.07	1.60-10.35	0.003	2.23	0.72-6.90	0.164
Tumour Grade						
Low	1.00			1.00		
High	2.51	1.31-4.81	0.005	3.09	1.39-6.85	0.005
Adjuvant Chemotherapy						
No	1.00			1.00		
Yes	0.51	0.29-0.90	0.021	0.49	0.26-0.93	0.030

Study IV

Division of the study population into exploration and validation cohorts is presented in Fig 12.

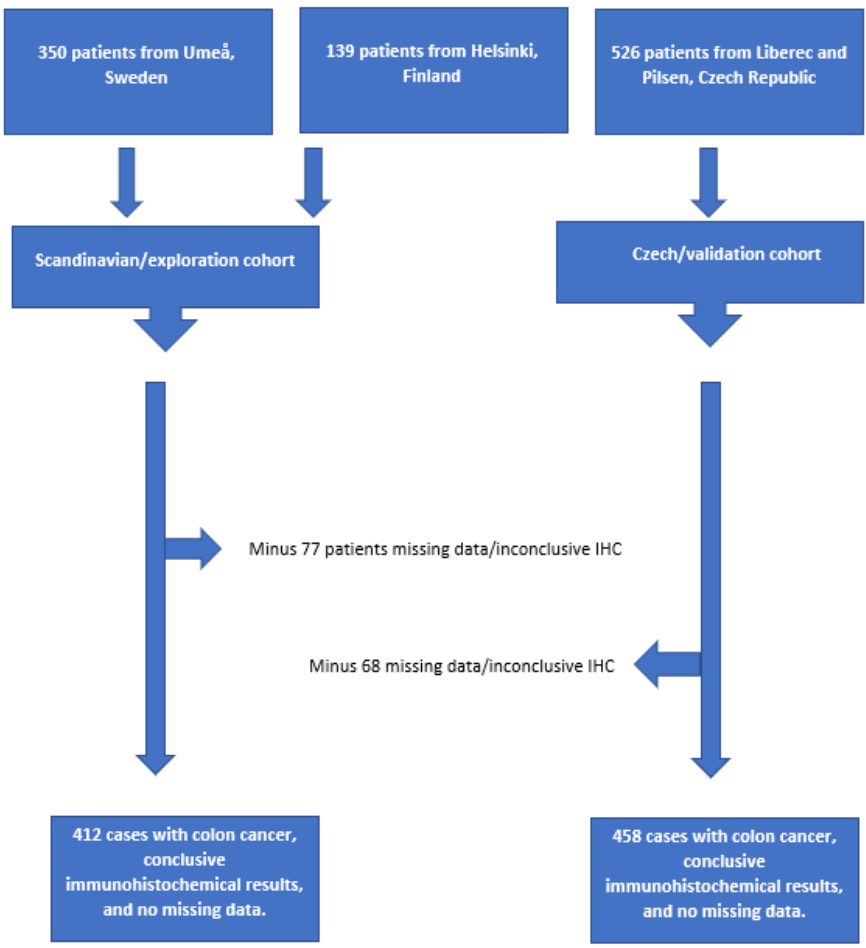

Figure 12. *Inclusion and exclusion of cases, and division of the entire population into a Scandinavian exploration and a Czech validation cohort*.

In all, 870 patients met the inclusion criteria. Of these, 399 were women (46%) and the mean age was 69 years. Patients were dichotomised at 70 years of age, and 487 patients (56%) were older than 70. Of the total cohort, 190 cases (22%) were identified as dMMR tumours, and right colon was the site in 53% (460 patients). Acute presentation and surgery occurred in 179 patients (21%), where 57 of cases were categorised as dMMR. The Scandinavian exploration cohort (412

patients from Finland and Sweden) was validated against a total of 458 patients from the Czech Republic. In the Scandinavian exploration cohort, 91 (22%) patients were dMMR cases while in the Czech validation cohort, 99 (22%) were dMRR. Acute surgery was performed in 103 cases (25%) in the Scandinavian exploration cohort and in 76 cases (17%) of the Czech validation cohort, where 33 (32%) and 24 (31%) were dMMR tumours (p=0.005 and p=0.021 respectively), (Table 13).

