Folates in the Treatment of Colorectal Cancer

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Gothenburg 2014

Cover illustration: 5,10-Methylenetetrahydrofolate by Christi Kogler

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ISBN 978-91-628-9212-8
http://hdl.handle.net/2077/36748

Printed in Gothenburg, Sweden 2014
Ineko AB

To my lively, loving family

"In the middle of the forest there is an unexpected glade that can only be found by someone who is lost."

Tomas Tranströmer

ABSTRACT

Folates in the Treatment of Colorectal Cancer

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Background: Colorectal cancer is one of the most common cancers in the world, and radical surgery with total removal of the tumour (RO-resection) is the single most important treatment. However, chemotherapy is recommended for patients with risk factors and patients with metastatic disease. 5-fluoruracil (5-FU) is the cornerstone of chemotherapy, used either as a single drug or in combination with other drugs. 5-FU it is almost always combined with the folate leucovorin (LV). The aim of this thesis was to examine the role of polymorphisms in genes involved in folate metabolism in relation to treatment and to examine the levels of various folate forms in the tumours, mucosa, and plasma of patients who received LV or Modufolin® which is the biological isomer of 5,10-methylenetetrahydrofolate.
Methods: Polymorphisms in the methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR), and thymidylate synthase (TYMS) genes were analysed using real-time PCR and TaqMan chemistry. The various folate forms were analysed in tumours, mucosa, and plasma using a sensitive liquid chromatography electrospray ionization tandem mass spectrometry technique.
Results: There was interdependency between polymorphisms in the MTFHR and MTR genes, which was associated with risk of side effects and overall survival in patients with stage III colorectal cancer receiving adjuvant chemotherapy. Total folate levels, all well as tetrahydrofolate (THF) and 5,10-methyleneTHF levels were significantly higher in tumours than in mucosa tissue. The individual variation in folate levels in both tumours and mucosa was greater than the variation found when the patients were subgrouped by gene polymorphisms. Only half of the patients who received 60 mg/m^2 LV had higher levels of 5,10-methyleneTHF in tumours than patients who received 0 mg/m^2 LV. Patients with rectal cancer had significantly lower levels of 5,10-methyleneTHF compared with patients with colon cancer. 5,10-methyleneTHF and THF concentrations were significantly higher in mucosa ($p<0.003$, both dosages) and tumours ($p<0.015$) 200 mg/m^2) after Modufolin® administration than after LV (Isovorin®) administration.
Conclusions: Polymorphisms in folate-associated genes can affect the risk that patients with colorectal cancer suffer from side effects during treatment with 5-FU-based chemotherapy. There is wide interindividual variation in 5,10-methyleneTHF levels in tumour tissue and mucosa after administration of standardised doses of LV. The doses of LV used in Nordic FLV-treatment may result in suboptimal levels of 5,10-methyleneTHF, especially in patients with rectal cancer. Modufolin® administration resulted in significantly higher 5,10-methyleneTHF levels than the natural l-form of LV, Isovorin®, and may potentially increase the efficacy of 5-FU-based chemotherapy.

Keywords: Folates, colorectal cancer, leucovorin, Modufolin®, polymorphisms, MTHFR; Methionine Synthase, TS, side effects, adjuvant chemotherapy LS-MS/MS

ISBN:978-91-628-9212-8 http://hdl.handle.net/2077/36748

POPULÄRVETENSKAPLIG SAMMANFATTNING (SUMMARY IN SWEDISH)

Bakgrund

Cancer i tjocktarm och ändtarm (kolorektal cancer) är en av de vanligaste cancerformerna och i Sverige diagnosticeras över 6000 nya fall årligen. Cirka 1/3 av tumörerna diagnosticeras i ändtarmen medan 2/3 befinner sig i tjocktarmen.

Flertalet av de patienter som har en kolorektal cancer kommer att genomgå kirurgi och operera bort sin tumör. Kirurgi är idag den enda möjligheten till bot. Även om man radikalt kan operera bort tumören, finns en risk att det kan finnas tumörceller kvar, så kallad mikrometastasering. Detta kan leda till att man får ett återfall i sin cancersjukdom och för att minska risken för återfall rekommenderas att riskpatienter skall behandlas med cytostatika. Hos cirka 20 % av alla patienter som diagnosticeras med en kolorektal cancer, har sjukdomen redan hunnit sprida sig utanför tarmen vid diagnostillfället. Om cancern är spridd används cytostatika för att bromsa sjukdomsförloppet.

En av hörnstenarna i cytostatikabehandlingen är 5-fluorouracil (5-FU). Denna substans, som använts i mer än 50 år, ges alltid tillsammans med leukovorin (LV) som är ett B-vitamin (folat). LV har sannolikt ingen effekt på tumörcellerna i sig själv men bidrar till att öka 5-FU-behandlingens effekt och minska biverkningar. Olika patienter svarar dock olika effektivt på behandlingen 5-FU/LV. Detta kan delvis bero på att man har mer eller mindre verksamma enzymer som deltar i metabolismen av folater. För att man skall kunna få en optimal effekt av 5-FU är det viktigt att det finns adekvata mängder av den aktiva metaboliten 5,10-metylentetrahydrofolat (5,10-metylenTHF).

I min avhandling har jag undersökt om det finns genetiska varianter av folatassocierade gener som kan förklara varför vissa patienter får mindre effekt av given cytostatikabehandling, eller mer biverkningar. Vi har vidare undersökt hur LV tas upp i tumörvävnad och närliggande tarmvävnad. I det sista arbetet har vi jämfört en ny substans, den naturliga isomeren av 5,10-metylenTHF, Modufolin®, för att se om denna variant mer effektivt tas upp i tumören jämfört med dagens behandling. Fördelen med Modufolin®, jämfört med LV, är att Modufolin® inte behöver omvandlas till aktiv form inne i cellen.

Metod

I delarbete I har vi studerat genetiska varianter av två enzymer; metylentetrahydrofolatreduktas (MTHFR) och metioninsyntas (MTR) hos 150 patienter som behandlats med cytostatika efter operation.

I delarbete II studerade vi om de olika genetiska varianterna av enzymerna MTHFR, MTR och tymidylatsyntas (TS) påverkade nivåerna av tre olika folatformer; tetrahydrofolat (THF), 5-metylTHF och 5,10-metylenTHF, i tumör och närliggande makroskopiskt normal tarmvävnad hos 53 patienter med kolorektal cancer. Analys av folatinnehållet utfördes med den känsliga metoden LC-MS/MS, vilken möjliggör att man kan mäta och kvantifiera olika former av folater i vävnad.

I delarbete III studerades hur nivåerna av de tre ovanstående folatformerna varierade när man gav eskalerande doser LV till 75 patienter som genomgick operation för kolorektal cancer.

Slutligen, i delarbete IV, jämfördes två grupper om vardera 16 patienter, som erhöll antingen Modufolin® eller Isovorin®, i två olika doser. Analyser av tumör, närliggande tarmvävnad och plasma genomfördes och innehållet av fyra olika folatformer (THF, 5-metylTHF, 5,10-metylenTHF och 5-formylTHF) kvantifierades med hjälp av LC-MS/MS teknik.

Resultat och slutsatser

Analyserna i delarbete I visade att patienterna med kolorektal cancer svarade olika på behandling med 5-FU/LV beroende på vilken genetisk variant av MTHFR respektive MTR de hade. De olika polymorfierna var associerade med varierande risk för biverkningar under behandlingen, och även skillnader i biverkningsmönster. Vissa kombinationer av MTHFR och MTR polymorfier visade sig också leda till signifikant fler avbrutna behandlingar och sämre överlevnad.

Delarbete II visade att den procentuella fördelningen av den biologiskt aktiva formen av folat, 5,10-metylenTHF, i tumör och närliggande tarmvävnad, skiljde sig åt hos patienter med olika genetiska varianter av MTHFR och TS.

I delarbete III fann vi att det var stor skillnad/spridning i mängden uppmätt 5,10-metylenTHF i tumör och närliggande tarmslemhinna hos patienter som fått samma dos av LV. För att alla patienter skulle komma upp i en mängd 5,10-metylenTHF som översteg mängden hos obehandlade patienter krävdes att den dos som gavs var minst 200 mg/m^2, vilket är högre än normal

standardbehandling. Det var vidare signifikant fler patienter med rektalcancer än koloncancer som låg lågt i koncentration.

Slutligen, i delarbete IV, fann vi signifikant högre koncentrationer av 5,10-metylenTHF i tumörvävnad efter tillförsel av Modufolin® jämfört med vad som uppmättes efter att Isovorin® tillförts. Inga allvarliga biverkningar som bedömdes vara var kopplade till Modufolin® förekom i gruppen.

Våra studier har bidragit till att öka kunskapen om hur folatmetabolismen påverkas av genetiska variationer hos patienter med kolorektal cancer. Vidare att det intracelluära folatinnehållet ökar efter det att högre doser folat i form av LV tillförs. Genom att tillföra folat i form av Modufolin® istället för Isovorin® kan mängden av den metaboliska aktiva formen av folat; 5,10-metylenTHF ytterligare ökas i vävnaden. Kompletterande studier behövs för att se hur Modufolin® kan påverka, och om möjligt förbättra, behandlingssvaret hos patienter med kolorektal cancer som behandlas med 5-FU.

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LIST OF PAPERS

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

I. Taflin H, Wettergren Y, Odin E, Carlsson G, Derwinger K.

Gene polymorphisms MTHFRC677T and MTRA2756G as predictive factors in adjuvant chemotherapy for stage III colorectal cancer.
Anticancer Res. 2011 Sep:31(9):3057-62.

II. Taflin H, Wettergren Y, Odin E, Carlsson G, Derwinger K

Folate Levels and Polymorphisms in the Genes MTHFR, MTR, and TS in Colorectal Cancer.
Clin Med Insights Oncol. 2014 Feb:17(8):15-20.

III. Taflin H, Wettergren Y, Odin E, Derwinger K

Folate levels measured by LC-MS/MS in patients with colorectal cancer treated with different leucovorin dosages.
Cancer Chemoth Pharma. 2014 Sep 20. Epub ahead of print. DOI: 10.1007/s00280-014-2591-9.

IV. Wettergren Y, Taflin H, Odin E, Kodeda K, Derwinger K

A pharmacokinetic and pharmacodynamic investigation of Modufolin® compared to Isovorin® after single dose intravenous administration to patients with colon cancer: a randomized study.
Cancer Chemoth Pharm. 2014 Oct. 24. Epub ahead of print. DOI: 10.1007/s00280-014-2611-9.

CONTENT

ABBREVIATIONS

5 -FU	5-Fluorouracil
5-methylTHF	5-Methyltetrahydrofolate
AJCC	American Joint Committee on Cancer
AUC_{last}	Longer lasting Area Under Curve
C_{max}	Maximal plasma concentrations
CIN	Chromosome Instability
CRA	Colorectal Adenoma
CRC	Colorectal Cancer
CRM	Circumferential Resection Margin
CT	Computed Tomography
cTNM	clinical TNM
DFS	Disease-Free Survival
DPD	Dihydropyrimidine dehydrogenase
dTMP	deoxythymidine monophosphate
dUMP	deoxyuridine monophosphate
ECOG	Eastern Cooperative Oncology Group
EGFR	Epidermal growth factor receptor
EORTC	European Organization for Research and Treatment of Cancer.
FAP	Familial Adenomatous Polyposis
FDG-PET	fluorodeoxyglucose positron emission tomography
FdUMP	5-fluorodeoxyuridine monophosphate
FLOX	5-FU, Leucovorin and oxaliplatin
FLV	5-Fluorouracil and Leucovorin
GCP	Good Clinical Practise
GLP	Good Laboratory Practise
HNPCC	Hereditary Non Polyposis Colorectal Cancer
i.v.	Intravenous
K-RAS	Kirsten Rat Sarcoma viral oncogene
LC-MS/MS	Liquid chromatography electrospray ionization tandem mass spectrometry
LLOQ	Lower Limit Of Quantification
LMW	Low Molecular Weight
LV	Leucovorin
MAP	MutYH associated polyposis
mCRC	metastatic Colorectal Cancer
MDT	Multidisciplinary Team
MRI	Magnetic Resonance Imaging
MSI	Microsatellite instability
MSS	Microsatellite stable.
MTHF	Methylenetetrahydrofolate
MTHFR	Methylenetetrahydrofolate reductase
MTR	Methionine synthase
NTDs	Neural tube defects
OS	Overall Survival
PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography
PFS	Progression Free Survival

PGA	Polyglutamic Acid
pTNM	Final pathology report TNM
RFC	Reduced Folate Carrier
SAM	S-adenosylmethionine
SNP	Single Nucleotide Polymorphism
TEM	Transanal Endoscopic Microsurgery
THF	Tetrahydrofolate
TME	Total Mesorectal Excision
TS	Thymidylate Synthase
TTP	Time to progression
UFS	Upstream stimulating factors
UICC	Union Internationale Contre le Cancer

1 INTRODUCTION

1.1 The history of folate

In order to discuss the role of folates in the treatment of colorectal cancer (CRC), one must first define and describe the role of folates themselves.

The history of folates started with Lucy Willis (1888–1964), a British pathologist who went to Bombay in 1928 to investigate macrocytic anaemia in pregnancy.

This anaemia was more frequent in poor textile workers, whose diet was deficient in fruit, vegetables, and protein. When treated with a commercial yeast extract, Marmite, the patient's anaemia was cured.

Figure 1. Dr. Lucy Willis (date and location unknown)

Some patients with macroscopic anaemia who were not cured after being treated with yeast extract were cured after receiving an injection of liver extract. The reason for the positive response is that liver is extremely rich in cobalamin, also known as vitamin B12. Intense research that aimed to find a cure for all types of macroscopic anaemia finally led to the isolation of a substance, folate, from spinach (folium is the Latin name for leaf) in 1941.

Figure 2. Typical British spreads.

The substance was synthesised in pure crystalline form in 1943 by Bob Stockland, working at Lederle. An aromatic pteridine ring linked to p-amino benzoic acid and a glutamate residue composed the new substance, which was called polyglutamic acid (PGA)[1].

It was soon discovered that the folates that occurred naturally differed from PGA, usually in three aspects: additional glutamate residues (i.e.

polyglutamates), reduction to di- or tetrahydroforms, and additional single carbon units attached to the N5 or N10 nitrogen atoms. The term folic acid (PGA) is now used only for the fully oxidised chemical compound and is therefore not applicable to folates found in biological active tissues or in natural food[2].

The term "folates" is thus used as an umbrella term for a large group of compounds with the same vitamin activity, that is, substituted/unsubstituted, oxidised/reduced, and mono/polyglutamate forms of pteroyl-L-glutamic acid, including the synthetic form, folic acid. The latter is called vitamin B9 in France and vitamin B11 in the Netherlands and Hungary[3].

1.2 Dietary intake and recommendations

Folates are water-soluble vitamins. Humans cannot produce them *in vivo*, and therefore, depend on adequate levels in the diet. The major sources of folates are vegetables, especially leafy greens, as well as fruits, beans, citrus juices, liver, and grains[3]. However, a substantial amount of the required intake of folates is received by fortified products and supplements, although there are national variations[4].

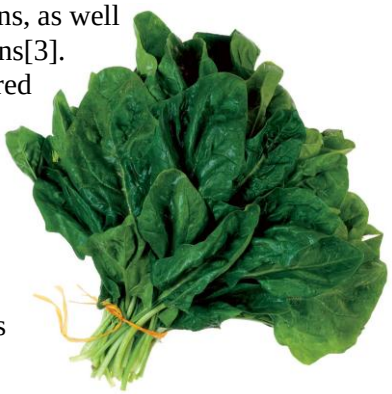

Figure 3. *Folates*

Digested folates are absorbed in the brush border surface membranes of enterocytes. Monoglutamates are then transported across the enterocytes into the bloodstream as the reduced methylated form of folate, 5-methyltetrahydrofolate (5-methylTHF) which is bound to a variety of folate-binding proteins. The entry of 5-methylTHF into the cells of the body is mediated by a specific folate carrier located in the cell membrane. Once inside the cell, it is demethylated by methionine synthase (MTR) to produce tetrahydrofolate (THF). The monoglutamate form is converted to the pentaglutamate form by a ligase enzyme. If demethylation does not occur, the 5-methylTHF molecule leaves the cell, because 5-methylTHF is a poor substrate for the ligase enzyme. Synthesis of THF is necessary to stabilise the presence of folate in the cell[5].

