Biomarkers of One-carbon Metabolism in Colorectal Cancer Risk

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Tillägnad:

Alla de människor i Västerbotten som valt att delta i Northern Sweden Health and Disease Study. Tack för er tid och ert engagemang.
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Abstract

One-carbon metabolism, a network of enzymatic reactions involving the transfer of methyl groups, depends on B-vitamins as cofactors, folate as a methyl group carrier, and amino acids, betaine, and choline as methyl group donors. One-carbon metabolism influences many processes in cancer initiation and development such as DNA synthesis, genome stability, and histone and epigenetic methylation. To study markers of one-carbon metabolism and inflammation in relation to colorectal cancer (CRC) risk, we used prediagnostic plasma samples from over 600 case participants and 1200 matched control participants in the population-based Northern Sweden Health and Disease Study cohort.

This thesis studies CRC risk with respect to the following metabolites measured in pre-diagnostic plasma samples: 1) folate, vitamin B12, and homocysteine; 2) components of one-carbon metabolism (choline, betaine, dimethylglycine, sarcosine, and methionine); and 3) three markers of different aspects of vitamin B6 status. In addition, this thesis examines three homocysteine ratios as determinants of total B-vitamin status and their relation to CRC risk.

In two previous studies, we observed an association between low plasma concentrations of folate and a lower CRC risk, but we found no significant association between plasma concentrations of homocysteine and vitamin B12 with CRC risk. We have replicated these results in a study with a larger sample size and found that low folate can inhibit the growth of established precancerous lesions.

Using the full study cohort of over 1800 participants, we found inverse associations between plasma concentrations of the methionine cycle metabolites betaine and methionine and CRC risk. This risk was especially low for participants with the combination of low folate and high methionine versus the combination of low folate and low methionine. Well-functioning methionine cycle lowers risk, while impaired DNA synthesis partly explains the previous results for folate.

We used the full study cohort to study associations between CRC risk and the most common marker of vitamin B6 status, pyridoxal' 5-phosphate (PLP), and two metabolite ratios, PAr (4-pyridoxic acid/(PLP + pyridoxal)) estimating vitamin B6 related inflammatory processes and the functional vitamin B6 marker 3-hydroxykynurenine to xanthurenic acid (HK:XA). Increased
vitamin B6-related inflammation and vitamin B6 deficiency increase CRC risk. Inflammation was not observed to initiate tumorigenesis.

Total B-vitamin status can be estimated by three different recently introduced homocysteine ratios. We used the full study cohort to relate the ratios as determinants of the total B-vitamin score in case and control participants and estimated the CRC risk for each marker. Sufficient B-vitamin status as estimated with homocysteine ratios was associated with a lower CRC risk.

These studies provide a deeper biochemical knowledge of the complexities inherent in the relationship between one-carbon metabolism and colorectal tumorigenesis.

**Keywords**

Colorectal cancer, one-carbon metabolism, biomarkers, folate, epidemiology.
Original Papers

This thesis is based on the following papers:


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Abbreviations

BHMT, betaine-homocysteine methyltransferase; CIN, chromosomal instability pathway; CUP, The Continuous Update Project; CIMP, CpG island methylator phenotype; CRC, colorectal cancer; CRP, C-reactive protein; CBS, cystathionine β-synthase, CSE, cystathionine γ-lyase, dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; DHFR, dihydrofolate reductase; DMG, dimethylglycine; EPIC, European Prospective studies into Cancer and Nutrition; FAP, familial adenomatous polyposis; 5-FU, fluorouracil; f-THF, 10-formyltetrahydrofolate; HNPCC, Hereditary non-polyposis colorectal cancer; HAA, 3-hydroxyanthranilic acid; HK, 3-hydroxykynurenine; KYNU, kynureninase; KAT, kynurenine aminotransferase; MSP, Mammography Screening Project; me-THF, methylene-THF; MSI microsatellite instability; MSS, microsatellite stable tumors; MSI-H, microsatellite instability; NSHDS, Northern Sweden Health and Disease Study; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; MMA, methylmalonic acid; m-THF, 5-methyltetrahydrofolate; MONICA, the Northern Sweden Monitoring of Trends and Determinants in Cardiovascular Disease cohort; NTD, neural tube defects; PEMT, phosphatidylethanolamine N-methyltransferase; PL, pyridoxal; PLP, pyridoxal’ 5-phosphate; PA, 4-pyridoxic acid; ROC, receiver operating characteristics; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SHMT, serine hydroxymethyltransferase; SNPs, single nucleotide polymorphisms; SDMA, symmetric dimethylarginase; THF, tetrahydrofolate; tHcy, total homocysteine; TYMS, thymidylate synthase; WHI-OS, Women’s Health Initiative Observational Study; VIP, the Västerbotten Intervention Programme; XA, xanthurenic acid.
Sammanfattning på svenska

Tjocktarmcancer (Kolorektal cancer (CRC)) är en av de vanligaste cancerformerna och också en av de vanligaste anledningarna till dödlig utgång efter en cancerdiagnos. Den typiska patienten är diagnostiserad i 60-70 årsåldern men det finns även människor som bär på olika former av CRC med en stark ärtftlig del och dessa människor är ofta diagnostiserade tidigare i livet. Det tar många år för CRC att utvecklas från en polyp till cancer som kan sprida sig till andra delar av kroppen. Detta gör att screening för CRC med hjälp av koloskopi och feces-prover har goda möjligheter att kunna hitta en tidig cellförändring och verkar därmed kunna minska risken för dödlighet på grund av CRC.