Table 13. *Comparison of clinical characteristics and tumour pathology of the Scandinavian exploration and Czech validation cohorts.*

Cohort	**Covariable**	**Total** (%)	**Elective** (%)	**Acute** (%)	**p value**
Scandinavian	**Sex**				
	Male	220 (53)	161 (52)	59 (57)	0.362
	Female	192 (47)	148 (48)	44 (43)	
Czech	**Sex**				
	Male	251 (55)	212 (55)	39 (51)	0.504
	Female	207 (45)	170 (45)	37 (49)	
Scandinavian	**MMR status:**				
	Deficient	91 (22)	58 (19)	33 (32)	0.005
	Proficient	321 (78)	251 (81)	70 (68)	
Czech	**MMR status:**				
	Deficient	99 (22)	75 (20)	24 (32)	0.021
	Proficient	359 (78)	307 (80)	52 (68)	
Scandinavian	**Tumour stage**				
	I+II	204 (50)	171 (55)	33 (32)	<0.001
	III+IV	208 (50)	138 (45)	70 (68)	
Czech	**Tumour stage**				
	I+II	222 (49)	194 (51)	28 (38)	0.032
	III+IV	229 (51)	183 (49)	46 (62)	
	Not determinable	7	5	2	

In the 412 patients in the Scandinavian exploration cohort, a strongly significant association was seen between dMMR tumour status and need for acute surgery in the uni- (OR=1.82, 95% CI 1.114-3.021, p=0.017) and multivariable (OR=2.21, 95% CI 1.281-3.951, p=0.005) models. The same relationship was seen in the 458 patients in the Czech validation cohort, where a significant association was seen between dMMR tumour status and need for acute surgery in both the uni- (OR=1.94, 95% CI 1.095-3.260, p=0.022) and the multivariable (OR=1.77, 95% CI 1.154-3.187, p=0.021) models (Table 14). In the Scandinavian exploration cohort, other variables associated with the need for acute surgery were tumour Stages III and IV in the uni- (OR= 6.18, 95% CI 2.037-18.342, p= 0.001 and OR= 5.42, 95% CI 1.818-16.545, p= 0.003 respectively) and multivariable (OR= 6.01, 95% CI 1.992-18.244, p= 0.001 and OR= 5.33, 95% CI 1.739-16.135, p= 0.003 respectively) models, respectively. Even in the Czech validation cohort, tumour stages III and IV were associated with the need for acute surgery in the uni- (OR= 3.26, 95% CI 1.743-14.607, p= 0.016 and OR= 3.66, 95% CI 1.771-16.956, p= 0.031 respectively) and multivariable (OR= 3.01, 95% CI 1.673-13.661, p= 0.022 and OR= 3.71, 95% CI 1.802-17.930, p= 0.039 respectively) models.

Table 14. *Risk for acute surgery as OR in the univariable and multivariable models.*

		Univariable analysis			**Multivariable analysis**		
Cohort	**Covariable**	**OR**	**95% CI**	**p value**	**OR**	**95% CI**	**p value**
Scandinavian	**MMR status**						
	Proficient	1.00			1.00		
	Deficient	1.82	1.11-3.02	0.017	2.21	1.28-3.95	0.005
Czech	**MMR status**						
	Proficient	1.00			1.00		
	Deficient	1.94	1.09-3.26	0.022	1.77	1.15-3.18	0.021
Scandinavian	**Tumour stage**						
	I	1.00			1.00		
	II	2.52	0.85-7.68	0.093	2.38	0.77-7.10	0.130
	III	6.18	2.03-18.34	0.001	6.01	1.99-18.24	0.001
	IV	5.42	1.81-16.54	0.003	5.33	1.73-16.13	0.003
Czech	**Tumour stage**						
	I	1.00			1.00		
	II	2.12	0.47-9.36	0.141	1.89	0.42-8.68	0.198
	III	3.26	1.74-14.60	0.016	3.01	1.67-13.66	0.022
	IV	3.66	1.77-16.95	0.031	3.71	1.80-17.93	0.039

Study V

From the 2107 potential cases, 141 revealing inconclusive immunohistochemistry or missing data, and 67 revealing inconclusive BRAF analysis were excluded from further analysis. The remaining study population was considered as sporadic dMMR and matched against the Swedish and Finnish National Cancer Registers while a detailed review of medical records was performed for the Czech population to identify the occurrence of non-colorectal malignancies prior to and

after (until end of follow-up) diagnosis of the CRC. From this population, 62 patients with a second or recurrent CRC, and 40 cases registered as non-melanoma skin cancer were excluded. Furthermore, an additional 44 patients with *BRAF*^{V600} wild-type were excluded since they were categorised as having a positive Lynch screening status (Fig 13).