Thus, cobalamin-dependent MTR is essential for the cellular conversion of 5-methylTHF. When there is a cobalamin-deficient, intracellular folate

levels, which is measured as red cell folate are low[6, 7] In addition, folic acid undergoes conversion to 5-methylTHF, but this process becomes saturated at doses of 270 micrograms and at higher levels, folic acid is transported directly into the plasma as folic acid[8].

In Sweden, the National Food Agency recommends that an adult man should have a daily folate intake level of at least 300 micrograms, and an adult woman should have 400 micrograms. The recommended intake for pregnant and lactating women is at least 500 micrograms daily. This recommendation is more or less the same in the rest of Europe[9]. However, folate uptake and intake can be affected by various circumstances. It has been shown that elderly people often have low folate levels due to reduced appetite, slow gastric emptying, and sometimes malnutrition[10, 11]. Smoking and chronic inflammatory diseases, which lead to malabsorption, also affect folate levels [12, 13], as does excessive use of alcohol[14, 15]. There are no absolutely clear cut-offs regarding the upper limit of folate intake. The American Institute of Medicine recommends a tolerable upper intake level of folic acid from supplements or fortified foods of 1,000 micrograms daily for adults and 300–400 micrograms daily for children between the ages of 1 and 8 years[16]. The reason for these limits is to avoid masking anaemia related to B12 deficiency, which presents a risk of developing neurological pathology.

1.3 The role of folates

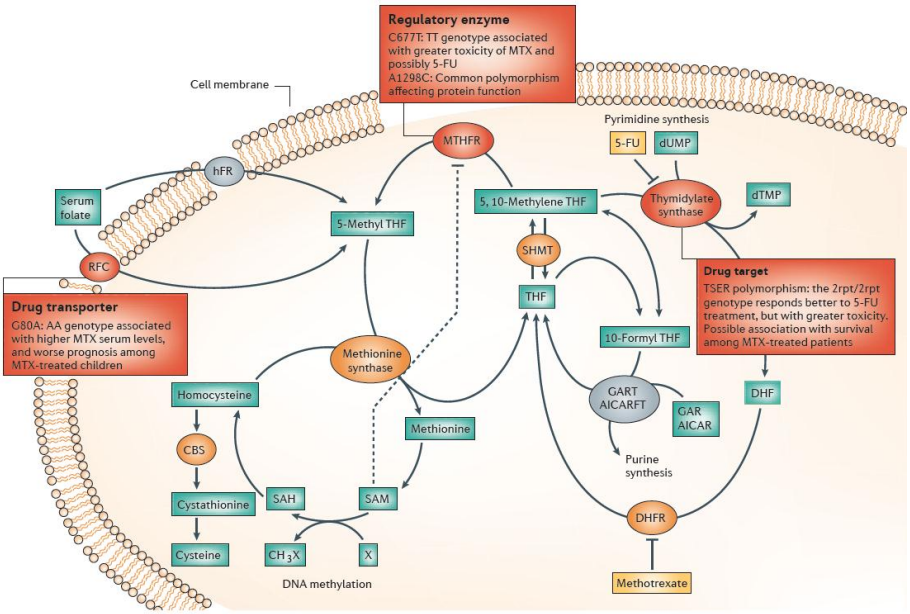

Figure 4. This simplified figure illustrates the interconnectedness of folate metabolism and proteins for which functional polymorphisms have been identified. Polymorphisms have been found that are associated with pharmacogenetic outcomes in three key proteins in these pathways: the drug transporter protein reduced folate carrier (RFC); the regulatory enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR); and the drug target thymidylate synthase. Key enzymes are denoted as ovals, substrates as rectangles. Red ovals denote enzymes with genetic polymorphisms that have been investigated in pharmacogenetic studies. Orange ovals denote enzymes for which functional genetic polymorphisms have been described. 5-FU, 5-fluorouracil; AICAR, 5-aminoimidazole-4-carboxamine ribonucleotide; AICARFT, AICAR formyltransferase; CBS, cystathionine-β-synthase; DHF, dihydrofolate; DHFR, DHF reductase; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; GAR, glycinamide ribonucleotide; GART, phosphoribosylglycinamide formyltransferase; hFR, human folate receptor; MTX, methotrexate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; X, various substrates for methylation.

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Cornelia M et al. Cancer pharmacogenetics: polymorphisms, pathways and beyond pharmacogenetics: polymorphisms, pathways and beyond.2003

Folates inside the cell act as donors and acceptors of methyl groups (-CH_3), in the biosynthesis of nucleotide precursors used for DNA synthesis. Folate-dependent enzymes also provide methyl groups for methylation of DNA, RNA, and proteins. These are important functions, as aberrations in the methylation of macromolecules, particularly DNA, as well as disruption in DNA synthesis and repair, are thought to play major roles in carcinogenesis.

These important cellular processes lie at opposite ends of the folate metabolism, linked by the enzyme methylenetetrahydrofolate reductase (MTHFR), which catalyses the irreversible conversion of 5,10-methyleneTHF to 5-methylTHF. The MTHFR substrate, 5,10-methyleneTHF, is also a cofactor for the thymidylate synthase (TS) enzyme in the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), which is the sole *de novo* source of thymidine and the rate-limiting step in DNA synthesis in mammalian cells.

5,10-methyleneTHF is also used in the production of 10-formylTHF, which in turn is used in *de novo* purine synthesis. The MTHFR product, 5-methylTHF, is the methyl group donor for the remethylation of homocysteine to methionine catalysed by the enzyme MTR. This is a reaction in which 5-methylTHF serves as both a cofactor and a substrate. Methionine is adenylated to form S-adenosylmethionine (SAM), which is the universal methyl group donor in methylation reactions. SAM inhibits the MTHFR enzyme, providing a negative feedback control loop [17, 18]. As shown in Figure 4 and 5, the regulation of folate enzymes is extremely complex, with many feedback control loops, reflecting the importance of these vital reactions.

1.3.1 The enzymes

There are several enzymes involved in folate metabolism, as described above. Folate transporters, such as the reduced folate carrier (RFC), involved in the early steps of uptake and processing are not described in detail in this thesis. The efficiency of the enzymes is not only regulated by local folate substrate levels, but also by genetic variations in the form of functional polymorphisms[19]. It has been suggested that these genetic variations, which also have geographically associated distributions, affect most processes, ranging from cancer risk to treatment effects. The variations in efficiency could be of importance when using chemotherapeutic regimes that affect the folate-associated enzymes. The possibility of future discovery of more functional polymorphisms should also be acknowledged.

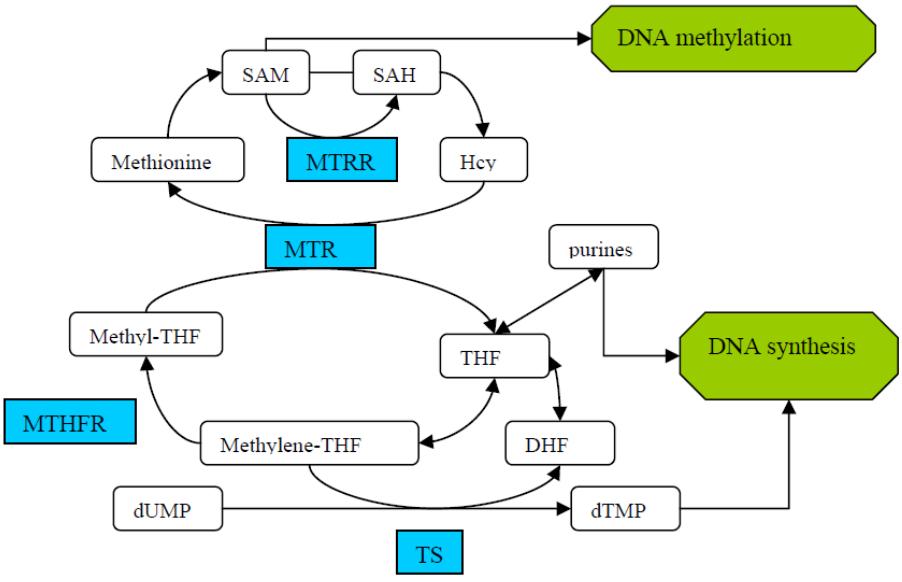

Figure 5. A simplified overview of folate metabolism showing the enzyme steps catalyzed by MTHFR, MTR, and TS.

Notes: Within the cells, folate polyglutamates are converted to 5,10-methyleneTHF, which is required as a methyl donor in the synthesis of dTMP from dUMP. The reaction requires the catalytic activity of the enzyme TS. In addition, 5,10-MethyleneTHF is also the precursor of metabolically active 5-methyl- THF, utilized in the remethylation of the amino acid HCy to methionine. This reaction is catalyzed by MTR. Endogenous methionine is then catabolized to produce the universal methyl donor SAM. The conversion of 5,10-methylene-THF to 5-methyl-THF is dependent on the enzyme MTHFR. Abbreviations: SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; HCy, homocysteine; DHF, dihydrofolate; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate.

1.3.2 Thymidylate synthase

Thymidylate synthase (TS) is an enzyme that consists of two identical subunits and acts intracellularly. The gene that codes for TS (TYMS) is located on chromosome 18. The enzyme is found in every living species and is rate-limiting for synthesis of thymidine, and hence, is necessary for DNA synthesis. TS is also the main target for chemotherapeutic agents such as the fluorouridine pyrimidines 5-FU and capecitabine. Because TS is a key enzyme in 5-FU treatment, it has been suggested that it is both a prognostic factor and a predictive factor[20-25].

Several gene polymorphisms have been described in the TYMS gene, and there is also an ethnical variation, making studies of TS highly complex [22, 23, 26]. TS expression seems to be affected by highly polymorphic tandem repeats in the TYMS promoter enhancer region (TSER). Horie et al. were the first to describe a germ-line polymorphism upstream of the TS translational start site, containing either double (*2R*) or triple (*3R*) tandem repeats of a 28-bp sequence[26].

Additional functional variants within the 5′-UTR region of the TYMS gene have been identified. Mandola et al. showed that the 28-bp TSER tandem repeats contain elements called upstream stimulating factors (USF) and that ligand binding by USF-1 and USF-2 enhances transcriptional activity of the TYMS gene[27]. A third polymorphism of the TYMS gene is a *6-bp* deletion in the 3′-UTR region of the gene. A review article by Lurje et al. states, "The possibility of three different polymorphisms in the same gene obviously complicates effort aimed at understanding the functional significance of each individual polymorphism. In the case of TYMS gene, there are 18 different allele combinations possible, all of which may theoretically influence clinical outcome."[23].

1.3.3 Methylenetetrahydrofolate reductase

The gene encoding MTHFR is located on chromosome 1 in humans. The MTHFR enzyme is responsible for determining whether reduced folates are directed towards the DNA methylation pathways or pyrimidine and purine synthesis. A possible consequence of the resulting increase in the substrate methylenetetrahydrofolate due to decreased enzymatic capacity might be an increased sensitivity to cytotoxic agents, and thus, increased risk of toxic reactions due to impairment of DNA synthesis and repair during chemotherapy. Another consequence might be a decrease in the availability of produced methyl groups, resulting in aberrant gene regulation[28]. This could have an effect on both the development of CRC and the way the tumour reacts to 5-FU-based chemotherapy [29]. MTHFR is less studied than TS, but two functional polymorphisms have been described [30].

A common functional polymorphism in the MTHFR gene is the C677T variant. The C677T polymorphism in exon 4 of the MTHFR gene leads to a replacement of a highly conserved alanine residue with a valine in the amino acid sequence. This biochemical change produces higher thermolability in the enzyme and a reduction in the activity rate being 65% in heterozygotes (CT) and 30% in homozygotes (TT)[28]. The frequency of CC, CT, and TT

in the population varies, but they are around 40–45% each for CC and CT and around 10% for TT.

A second variant of the MTHFR enzyme, with a substitution of A to C at nucleotide 1298, has also been identified. Unlike the MTHFR C677T polymorphism, the enzyme activities of the variants of MTHFR A1298C polymorphism are not thermolabile, but the enzyme activity is reduced by approximately 40% of the wild type (AA genotype) in the variant genotype [7]. Zhang et al. presented the A1298C polymorphism in MTHFR as a prognostic marker in female patients with metastatic colon cancer. The A1298C polymorphism showed statistically significant differences in overall survival (OS) rates in female, but not male patients with metastatic colon cancer. The OS was higher in patients with the AA genotype compared with patients with the A/C genotype (p=0.038) [31].

1.3.4 Methionine synthase

MTR, a gene located on chromosome 1, was previously described in terms of the conversion of 5-methylTHF to THF for the provision of THF for use in nucleotide synthesis. However, MTR is also essential for the provision of SAM, the universal donor of methyl groups. A common MTR variant consists of an A-to-G transition at base-pair 2756 that leads to a change from aspartic acid to glycine at codon 919 (D919G). Although the direct functional impact of this polymorphism has not been established, there is some evidence that this may be an activating polymorphism; in some studies, individuals with the GG genotype have higher serum folate concentrations [15] and lower homocysteine concentrations [16,17]. An association between the MTR A2756G polymorphism and genetic susceptibility to CRC and colorectal adenomas (CRA) has been widely documented, but with inconsistent results. However, there may be interactions between the MTR polymorphism and other risk factors, such as smoking and excessive alcohol use [32].

1.4 Folate deficiency

The diagnosis of folate deficiency is not easy to verify, but red blood cell folate concentration has been defined as the primary indicator of adequacy due to its correlation with liver folate and tissue stores[33]. The reference level of folate deficiency is not easy to define, and the result might be overtreatment of diffuse symptoms with folate supplements[34, 35]. General deficiency of folate has been associated with a number of different illnesses[36]. Anaemia, neural tube defects (NTDs), and CRC are discussed

in further detail in the following sections. These particular disorders are included because they have a part in folate research and the associated discoveries. It is important to realise that folate deficiencies associated with local deficiencies that are due, to a focal inflammatory process, cancer, or medical treatment such as methotrexate, are not included in this section.

1.4.1 Anaemia

As mentioned previously, it was a severe form of anaemia that led to the discovery of folate. Clinically, severe folate deficiency yields a specific type of anaemia, megaloblastic anaemia[37]. Symptoms of megaloblastic anaemia include fatigue, muscle weakness, tender tongue, and neurological symptoms. This anaemia includes large, abnormally nucleated erythrocytes that are not divided normally and that assemble in the bone marrow. It also affects the white blood cells and platelets.

1.4.2 Neural tube defects

NTDs are the most frequent and most tragic congenital abnormalities of the central nervous system. The brain and spinal cord develop from the neural tube, which is formed by dorsal folding of the neural plate after the 15th post-conception day[3].The critical period for anencephaly is between the 35th and 40th gestational days, and that of spina bifida is between the 37th and 42nd gestational days [38]. Therefore, in order to be effective, early supplementation is needed, preferably before conception.

The role of folate deficiency was described in 1964, when Hibbard et al. reported a higher rate of congenital abnormalities (3.0%) in the infants of folate-deficient mothers than in controls (1.6%) [39]. Smithell et al. reported a relationship between human embryopathy and a deficiency of folate metabolism and the role of vitamin deficiency in the development of NTDs [40].

In 1992, Czeizel et al. presented an important study in the New England Journal of Medicine. Women planning a pregnancy were randomly assigned to receive a single vitamin containing 0.8 mg of folic acid or a mineral tablet. In the group receiving the minerals, 6 of 2052 delivered babies were diagnosed with NTDs. In the group receiving the vitamin supplements, none of the 2014 babies was diagnosed with NTDs[41]. These results led to nationwide fortification of enriched uncooked cereal grains with folic acid, beginning in 1996 in the United States and in 1997 in Canada and becoming mandatory in 1998. However, this is not the case in Europe[3].

1.4.3 Cardiovascular disease

Folate deficiency results in a decreased capability to degrade the amino acid methionine in the liver, which leads to elevated homocysteine levels in plasma. Previously, a high homocysteine level was thought to be only a sign of low folate level, but research has shown that even a moderate elevation of homocysteine is an independent risk factor for cardiovascular disease[42-44] and stroke[44]. However, there are no conclusive results regarding the role of fortification with folates, in risk reduction of major cardiovascular events [45, 46].