Det finns flera etablerade riskfaktorer för CRC, de med starkast påverkan är ålder, manligt kön och en familjehistoria där flera andra i den närmsta släkten blivit diagnostiserade med CRC. Flera livsstilsfaktorer spelar också roll, som rökning, stillasittande, bukfetma, stress och sömnstörningar. En diet med stort intag av rött kött och processat rött kött, men lågt intag av grönsaker, fibrer, mjölkprodukter och fullkorn verkar öka risken för att drabbas av CRC.

Trots att vi vet om många riskfaktorer för CRC så är det ingen av dessa som är väldigt starkt kopplat till risk för CRC, som rökning vid lungcancer, humant papillomvirus vid livmoderhalscancer, eller hög alkoholkonsumtion vid levercancer. Vi vet också att även om genetik och ärftlighet spelar roll, så spelar livsstilsvariabler i de flesta fall en större roll. Det finns därför ett outforskat glapp som inte kan förklaras av genetiska orsaker och som ej heller fullt ut förklaras av de livsstilsfaktorer som identifierats som riskfaktorer.

En av de faktorer som har setts ha en potential för att förklara att vissa människor får CRC och andra inte är folat eller folsyra (den syntetiska formen av folat). Folat är en B-vitamin som samverkar med flera andra B-vitaminer (vitamin B2, B6 och B12) i ett metabolt nätverk i kroppen kallat enkolsmetabolism. Enkolsmetabolism är ett system för hantering och överföring av enkolsglutar (även kallade metylgrupper). Flera andra metaboliter spelar också roll, som kolin, betain och flera sorts proteinbyggstenar (aminosyror). Nätverket består också av flera enzymer (proteiner som påskyndar metabola reaktioner i cellen) som kan påverka balansen av olika metaboliter inom enkolsmetabolism. Förenklat så har enkolsmetabolism tre huvudsakliga slutmål: byggstenar för DNA och RNA, metylgrupper för metylgruppsdonatorn S-adenosylmetionin (SAM) som ansvarar för huvuddelen av metyleringsreaktioner i kroppen och nybildning
av aminosyran cystein som används för den kroppsegna antioxidanten glutation.

Både metylering och DNA-syntes har en direkt påverkan på cancercellers tillväxt. Störningar i metylерingsprocesser kan leda till att gener som ökar cancercellers förmåga att överleva och sprida sig uttrycks mer, och att gener som hindrar cancerutveckling tystas. Göt tillgång till byggstenar för DNA ger ett mer stabilt genom, men kan även vara nödvändiga för att snabbt delande celler, som cancerceller, ska kunna fortsätta sin tillväxt. Just folat är en av de faktorer som har diskuterats kunna ha en dubbel roll vid cancerutveckling, minskar risken att normala celler utvecklas till cancerceller, men öka livskraften hos redan existerande pre-cancerösa celler.


Vi har använt blodprover insamlande före diagnos av CRC hos över 600 deltagare och runt 1200 matchande friska kontrolldeltagare som deltagit i Northern Sweden Health and Disease Study (NSHDS). Vi har jämfört nivåerna av enkolsmetaboliter i blodet hos de som utvecklat CRC och de som inte gjort det för att bedöma om det finns en koppling till CRC-risk. Vi gjorde först en valideringsstudie på en tidigare studie som visade minskad risk för CRC kopplat till låga nivåer av folat och kunde reproduera de tidigare fynden. Därefter studerade vi ett flertal metaboliter inblandade i enkolsmetabolism och såg en koppling mellan höga nivåer av betain och metionin till lägre risk för CRC. I en tredje studie undersökte vi olika aspekter av vitamin B6 i relation till CRC-risk. Vitamin B6 i blodet är inte bara en markör för intag av födoämnen med vitamin B6 utan är även känt att minska vid inflammatoriska processer i kroppen. Vi såg att låga nivåer av vitamin B6 var associerade med högre CRC-risk. Vi såg också att detta samband kunde tänkas ske via interaktioner med inflammatoriska processer, då en markör för vitamin B6 vid inflammation var kopplat till en högre risk för CRC. I vår avslutande studie undersökte vi flera markörer för total B-vitaminstatus i relation till CRC-risk. Vi fann att dessa markörer på ett tillfredsställande sätt speglade total B-
vitaminstatus och att total B-vitaminstatus samt dessa markörer var kopplade till en lägre risk för CRC.