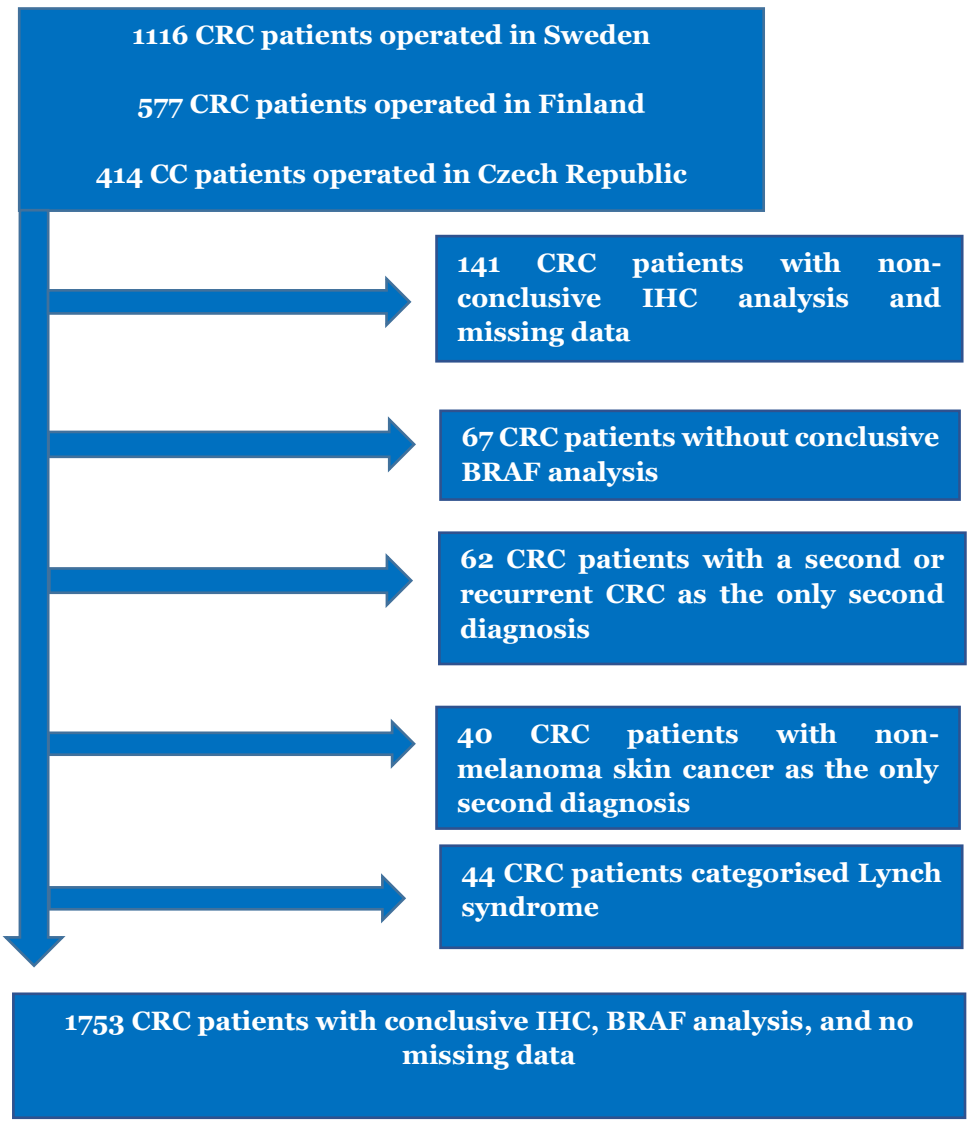

Figure 13. *Initial study population and exclusions.*

Demographic and clinical characteristics for 1753 patients are shown in Table 15. Among cases included, 838 were females (48%) and 539 patients (33%) were diagnosed with a rectal tumour. Median age at diagnosis was 67 (interquartile range [IQR] 60-75) years, and patients were dichotomised at 70 years of age. Sporadic dMMR status was found in 236 patients (13%). Of these patients 327 (19%) had least one non-colorectal malignancy within 20 years before or after (until end of follow-up) the diagnosis of the CRC, where dMMR status was detected in 56 cases (17%, p=0.031).

Table 15. *Occurrence of non-colorectal cancer according to clinical characteristics and tumour pathology of the entire study population.*

Covariable	**Total(n= 1753)**	**Other cancer (n=327)**	**No other cancer (n=1426)**	**p value**
Age				
Years median	67	70	67	NA
Range	29-105	29-97	29-105	
IQR	60-75	63-77	60-75	
Dichotomised 70 years				
<70	1007 (57)	149 (46)	858 (60)	<0.001
≥ 70	746 (43)	178 (54)	568 (40)	
Sex ratio				
Male	915 (52)	161 (49)	754 (53)	0.235
Female	838 (48)	166 (51)	672(47)	
MMR status				
Deficient(sporadic)	236 (13)	56 (17)	180 (13)	0.031
Proficient	1517 (87)	271 (83)	1246 (87)	
Tumour location				
Colon	1214 (69)	222 (68)	992 (70)	0.553
Rectum	539 (33)	105 (32)	434 (30)	
Tumour Stage				
I	157 (9)	35 (11)	122 (9)	0.074
II	708 (40)	146 (45)	562 (39)	
III	670 (38)	112 (34)	558 (38)	
IV	218 (13)	34 (10)	184 (14)	

The occurrence of non-colorectal malignancy according to tumour category are listed in Table 16.