1.4.4 Colorectal cancer

The role of folates in carcinogenesis of the colon and rectum has been the objective of many studies. In an analysis of two American cohort studies, the Nurses' Health Study and the Health Physicians Follow-Up Study, significantly reduced risks were observed in both women and men in the highest quintile of total folate intake compared with the lowest[47]. Kato et al. performed a nested case–control study at Sloan Kettering Cancer Centre based on the New York University Women's Health Study cohort. The study reported a significant 50% reduction in CRC risk in women in the highest quartile of plasma folate compared with the lowest[48].

Giovannucci et al. published a review article in 2002 that included cumulative data indicating that maintaining adequate folate levels may be important in lowering the risk of CRA, which is the major precursor lesion for most CRCs. The article also provided support for an inverse relationship between folate exposure and colorectal neoplasia risk[36]. Kim et al. showed that folate deficiency has an inhibitory effect, whereas folate supplementation has a promoting effect, on the progression of established neoplasms[49].

In contrast, folate deficiency in normal epithelial tissues appears to predispose them to neoplastic transformation, and modest levels of folate supplementation suppress the development of tumours in normal mucosa[50]. However, more recent studies have not supported an inverse association between plasma folate and CRC risk. A nested case–control study in the Northern Sweden Health and Disease Cohort reported that subjects with over four years of follow-up and plasma folate levels in the highest quintile were at a four-fold increased risk of CRC compared with those in the lowest quintile[51], whereas a Japanese cohort study of 375 individuals diagnosed with CRC provided no evidence of a relationship between plasma folate and CRC risk in either men or women[52].

1.5 Folate supplementation

As described in the section regarding NTDs, there is an obvious advantage to ensure that an adequate folate intake is provided to women in their childbearing years. However, supplementation to a whole population without any signs of deficiency or risk factors might be questioned, especially regarding the preventive effect on CRC.

A health technology assessment by Cooper et al. evaluated the clinical effectiveness and cost effectiveness of drugs and micronutrition interventions in the prevention of CRC and/or adenomatous polyps in populations with different risk levels of developing CRC. In six randomised clinical trials regarding folic acid and CRC identified by the researchers, there was no significant effect of folic acid versus placebo on adenoma recurrence (RR 1.16, 95% CI, range 0.97–1.39) or advanced adenoma incidence in individuals with a history of adenomas. In the general population, there was no significant effect of folic acid on risk of CRC (RR 1.13, 95 % CI, range 0.77–1.64), although the studies were of relatively short duration [53].

Mason et al. highlighted a temporal relationship between the onset of folic acid fortification and rises in the incidence of CRC in both the United States and Canada[54]. Two large multicentre phase III studies; the Aspirin/Folate Polyp Prevention Study by Cole et al. [55] and the United Kingdom Colorectal Adenoma Prevention Study by Logan et al. [56], investigated the role of folic acid supplementation together with aspirin.

The US-based study reported by Cole et al. 2007, randomised individuals to 1000 micrograms of folic acid or placebo daily for three years, and recurrence data were available for 987 individuals. The incidence of at least one CRA, the primary endpoint of the study, was essentially the same in the folic acid and placebo arms. However, the folic acid arm showed non-significant increases of 32% and 20% of patients with advanced CRA and number of subjects with more than three CRAs, respectively. In the second follow-up interval, the individuals in the folic acid arm showed a non-significant 13% increase in the incidence of at least one CRA recurrence and a 63% increase in advanced CRA, and the number of subjects with three or more CRAs more than doubled. While the authors concluded that the results indicated that a daily dose of 1000 micrograms of folic acid did not result in a reduction in CRA recurrence, the more concerning conclusion was a possible increased risk of advanced CRA or multiple CRAs[55].

The United Kingdom Colorectal Adenoma Prevention Study randomised individuals to 500 micrograms of folic acid or placebo daily for three years, using similar endpoints, and reported recurrence data for 853 participants. The subjects who received folic acid had only a very minor increase in incidence of one or more CRA, and no increase in advanced CRA [56]. Possible reasons for the discrepancies in the findings of the two trials are the lower dose of folic acid used in the UK trial (500 micrograms compared to 1000 micrograms) and a likely lower dietary intake of folate due to the absence of mandatory folate fortification of flour in the United Kingdom [4].

The two studies suggest that although there is evidence that folate deficiency is associated with higher risk of CRC, supplementation in healthy individuals might not be beneficial. In addition, in the study by Cole et al., an elevated risk of prostate cancer was noted in the group that received 1000 micrograms of folic acid [55].

The fact that supplementation with a high dosage of folates might promote carcinogenesis has been reported previously [57, 58]. As discussed above, folate is important in biochemical reactions that provide nucleotides for DNA synthesis and DNA methylation. Rapidly growing tissues, such as tumours, have an increased demand for nucleotides and could benefit from folate supplementation. This means that the timing of supplementation is of importance. A prospective, population-based colonoscopy study conducted by Forsberg et al. with 745 individuals (aged 19–70 years) born in Sweden discovered that adenomas were present in 10% of the individuals, and that the presence of adenomas was positively correlated with higher age. Of the participants (mean age 51.1 years), 15% of the men and 6% of the women had adenomas; advanced adenomas were seen in 2.8% of the study participants [59]. The results indicate that asymptomatic CRAs are not rare, especially in the elderly population. Folate administration prior to the existence of pre-neoplastic lesions can prevent tumour development, whereas folate administration after early lesions have been established appears to increase tumourigenesis [49, 60, 61].

2 COLORECTAL CANCER

2.1 Epidemiology

According to the World Health Organisation International Agency for Research on Cancer, there were 14.1 million new cancer cases, 8.2 million cancer deaths, and 32.6 million people living with cancer (within five years of diagnosis) in 2012 worldwide. CRC is the third most common cancer form, with 1.2 million new cases every year. The overall age-standardised cancer incidence rate is almost 25% higher in men than in women, with rates of 205 and 165 per 100,000, respectively. Male incidence rates vary almost five-fold across the different regions of the world, with rates ranging from 79 per 100,000 in Western Africa to 365 per 100,000 in Australia/New Zealand (with high variation in the rate of prostatic cancer diagnosis). There is less variation in female incidence rates (almost three-fold), with rates ranging from 103 per 100,000 in South-Central Asia to 295 per 100,000 in North America.

In Sweden, where CRC is the third most common cancer form after breast and prostate cancer, 4000 new cases of colon cancer and around 1900 new cases of rectal cancer are diagnosed every year according to The National Board of Health and Welfare in Sweden. The incidence of colon cancer is 42 per 100,000 in both sexes, although rectal cancer is more often diagnosed in men (25 per 100,000 in men compared to 17 per 100,000 in women).

2.2 Risk factors for developing CRC

A risk factor refers to the chance of developing a disorder, in contrast to prognostic or predictive terminology. The single most important factor for developing CRC is advanced age. CRC is a very rare disease in individuals under the age of 40 years, and the mean age for diagnosis is 72–74 years. Adenomas in the colon and rectum are a risk factor for developing CRC, and studies have shown that endoscopic removal of adenomas and surveillance reduce the risk of CRC[62]. Chronic inflammatory disease of the colon and rectum, such as ulcerative colitis and Crohn's disease, is a strong risk factor for developing CRC[63, 64].

Lifestyle and habit-related factors such as cigarette smoking, high intake of alcohol, low socioeconomic status, low rate of physical activity, high body mass index, and a diet high in processed food and red meat, and low in fruits

and vegetables are all factors that are associated with a higher risk of developing CRC [65, 66].

Hereditary factors are also strongly associated with an increased risk of developing CRC. Hereditary factors are involved in around 20–30% of all CRCs [67, 68]. Besides mutation disorders, individuals with one first-degree relative with CRC double their lifetime risk of developing CRC, and with two relatives, the risk is even higher[68]. If an individual is diagnosed with CRC before the age of 50 years, genetic screening should be offered.

There are two major dominantly inherited autosomal syndromes of CRC in which the gene mutation is known: Lynch syndrome (formerly called hereditary nonpolyposis colorectal cancer) and familial adenomatous polyposis coli (FAP). Around 2–3% of all CRCs are associated with Lynch syndrome, and less than 1% are associated with FAP. Lynch syndrome is characterised by a very high risk of developing a tumour. While there is an estimated 80-fold increased risk of CRC, there is also a high risk of endometrial and ovarian cancers. Lynch syndrome should be suspected in patients diagnosed with cancer at an early onset and in patients who develop multiple cancers.

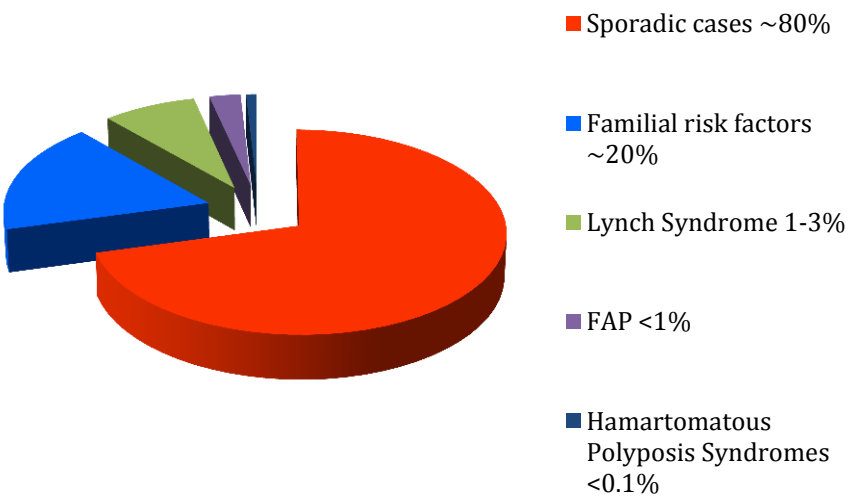

Figure 6. Etiology of colorectal cancer. Numbers taken from the Swedish National Guidelines 2014.

FAP is associated with a massive number of adenomas in the entire gastric canal. If left untreated (i.e. without surgery), 100% of patients will develop CRC and therefore, prophylactic surgery is recommended [69]. Families with these monogenic mutations are often known and under surveillance in accordance with standardised protocols. MutYH-associated polyposis (MAP) is caused by an autosomal recessive mutation, and is also associated with multiple adenomas. The mutation should be suspected when a patient develops FAP symptoms without signs of an autosomal-dominated inheritance. Around 1% of the normal population are heterozygote carriers of a MutYH mutation.

2.3 Diagnosis, medical investigation, and staging

The most common signs of CRC are faecal blood, changes in bowel habits, and iron-deficiency anaemia. Pain and tumours that are palpable are late signs. Diagnosis is mainly by bowel examination, colonoscopy, or colonography, and it can be confirmed with biopsies.

In order to provide the correct treatment for the patient, it is necessary to classify the stage of the disease. The anatomical extent of the disease is the single most important prognostic factor[70, 71]. The TNM system, developed by the American Joint Committee on Cancer and the Union for International Cancer Control has replaced the former Dukes' staging system. The anatomically based TNM system classifies the local invasiveness of the primary tumour (T), the regional spread (i.e. the number of lymph node metastases [N]), and the presence of distant metastasis (M).

It is important to understand that staging the cancer disease is a continuous process that starts at the diagnosis of the patient i.e., known as clinical TNM (cTNM). Preferably, this is performed before any treatment modality starts, as a patient with advance disease might need neoadjuvant treatment or might not benefit from surgery at all. It is important to realise that almost 20% of all colonic cancers are diagnosed in an emergency setting and require rapid staging that is limited by the patient's condition.

Radiology examination to provide information on M-status is mandatory. Usually, computed tomography (CT) of the thorax and abdomen is performed, but contrast-enhanced ultrasound, magnetic resonance imaging (MRI), or fluorodeoxyglucose positron emission tomography (FDG-PET)

sometimes provides useful information [72]. The cTNM-staging procedure is extremely important in rectal cancer, as the information is used to decide whether or not the patient will receive preoperative radio-chemotherapy. MRI is always used, and sometimes, transrectal ultrasound [73, 74].

If the patient undergoes surgery, there is a clinical intraoperative evaluation, and the removed tumour provides specimen for the final pathology report TNM (pTNM).

The T-staging describes the extent of spread through the layers that form the wall of the colon and rectum. T4 indicate that the tumour is advanced and has grown through the wall of the colon or rectum and into nearby tissues or organs. T4a means growth into another organ, whereas T4b indicates growth through the serosa layer. Preoperative information about T-stage is very useful, as it could affect the surgical procedure, as well as the need for neoadjuvant therapy.

T-status is closely related to N-status. In different studies regarding rectal cancer, T1, T2, T3, and T4, carry 0–12%, 12–28%, 36–66%, and 53–79% risk of lymph node metastasis [74, 75]. The analysis of the lymph nodes (N) is one of the most important prognostic factors in CRC. It is also important because the indication for adjuvant therapy is mainly by the presence of regional lymph node metastasis [76]. A minimum of 12 lymph nodes should be accessed in order to avoid under-staging [77, 78].

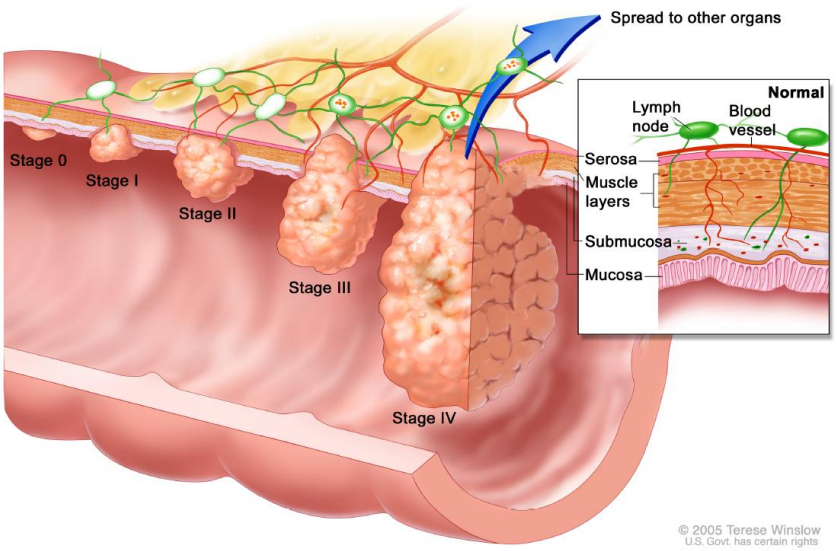

Figure 7. For the National Cancer Institute © 2005 Terese Winslow, U.S. Govt. has certain rights. Used with permission.

The separate TNM factors are converted into an overall stage. The main characteristic of stage I is early local cancer; stage II are locally advanced cancers; stage III are cancers with lymph node metastasis; and stage IV are cancers with distant metastasis. These traits are similar to the older Dukes' system.

2.4 Treatment

The Swedish National Board of Health has declared, in the national guidelines for 2014, that every patient with a CRC diagnosis should be discussed in a multidisciplinary team (MDT) conference. The members of the team should consist of at least a colorectal surgeon, a radiologist, an oncologist, a pathologist, and a contact nurse. The patient should be discussed at least before and after surgery and in case of recurrence in order to secure the best treatment.

Radical surgery with total removal of the tumour is the single most important treatment for CRC. Although some centres have reported complete clinical response after radio-chemotherapy[79], this treatment should be considered suitable only in a study population.

2.4.1 Surgery for colon cancer

The aim of the surgery is to perform a radical resection with tumour-free margins. In colon cancer, a distant margin of at least 5–10 cm is preferred, which usually is not a technical problem. The involved vessels and the placing of the ligatures often govern the extent of the bowel resection. In colon cancer, standardised procedures that take into account the blood and lymph supplies of the colon are the most common. These include ligation one arcade away from the tumour. If the cancer is situated in the right colon, ligation of the *a.ileocolica*, *a. colica dextra*, and *a. colica media* is performed. A colon cancer in the left colon requires ligation of the *a.mesenterica inferior*, and if the cancer is situated in the sigmoideum, a ligation of the *a.rectalis superior* must be added. Theoretically, one can argue that an approach where the ligature of the supporting vessels, both arteries and veins, is placed as close to the aorta as possible should be beneficial from an oncological point of view [80], but the "high tie" method has more associated morbidity, and no clear consensus about the most optimal method has been reached thus far [81, 82]. In open colon cancer surgery, dissection of the bowel is performed before the vessels are ligated. Several studies have shown that the oncological results after laparoscopic resection, with or without the assistance of a robot technique, are the same as

after open surgery [83-85]. In the laparoscopy technique, the vessels are ligated before the tumour dissection is performed, and the ischemic time from the ligation of the blood supply until the tumour is removed is prolonged.