Sammantaget har våra studier visat att enkolsmetabolism är associerat med CRC-risk. De flesta metaboliter inom enkolsmetabolism verkar vara skyddande eller neutrala gentemot risk för CRC, förutom folat, där låga nivåer verkar minska risken. Hög total B-vitaminstatus, vilket inkluderar folat, verkar dock vara sammankopplat med en minskad risk. Våra resultat visar att det är tänkbart att det är folats roll vid syntes av DNA som kan öka risken för CRC. Detta överensstämmer med hypotesen om att folat skulle ha en dubbel roll vid cancerutveckling.
Background

Colorectal Cancer

Incidence and Mortality

Colorectal cancers (CRC) are malignant epithelial tumors of the colon and rectum, also called adenocarcinomas. Globally, colorectal cancer is the third most commonly diagnosed cancer. Each year 1.4 million new cases are diagnosed and 600,000 individuals die from CRC, making it the fourth most common cause of cancer death. In Europe, CRC is the second most common cause of cancer death, although mortality rates range from comparably low in Scandinavia and Western Europe to more prominent in parts of Central Europe. The cumulative incidence and mortality of CRC in Sweden and in the Northern Sweden Health and Disease Study (NSHDS) cohort are depicted in Figure 1. In Sweden, as in the rest of the world, CRC incidence increases sharply after age 50. However, a recent increase in CRC incidence among younger persons has been observed, especially in rectal cancers. Men are almost twice as likely as women to be diagnosed with rectal cancers and are also more likely to be diagnosed with distal colon cancer, while both men and women carry the same risk for proximal colon cancer diagnosis.

![Cumulative incidence and mortality of CRC](image)

**Figure 1.** Cumulative risk of incidence and mortality in men and women in Sweden and in the NSHDS cohort across different age categories. Data from the NSHDS and NORDCAN © 2017 Association of the Nordic Cancer Registries, IARC (Assessed 8/7/17).
**Staging and Prognosis**

The overall five-year survival after CRC diagnosis has gradually improved over the last several decades, especially in wealthier countries. In many parts of Europe, Australia, and the US, the five-year survival is approximately 65%, although in lower income countries the five-year survival is still below 50%. Prognosis after CRC diagnosis varies depending on tumor stage. Stage is classified based on local spread (T stage), lymph node involvement (N stage), and distant metastasis (M stage). These factors are combined into an overall measure called the TNM stage (ranging from I to IV with several subclasses). The TNM stage provides valuable prognostic and treatment information. Most importantly, survival varies according to TNM staging. Patients diagnosed with stage I CRC have excellent survival, but only a small percentage of patients diagnosed with stage IV tumors are still alive five years after diagnosis. Figure 2 shows the five-year cancer-specific survival according to stage in over 100 000 CRC case participants diagnosed between 1991 and 2000. For cases diagnosed between 2006 and 2012, five-year cancer-specific survival for all stages combined has increased from 65.2% to 66% for colon cancer and 68% for rectal cancer; however, information about stage is less precise in late surveys.

Several molecular features in the CRC tumors, described in more detail below, have been associated with CRC prognosis. Better clinical outcome is associated with a higher density of tumor-infiltrating T-cells. T-cell density is also associated with microsatellite instability (MSI), a positive prognostic marker. On the other hand, mutations in the *BRAF* and *KRAS* oncogenes are associated with poor overall prognosis, but this association is confined to tumors with microsatellite stable tumors (MSS). Cancer-specific survival in patients with mutations in the *PIK3CA* oncogene has been observed to be longer in patients who used aspirin regularly after diagnosis although this association was not observed in patients with wild type *PIK3CA*. Survival also varies depending on patient characteristics. For example, cancer cachexia – muscle and fat storage wasting stimulated by tumors – is reported to lead to death in at least 20% of cancer patients. As cachexia often sets in before the diagnosis, unexplained weight loss should be seen as a warning sign of cancer and could explain the better clinical outcome associated with being overweight (BMI 25-30) at diagnosis. In addition, as with many other cancers, smoking is associated with poor clinical outcome.
**Figure 2.** Sankey graph showing the distribution of TNM stages in CRC (left) and five-year CRC-specific survival depending on TNM staging (right). **BLUE** represents patients who are alive or dead from causes other than CRC five years after diagnosis. **ORANGE** represents patients who died from CRC within the same period. Tumors with unknown stage are excluded. Adapted from O’Connell et al.15

**Colorectal Tumorigenesis and Heterogeneity**

In 1990, Fearon and Vogelstein published a groundbreaking article that described specific pathways in the progress from colorectal adenoma to carcinoma. They found that a stepwise pattern of genetic and epigenetic alterations activating oncogenes and silencing tumor suppressor genes ultimately leads to cancer. The great bulk of sporadic colorectal tumors was proposed to progress through the chromosomal instability pathway (CIN), which is characterized by mutations in oncogenes and tumor suppressor genes.26 Furthermore, around 15-20% of tumors are categorized by the loss
of expression of DNA mismatch repair proteins. These mismatch repair
deficient tumors display a high level of microsatellite instability (MSI-H) due
to deletion and insertion mutations at microsatellites spread throughout the
genome.\textsuperscript{1} \textsuperscript{17}