Table 16. The occurrence of *non-colorectal malignancy comparing patients with sporadic dMMR and pMMR CRC tumours.*

	Total (%) 363(100)	**Sporadic dMMR (%) 60(100)**	**pMMR (%) 303(100)**	**p value**
Prostate cancer	91(25.1)	18(30)	73(24.1)	0.337
Breast cancer	49(13.5)	9(15)	40(13.2)	0.712
Endometrial cancer	46(12.7)	4(6.6)	42(13.7)	0.126
Haematological cancer	38(10.7)	9(15)	29(9.6)	0.211
Urinary tract cancer	31(8.5)	7(11.7)	24(7.9)	0.342
Melanoma	17(4.9)	1(1.6)	16(5.3)	0.226
Endocrine cancer	14(3.8)	3(5)	11(3.6)	0.617
Ovarian	13(3.6)	1(1.6)	12(3.9)	0.384
Stomach/oesophagus	12(3.3)	2(3.3)	10(3.4)	0.992
Hepatobiliary cancer	11(3.0)	1(1.8)	10(3.4)	0.502
Lung cancer	11(3.0)	2(3.3)	9(3.0)	0.880
Brain cancer	10(2.6)	2(3.3)	8(2.8)	0.764
Pancreas cancer	8(2.2)	1(1.8)	7(2.4)	0.756
Head and neck cancer	5(1.2)	0(0)	5(1.6)	0.317
Non-specific	4(1.1)	0(0)	4(1.4)	0.373
Small bowel cancer	2(0.5)	0(0)	2(0.7)	0.528
Skeletal cancer	1(0.2)	0(0)	1(0.3)	0.652

IRR for uni- and multivariable Poisson regression analyses within 20 years before and after (until the end of follow-up) CRC diagnosis.

A higher IRR, for the occurrence of a non-colorectal malignancy within 20 years before and after (until end of follow-up) diagnosis of CRC, was seen in the uni- (IRR=1.46, 95% CI 1.11-1.93, p=0.008) as well as in the the multivariable (IRR=1.45, 95% CI 1.10-1.96, p=0.010) models for sporadic dMMR compared to pMMR tumours. Age was seen to be an independent significant predictor for the

occurrence of other malignancy both in the uni- (IRR= 1.03, 95% CI 1.02-1.04, p< 0.001) and the multivariable (IRR=1.03, 95% CI 1.02-1.04, p< 0.001) analyses. Furthermore, patients with a left colonic tumour had a significantly lower risk than those with a rectal tumour for the occurrence of a non-colorectal malignancy in the uni- (IRR=0.72, 95% CI 0.54-0.89, p=0.029) and multivariable (IRR= 0.67, 95% CI 0.50-0.91, p= 0.009) analyses (Table 17)**.**

Table 17. *IRR for the occurrence of a non-colorectal malignancy grouped for sporadic dMMR and pMMR in uni- and multivariable models, within 20 years before and after (until end of follow-up) diagnosis of CRC.*

	Univariable analysis			**Multivariable analysis**		
	IRR	**95% CI**	**p value**	**IRR**	**95% CI**	**p value**
Age Continuous	1.03	1.02-1.04	<0.001	1.03	1.02-1.04	<0.001
Sex						
Male	1.00			1.00		
Female	1.05	0.85-1.30	0.647	0.98	0.78-1.21	0.853
MMR status						
Proficient	1.00			1.00		
Deficient	1.45	1.10-1.92	0.009	1.46	1.09-1.95	0.011
Tumour location						
Right Colon	0.98	0.77-1.25	0.855	0.85	0.66-1.09	0.194
Left Colon	0.73	0.54-0.97	0.032	0.68	0.50-0.91	0.010
Rectum	Ref			1.00		
Tumour stage						
I	1.31	0.83-2.08	0.248	1.31	0.82-2.08	0.258
II	1.21	0.83-1.77	0.317	1.20	0.82-1.75	0.339
III	1.01	0.69-1.40	0.950	1.06	0.72-1.56	0.774
IV	1.00			1.00		

IRR for uni- and multivariable Poisson regression analyses within 20 years before the diagnosis of CRC.

In the sporadic dMMR group, there was a non-significant relationship for the occurrence of a non-colorectal malignancy within 20 years before and after the diagnosis of CRC, in both the uni- (IRR=1.41, 95% CI 0.91-2.20, p=0.124) and multivariable (IRR=1.29, 95% CI 0.82-2.04, p=0.271) model, compared to pMMR tumours. The only independent variable that showed a significant association with non-colorectal malignancy in both the uni- (IRR= 1.05, 95% CI 1.03-1.06, p= 0.000) and multivariable (IRR= 1.04, 95% CI 1.03-1.06, p< 0.001) analyses was age.

IRR for uni- and multivariable Poisson regression analyses for the occurrence of a non-colorectal cancer after the index CRC.