2.4.2 Surgery for rectal cancer

Rectal cancer surgery is often more complicated than colon cancer surgery and presents a greater risk of postoperative complications. The anatomy poses challenges due to limitation of the pelvis and the proximity to sensitive structures such as nerve bundles and the presacral venous plexus. The blood supply to the rectum is deviated from both the *a. mesenterica inferior* (*a. rectalis superior*) and the *a. iliaca interna* (*a. rectalis media*, *a. rectalis inferior*). In both open and laparoscopic rectal cancer surgery, the vessels are ligated prior to tumour dissection. An important parameter in rectal cancer is tumour height, meaning the distance from the anal verge to the lower neoplastic limit. The height is determined with a straight rectoscopy as it is withdrawn. A rectal cancer is clinically defined as a cancer within 15 cm from the anal verge.

In rectal cancers, a distal resection margin of at least two cm is desirable, but a very low anastomosis could end up damaging sphincter capacity. Therefore, for low rectal cancers, at 6–7 cm, it is common in Sweden to perform an abdominoperineal resection. The procedure is often called an amputation, as the low placement leaves no margin for anastomosis, and thus, requires a terminal colostomy.

For mid-range tumours, at 8–13 cm, a total mesorectal excision (TME) anterior resection is the standard [86]. There is a risk of poor healing and leakage with a low anastomosis, and thus, the patient usually gets a temporary diverting stoma. Very high tumours bordering the rectosigmoid colon can often be treated with the anterior TME technique, with the possibility of limiting the dissection of the distal rectum.

If the cancer is detected early, the tumour is small, and there are no signs of lymph node involvement in the preoperative examination, one treatment option is local excision, by trans anal endoscopic microsurgery (TEM)[87]. However, this technique only removes the tumour and a limited amount of the surrounding tissue. Because no information on lymph node metastasis is provided, the staging might be less accurate[75].

Because 2–4% of all patients diagnosed with CRC have synchronous tumours, it is mandatory to examine the entire large bowel[88]. If for any reason a "clean colon" is not established prior to surgery, the examination has to be performed after recovery.

2.5 Other treatment options

As previously described, surgery is the most important method to achieve a cure in treating colorectal cancer. However, additional treatment has been shown to reduce the risk of local metastasis and metastatic disease, as well as increase overall survival. Treatment can be divided into neoadjuvant, adjuvant, and palliative treatments.

Neoadjuvant treatment is the administration of therapeutic agents before a main treatment. The term "conversion therapy" is closely related, and the aim is to make a cancer operable. *Adjuvant* treatment is defined by the National Cancer Institute as an additional cancer treatment given after the primary treatment to lower the risk that the cancer will return. It is important that all visible tumours have been removed and that none further is seen by radiology—otherwise, the term "palliative" or "first-line treatment" should be used. The indication is therefore more relative and based on risk assumptions extrapolated from epidemiological data, and the exact risk to the individual is unknown. Adjuvant therapy may include chemotherapy, radiation therapy, hormone therapy, targeted therapy, or biological therapy. Finally, *palliative* treatment is designed to relieve symptoms and improve the patient's quality of life. It can be used at any stage of an illness if there are troubling symptoms, such as pain or sickness. Palliative treatment can also mean using medicines to reduce or control the side effects of cancer treatments. In advanced cancer, palliative treatment may help patients to live longer and to live comfortably, even if they cannot be cured.

2.5.1 Neoadjuvant treatment

The need for neoadjuvant treatment should be discussed during the preoperative MDT conference. With colon cancer, neoadjuvant treatment is sometimes used for locally advanced cancers that are associated with very high risk of recurrence and with high degrees of node metastasis.

Neoadjuvant treatment is more commonly used in rectal cancer. Radiotherapy has been shown to reduce the dreaded complication of local recurrence. However, radiotherapy has many side effects associated with high morbidity, and it is important to select patients who will benefit the

most from radiotherapy[89]. Detailed preoperative staging using high-resolution MRI and clinical examination enables the selection of patients who require preoperative therapy for tumour regression or reducing the risk of local recurrence.

In Scandinavia, rectal cancers are categorised as follows[90, 91]

Good – early rectal cancers that will not benefit from neoadjuvant treatment, as the risk for local recurrence after surgery is low.

Bad – internationally named, locally advanced. In this group, the recommendation is often five days of radiotherapy with a dose of 5 Gray each time, followed by immediate surgery.

Ugly – locally advanced tumours in which a downsizing effect is wanted. These patients receive long-term radiotherapy, usually for five weeks, in combination with 5-FU/LV or capecitabine. The surgery is scheduled after an additional numbers of weeks in order to allow the tumour to shrink and the patient to recover[92]

2.5.2 Adjuvant treatment

After radical surgery for colon cancer, adjuvant treatment with chemotherapy has been shown to reduce the risk of recurrence and increase the chance of survival[93, 94]. The evidence is high regarding colon cancer stage III, but not as significant for stage II. Risk factors are used in addition to TNM stage in order to select patients who will benefit from treatment. Acute operation due to tumour perforation, ileus, peri-vessel or nerve bundle involvement, low differentiation grade involvement, positive circumferential margin, and low numbers of examined lymph nodes are examples of risk factors that should be taking into consideration during the MDT conference when deciding whether to recommend adjuvant treatment for a patient [95, 96].

Adjuvant treatment with 5-FU/LV or capecitabine in stage III colon cancer reduces the relative risk of recurrence[89, 93, 97, 98]. The effect increases if oxaliplatin is added, but the combination is associated with increased side effects, especially in patients older than 70 years of age [99, 100]. The current recommendation in Sweden for colon cancer is Nordic FLOX, which stands for a combination of oxaliplatin 85 mg/m^2 as a 2-h infusion followed by a 3-min bolus injection with 5-FU 500 mg/m^2 and a bolus injection with LV 60 mg/m^2 30 min later, given on days 1 and 2. The treatment is followed

by rest for 12 days. The term Nordic FLV is used for the same regimen, without oxaliplatin.

The standard regimen period is six months, although on-going studies (SCOT) might find that it is possible to reduce this period. The mechanisms of 5-FU and LV will be discussed separately.

The evidence for the use of adjuvant chemotherapy for rectal cancer is considerably weaker than for colon cancer. Since the introduction of radiotherapy, the incidence of local recurrence has reduced. Rectal surgery is also associated with higher surgery-related morbidity, which might make it difficult to start with chemotherapy in the recommended timeframe. There is strong support for that starting adjuvant chemotherapy early after surgery[101-103]. The use of radiotherapy might also affect the lymph nodes, which is the strongest factor when deciding whether adjuvant chemotherapy should be recommended. A systematic Cochrane review showed support for the use of 5-FU-based postoperative adjuvant chemotherapy for patients undergoing radical surgery for non-metastatic rectal carcinoma, but much of the material was old and had been collected prior to the implementation of radiotherapy [104]. A systematic review of modern studies by Bujko et al. could not confirm those results. A non-protocoled subgroup analysis of one study indicated a beneficial effect of adjuvant chemotherapy for high rectal tumours and for patients downstaged to T0-2N0, but no effect for low-lying rectal tumours [105]. A newly published report from the EORTC 22921 randomised study stated that adjuvant 5-FU-based chemotherapy after preoperative radiotherapy (with or without chemotherapy) did not affect disease-free survival or overall survival [106]. In contrast, Tiselius et al. analysed 436 Swedish patients with stage III rectal cancer, the majority of whom had been operated on using the TME technique. In this cohort, the patients who received adjuvant chemotherapy had a significantly longer overall survival [107]. The Swedish National Board of Health has given the use of adjuvant treatment in rectal cancer a 5 in priority due to little scientific evidence. However, patients with stage II rectal cancer with risk factors and stage III that has not been treated with neoadjuvant radiotherapy are recommended for adjuvant treatment with Nordic FLV.

2.5.3 Chemotherapy in metastatic disease /Palliative chemotherapy

Approximately 40% of patients with CRC are left with palliative treatments, either primarily or because of a recurrence later during the course of the disease—a metastatic situation (mCRC). In the absence of tumour-controlling treatments, the prognosis for patients with mCRC is poor, with, on a population level, a median survival of less than six months and a very low probability of surviving beyond one or two years [108, 109].

In modern clinical studies, the median survival is now almost two years, but in a population-based context, it is closer to one year. Three-year survival for patients of all ages with mCRC is now 21%, and five-year survival is 9%. It is important to evaluate the effect of chemotherapy with CT scans. A common rule is to evaluate after two months of treatment. If there is regression or stable disease, the treatment should continue for another couple of months, but if there is progression, a different protocol must be evaluated.

Surgical treatment should always be considered. Liver metastases are the most common manifestation of visible metastatic disease in patients with CRC. About 15–20% of patients have liver metastases at the time of diagnosis. In addition, up to 50% of patients in stage III disease later develop liver metastases. Some of those patients can be treated by surgically removing a part of the liver; other equivalent options include cryosurgery and radio frequency ablation. Surgery might also be performed to remove metastases in the lungs or for local recurrences. Five-year survival rates have been reported in 25–30% of patients in whom radical surgery was performed for liver metastasis, lung metastasis, or peritoneal metastasis [110-112].

The chemotherapeutic regimens used in palliative settings are 5-FU in combination with LV, capecitabine, irinotecan, and oxaliplatin. These drugs are used alone or in different combinations; patients often receive both second- and third-line therapy regimens.

New target drugs that have been released are being used in combination with the established chemotherapeutic agents. Four of the new drugs are used in CRC: two antibodies that affect the angiogenesis process—bevacizumab (Avastin) and aflibercept (Zaltrap)—and two antibodies that affect the epidermal growth factor receptor in K-RAS wild type tumours—cetuximab (Erbitux) and panitumumab (Vectibix). The effects of these drugs are well documented, and although the cost is significant and the effect can sometimes be quite limited, they are recommended for downsizing prior to

surgery as well as in selected cases in adjuvant and palliative settings [113-116].

2.5.4 Side effects

Although the Nordic FLV regimen is considered to be a fairly mild chemotherapy treatment, it is important to realise that many patients experience considerable side effects. These can affect many issues, ranging from quality of life to the chance of completing the adjuvant therapy, and ultimately, even the chance of survival. The most common side effects of FLV are diarrhea, nausea, anorexia, and depression of the bone marrow. While the side effects are not permanent once the treatment is finished, some patients are affected to the extent that they have to have a dose reduction or even have to be admitted to the hospital for inpatient care. Oxaliplatin is neurotoxic and carries the risk of causing peripheral neuropathy, especially in the hands and feet; in this case, the effects can be permanent [117].

Because the number of patients being offered surgery for liver metastasis is increasing, the issue of chemotherapy-induced liver injury is growing. The timing between chemotherapy and surgery is of great importance [118-120].

There is relatively little experience in treatment with cytotoxic drugs in elderly patients, and approximately 30–40% of patients diagnosed with CRC are 75 years of age or older [121]. In fact, age is a barrier to inclusion in clinical trials with new cancer therapies. In the most relevant studies on CRC, less than 20% of the patients were above the age of 70 years [122]. However there are now many studies showing that patients without significant comorbidity older than 75 and even 80 years could benefit from chemotherapy [121, 122].

Considering the number of patients receiving 5-FU-based treatment, not only for CRC but also for other cancers, such as breast cancer, finding a predictive factor for side effects would be very valuable for the patients as well as for society. According to the results, MTHFR and MTR polymorphisms can affect the risk of toxicity during adjuvant 5-FU-based chemotherapy treatment. However, folate metabolism is very complex, and it is highly plausible that polymorphisms in other genes, such as TS, affect the results further. There is also another polymorphism in MTHFR, A1298G, which was not examined in this study. Thus, further studies must be performed in order to find predictive markers for 5-FU-based chemotherapy [123].

Any side effects from administered chemotherapy are classified according to the National Cancer Institute Common Terminology Criteria for Adverse Events. In our studies, in order to obtain an accurate classification, the charts were assessed by research nurses with a lot of experience classifying side effects.

In clinical studies, the term *adverse event* is used instead of side effect. However, the definition of an adverse event is much wider than the definition of a side effect, and it is defined by the WHO as "medical occurrence temporarily associated with the use of a medicinal product, but not necessarily causally related".

2.5.5 Outcome

There are several ways to assess outcome in CRC treatment, each with its strengths and limitations. Overall survival is a concept that is easy to use but more difficult to assess in an aging patient population. Cancer-specific survival might be a better parameter, but it mandates solid follow-up or cause of death data, which can be hard to obtain. Other treatment outcome measurements are time to progression and the related progression-free survival.

In summary, prognosis is strongly stage-dependent, with very good prognoses in the early stages. Outcomes by quality of life and functional results are of increasing interest, which might be an indication that cancer-related survival is improving.

2.6 Pharmacy

The following sections include short descriptions of some of the main drugs mentioned in this thesis.

2.6.1 5-fluoruracil

5-FU was developed in 1957 by Charles Heidelberger. In 1954, Rutman et al. had discovered that rat hepatomas were consuming the pyrimidine uracil more rapidly than normal rat liver tissue and uracil was identified as a target molecule for chemotherapy[124]. 5-FU is an analogue of uracil in which the hydrogen at position 5 is replaced by fluorine. It enters the cell rapidly, using the same facilitated transport mechanism as uracil. 5-FU is converted intracellularly to several active metabolites: 5-fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate, and fluorouridine triphosphate. These metabolites disrupt RNA synthesis and the action of TS. The rate-limiting enzyme in 5-FU catabolism is dihydropyrimidine dehydrogenase (DPD), which converts 5-FU to dihydrofluorouracil. More than 80% of administered 5-FU is catabolised primarily in the liver, where DPD is abundantly expressed. The activity of DPD has been shown to be associated with side effects during 5-FU-based chemotherapy[125].

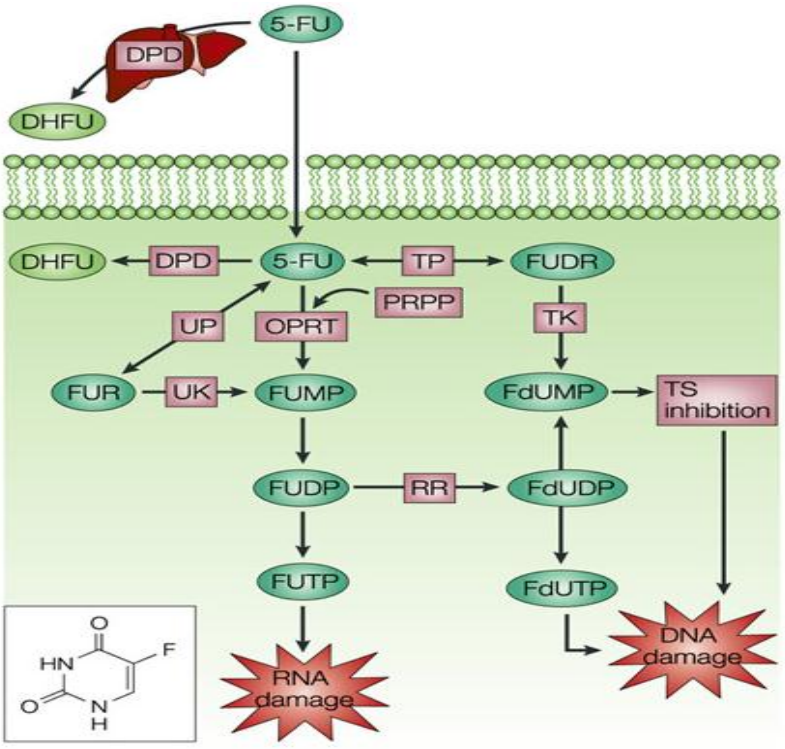

Figure 8. 5-Fluorouracil (5-FU; see structure) is converted to three main active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). The main mechanism of 5-FU activation is conversion to fluorouridine monophosphate (FUMP), either directly by orotate phosphoribosyltransferase (OPRT) with phosphoribosyl pyrophosphate (PRPP) as the cofactor, or indirectly via fluorouridine (FUR) through the sequential action of uridine phosphorylase (UP) and uridine kinase (UK). FUMP is then phosphorylated to fluorouridine diphosphate (FUDP), which can be either further phosphorylated to the active metabolite fluorouridine triphosphate (FUTP), or converted to fluorodeoxyuridine diphosphate (FdUDP) by ribonucleotide reductase (RR). In turn, FdUDP can either be phosphorylated or dephosphorylated to generate the active metabolites FdUTP and FdUMP, respectively. An alternative activation pathway involves the thymidine phosphorylase catalysed conversion of 5-FU to fluorodeoxyuridine (FUDR), which is then phosphorylated by thymidine kinase (TK) to FdUMP. Dihydropyrimidine dehydrogenase (DPD)-mediated conversion of 5-FU to dihydrofluorouracil (DHFU) is the rate-limiting step of 5-FU catabolism in normal and tumour cells. Up to 80% of administered 5-FU is broken down by DPD in the liver.