Molecular subtyping of CRC tumors has revealed several important tumor
characteristics besides MSI, and mapping the heterogeneity of CRC tumors
has been based on mutations in the \textit{BRAF} and \textit{KRAS} oncogenes as well as CpG
island methylator phenotype (CIMP) status.\textsuperscript{27} CIMP tumors are characterized
by hypermethylation of cytosine in the symmetrical dinucleotide CpG,
specifically in promoter regions in the DNA, leading to silencing of tumor-
suppressor genes.\textsuperscript{28} Mutations in \textit{KRAS} and \textit{BRAF} are oncogenic driver
mutations that provide CRC cells with self-sufficient growth signals via the
MAPK pathway. MAPK signals regulate proliferation, differentiation, and cell
motility through phosphorylation of transcription factors.\textsuperscript{28} \textsuperscript{29} Almost all
sporadic MSI-H tumors have mutations in the \textit{BRAF} oncogene and extensive
DNA methylation, although patients with Lynch syndrome have MSI-H
tumors but no \textit{BRAF} mutation. These characteristics mean that a combination
of the two tests can help identify affected patients and families.\textsuperscript{30}

In 2015, the CRC Subtyping Consortium established four molecular subtypes
for CRC: MSI immune (14%), canonical (37%), metabolic (13%), and
mesenchymal (23%).\textsuperscript{31} The MSI immune subgroup is characterized by MSI,
CIMP-high, and \textit{BRAF} mutations, the metabolic subgroup by \textit{KRAS} mutation
and CIMP-low, and the canonical and mesenchymal subgroups by frequent
somatic copy alterations and by Wnt signaling pathway activation and
amplification of the Myc transcription factor or stromal infiltration,
respectively.\textsuperscript{31} That is, compared to the classification described by Fearon and
Vogelstein, the MSI immune subgroup represents the mismatch repair
deficient subtype and the CIN is divided into three distinct groups.

\textbf{Treatment}

Localization in the colorectum and TNM stage are important factors when
deciding on treatment strategies. Preoperative T-stage is best determined with
MRI in rectal cancers, \textsuperscript{32} \textsuperscript{33} and more advanced stages are treated with
neoadjuvant radiotherapy.\textsuperscript{34} For rectal cancers, the rectum, mesorectum, and
surrounding fascia are surgically removed;\textsuperscript{35} however, for colon cancers, a
colectomy is performed where the tumor and corresponding lymph nodes are
resected.

The use of chemotherapy depends on the TNM stage of the tumor. Stage I
tumors are often completely removed and require no adjuvant chemotherapy
to decrease the risk of recurrence. More advanced colonic tumors have a high chance of recurrence after surgical removal and adjuvant chemotherapy is recommended. The antifolate agent fluorouracil (5-FU) functions by inhibiting thymidylate synthase (TYMS) and is the basis of chemotherapeutic practice in CRC.36, 37 Inhibition of TYMS deprives cells of the only de novo source of the DNA nucleoside thymidine.38 TYMS uses 5,10-methylenetetrahydrofolate (me-THF) as a methyl group donor and the reduced me-THF derivative leucovorin (folinic acid, not to be confused with folic acid) increases the inhibition of TYMS by 5-FU and improves clinical outcome parameters compared to 5-FU alone.36, 37, 39 Stage IV tumors are tumors with distant metastasis at one or more sites, most often in the liver (~13% of all CRC tumors), lung (~5%), bone (<1%), and brain (<1%).40 Prognosis, especially for patients with bone or brain metastasis, is poor and treatment is a combination of cytotoxic drugs.40

Future oncological treatments may target the molecular characteristics of specific tumors.41 Already, patients with mutations in the oncogenes BRAF or KRAS are known to be poor responders to epidermal growth factor inhibitor therapy.42-45

Screening

The transition time through the adenoma-carcinoma sequence is slow, and the mean sojourn times (the period from when the tumor first could be detected by screening until diagnosis) for advanced adenoma to CRC diagnosis has been estimated to vary between five and 20 years, as the average annual transition rate is between 5% and 20%.46-48. Given this slow progression and the relative ease of curative surgery, screening for CRC has great potential compared to other cancers.48 Randomized trials on yearly screening with fecal occult blood tests show a decrease in CRC mortality, but not on all-cause mortality.49 Screening with flexible sigmoidoscopy also decreases CRC incidence and mortality, specifically in men and in women under 60-years-old.50-52 No randomized trials on screening with colonoscopy have been published, but observational studies show promise.53, 54 Screening with colonoscopy is common in the ages of 50-75 in the US and Germany.55 In Sweden, two study cohorts have been established to evaluate CRC screening with a fecal blood test and colonoscopy.56-58 Before the start of these studies, screening for CRC was uncommon in Sweden, and opportunistic screening was observed to be almost nonexistent.59
Risk Factors and Preventive Factors

There is no single environmental factor that greatly increases CRC risk. However, several factors that modulate CRC risk have been observed. The Continuous Update Project (CUP), which reviews the evidence from randomized trials and cohort studies regarding different cancers, published a report on CRC in 2010 and an updated report in 2017. The CUP found convincing evidence for increased CRC risk for body fatness, adult height, consumption of red and processed meat, and for men with a high intake of alcohol. The CUP also found that physical activity and foods containing fiber decreased CRC risk. The 2017 CUP update reinforced the results for red and processed meat and alcohol and further found convincing evidence for a lower CRC risk associated with higher intake of dairy products and whole grains. Furthermore, the following additional risk factors have been identified: inflammatory bowel diseases (particularly when poorly controlled), smoking, and type II diabetes.