As seen in Table 18, a significantly higher IRR for the occurrence of a non-colorectal malignancy after (until end of follow-up) the event of the index CRC was seen in both the uni- (IRR=1.68, 95% CI 1.16-2.42, p=0.005) and multivariable (IRR=1.62, 95% CI 1.10-2.40, p=0.013) models for sporadic dMMR compared to pMMR tumours. Furthermore, age was a significant predictor for the occurrence of a non-colorectal malignancy in both the uni- (IRR= 1.04, 95% CI 1.02-1.05, p<0.001) and multivariable (IRR= 1.04, 95% CI 1.02-1.05, p= 0.000) analyses.

Table 18. *IRR for the occurrence of a non-colorectal malignancy after diagnosis of CRC according to sporadic dMMR or pMMR cases in the uni- and multivariable models.*

	Univariable analysis			**Multivariable analysis**		
	IRR	**95% CI**	**p value**	**IRR**	**95% CI**	**p value**
Age	1.04	1.02-1.05	<0.001	1.04	1.02-1.05	<0.001
Sex						
Male	1.00			1.00		
Female	1.07	0.81-1.41	0.627	0.97	0.73-1.29	0.850
MMR status						
Proficient	1.00			1.00		
Deficient	1.64	1.15-2.36	0.007	1.61	1.10-2.35	0.014
Tumour location						
Right Colon	0.96	0.70-1.30	0.773	0.81	0.58-1.12	0.256
Left Colon	0.76	0.52-1.10	0.141	0.72	0.49-1.05	0.104
Rectum	1.00			1.00		
Tumour stage						
I	0.79	0.48-1.46	0.449	0.77	0.41-1.42	0.395
II	0.75	0.44-1.29	0.299	0.72	0.42-1.24	0.241
III	0.79	0.46-1.36	0.394	0.82	0.48-1.40	0.457
IV	1.00			1.00		

Discussion

Study I

The role played by MSI in stage II colon cancer is still controversial despite several studies on the issue. The purpose of this meta-analysis was an attempt to define the role of MSI.

This was the first meta-analysis to specifically evaluate studies on stage II CC only. The analysis revealed no significant association between MMR status and OS or DFS, so the optimal treatment algorithm for these patients remains unclear. About 20% of these patients experience relapse and finally die of disseminated disease[75]. Chemotherapeutic regimens used for stage III CC have been investigated in several clinical trials and systematic reviews with contradicting results. Several factors increasing the risk of recurrence have been identified and this has led to the recommendation that these patients should receive postoperative adjuvant chemotherapy. Due to low discrimination capability, these studies were not evaluated within the framework of a prospectively designed clinical trial. MMR status has been one promising candidate. The first meta-analysis summarised results from 32 studies including all cancer stages and the estimated HR for OS was 0.65 (95%CI=0.59-0.71)[12]. The second meta-analysis performed on 3690 patients revealed no benefit from chemotherapy in patients with a MSI tumour (HR 0.70 (95%CI=0.44-1.09)[76]. The third meta-analysis showed significant benefit from 5-FU-based treatment of MSS tumours, though patients with stages II and III were analysed as one cohort[77].

This meta-analysis, strictly focusing on stage II CC only, revealed no prognostic significance of MSI in these patients. There are several possible reasons behind this negative result such as selection bias in the source articles and the high proportion of patients (47.3%) receiving adjuvant chemotherapy *i.e.*, twice as many as in the population-based SEER database[78]. This could mitigate the prognostic effect of MSI since the higher survival rate in MSS patients could have due to chemotherapy while at the same time it is well-known that chemotherapy does not improve outcome in MSI cases. Patient selection could also have a negative effect on the possibility to estimate MSI significance. One study included exclusively patients who had never received chemotherapy[43]. Furthermore, the non-significant results and broad CI could be explained by contradicting results of the studies included, emphasising the need for further well-designed studies with adequate power[75,40,41].

Study II

The exact role of dMMR in the prognosis of CC is still unclear due to contradicting results. This study aimed to determine the prognostic impact of dMRR in stage II colon cancer patients only.

In this study, no significant association between dMMR and OS was found. On the other hand we detected that dMMR is a strong prognostic factor associated with reduced risk for relapse and CRC-specific death, findings particularly important for CC treatment. The relatively good prognosis for stage II patients after surgery is the main reason for the failure to detect a clinically relevant association between OS and TTP[66]. As previous studies have shown, microsatellite-instable CRC tumours are generally diagnosed in older patients, and this is probalby one reason why OS was not primarily affected by MMR status. MSI tumours correlate with non-CRC-related deaths that occur more frequently in older patients. In this study, patients with dMMR tumours had a higher mean age at diagnosis ($p = 0{\cdot}009$). dMMR is a strong prognostic factor for relapse and colon cancer-specific death in colon cancer stage II, as revealed in the analysis of time to relapse. Though there are studies where the significance of dMMR status is doubtful, this factor is likely to be useful in clinical decision-making regarding postoperative adjuvant treatment. The strength of this study is that it includes strictly selected stage II CC patients only. In addition, all surviving patients were followed for at least 5 years with minimal loss to follow-up.