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TS catalyses the reductive methylation of dUMP to dTMP, using 5,10-methyleneTHF as the methyl donor. This reaction provides the sole source of thymidylate, which is necessary for DNA replication and repair. The 36-kDa TS protein functions as a dimer, both subunits of which contain a nucleotide-binding site and a binding site for 5,10-methyleneTHF. The 5-FU metabolite FdUMP binds to the nucleotide-binding site of TS, forming a stable ternary complex with the enzyme and 5,10-methyleneTHF, thereby blocking binding of the normal substrate dUMP, and thus, inhibiting dTMP synthesis. The greatest impact of 5-FU is on highly proliferative cells such as tumour epithelial cells. The level of inhibition of TS by FdUMP has been shown to correlate with the clinical response of 5-FU treatment[126, 127]. However, as a single drug for advanced CRC, 5-FU treatment has a very modest effect, with a response rate of around 10% [128].

2.6.2 Leucovorin

In order to have an effect, 5-FU therapy must lead to the formation of the ternary complex between FdUMP and TS. The binding of FdUMP to TS is reversible in the absence of adequate amounts of 5,10-methyleneTHF. In 1977, Danenberg et al showed that the inhibition of TS by 5-FU could be potentiated by reduced folates, which are converted intracellularly to 5,10-methyleneTHF [129]. LV enters the cell via the reduced folate carrier and is anabolised to 5,10-methyleneTHF, which is then polyglutamated by the enzyme folylpolyglutamate synthetase. This process not only increases the cellular retention of 5,10-methyleneTHF, but it also enhances the stabilisation of the ternary complex with TS and FdUMP[130].

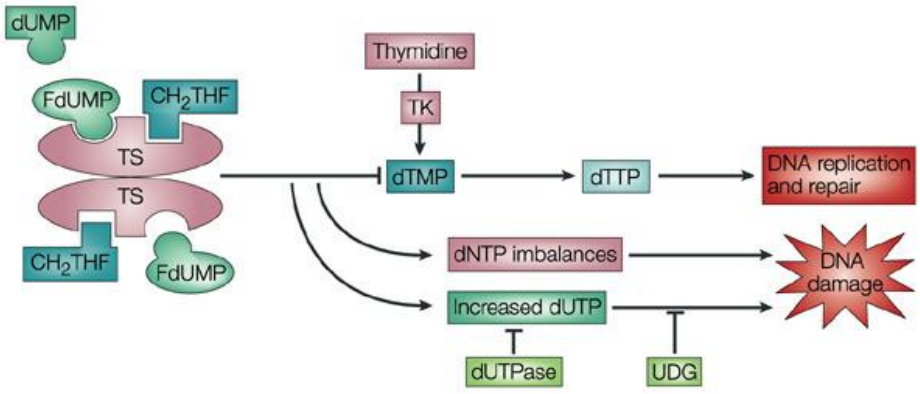

Figure 9. Thymidylate synthase (TS) catalyses the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) with 5,10-methylene tetrahydrofolate (CH2THF) as the methyl donor. The 5-fluorouracil (5-FU) active metabolite fluorodeoxyuridine monophosphate (FdUMP) binds to the nucleotide-binding site of TS and forms a stable ternary complex with TS and CH2THF, blocking access of dUMP to the nucleotide-binding site and inhibiting dTMP synthesis. This results in deoxynucleotide (dNTP) pool imbalances and increased levels of deoxyuridine triphosphate (dUTP), both of which cause DNA damage. The extent of DNA damage caused by dUTP is dependent on the levels of the pyrophosphatase dUTPase and uracil-DNA glycosylase (UDG). dTMP can be salvaged from thymidine through the action of thymidine kinase (TK).

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The polyglutamate derivates of 5,10-methyleneTHF were shown to be more effective than the monoglutamate forms, and in 1978, Ullman et al presented an article that stated that 20 micrograms of the stable, reduced form 5-formyltetrahydrofolate (5-formylTHF, i.e., leucovorin) enhanced 5-FU cytotoxicity five-fold in leukemic cells. These preclinical data were later confirmed in clinical studies that showed that by adding LV to 5-FU treatment, the tumour response rate improved to 21% [131, 132]. Since the beginning of 1990 5-FU/LV has been a cornerstone in neoadjuvant, adjuvant, and palliative CRC treatment, and it may be used as a single or combination therapy.

In clinical practice, LV is manufactured as calcium folinate, which is the calcium salt of 5-formylTHF. It is a racemic mixture that is a combination of two isomers: the levo (l)-isomer and the dextro (d)-isomer. The term "racemic mixture"means that there are equal amounts of l- and d-isomers. The l-isomer is the biologically active form, whereas the d-isomer of racemic LV has no biological activity at all. The drug is administered as an intravenous bolus injection or as an infusion. In paper IV, LV was administered in the form of Isovorin®. This product is the calcium salt of levofolinate, also known as levoleucovorin, l-leucovorin (l-LV). l-LV is composed purely of the biologically active l-isomer. After absorption, l-LV (l-5-formylTHF) is metabolised to 5,10-methyleneTHF. By comparison, 30 micrograms of l-LV is as effective as 60 mg of the racemic form, LV (calciumfolinate)[133].

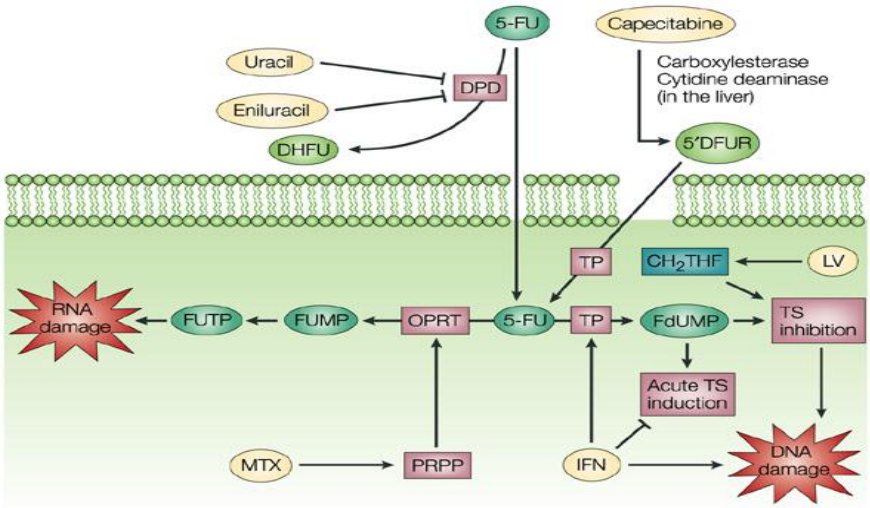

Figure 10. Summary of some of the strategies that have been investigated for increasing the anticancer activity of 5-fluorouracil (5-FU). Leucovorin (LV) increases the intracellular pool of 5,10-methylene tetrahydrofolate (CH_2THF), thereby enhancing thymidylate synthase (TS) inhibition by fluorodeoxyuridine monophosphate (FdUMP). Eniluracil and uracil inhibit DPD-mediated degradation of 5-FU. Methotrexate (MTX) is thought to increase 5-FU activation by increasing phosphoribosyl pyrophosphate (PRPP) levels. Interferons (IFNs) have been reported to enhance thymidine phosphorylase (TP) activity, abrogate acute TS induction caused by 5-FU treatment and enhance 5-FU-mediated DNA damage. Capecitabine is a 5-FU pro-drug that is converted to 5'-deoxy-5-fluorouridine (5'DFUR) in the liver by the sequential action of carboxylesterase and cytidine deaminase. 5'DFUR is converted to 5-FU by TP.

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The most commonly used 5-FU/LV regimens for CRC are the Mayo Clinic regimen, the Roswell Park regimen, the de Gramont regimen and the Nordic FLOX regimen. In all of these regimens, LV is administered in the form of calciumfolinate.

In view of the fact that millions of doses of 5-FU have been administered worldwide, it is interesting to note that no clinical study evaluating the optimal dose, route, or duration of LV has yet been published. Furthermore, current 5-FU/LV dosing is based on the traditional use of body surface area (mg/m^2), which allows for convenient dose calculation but is not supported by any scientific evidence. Furthermore, studies have shown a high degree of inter- and intraindividual variability in resulting plasma levels in patients receiving 5-FU/LV, which could indicate that some patients receive suboptimal chemotherapy and/or LV concentrations[134].

2.6.3 5,10-methyleneTHF (Modufolin®)

The intracellular conversion of LV to 5,10-methyleneTHF involves multiple metabolic steps and is dependent on the efficacy of the involved enzymes. The need for metabolic activation of LV could result in interindividual differences in its utilisation, which could affect the potential benefit gained from the LV supplementation.

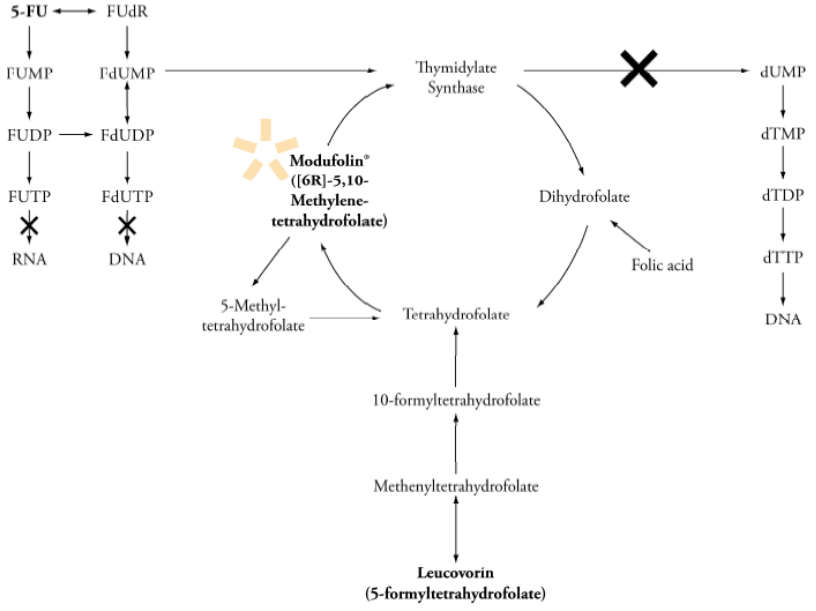

Figure 11. Mechanism of Modufolin

As 5,10-methyleneTHF is the only natural folate that directly binds the ternary complex, it could have advantages over LV. However, as a substance, 5,10-methyleneTHF is very sensitive to oxidation, and therefore, it is difficult to produce. After some effort a substance, Modufolin® is now available for early trials. Modufolin® is the biological isomer of 5,10-methyleneTHF; and this patented drug was administered in the study presented in paper IV.

2.7 Predictive and prognostic factors

The term "prognostic factor" refers to the outcome of a person with a disease, whereas the term "risk factor" refers to the likelihood of a healthy individual being affected by illness. The terms "prognostic factors" and "predictive factors" are often confused. In the Journal of Clinical Oncology, Italiano et al. (2011) presented a useful definition:

"A prognostic factor is a clinical or biological characteristic that is objectively measurable and that provides information on the likely outcome of the cancer disease in an untreated individual. Such prognostic markers are helpful for identifying patients with cancer who are at high risk of metastatic relapse and therefore potential candidates for adjuvant systemic treatments. In contrast, a predictive factor is a clinical or biological characteristic that provides information on the likely benefit from treatment (either in terms of tumour shrinkage or survival). Such predictive factors can be used to identify subpopulations of patients who are most likely to benefit from a given therapy. Importantly, prognostic factors define the effects of patient or tumour characteristics on the patient outcome, whereas predictive factors define the effect of treatment on the tumour"[135].

Some examples of prognostic factors for CRC are age, tumour stage, tumour histological grade, comorbidity, and whether or not it was possible to perform a radical surgical removal of the tumour.

Identifying predictive markers for CRC is closely linked to the increasing knowledge regarding the process of carcinogenesis. A better possibility of prediction is a necessary step towards a fully individualised and tailored cancer treatment, and it will be discussed later. Some examples of predictive factors used in clinical practice today are KRAS-status in CRC and HER-status in breast cancer.

3 AIM

The aims of this thesis were to:

- Explore the effect of functional polymorphisms in the genes encoding methylenetetrahydrofolate reductase (MTHFR C677T) and methionine synthase (MTR A2756G) on outcome and risk of toxicity during adjuvant chemotherapy administered in stage III CRC.

- Explore and describe the effect of polymorphisms in folate-associated genes on the levels of different folate forms and their distribution in tumours and mucosa in patients with CRC.

- Determine the levels of THF, 5,10-methyleneTHF, and 5-methylTHF in tumours and mucosa of CRC patients who received different dosages of LV intravenously at the time of surgery.

- Compare the concentration of folate metabolites in tumours, mucosa, and plasma in patients with colon cancer after administration of Modufolin® or Isovorin® (levo-leucovorin).

4 PATIENTS

4.1 The clinical database

In Gothenburg, all CRC operations, with a very few exceptions (e.g. emergency surgery without previous diagnosis or a combination of liver metastasis surgery and colon resection) are performed at Sahlgrenska University Hospital/Östra.

In 1999, a local CRC database was established that includes clinical data, pathology reports, and information regarding chemotherapy and radiotherapy. A record is kept on how the registrations should be made to keep them consistent over time. The database is continually updated by research nurses and internally validated twice yearly. Since 2002, the clinical database has been linked to a biobank (No 242), and consecutive collection of tissue samples from tumours and macroscopically normal mucosa from almost all patients undergoing elective cancer surgery has been performed. Tissues from patients undergoing acute surgery for CRC during office hours have also been retrieved. Today, data from over 4000 patients with CRC can be found in the database.

The tissue samples are collected directly by research nurses in a standardised manner at the time of tumour removal, and immediately snap-frozen at -80°C. Blood, saliva and urine samples are also collected from the patients and stored in freezers.

4.2 Ethical considerations

All studies in this thesis were performed according to the principles stated in the Declaration of Helsinki. In papers I and II, as part of the clinical routine and with the aid of research nurses, the patients were informed of the projects and the aim. Acceptance of participation was documented and all of the patients had the opportunity to decline participation, according to Good Clinical Practice (GCP), which is an international ethical and scientific quality standard for designing, conducting, recording, and reporting trials that involve the participation of human subjects. Compliance with this standard provides public assurance that the rights, safety, and wellbeing of trial subjects are protected, consistent with the principles that have their origin in the Declaration of Helsinki, and that the clinical trial data are

credible. The Regional Ethical Review Board of Gothenburg gave its approval before the database was set and tissue gathering began (Ö445-00).

In paper III, all the patients were informed of the study and they volunteered participation in accordance with ICH GCP. The study was also approved by the Regional Ethics Committee in Gothenburg.

In study IV, all of the doctors who included patients in the study were educated in GCP. The patients were informed about the study and acceptance of participation was documented. The protocol complied with local regulations and was approved by the institutional review board, by the Swedish national competent authority, Medical Products Agency, and by the Regional Ethics Committee in Gothenburg (ISO-CC-002).

4.3 Patients

A summary of the included patient cohorts is presented in the following table.

Table 1.

Characteristics	Paper I	Paper II	Paper III	Paper IV
Number of patients	150	53	75	32
Median age (range)	66 (32–82)	73 (30–92)	70 (37–89)	73 (41–93)
Gender (male/female)	88/62	28/25	39/36	16/15
Tumour location (colon/rectum/synchronous tumours)	75/75/0	39/14/0	38/34/3	29/0/0
TNM stage	3	1–4	1–4	1–3

Paper I

A total of 649 patients were operated on for stage III CRC in the period 1999–2006. Of those patients, 429 (66.1%) received postoperative adjuvant chemotherapy, and 150 patients were randomly selected and analysed. As seen in Table 1, surprisingly, the tumour locations were 75 colon and 75 rectal cancers. This was not the intent, but merely coincidence. Our interpretation is that this even breakdown reflects the fact that because Sahlgrenska University Hospital/Östra is a Centre for rectal surgery in the region, the proportion of rectal cancer in relation to colon cancer is not the same as it is in the population.