Overweight measured by BMI is associated with increased CRC risk; when stratified by sex and tumor localization, BMI is associated with colon cancer in both men and women while in rectal cancer the association is only observed in men. Waist-to-hip ratio is a better measurement of abdominal obesity and is more strongly associated with colon cancer risk than BMI. In Mendelian randomization studies, which aim to strengthen the evidence for causality of risk relationships, gene variants associated with high BMI were associated with overall CRC risk, colon cancer risk in women, and rectal cancer risk in men. Red and processed red meat have a similar heterogeneous CRC risk distribution as body fatness when examining male and female sex separately. Dietary intake of red and processed red meat is only significantly associated with CRC risk in men. Moreover, the associations to the risk of CRC and other diseases have been observed to differ for unprocessed and processed red meat.

Additional preventive factors include estrogen therapy, and higher plasma concentrations of vitamin D, but not vitamin D intake. Regular aspirin use is associated with a lower CRC risk and a lower risk of distant metastasis. An association with a lower risk of new adenomas in colorectal cancer patients has also been observed.

Although no single environmental factor contributes greatly to CRC risk, taken together environmental factors substantially contribute to CRC risk and a large Scandinavian twin study found that only 35% of CRC incidence is due to hereditary factors, including the two most common forms of hereditary CRC – Lynch syndrome (Hereditary non-polyposis colorectal cancer, HNPCC).
and familial adenomatous polyposis (FAP). These two cancers represent 3-5% of the total CRC incidence. A family history of CRC, combining both environmental and hereditary factors, is one of the strongest risk factors for CRC.\textsuperscript{80}

Further evidence of a strong environmental component in CRC risk comes from comparing changes in CRC incidence in different populations and over time. While incidence is stable or declining in Europe and the United States, a rapid increase in CRC has been observed in populations that have made a transition from a relatively low median income to higher income, such as in Japan and in Eastern European countries.\textsuperscript{81, 82} Populations that have emigrated from low median income countries to more prosperous societies have also experienced a transition to the higher incidence rates in their new homelands.\textsuperscript{81, 82} A classic example is the increased incidence of CRC observed in the emigrated Japanese communities in Hawaii and California compared to the incidence rate in Japan.\textsuperscript{83} Taken together, these epidemiological considerations point to a combination of environmental risk factors associated with a Western lifestyle converging in an increased CRC incidence. In addition to the previously mentioned risk factors, it has been suggested that stress, sleep deprivation and circadian rhythm disturbances, and an energy dense diet could explain the higher risk in Westernized societies.\textsuperscript{81, 82}
One-Carbon Metabolism

Enzymatic Reactions and Metabolites

One-carbon metabolism, a complex network of enzymatic reactions (Figure 3), uses different methyl group sources as enzymatic cofactors, including certain amino acids, choline, and betaine and B-vitamins riboflavin (B2), pyridoxal phosphate (B6), folates (B9) and cobalamin (B12).\textsuperscript{84–87} One-carbon metabolism is essential for the synthesis of nucleotides and proteins and uses the universal activated methylator co-substrate S-adenosylmethionine (SAM) as the methyl group donor. Methylation reactions are important for genome stability and gene translation, and ultimately risk of tumorigenesis.\textsuperscript{84, 85, 88}

Figure 3. One-carbon metabolism. PURPLE circles signify enzymes with their respective B-vitamin co-factors in BLACK. Methylation reactions, redox reactions, and nucleotides in ORANGE are the three primary outputs of one-carbon metabolism. Abbreviations: BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; DMG, dimethylglycine; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; DHFR, dihydrofolate reductase; f-THF, 10-formyltetrahydrofolate; MS, methionine synthase; me-THF, 5,10-methylene-THF; MTHFR, methylenetetrahydrofolate reductase; m-THF, 5-methyltetrahydrofolate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; tHcy, total homocysteine; TYMS, thymidylate synthase.
The main constituents of one-carbon metabolism are the folate and methionine cycles. Labile methyl groups are mainly provided through serine in the folate cycle and to a lesser extent via the choline oxidation pathway. Folic acid – the stable and synthetic form of folate – is commonly added to food (folic acid fortification) to decrease the risk of neural tube defects during pregnancy. Folic acid has a higher gastrointestinal bioavailability than naturally occurring folates and needs to be reduced twice in order to enter the folate circle as tetrahydrofolate (THF). This conversion, catalyzed by dihydrofolate reductase (DHFR), takes place almost exclusively in the liver, and not as previously thought in the bowel wall. Consequently, a folic acid bolus taken up in the duodenum passes unmodified into the portal vein. In humans, the conversion of folic acid to THF by DHFR can be slow and DHFR is easily saturated. In populations that consume folic acid that has been added to foods, unmetabolized folic acid is found in serum, maternal blood, and in umbilical cord blood.