This was a population-based cohort, reflecting the normal clinical situation where the proportion of dMMR patients was in line with the numbers found in previous meta-analyses[77,79,80]. Stage II colon cancer remains a surgically treated disease. Ten-year OS reached 71.2% for patients who received adjuvant chemotherapy vs. 50.3% among those who did not, and the median survival time increased from 7.0 years to 13.2-years. Adjuvant chemotherapy is reserved for patients with a high-risk tumour. These tumours are defined as those presenting with ileus, T4 tumours, tumours with vascular and lymphatic invasion, and perforating tumours. Inadequate lymphadenectomy is a further indication for adjuvant treatment. In this study, 25% of stage II CC patients received adjuvant chemotherapy which reflects the modern trend in oncology[81].

Determination of MMR status has been used to improve patient selection for adjuvant treatment. Recent clinical studies as well as one meta-analysis found dMMR to be a negative predictor of adjuvant chemotherapy efficacy, indicating

that chemotherapy should be omitted in high-risk dMMR patients [30,82]. However, it should be mentioned that other meta-analyses and retrospective studies on cohorts of similar patients have indicated a possible positive prognostic value of dMMR-status[79], but none have specifically investigated the impact of dMMR on prognosis in stage II CC . Most studies base their results on data from mixed non-selected cohorts of patients with stages II and III disease, or colon and rectal cancers analysed together. Reports are sometimes biased by the selection of low-risk patients that have not received chemotherapy, while others present results from stage II colon cancers where all patients have received adjuvant treatment regardless of the presense of high-risk factors or not[83].

Study III

CC stage II remains primarily a surgically treated disease. The relationship between exposure to adjuvant chemotherapy and survival after relapse is not fully understood. A number of studies suggest an association between dMMR and survival prognosis in CRC, but the exact predictive role is still not fully understood.

Survival after the administration of adjuvant chemotherapy in patients with stage II CC was found to be dependent on MMR status. No significant effect was observed in patients with a dMMR tumour, while patients in the pMMR group showed a significantly better OS and significantly longer survival after relapsing. These results were significant in both the uni- and multivariable analyses, clearly showing that the functional status of the MMR system in CC tumours corresponds to different clinical phenotypes with completely different behaviour in response to adjuvant chemotherapy. These patients are generally considered to have a favourable outcome after surgery, but even so about one out in four patients relapse within 5 years[84]. So far, zero or limited clinical benefit from adjuvant chemotherapy has been observed, probably due to the relatively good prognosis after surgery alone and the low statistical power of previously published randomised clinical trials. Much larger multicentre randomised trials are needed to detect a statistically significant survival benefit.

Debate on the best postoperative strategy in stage II CC continues, and most biomarkers used today serve only as prognostic factors. This study supports the idea that determination of MMR status can have predictive value in this category of patients, in agreement with other retrospective analyses and randomised trials[20,77,85]. The strength of this study is that it does not have the weaknesses commonly seen in previous studies such as inclusion of patients with rectal cancer or stage III disease, stage migration, and small sample size.

Our results revealed an interesting interaction between adjuvant treatment and post-relapse survival of patients with respect to MMR status. This is the first study that has discovered different clinical outcome of patients after relapse in the pMMR cohort with respect to postoperative treatment (adjuvant chemotherapy given or not) and our study population is sufficiently large to allow for these conclusions. Patients treated with adjuvant chemotherapy experienced significantly longer PRS and this relationship was significant in both uni -and multivariable models. One possible explanation for the favourable outcome in patients with a pMMR tumour who relapse after adjuvant chemotherapy could be a chemotherapeutic effect leading to the higher conversion rate, better resectability, and more frequent metastasectomy. It is known that metastasectomy, especially disease in the lungs and liver, has a favourable effect on survival. dMMR tumours, however, have a tendency to relapse with peritoneal carcinomatosis, a scenario that excludes such patients from surgical resection of oligometastases[86].

This study suffers the disadvantages of a retrospective study such as the differences between the older and more recent cohorts due to progress in the field of adjuvant chemotherapy in recent years. One aspect of this is the use of combined chemotherapy regimens that has been standard since 2002 and the U.S. FDA's approval in 2004 of the use of oxaliplatin for adjuvant chemotherapy of CC. Despite this, 25% of stage II CC patients in our study had received adjuvant chemotherapy which reflects the modern trend in oncology. Another limitation of this study is that we did not evaluate the relationship between survival and *BRAF* and MMR status interaction.