All of the patients in paper I received chemotherapy in accordance with the Nordic FLV regimen based on 500 mg/m^2 5-FU in combination with 60 mg/m^2 LV. Sixteen of the included patients received oxaliplatin in the standard dose of 85 mg/m^2 as an intravenous infusion every other week in combination with the FLV-treatment (i.e. Nordic FLOX). There was a predominance of male patients, reflecting the fact that rectal cancer is more common in males.

Paper II

Blood samples and tumour and mucosa tissue are routinely collected from patients with CRC at Sahlgrenska University Hospital/Östra. Fifty-three of these patients were randomly included in the study. All tumours were adenocarcinomas, and the TNM stages varied from I to IV.

Paper III

This study was designed to determine the levels of different folate forms in tumours, blood, and mucosa of patients with CRC who received different dosages of LV in the form of calcium folinate. The LV was administered as a bolus injection immediately at the beginning of the surgical procedure. Eighty patients scheduled for a colorectal resection with a cancer indication were enrolled in the study between January 2011 and January 2012. There were no exclusion criteria, except for the inability to understand the study information or inability to provide written informed consent. The patients were preoperatively randomised into four groups. The first group served as a control group and received no LV, while Groups 2, 3, and 4 received 60, 200, and 500 mg/m^2 LV, respectively.

Based on the routine pathology reports, four patients were excluded from the study because the analysis revealed a lack of adenocarcinoma tissue; two patients had an obstruction related to diverticulitis, one had a squamous epithelial cancer, and one had a non-malignant adenoma. During the analysis

of the blood samples, we discovered that one patient in treatment group two had received an LV dose that was not according to the protocol, and that patient was also excluded from the study.

Paper IV

Paper IV was a randomised, single-blinded, phase I/II study at a single centre (Sahlgrenska University Hospital/Östra, Gothenburg, Sweden).

Between September 2012 and July 2013, 32 patients scheduled for colon resection due to colon cancer were screened and asked to participate in the study. The main inclusion criteria were age ≥18 years, ECOG performance status of 0–1, and resectable colon cancer/curative intent of surgery. The main exclusion criteria were concurrent other anti-tumour therapy, other malignant disease, severe systemic disease, and medications that could influence homocysteine, folate, and vitamin B12 status taken within 30 days of surgery. Included patients were randomly assigned (in a 1:1:1:1 ratio, with a block size of eight) to receive either Modufolin® or Isovorin® at a low (60 mg/m^2) or high (200 mg/m^2) dosage.

Treatment group assignment was based on a computer-generated randomisation list. One patient randomised to Isovorin® (60 mg/m^2) was withdrawn prior to administration of the study drug due to an adverse reaction towards the administered epidural anaesthesia. This patient was excluded from both the per protocol and safety analyses. One patient each from the Modufolin® (60 mg/m^2) and the Isovorin® (60 mg/m^2) groups were excluded from per protocol analysis because the pathological evaluations of the specimens yielded diagnoses of adenoma, and thus, no malignant tissue was available for analysis. However, the two patients were included in the safety analysis.

5 METHODS

The laboratory methods used in the papers are genotyping by real-time PCR and TaqMan chemistry, SNP-analyses, and LC-MS/MS, as detailed below. The analyses were performed according to Good Laboratory Practice, GLP, which refers to a quality system of management controls for research laboratories and organisations that aims to ensure the uniformity, consistency, reliability, reproducibility, quality, and integrity of chemical non-clinical safety tests.

5.1 Real-time polymerase chain reaction (PCR) with TaqMan chemistry

The man behind the polymerase chain reaction technique, Dr Kary B Mullis, presented the method in 1985 and received the Nobel Prize in 1993. Today, PCR is the gold standard method for amplifying DNA.

There are different reasons why one might want to amplify DNA. One reason is to simply create multiple copies of a rare piece of DNA, for example, in forensic medicine. However, in clinical medicine, the main reason is often that DNA analysis requires amplification in order to obtain enough DNA to provide a detectable signal for quantification. In ordinary PCR, a high temperature is used to obtain single-stranded DNA, after which the temperature is lowered in order to enable the primers to bind to the DNA sequence of interest. Four nucleotides, buffer solution, and a DNA polymerase enzyme are added, and DNA copying can begin. The reaction is usually performed in 40 cycles.

In ordinary PCR, the amplified DNA is run on an agarose gel, which is stained so that the gene of interest becomes visible as a band. The size and intensity of the band can then be measured. In real-time PCR, instead of looking at gel at the end of the reaction, the reaction takes place in an instrument that monitors the reaction with a detector that measure fluorescence.

When using TaqMan chemistry, a probe is marked with a fluorescent "reporter-fluorophore" at the 5´-end and a "quencher" at the 3´-end. TaqMan probes are designed in such a way that they anneal within a DNA region amplified by a specific set of primers. The principle relies on the 5´–3´ exonuclease activity of Taq polymerase to cleave a dual-labelled probe

during hybridisation to the complementary target sequence and fluorophore-based detection as the Taq polymerase extends the primer and synthesises the nascent strand. The 5' to 3' exonuclease activity of the Taq polymerase degrades the probe that has annealed to the template. Degradation of the probe releases the fluorophore from it and breaks the close proximity to the quencher, thus relieving the quenching effect and allowing fluorescence of the fluorophore. Hence, fluorescence detected in the quantitative PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR. The results are measured directly in the test tubes and presented as a graphic curve.

In the analysis, a known threshold value is programmed in the system, which stands for the level of fluorescence that the curve should pass in order to obtain a positive result.

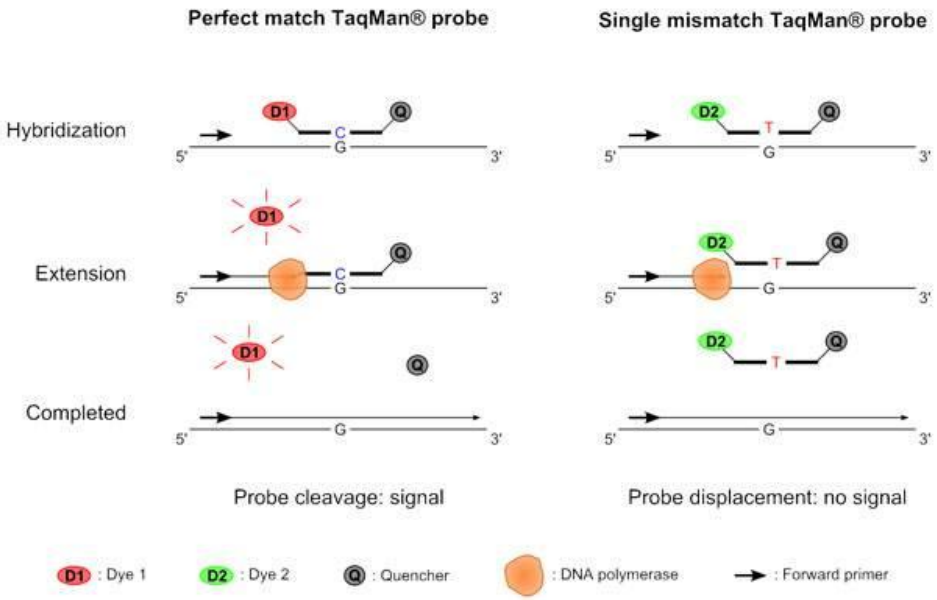

Figure 12. Real-time polymerase chain reaction (PCR) with TaqMan chemistry.

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5.2 SNP assays

All genotype analyses were performed by the Genomics Core Facility in Gothenburg, Sweden. The SNP assays (Applied Biosystems, Foster City, CA) and the TaqMan PCR master mix (Applied Biosystems) were aliquoted into a 384-well plate using a liquid-handling Biomek FX robot (Beckman Coulter Inc., San Diego, CA). Reactions were characterised by comparing the threshold cycle (CT) values as described by the manufacturer. Laboratory staff members involved in the genotyping were blinded to the clinical outcomes.

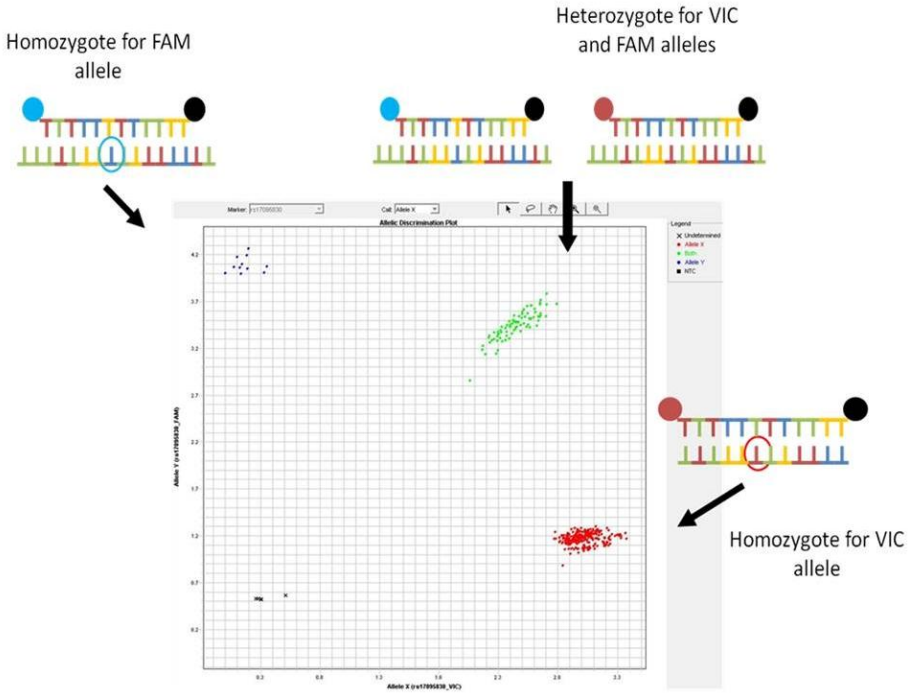

Figure 13. Illustration of SNP assays.

5.3 Liquid chromatography electrospray ionization tandem mass spectrometry

In papers II–IV, the specific folate forms in both mucosa and tumours were analysed. It is challenging to quantify different folates in tissues, due to their instability and sensitivity to the environment and the complexity in which they exist. A particular problem complicating the analysis is the partial conversion of methyleneTHF to THF induced by heat, light, oxygen, pH, and formaldehyde. LC-MS/MS is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (LC) with the mass analysis capabilities of mass spectrometry (MS). In the method used in papers II–IV, two mass spectrometers were coupled to the LC- equipment.

The LC-MS/MS technique that was used in paper III and IV was developed to analyse different folate forms by Odin et al. [136]. In short, the folate extraction method included homogenisation, heat, and folate conjugase treatment to hydrolyse polyglutamyl folate to monoglutamyl folate. The samples were then purified with ultrafiltration and inserted in the machine, where the various forms of folates were detected and quantified using positive electrospray. The analyses in papers II and III were performed locally, and the analyses in paper IV were performed both locally (tissue samples), and at the Charles River Laboratories; Edinburg, Great Britain (plasma samples).

5.4 Statistical analysis

There are many ways of describing data spread and distribution. Taken into consideration is also the art and type of data, such as categorical or numerical data, which have to be treated differently. Detailing the mean or median values follows a common manner, using a range of lowest/highest values. At times, interquartile range or standard deviations (the average of all differences from the mean) provides better information on the degree of variation. In paper I, results regarding side effects are presented as the risk and relative risk (odds ratio), and the overall survival is presented with a Kaplan–Meier estimator.

Yet another method was used in paper II, wherein 5,10-methyleneTHFMTHF percentages of the total folate amount were used as the interindividual variation in concentration. We also assumed more

equilibrium in the tissue compared with the potential dynamics in papers III and IV, where the folates were supplemented.

Standard statistical testing procedures involve the development of a null hypothesis, which is a general statement or default position that states there is no relationship between two quantities. When testing whether the result of a study is significant, different tests can be used.

Student's t-test is used when there are two independent groups and the researcher wishes to compare mean values between the two groups. The test demands that the data are normally distributed. Only data from an interval or ratio scale can be normally distributed. For larger numbers of numerical data, it can be assumed that there is a normal distribution. Deviations for normal distribution can be analysed and tested for skewness. If there are more than two groups that are analysed, an analysis of variance (ANOVA) is used.

If the data are not distributed normally, for small samples, or when the data are non-parametric, Fisher's exact test or the Mann–Whitney test can be used. These tests not only compare mean values, but the input values are also ranked after size. The Kruskal–Wallis test is the corresponding equivalent to the ANOVA testing if there are multiple groups.

The statistical power of a test is the probability that it correctly rejects the null hypothesis when the null hypothesis is false. Multiple problems have come to be associated with this framework, ranging from obtaining a sufficient sample size to specifying an adequate null hypothesis. Measurement processes that generate statistical data are also subject to error. Many of these errors are classified as random (noise) or systematic (bias), but there are also other important types of errors. In Type I errors, the null hypothesis is falsely rejected, giving a "false positive", and in Type II errors, the null hypothesis fails to be rejected and an actual difference between populations is missed, giving a "false negative".

In the presented work, descriptive statistics of demographic data are shown using contingency tables. SAS/JMP statistical software was used for the statistical analysis (SAS Institute Inc./.CA). The significance level set throughout the studies was 95%.

6 RESULTS

6.1 Paper I

There was no association between any polymorphism and demographic factors or pathological data, such as local tumour stage or differentiation grade. Furthermore, there was no association between MTHFR and MTR genotype prevalence.

Patients with MTHFR CC genotype had a lower risk of suffering nausea (p=0.027) and paraesthesia (p=0.0042) and needed to have their drug dose reduced less often (p=0.025) than patients with the CT/TT genotype. The paraesthesia side effect was clearly also associated, to a large extent, with the oxaliplatin combination therapy. Patients with the MTHFR CC genotype in combination with MTR 2756 AA genotype experienced better survival after adjuvant chemotherapy (p=0.034).

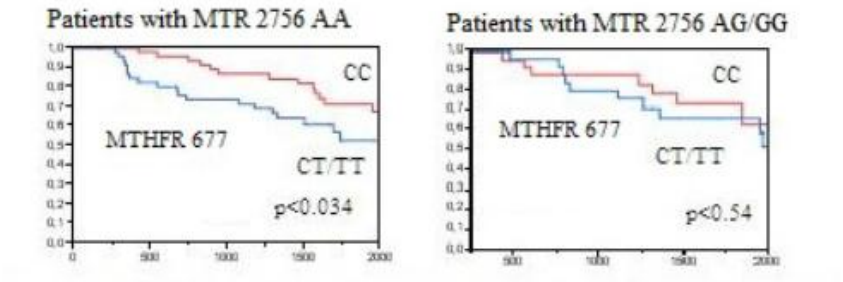

Figure 14. Survival after adjuvant chemotherapy in stage III colorectal cancer correlated to gene polymorphysis in functional polymorphisms in MTHFR and MTR.

The risk of suffering side effects was dependent upon the combination of genotypes, where patients with a MTHFR 667 CC and MTR 2756 AA genotype carried the lowest risk of 5-FU-related toxicity. There was no statistical association between MTR 2756 genotype by itself and survival or risk of side effects. A sub-group analysis, excluding patients who started with a lower dose due to high age or co-morbidity, showed that patients with the genotype MTR AG/GG were more likely to need a reduction in dose (58.6% vs. 76.6%, p=0.038) and pre-emptive treatment termination than those with the AA genotype (relative risk 2.1, p<0.03).

6.2 Paper II

The distribution and levels of THF, 5-methylTHF, and 5,10-methyleneTHF in the tumour and mucosa tissues are presented below. The results display large individual variation in folate levels in both tumour and mucosa tissue. The genotype frequencies of the different polymorphisms, as well as both the actual folate concentrations and their distribution by percentages are shown.