THF is converted by serine hydroxymethyltransferase (SHMT) into 5,10-methylene-THF (me-THF). SHMT catalyzes the conversion of serine to glycine and THF to me-THF in a vitamin B6 dependent reaction. me-THF can be converted back to THF by thymidine synthase (TYMS) in a reaction where deoxothymidine monophosphate (dTMP) is formed from deoxouridine monophosphate (dUMP). There are two additional possible pathways for me-THF: it can either be further reduced to the predominating folate form found in blood, 5-methyltetrahydrofolate (m-THF), by the vitamin B2-dependent enzyme methylenetetrahydrofolate reductase (MTHFR) or undergo a series of reactions to form 10-formyltetrahydrofolate (f-THF). While f-THF is a precursor for nucleotide synthesis, m-THF exits the folate cycle and enters the methionine cycle by methyl group transfer by the vitamin B12-dependent methionine synthase (MS) enzyme. The methyl group provided by m-THF converts homocysteine into methionine and m-THF back into the folate cycle as unmethylated THF. Alternatively, methyl groups for remethylation of homocysteine can be provided via the choline oxidation pathway and the enzyme betaine-homocysteine methyltransferase (BHMT). Homocysteine can also be diverted through the pyridoxal’ 5-phosphate-dependent (PLP) transsulfuration pathway, where homocysteine provides sulfur for cysteine formation. The methionine formed by BHMT or MS is the substrate for synthesis of S-adenosylmethionine (SAM) carrying an activated methyl group. SAM, involved in the majority of methylation reactions, is transformed into S-adenosylhomocysteine (SAH). SAH can be transformed into homocysteine by S-adenosylhomocysteine transferase. SAM carries the role of a universal reactive methylator and constitutes one of the most common cofactors in enzymatic reactions in the body.
The main outputs from one-carbon transfer reactions (dTMP, f-THF, methionine, and cysteine) is involved in three distinct functions: nucleotide synthesis (dTMP and f-THF), methylation reactions (methionine), and redox balance and glutathione synthesis (cysteine).84-86

One-carbon Metabolism in Cancer and Genomic Instability

The history of one-carbon metabolism is intertwined with cancer research and treatments since the late 1940s when Sidney Farber and colleagues observed a temporary remission of acute leukemia in children treated with the folic acid antagonist aminopterin.101, 102 Additional chemotherapies based on the antifolate concept followed, such as methotrexate and 5-FU.96 Many of these inhibit TYMS, resulting in decreased synthesis of thymine and a build-up of uracil. This leads to uracil misincorporation into DNA and DNA instability, which induces cell cycle arrest and apoptosis.38,103 f-THF, similar to the TYMS product dTMP, is an essential co-factor and co-substrate for nucleotide synthesis and is necessary for sufficient nucleotide supply and increases genome stability.104

Much of research into one-carbon metabolism has focused on polymorphisms that impact the activity of MTHFR, the central hub linking the folate cycle to the methionine cycle. A common polymorphism in the gene encoding MTHFR, MTHFR 677TT, results in thermolability and lower activity of the enzyme in the presence of low folate concentrations.97,105 The MTHFR 677TT polymorphism has convincingly been associated with a lower risk of CRC,106 with a possible exception in populations with a poor folate status.107

Genome instability and increased mutation rate are one of the hallmarks of cancer, and tumors are characterized by aberrant DNA methylation and histone modification.108 DNA methylation, one of several epigenetic mechanisms that control gene expression, depends on the availability of the universal methyl donor SAM. SAM and several other factors in one-carbon metabolism are involved in DNA and histone methylation. These factors include polymorphisms in the MS and/or MTHFR genes and dietary intake of folate and methionine.109-113

Tumor cells are unable to proliferate when methionine is replaced by the immediate precursor homocysteine in cell media, whereas normal cells can synthesize necessary methionine from homocysteine, a phenomenon known as methionine dependency.114 Methionine is an essential amino acid involved in DNA methylation, protein synthesis, and synthesis of polyamines and glutathione.84,86 In animal models, low methionine diets inhibit tumor growth and size and synergizes with cytotoxic treatments.115 However, in humans
dietary methionine restrictions do not cause a reliable decrease of plasma concentrations of methionine, and the tumor cells scavenge amino acids from the tumor stroma, potentially limiting the impact of dietary methionine restrictions.115

**Folate Cycle Components and Folic Acid in CRC**

The folate cycle comprises different folate forms (Figure 4). Folate is a generic term for several compounds, including folic acid and its derivatives at varying reduction states. These include 5-methyltetrahydrofolate (me-THF), 10-formylTHF (f-THF), 5,10-methyleneTHF (m-THF), and unsubstituted THF.87, 116 Although the amino acids serine and glycine are important for feeding the folate cycle with one-carbon units,84, 117 research has mainly focused on folate and comparatively little on the methyl donors serine and glycine.85

![Folate cycle](image)

**Figure 4.** Folate cycle. PURPLE circles signify enzymes with their respective B-vitamin co-factors in BLACK. Nucleotides in ORANGE are the primary outputs of the folate cycle, m-THF is integral. Abbreviations: dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; DHFR, dihydrofolate reductase; f-THF, 10-formyltetrahydrofolate; MS, methionine synthase; me-THF, methylene-THF; MTHFR, methylenetetrahydrofolate reductase; m-THF, 5-methyltetrahydrofolate; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; tHcy, total homocysteine; TYMS, thymidylate synthase.