Well-designed prospective trials are needed to determine which patients with these alterations are likely to gain most benefit from the cytotoxic treatments available and which patients should receive biological therapy. Today, this topic is of great interest since the recent introduction of PD-1 and PD-L1 inhibitors in the treatment of MSI-H tumours[86,87].

Study IV

To our knowledge differences between CC tumours arising from different carcinogenetic pathways have not been studied systematically, in particular the effect of high immune cell infiltration of tumours arising from dMMR alteration causing the same predisposition to acute surgery in cohorts different in origin and time. The complex interactions between variables that influence the oncologic outcome of colon cancer presenting as acute cases requires further research before being applied in everyday clinical practice.

In this study, a strongly significant association between MMR status and presentation as acute CC surgery was seen, supporting a genetic background theory whereby impaired ability to correct errors in DNA replication predisposes to emergency presentation. dMMR status seems to be a prognostic predictor for emergency surgery during the same admission as when the malignancy is diagnosed. This strong relationship was seen in both the Scandinavian exploration and the Czech validation cohorts. We believe this originates from the relationship between the tumour cell biology and the tumour microenvironment[88]. It is well-known that dMMR tumours accumulate multiple mutations that can give rise to frameshift peptide neoantigens. The high mutational neoantigen load in these tumours is reflected by pronounced anti-tumour immune responses, high intratumour and intermediate stromal infiltration comprising mainly Th1 and cytotoxic T-cells[10,89].

Patient characteristics such as tumour stage and radicality seems to be the main determinants of oncologic outcome. At the same time, it is well-accepted that patients operated acutely have a poorer outcome in both the short- and long-term perspective, and that these outcomes seem independent of the surgeon case-load, surgeon specialisation, hospital type, or time of the day when surgery is performed[90-93]. This supports the theory that suboptimal surgical technique is not the main reason for the unfavourable outcome of patients who need emergency surgery. Instead, a more aggressive tumour biology and diagnosis at a late stage due to delay on the part of the patient or healthcare system are probably main reasons for impaired oncological outcome. We believe that these variables have a predominant effect on the outcome of cases presenting as emergency surgery rather than MMR status *per se*. These interactions result in dMMR cases presenting more often as acute cases, but the favourable prognostic effect

disappears due to more dominant and potentially cooperating variables as mentioned above. Other studies have evaluated the use of MMR status as a prognostic and predictive factor in CC [94], and to our knowledge this relationship is much more complex in acute cases. This suggests not administering classic adjuvant chemotherapy to elderly dMMR patients, even if they present as an emergency. The combination of high age and other cause mortality together with the poor response of dMMR tumours to adjuvant chemotherapy is one logical reason for such a suggestion[94,95].

This study showed a strongly significant association between dMMR tumour and risk for acute colon cancer presentation, regardless of bias of retrospective studies and possible heterogeneity between more recent and older cohorts or differences between numbers of patients included. This relationship was equally significant in both exploration and validation cohorts. In view of the complex relationships and labyrinth of interactions between variables that influence the oncologic outcome of acute CC, further research is required before our results can be applied in everyday clinical practice.

Study V

Sporadic dMMR CRC patients has a higher IRR for the occurrence of non-colorectal malignancy within 20 years prior to or after diagnosis of CRC. This relationship between sporadic dMMR status and higher incidence of non-colorectal malignancy may originate from failure in the system ensuring integrity of DNA replication, i.e., the same mechanism as in the familial Lynch syndrome.

This multicentre study, including patients from three different European medical centres, revealed a positive association between sporadic dMMR tumours and non-colorectal malignancy. This relationship was more obvious after the index CRC event. Our results suggest that the genetic background, *i.e.* impaired ability to correct errors in DNA replication, may be associated with the development of a non-colorectal tumour not only in the familial type but also in sporadic cases. The higher incidence rate ratio for sporadic dMMR, especially after the diagnosis of CRC, may originate from the fact that these tumours are usually detected in cancer patients with MMR abnormalities. This study is the first to investigate the relationship between sporadic dMMR tumours and the occurrence of non-

colorectal malignancy as opposed to well-studied hereditary cases. Further research is needed to determine the necessity of more meticulous surveillance strategies for sporadic dMMR cases. It is possible that the surveillance strategy for hereditary dMMR is applicable in sporadic cases, but it could be that a tailored strategy is more suitable for these patients.