Table 2. Folate levels in tumour and mucosa

	TUMOR MEAN ± SD (RANGE)	MUCOSA MEAN ± SD (RANGE)	*P*-VALUE[B]
Total folate amount[a]	2705 ± 2006 (280–10870)	2149 ± 1206 (540–5697)	0.012
THF[a]	1154 ± 809 (120–4400)	891 ± 427 (364–2153)	0.014
5-methylTHF[a]	552 ± 568 (40–2250)	528 ± 436 (65–2200)	0.31
5,10-methyleneTHF[a]	985 ± 714 (24–3454)	768 ± 507 (41–2683)	0.0045

Notes: [a]The folate concentration equals pmol/g wet weight tissue. [b]*P*-value was calculated by Wilcoxon signed rank test.

Folate forms that represent the substrates and products of the MTHFR and MTR enzymes were analysed according to the genotypes MTHFR C677T and MTR A2756G, respectively, whereas 5,10-methyleneTHF (the cofactor that binds to TS) and THF (the precursor of 5,10-methyleneTHF) were analysed according to the 5'-TSER 28 bp and 3'-TSUTR 6 bp deletion/ insertion genotypes.

The results showed that there were no statistically significant differences in the mean concentration of any folate in the mucosa or tumour tissue in relation to the analysed polymorphisms (data not shown).

Table 3. Percentage levels of 5,10-methyleneTHF and 5-methylTHF by the MTHFR C667T polymorphism.

MTHFR C677T		5,10-METHYLENETHF (% ± SD)		5-METHYLTHF (% ± SD)	
GENOTYPE	N	TUMOR	MUCOSA	TUMOR	MUCOSA
CC	30	39.1 ± 12.9	33.9 ± 12.8	18.2 ± 12.0	21.7 ± 11.0
CT	18	35.6 ± 13.3	34.4 ± 12.7	19.7 ± 10.4	22.2 ± 9.4
TT	4	20.8 ± 9.7	31.6 ± 7.6	26.2 ± 12.1	26.2 ± 9.5
P-value[b]		0.033	0.93	0.31	0.71

Note: [a]In relation to the total folate concentration. [b]*P* by Kruskal—Wallis tests.

When comparing the folate levels expressed as the mean percentage of the total folate amount, the level of 5,10-methyleneTHF in tumours was found to differ according to the MTHFR C677T polymorphism. The level was highest in patients with the MTHFR CC genotype and lowest in patients with the TT genotype (p=0.033). Furthermore, a significantly lower percentage level of 5,10-methyleneTHF was found in the tumours of patients with the 5'-TSER 3R/3R genotype (p=0.0031). The total folate level, as well as the THF and 5,10-methyleneTHF levels, were significantly higher in the tumour tissue when compared with the mucosa tissue.

Table 4. Percentage levels of 5,10-methyleneTHF and THF by the 5'-TSER 28bp polymorphis.

5'-TSER 28 BP		5,10-METHYLENETHF (% ± SD)		THF (% ± SD)	
GENOTYPE	N	TUMOR	MUCOSA	TUMOR	MUCOSA
2R/2R	11	38.5 ± 7.8	32.5 ± 13.0	45.2 ± 10.3	47.1 ± 12.9
2R/3R	26	41.2 ± 13.9	34.8 ± 12.8	41.9 ± 12.5	44.8 ± 12.7
3R/3R	16	28.0 ± 12.1	34.1 ± 11.6	46.5 ± 14.2	39.3 ± 10.1
P-value[b]		0.0031	0.60	0.64	0.25

Notes: [a]In relation to the total folate concentration. [b]*P* by Kruskal—Wallis tests.

6.3 Paper III

Folate levels in tumours and mucosa

The mean levels of 5,10-methyleneTHF, THF, and 5-methylTHF were analysed in both tumour and mucosa tissues obtained from patients in each treatment group. The mean level of each folate increased with increasing dosage of LV and showed large inter-patient variation in all treatment groups. The folate levels differed significantly between the mucosa and tumour tissues and were generally lower in the mucosa of the control group. Patients who received 60 or 200 mg/m^2 LV had significantly higher mean levels of 5,10-methyleneTHF in their tumours compared to the levels in their mucosal samples. After treatment with 500 mg/m^2 LV, the difference in 5,10-methyleneTHF level between the tumour and mucosa samples was no longer statistically significant.

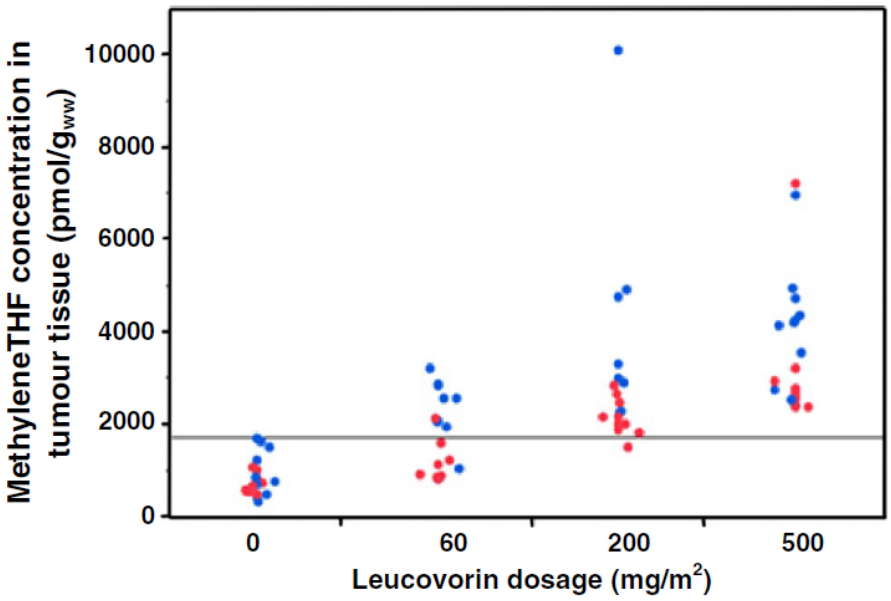

Figure 15. Comparison of the 5,10-methyleneTHF levels in the tumour tissues of patients with colon or rectal cancer after supplementation with 0, 60, 200, or 500 mg/m^2 LV in combination with 5-FU. Individual patients with colon cancer are represented by blue dots, and rectal cancer patients with red dots. The horizontal line marks the highest 5,10-methyleneTHF concentration found in the control patients (1714 pmol/g$_{ww}$).

The same pattern was seen when the THF concentration or the sum of 5,10-methyleneTHF and THF were analysed. No significant differences in the levels of 5-methylTHF were seen between the tumour and mucosa samples in any of the treatment groups. However, in contrast to the other folates, the 5-methylTHF level in the mucosa of the control group was significantly higher than the level in tumour tissue.

Folate levels and tumour location

During the data analysis, we discovered that there were differences between the folate levels in colonic and rectal tumours according to treatment doses. For all treatments groups, the mean 5,10-methyleneTHF levels in the rectal tumours were significantly lower than those in the colonic tumours.

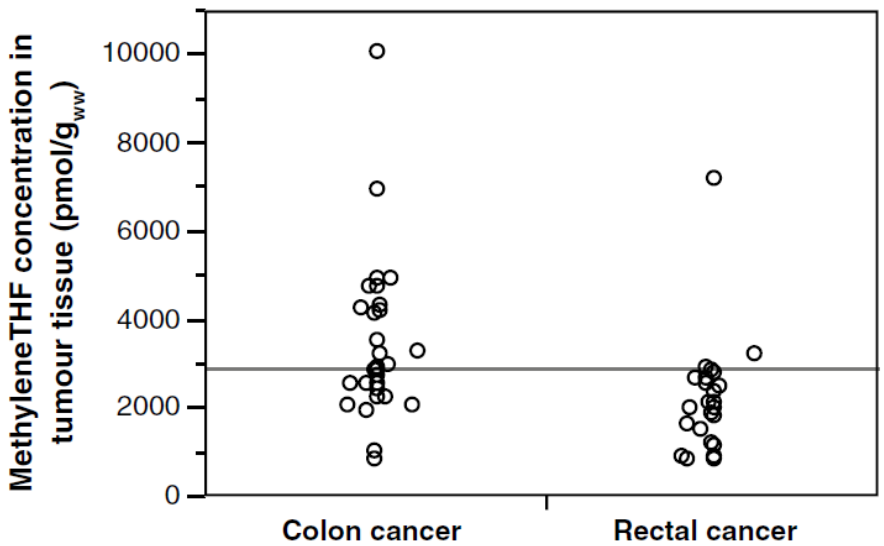

Figure 16. Comparison of the methyleneTHF concentration in tumour tissue of patients with colon (n = 29) or rectal (n = 28) cancer after FLV treatment. Each circle represents an individual patient. As shown, patients with rectal cancer had lower levels of methyleneTHF in their tumours. The horizontal line represents the grand mean.

The difference was significant in the groups that received 60 or 200 mg/m^2 LV. The THF level was significantly lower in the rectal tumours of patients who received 60 mg/m^2. The 5,10-methyleneTHF + THF levels were generally low in rectal tumours compared with colon tumours. In contrast, the 5-methylTHF levels were higher in the rectal tumour tissues of patients who were treated with LV, and a significantly higher level was seen after treatment with 60 mg/m^2. As shown in Figure 13, only 10 (50%) of the patients given 60 mg/m^2 LV achieved a 5,10-methyleneTHF level in their

tumour tissues that was above the highest value of any patient in the control group (1714 pmol/g_{ww}). At 200 mg/m^2 LV, all patients except one, reached 5,10-methyleneTHF levels >1714 pmol/g_{ww}, and at 500 mg/m^2 LV, all patients had 5,10-methyleneTHF levels above the level of that of the controls. Data was weighted according to time to vessel ligation, which was a parameter suspected to affect the tissue folate levels. However, this did not affect the significant differences between colon and rectal tumour tissue.

Blood analysis

A strong correlation was noted between the levels of LV in blood samples collected 10 and 30 minutes after the given dose of LV and the levels of the different folate forms in both tumour and mucosa samples. However, there was no correlation between the blood levels of LV and levels of folate in tissue in relation to the administered dosage of the drug.

6.4 Paper IV

6.4.1 Adverse Events (AE)

The total number of AEs, including five serious adverse events (SAEs), reported from the study (safety population, *n* =31) were 24, experienced by 14 patients. One patient could experience many AEs on the same occasion. For example, one patient in the group who received 200 mg/m^2 Modufolin® suffered from an anastomotic leak 5 days after surgery and hence experienced six different AEs, e.g., pyrexia, abdominal pain and vomiting. One patient, randomized to Isovorin® (60 mg/m^2), died of a cardiac arrest 2 days after surgery. This patient was included in both per protocol and safety populations since the patient was not in violation of the study protocol during the collection of plasma and tissue samples. One more patient was lost in follow-up 20 months after surgery, cause of death unknown. Postoperative complications including wound dehiscence, anastomotic leakage, and pulmonary embolism were among other SAEs. None of the AEs/SAEs were considered by the safety physician as having any suspected relationship with study treatments. No Suspected Unexpected Serious Adverse Reaction (SUSAR) occurred.

6.4.2 Pharmacodynamics

The differences in magnitude and significance of the analyzed folate metabolites after Modufolin® and Isovorin® administration, respectively, are shown by drug, dose and metabolite in Figure. 17.

Modufolin® administration resulted in significantly higher mucosa concentrations of 5,10-methyleneTHF ($p<0.01$ at both dose levels) and THF ($p<0.05$ at 60 mg/m^2, $p<0.01$ at 200 mg/m^2) than did Isovorin®. Higher concentrations of 5,10-methyleneTHF and THF were also observed in tumour tissue after Modufolin® administration. The concentration difference in tumour was statistically significant in favour for Modufolin® at 200 mg/m^2 while significance was not reached at the lower dose level for 5,10-methyleneTHF or THF.

A statistically significant linear correlation of tumour and mucosa concentrations was confirmed for all studied folate metabolites after Modufolin® (60 mg/m^2) treatment.

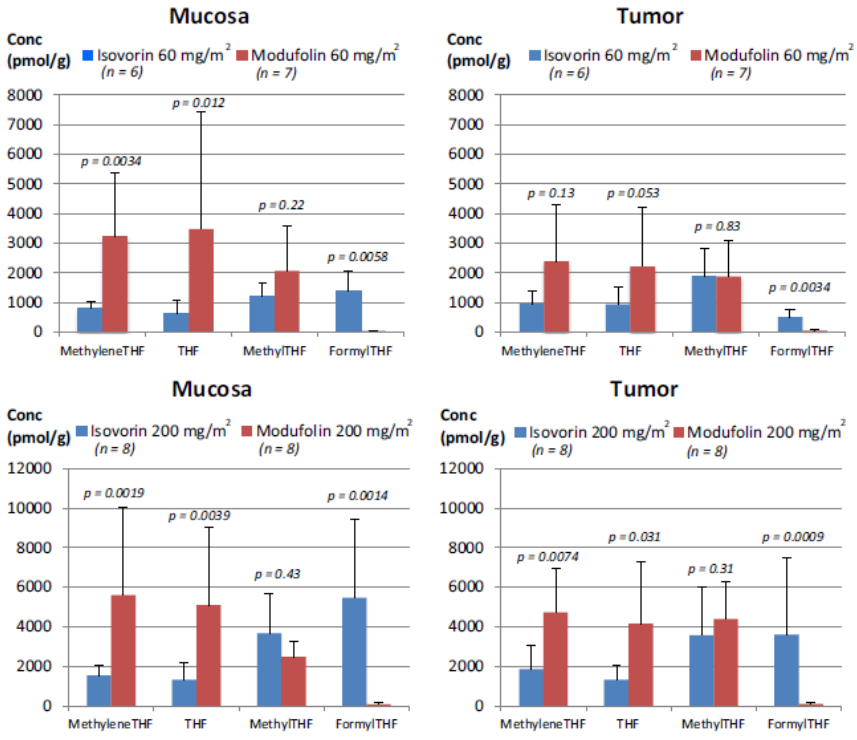

Figure 17. Comparison of mean concentrations (including SD) of methyleneTHF, THF, methylTHF, and formylTHF in mucosa and tumor after 60 or 200 mg/m^2 Isovorin® or Modufolin® (n=29, per protocol population).

6.4.3 Pharmacokinetics

The lower limit of quantification (LLOQ) for the folate metabolites in plasma ranged from 100-250 µg/L. The concentration of the folates in plasma samples collected 5-20 days before surgery, and on the day of surgery prior to drug administration, was below the LLOQ. The mean plasma concentrations of the folate metabolites versus time could not be fully evaluated for 5,10-methyleneTHF and THF after Isovorin® treatment or for 5-formylTHF after Modufolin® treatment because the major part of the obtained results for the assessed metabolites was below the LLOQ.

Administration of 200 mg/m^2 Modufolin® resulted in significantly higher maximal plasma concentrations (C_{max}) and longer lasting Area Under Curve (AUC_{last}) of 5,10-methyleneTHF ($p<0.01$) and THF ($p<0.001$) than did the same dose of Isovorin®. At the lower dosage, the resulting observations of both metabolites were below LLOQ for all patients except one in the

Isovorin® group, thus a statistical test of difference between treatments was not performed.

As in tissues, the 5-methylTHF levels in plasma were in the same order of magnitude after Modufolin® and Isovorin® administration. The calculated C_{max} and AUC_{last} ratios (Modufolin® to Isovorin®) were 0.7 at both dose levels. Following treatment with Isovorin®, 5-formylTHF was measured at high levels. Modufolin® on the other hand resulted in only one 5-formylTHF observation above LLOQ in the 60 mg/m^2 group and four in the 200 mg/m^2 group. Disregarding the relatively few observations for the Modufolin® groups the C_{max} and AUC_{last} Modufolin® to Isovorin® ratios were calculated to be 0.01 and 0.0017, respectively, at the high dose ($p<0.01$), and 0.026 and 0.0053, respectively, at the low dose (p not applicable).

6.4.4 Pharmacodynamics and Pharmacokinetics correlation

The presence of any linear correlations between tissue concentrations and plasma AUC_{last} observations of the different folate metabolites was explored for the different treatments. One statistically significant linear AUC_{last} correlation was identified in the Modufolin® (60 mg/m^2) group for 5-methylTHF in mucosa ($r=0.76$, $p<0.05$). No further correlations were found for any metabolite or treatment.