Important dietary sources of naturally occurring folate are leafy greens, fruit (including orange juice), and legumes.87 Foods from animal sources only contain small amounts of folate, with the exception of liver and egg yolks. Adequate folate status before and during early pregnancy protects the fetus against birth defects such as spina bifida, encephalocele, and anencephaly (collectively known as neural tube defects, NTD).89, 118 NTDs can result in death or severe life-long disability and NTD prevention represents a major
public health challenge. Consequently, in the early 1990s many countries introduced mandatory folic acid fortification of common foodstuffs. Countries with mandatory or widespread voluntary folic acid fortification are depicted in Figure 5. Notable exceptions are Russia, India, China, Europe (except Moldavia), and parts of Africa.

Figure 5. ORANGE signifies countries with a mandatory folic acid fortification of grains; BROWN signifies countries with a widespread voluntary folic acid fortification of grains. BEIGE signifies countries with no widespread fortification of grains. Adapted from the Food Fortification Initiative: http://www.ffinetwork.org/global_progress/ (assessed 6/20/17).

Following fortification, studies on pre-clinical models started to emerge that indicated that folate may have a double-edged role in CRC, with a protective role in the normal colorectal mucosa but accelerating the growth of already established pre-cancerous lesions. Subsequent studies on intake of folate and folic acid seemed to indicate a weak inverse association or no association with CRC risk, although two Norwegian randomized trials on folic acid supplementation, conducted with the primary aim to lower homocysteine to reduce the risk of heart disease, observed as a secondary outcome an increased risk of some types of cancers. However, a later published meta-analysis of randomized trials on folic acid supplementation only found a borderline, although nonsignificant, increase of some types of cancers. Prospective case-control studies on plasma folate concentrations and risk of CRC were generally underpowered and taken together provide no clear picture on the influence of folate status in CRC risk. A study from 2006, from a northern Swedish population with low folate intakes and no mandatory fortification, found that study participants with the lowest plasma concentrations of folate
also had the lowest risk association to CRC.\textsuperscript{132} A few years later, these findings were reproduced in a large US study,\textsuperscript{133} but the largest study to date with over 1300 cases concluded that plasma concentrations of folate were not associated with CRC risk.\textsuperscript{134} The same results were also observed concerning the risk of distal colorectal adenoma.\textsuperscript{135} There is still some remaining discussion regarding a temporary interruption in the trend of decreasing CRC incidence in the US and Canada coinciding with the introduction of folic acid fortification\textsuperscript{120, 136} and the potentially harmful role of unmetabolized folic acid.\textsuperscript{137-140}

Concerns have been raised that folate should not be studied in isolation due to the tightly intertwined reciprocal relationship and interactions with other one-carbon metabolites and polymorphisms.\textsuperscript{138, 139} In a recently published article that relies on analyses of a large panel of biomarkers and single nucleotide polymorphisms (SNPs) involved in one-carbon metabolism and using advanced statistical methods to investigate the full picture of one-carbon metabolism, we observed that plasma concentrations of folate (direct association) and vitamin B2 and B6 (inverse association) were most strongly associated with CRC risk.\textsuperscript{141}

Although serine and glycine are amino acids that are abundant in plasma, intake or circulating levels of these metabolites have not been studied in relation to clinical cancer risk.\textsuperscript{85} However, in cell lines and in an \textit{in vivo} model, serine starvation decreased tumor growth.\textsuperscript{142, 143}

\textbf{Methionine and Choline Oxidation Pathway Metabolites in CRC}

The enzyme BHMT catalyzes the remethylation of homocysteine into methionine and betaine into dimethylglycine (DMG).\textsuperscript{86} Methionine is the substrate for SAM synthase, and the product of the synthase reaction (i.e., SAM) contains an activated methyl group that is essential for most methylation reactions in the body.\textsuperscript{84, 86, 144} Betaine can be provided either through diet (the main sources are wheat and wheat bran)\textsuperscript{145} or through oxidation of choline.\textsuperscript{86} Betaine can be further sequentially oxidized to DMG and sarcosine (Figure 6). Choline is not only the precursor of betaine in the choline oxidation pathway but also involved in the hepatic synthesis of the neurotransmitter acetylcholine and structural lipoproteins.\textsuperscript{86, 146} Dietary choline deficiency may cause DNA strand breaks and altered epigenetic regulation of DNA expression and histone methylation.\textsuperscript{146} Choline can be supplied either through diet (the richest sources are organ meats and egg yolks)\textsuperscript{145} or through \textit{de novo} synthesis by the SAM-dependent enzyme phosphatidylethanolamine N-methyltransferase (PEMT).\textsuperscript{86} PEMT is upregulated by estrogens, and pre-menopausal women are less sensitive to
choline deficiency than men or post-menopausal women. Deficiency of the essential nutrient choline causes fatty liver disease and liver damage.