In this study, faulty mismatch repair processes and occurrence of other tumours were associated with age as an independent variable. This association is a result of synergic interaction between age and dMMR accrual and is reflected in the stronger relationship between dMMR and non-colorectal malignancy after the index CRC event. In other words, higher age accompanied by a higher frequency of hypermethylation of *MLH1* genes results in dMMR carcinogenesis, increasing the risk for other cancer types. In humans and in laboratory animals the incidence of cancer increases with age and different patterns of age-related distribution of tumours in different organs and tissues have been seen. Studies also show that aging may increase or decrease the susceptibility of various tissues to initiation of carcinogenesis and usually facilitates promotion of carcinogenesis and relapse [96]. Aging predisposes to cancer through several mechanisms including tissue accumulation of cells in late stage of carcinogenesis, changes in homeostasis, alterations in the immune and endocrine systems, and telomere instability[97]. This is why the effect of age on tumourigenesis cannot be restricted to dMMR cases only but should also apply to pMMR cases[98]. However, published reports have revealed that molecular and clinicopathological differences do not differ significantly between different age groups of patients with CRC, and that sporadic CRC shows similar molecular changes in different age groups[99]. This close relationship between age and dMMR tumours seems to be much more complex since DNA MMR plays a critical role in preserving the integrity of the genome in virtually all organisms[100]. The accumulation of DNA damage and the resulting genomic instability leads not only to carcinogenesis but is also thought to contribute to aging [101,102].

In this study a wide spectrum of malignancies was seen, including cancers of the breast and prostate, ovary, haematological, melanoma, stomach, urinary tract, pancreaticobiliary, small intestine, brain, head and neck, and endocrine system. All non-melanoma skin neoplasms were excluded because they are relatively common, have a different epigenetic background affected by UV radiation, and are usually non-fatal. Furthermore, registration of non-melanoma skin cancer (ICD-10 C44) is likely to be less complete than for other cancer categories[103]. This wide variety of other cancers is seen in most studies dealing with the heredity of Lynch syndrome. On the other hand, we found some forms of hematological malignancies not seen in Lynch studies[104,105]. A possible explanation for this discrepancy is that haematological malignancies mainly affect older patients[106]. We also saw fewer cases of endometrial cancers in our study population

compared to studies on hereditary malignancy[107]. This is probably due to the fact that most Lynch endometrial cancers carry a deleterious germline mutation in the *MSH2* gene, whereas sporadic cases carry an aberration in the *MLH1* gene[108]. It is known from previous reports that aberrations in different genes lead to different degrees of penetrance and different patterns of cancer expression[109].

Conclusions

This thesis on clinically relevant effects of MMR-status shows clearly its usefulness in prognostic and predictive strategies for CRC patients, as the disease remains a significant healthcare problem worldwide. Its treatment algorithm between patients with different genomic background can vary significantly. Our project supports the idea of using MMR-status as a prognostic and predictive tool in a subset of CC patients in everyday praxis, especially in stage II and acute cases.

The association between non-CRC and the subset of sporadic dMMR tumours is a novel finding that supports the usefulleness of MMR status as a predictive instrument for other tumours than CRC. This idea should challenge the algorithm for control and surveillance of dMMR patients. Because of the lack of clear guidelines for screening protocols, the results of this thesis should be the start for a better monitoring and a more careful follow up of this subset of cancer patients.

General discussion and future perspectives

Rather than being one homogenous disease, CRC is a heterogenous disease showing clinically and molecularly distinct carcinogenetic profiles. Many studies have pointed out that CRC types differ according to molecular subtypes defined by different molecular markers. These subtypes have been the subject of many research programmes, and researchers have changed their way of viewing this problem. All these efforts have aimed to improve patient outcome and minimise the side-effects of adjuvant chemotherapy in subgroups of patients that are at risk of being overtreated. Many pre- and postoperative treatment strategies have been changed since the start of this thesis, but MMR has still not found its place as a validated tool in routine diagnostic workups for CRC. This is strange considering this relatively simple clinical way to identify subsets of CRC patients who appear to have a better prognosis and thus be spared adjuvant treatment.

In this thesis I have tried to investigate the usefulness of MMR-status as a prognostic factor, especially in patients with stage II CC. This subgroup of CRCs suffers from a lack of guidelines on the most appropriate treatment strategy. Our studies have shown that the prognostic effect of MMR-status shown in previous work remains stable and much more obvious in stage II CC patients. This relationship is also valid and applicable from an international perspective as remarkable congruence was seen between Swedish, Finnish, and Czech populations. This international cooperation will be continued in a stronger and more effective way as new efforts are made to clarify the secrets behind the multi-

heterogeneity of carcinogenetic pathways. We were unable to escape the difficulties created by the COVID 19 pandemic. Insignificant though our problems were compared to the deaths and suffering caused by the disease, we must point out that this unexpected pandemic greatly affected the way in which our study group could meet and cooperate.

Despite COVID 19, this project dealed with two questions that have never been investigated before: 1. The association between genetic background originating from dMMR with acute presentation of CC; and 2. The relationship between sporadic dMMR and the risk for other non-CRC malignancy. Personally, I am most interested in continuing research into other cancer-associated questions and the appropriate treatment of cancers patients.

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