6.4.5 Time and demography

There was no statistical difference in folate levels related to age, gender or tumour location. Neither was there any relation between folate levels and tumour staging and pathology although there was a trend of higher folate levels along with a worse differentiation grade. Patients who suffered Severe Adverse Events, SAEs, had higher levels of THF in tumour tissue and mucosa ($p<0.05$). The time between ligation of main blood vessels and the biopsy sampling performed in the absolute connection with the removal of the tumour specimen did not affect the folate levels. The time from i.v. bolus of the study drug until vessel ligation did relate to tissue levels of 5,10-methyleneTHF ($p<0.05$) and 5-methylTHF ($p<0.01$) with shorter time being associated to lower folate levels. This was not seen in mucosa.

7 DISCUSSION

Although radical surgery is the current curative treatment for CRC, the focus of this thesis is directed at the use of chemotherapy. There are several different settings ranging from the adjuvant chemotherapy of stages II and III without visible remaining tumour, through neoadjuvant therapies that aim to ameliorate the chance of successful surgery, and palliative regimens for disseminated disease. Common to all of these settings are the risk of side effects and the fact that 5-FU is still a foundation for most of the treatment regimens. Given the large number of treatments worldwide and the fairly high risks encountered, including hospital stays and even mortality, any small step towards better efficacy or a decrease in side effects would be desirable.

The focus of paper I was the problem with side effects during treatment with 5-FU based chemotherapy. Although the Nordic FLV-regime is considered to be a fairly mild chemotherapy treatment, it is important to realise that many patients will end up with considerable side effects. Approximately 40% of the patients diagnosed with CRC are 75 years old or older [121]. There is relatively little experience in treatment with cytotoxic drugs in elderly patients. In fact, age represents a barrier for the inclusion of patients in clinical trials with new cancer therapies. In the most relevant studies on CRC, less than 20% of the patients were above the age of 70 years [122]. There is now solid data showing that elderly patients in good condition benefit from chemotherapy and do not suffer from more serious side effects than younger patients do [137, 138]. However, in elderly patients, it is most important to valuate comorbidity when chemotherapy is scheduled

The omnipresent problem of toxicity and growing insight in genomics and metabolomics leads to the suspicion that all individuals do not fully respond to LV as folate supplementation during 5-FU based chemotherapy and, therefore, do not experience an optimal treatment effect [89, 104, 139]. The search for predictive markers in order to select the patients who will benefit from 5-FU-based treatment is an important research topic. There have been many promising candidates with putative impact as both predictive as well as prognostic factors.

Previously, our own research group published a work wherein the gene expression of 18 interesting genes was examined in tumours of patients with CRC. Low thymidine phosphorylase (TP), dihydrofolate reductase (DHFR), dihydropyrimidine dehydrogenase (DPD), excision repair cross-

complementing 1 (ERCC1), and TS gene expression were associated with better time to progression in the entire study population. Low TP, DPD, and ERCC1 expression were independently associated with improved overall survival. Low TP gene expression was also predictive of response. This study suggests that TP gene expression in particular is a predictive as well as a prognostic biomarker for CRC patients with advanced disease. Gene panels assessing pre-treatment TP, DPD, ERCC1, DHFR, and TS gene expression may help improve the therapeutic potential of 5-FU as well as novel antifolate-based regimens [140].

While there are some biomarkers in use in clinical practise in other cancer areas, for example, HER 2 in breast cancer, few results have been as clear regarding CRC. Many suggestions have come up, such as the use of microsatellites, which are short repetitive DNA sequences that are common throughout the entire genome. However, DNA replication can result in mutations and variations in length, and if it accumulates, it can result in microsatellite instability (MSI). According to the extent of the instability, the tumours can be divided into MSI-high, MSI-low, and microsatellite stable (MSS). MSI-status has been suggested both as prognostic and predictive factor. Regarding 5-FU treatment, several studies have questioned whether MSI status can be regarded as a functional predictive factor [141, 142]. Another potential predictive marker is TP53; however analysis of TP53 is not used as a predictive marker today [123, 143, 144].

The only biomarker currently in use in CRC is K-RAS. This proto-oncogene is mutated in about 35% of all CRCs, and it is a predictive marker for the response to anti-EGFR-therapy, in which only patients with wild-type K-RAS benefit from treatment. However, whether K-RAS status has prognostic value remains controversial. Several studies have shown that K-RAS mutations are not predictive of response to 5-FU therapy [145, 146].

As discussed in paper I, CRC patients with the MTHFR 677 CC genotype had a significantly lower risk of chemotherapy-related toxicity and a better survival rate than those with CT/TT genotypes. These findings concur with those previously reported, including those of our own group. In paper I, there was a link between MTR genotype and the risk of a need of dose reduction due to toxicity. Previous findings on TS and MTHFR gene polymorphisms as predictive factors looked promising as described in several publications [19, 147]. However, the data have been hard to validate and have even occasionally been contradicted [148-150]. There are many difficulties in the search for predictive factors, as shown by the increased understanding about the molecular heterogeneity of CRC [151, 152]. The absence of validated

conclusive findings also suggests that the molecular heterogeneity makes the use of single markers more difficult. As noted by Tejpar et al., although there are many candidates, no real clinical use for any clinical marker, except for K-RAS, has yet been found [123].

This notion was also given some support in paper II. The large individual variation in the mean folate levels in both tumour and mucosa tissues found in the study could provide one explanation as to why the results are so difficult to reproduce and implement in a clinical setting. We expected to find larger differences in distribution of the folates related to the polymorphisms. However, complicating the interpretation of the data is the possibility that decreased enzyme activity could be compensated for by increasing the activity of other enzymes involved in the same folate pathway. As discussed in a study by Odin et al. high levels of folates might compensate for unfavourable genotypes [150]. This might lead to an overestimation of the clinical relevance of genetic polymorphisms.

In paper III, we wanted to analyse the actual levels of the enzymes THF, 5-methylTHF, and 5,10-methyleneTHF in tumour and mucosa tissue. As presented in the Results Section, the results are very interesting. In paper II, there was a difference between folate levels in tumour tissue compared to mucosa. In paper III, patients who received 60 mg/m^2 or 200 mg/m^2 LV had significantly higher mean levels of 5,10-methyleneTHF in their tumours, compared with the levels in their mucosal samples. After treatment with 500 mg/m^2 LV, the difference in 5,10-methyleneTHF level between the tumour and mucosa samples was no longer statistically significant. Comparing colon and rectal tumours, the difference in 5,10-methyleneTHF in tumours was significant in the groups that received 60 mg/m^2 or 200 mg/m^2 leucovorin, with rectal tumours being the lowest. The difference in folates between colon and rectum could be due to many factors. The first and second parts of the colon developed from the midgut, and the third distal part of the transverse colon and rectum developed from the hindgut. Differences in physiology and anatomy, environmental carcinogens, genetic mechanisms and the function of different parts of the colon and rectum might influence the cancer process[153].

The most interesting part of the results is the fact that among the patients receiving the standard dose of LV in the Nordic FLV regimen (i.e., 60 mg/m^2), only ten (50%) of the patients achieved a 5,10-methyleneTHF level in their tumour tissues that was above the highest value of any patient in the control group (1714 pmol/g_{ww}). At 200 mg/m^2 LV, all patients except one

reached 5,10-methyleneTHF levels >1714 pmol/g_{ww}, and at 500 mg/m^2 LV, all patients had 5,10-methyleneTHF levels above the level of the controls.

This study raises the question of whether the commonly used dosage of LV might result in a sub-optimal concentration of 5,10-methyleneTHF in the tumour tissue to provide an optimal effect of 5-FU treatment. Similar conclusions were drawn by Schlemmer et al. in a study published in 2008 that showed significantly higher values regarding reduced folates in tumour tissues as well as in liver metastases when doses of 200 mg/m^2 and 500 mg/m^2 were used [154]. The fact that the analysis showed that rectal tumours contained lower levels of 5,10-methyleneTHF compared to colon tumours after administration with LV might explain the lack of evidence regarding the effect of adjuvant 5-FU-based chemotherapy in rectal cancer.

Paper IV describes a phase I/II study in which we compared the two substances Modufolin® and Isovorin®. in patients with colon cancer. As mentioned previously, Modufolin® is commercially produced 5,10-methyleneTHF that does not need to be converted intracellularly in order to be active. 5,10-methyleneTHF is necessary in the formation of a ternary complex together with TS and the fluorinated metabolite of 5-FU, FdUMP. As seen in previous work, response to LV administration is associated with considerable intra- and inter-patient variability that could be explained by differences in folate metabolism [155-158]. The use of Modufolin® instead of LV might circumvent such metabolic obstacles. Thus, an abundance of 5,10-methyleneTHF in tumour tissue may be generated by using Modufolin®, which might lead to an increased response of 5-FU-based chemotherapy by stabilising the ternary complex [154, 159]. The possible influence on treatment effect using Modufolin® need to be addressed in future studies, as the current study was a pharmacological study of the folate and did not include 5-FU.

8 CONCLUSIONS

- The polymorphisms MTHFR C677T and MTR A2756G influence the activity of enzymes involved in folate metabolism. This could affect the risk of toxicity during adjuvant 5-FU-based chemotherapy and outcome in stage III CRC.

- Different combinations of gene polymorphisms may affect enzyme activity and could be a part of the explanation for some of the difficulties obtaining uniform results when using single gene polymorphisms as predictive markers.

- Total folate level was higher in tumour tissue than in mucosa tissue. No differences were found in the actual tissue folate levels or in their distribution, with respect to the polymorphisms in the MTHFR, MTR, or TS genes.

- There is a great deal of inter-patient variability in tissue folate levels in CRC patients after supplementation with LV at a standardised dosage.

- High LV doses were needed to exceed baseline 5,10-methyleneTHF values, especially in rectal cancer patients. The results indicate that the currently used LV dose might be insufficient to attain the full antitumoural effect of 5-FU.

- Modufolin® administration resulted in significantly higher 5,10-methyleneTHF levels than Isovorin® did. As 5,10-methyleneTHF is of importance for 5-FU effect, further studies will reveal whether Modufolin® may potentially increase the efficacy of 5-FU-based chemotherapy.

9 FUTURE PERSPECTIVES

CRC is a heterogenic disease. It has been shown that the development from a normal cell to a cancer cell in the colon and rectum takes place through many different pathways. The role of the immunological response, the influence of the microbiotic flora, and the genetic changes in the carcinogens process need to be examined further. According to its molecular features, CRC is not just one disease. So far, five different subtypes have been described, which might influence the cancer process [151, 152]. In addition, the metastatic process provides further molecular diversity [160].

Today, the possibility of individualising chemotherapy for patients with CRC is quite limited. In the future, new developing techniques will most likely facilitate the possibility of screening an individual tumour in order to have a "molecular fingerprint". However, the genetic pattern in CRC is very complex, and genetic changes will most certainly act co-dependently. As such, genetic mapping of the tumour is needed to investigate multiple genes in every patient. As technology develops, the cost and time involved with these tests will most certainly decrease. With individual genetic pattern for genes of interest for every tumour, patient selection when designing clinical studies has a possibility to be more specific. Although CRC is a common form of cancer, this development will demand a structured collaboration nationwide, and preferably, internationally, to ensure that the clinical study will reach enough power, and facilitate the development of new substances. This approach presents a realistic opportunity for personalized therapy.

However, we also have to focus on the drugs that are used today!

As pointed out several times, while 5-FU is an old substance, it is still the cornerstone of many cancer regimens. It is of great importance that enough 5,10-methyleneTHF is provided in the tissue to enhance the formation of the ternary complex. As shown in our study, Modufolin® administration will result in significantly higher levels of 5,10-methyleneTHF in tissues. The next task will be to investigate how Modufolin® functions together with 5-FU in a clinical setting. The optimal timing of providing LV in relation to the administration of 5-FU has been debated. A clinical use of Modufolin® will demand new pharmacological studies in order to optimise the timing of drug administration to provide for the best clinical outcome for the patients receiving 5-FU- based treatment.

10ACKNOWLEDGEMENT

No Man Is An Island.

This thesis would never have been made without a lot of support by many others. I would like to express sincere gratitude to all of those wonderful persons who have helped me during this process, in particular:

Kristoffer Derwinger associate professor, my fantastic supervisor, who has showed endless patience and support with me and my work and has also been a wise friend. Sometimes demanding, always results-oriented, always understanding. Thank you for letting me grow into research on my terms. "You know more than you think you do" is a typical Kristoffer answer. I could not have wished for a better supervisor.

Yvonne Wettergren, associated professor, my excellent co-supervisor, for being a fantastic researcher, always curious, positive, and full of ideas. You have a pedagogic talent that I envy and you have been tremendously helpful 24/7 over many years. Looking forward to many more projects!

Bengt Gustavsson, professor, head of the Surgical Oncology Laboratory at Sahlgrenska University Hospital. Thank you for being kind and calm and for always generously sharing your knowledge. Very few in the world know more about 5-FU and folates then you do. It took some time, but when I realised that one of my passwords was the name of an enzyme, I knew I was hooked!

Marianne Hall-Angerås, associate professor, former head of the Department of Surgery at Sahlgrenska University Hospital/Mölndal, for being a true role model and, for a short period due to administration reasons, also my supervisor! You are one of a kind!

Ulf Angerås, associate professor, former head of the Department of Surgery at Sahlgrenska University Hospital/Östra, for being a very encouraging boss. You were always supportive when I was a nervous young resident, and I miss your daily jokes during the morning reports.

Anders Hyltander, associate professor, former head of the Department of Surgery at Sahlgrenska University Hospital/Östra, for believing in me and allowing me to try my wings.

Per Lindnér, associate professor and present head of the Transplant Unit at Sahlgrenska University Hospital, for hiring me, being supportive of my research, and always creating a positive atmosphere.

Peter Naredi, professor, head of the Department of Surgery at Sahlgrenska Academy, for being skilled, inspiring, enthusiastic, and always friendly. You make people grow.

My collaborators at the Surgical Oncology Laboratory:

Hillevi Björkqvist and **Ann-Louise Helminen**, for being great research nurses, always on the run, collecting samples, and informing patients with a huge amount empathy and knowledge. Also warm-hearted, always listening, and comforting when I needed you to be.

Marianne Åkerström and **Jaquline Falch** for excellent technical skills and being so fun to be around!

Lena Munro, for keeping the clinical database in perfect shape and for being the first nurse who showed me how to take blood samples almost 25 years ago…

Elisabeth Odin, co-author, for your technical skill and trained research eyes, which helped me avoid many mistakes.

Göran Carlsson, co-author, Senior Consultant at the Department of Oncology, as well as surgeon, for sharing your great knowledge in chemotherapy treatment as well as science.

Elinor Bexe-Lindskog, my companion through this research journey, for being the one who always listens, laughs, and solves my administrative problems. I admire you for so many reasons.

My former colleagues at the Department of Surgery SU/Östra and SU/Sahlgrenska for encouragement clinical and academic support as well as friendship.

My present colleagues at the Transplant Unit at Sahlgrenska University Hospital, a team that always puts their own personal comfort aside and focus on the patients no matter what! For me, you are top knives. Looking forward to drinking more weak coffee in the middle of the night with you.

My friends in the Swedish Surgical Association, especially in the Education Committee, for broadening my perspective and for fruitful discussions on education, surgery and life.

Åsa, Katja, Annika, Marie, Christina, and **Katarina,** my friends from medical school as well as from the medical ballet... It takes a while to become old friends. Thank you for always being there.

The big and lovely Taflin family in Sweden and abroad. Life would be boring and quiet without you. Thank you for letting me be a part of your family!

Eva Gustafsson, for being a wonderful mother and grandmother, always telling me to relax and take it easy. Keep nagging; perhaps someday I will listen. Love you.

Bengt Gustavsson, my father (yes, it is the same man), for inspiring me and always letting me feel that I can do whatever I want. I am proud to be your daughter.

Sofie, Lotta, and **Anders,** my talented siblings, for being so hilarious to be around, never missing a single pun! I can't understand why our spouses don't find it as much fun as we do. It's a privilege to have you as my family, always debating, always caring.

Kristian Taflin, love of my life and soul mate. It's easy to fly when one has a co-pilot like you. Thank you for always being supportive and encouraging and a wonderful husband and father. There is never a dull moment together with you and I love it.

Finally: Hanna, Victor, and Julia—Team Taflin.

Finaste barnen, älskar Er härifrån till universums slut och tillbaka. Kram, mamma.

This work was supported by grants from the Swedish Cancer Society, the Swedish state under the LUA-ALF agreement, theAssar Gabrielsson Foundation for Cancer Research, the Gothenburg Medical Society, The Lions Cancer Foundation West, and the Bror och Anna-Lisa Björnsson Foundation.

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