**Figure 6.** The methionine cycle and the choline oxidation pathway. **PURPLE** circles signify enzymes with their respective B-vitamin co-factors in **BLACK**. Methylation reactions in **ORANGE** are the primary outcomes of the methionine cycle. Abbreviations: BHMT, betaine-homocysteine methyltransferase; MS, methionine synthase; m-THF, 5-methyltetrahydrofolate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate; tHcy, total homocysteine.

Study results on dietary intake of choline and betaine in relation to CRC risk are inconsistent. Increased dietary intake of choline appeared to be linearly associated with colorectal adenoma risk while dietary intake of betaine was inversely associated in a study nested in the all-female Nurses’ Health Study. Similar results for betaine, but not choline, were observed in the Norwegian Colorectal Cancer Prevention cohort. In the all-male Health Professional Follow-up Study, neither dietary intake of choline or betaine was observed to be associated with CRC risk, but in a Chinese case-control study higher dietary intake of choline, but not betaine, was associated with a decreased CRC risk in both men and women.

High dietary intake of methionine is consistently associated with a lower CRC risk. Plasma concentrations have been investigated in two studies. A study nested in the Women’s Health Initiative Observational Study (WHI-OS) found that plasma concentrations of betaine were associated with a lower risk of CRC risk, while plasma concentrations of choline and dimethylglycine were not significantly associated with CRC risk. Similarly, a case-control study comprising over 1300 men and women with diagnosed CRC nested in the European Prospective studies into Cancer and Nutrition (EPIC) found that plasma betaine was associated with a decreased CRC risk and observed an interaction with folate status: in participants with a low folate status, plasma betaine was observed to be inversely associated with CRC risk. In addition, plasma concentrations of choline and methionine were associated with a
lower overall CRC risk, but in the case of choline, the association was mainly driven by a lower risk in women. In the same study, plasma dimethylglycine was not associated with CRC risk. Plasma concentrations of methionine and betaine are also associated with a lower risk of distal colorectal adenoma.\textsuperscript{135}

**Transsulfuration Pathway Metabolites in CRC**

Over the past several decades, homocysteine emerged as a potential causal risk factor in atherosclerosis; during the late 1990s, several randomized trials were launched to evaluate whether a homocysteine-lowering therapy with B-vitamins (vitamin B6, folate, and vitamin B12) could lower the risk of cardiovascular incidents.\textsuperscript{154} Despite significant reductions in homocysteine concentrations, the expected lower risk of cardiovascular incidents could largely not be verified.\textsuperscript{154, 155} Circulating homocysteine is also implicated in cognitive disease and NTDs, but only limited evidence suggests that homocysteine could be a risk factor in itself. Instead, homocysteine could be considered a marker of poor status of one-carbon metabolism or secondary to underlying disease.\textsuperscript{99}

![Figure 7. The transsulfuration pathway. PURPLE circles signify enzymes with their respective B-vitamin co-factors in BLACK. Redox reactions in ORANGE are the primary outcomes of the transsulfuration pathway. Abbreviations: CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; tHcy, total homocysteine.](image)

Homocysteine can either be remethylated to methionine by vitamin B12 dependent MS using m-THF as a cosubstrate or by the betaine-dependent BHMT.\textsuperscript{84, 99, 156} Higher dietary methionine intake increases homocysteine concentrations and the transformation of excess homocysteine to cysteine by the two vitamin B6 dependent enzymes cystathionine β-synthase (CBS, homocysteine to the intermediate cystathionine) and cystathionine γ-lyase (CSE, cystathionine to cysteine).\textsuperscript{98, 99, 156} CBS and CSE are part of the transsulfuration pathway depicted in Figure 7. Elevated plasma
concentrations of homocysteine mainly reflect deficiencies in folate and/or vitamin B12, but could also reflect impaired clearance due to insufficient vitamin B6 concentrations or genetic variants of genes involved in homocysteine metabolism (MTHFR, MS, CBS, and CSE).\textsuperscript{97, 99, 100, 156} Supplementation with folic acid, vitamin B6, vitamin B12, and the methyl group donor betaine can decrease plasma homocysteine concentrations,\textsuperscript{157} and for vitamin B2 the same decrease has been found in individuals with the MTHFR 677TT polymorphism.\textsuperscript{158} Recently, three homocysteine ratios were introduced that could more accurately than homocysteine determine deficiencies in total B-vitamin status.\textsuperscript{159} Increases in the ratios of homocysteine to cysteine (hcy:cys) and to creatinine (hcy:cre) and hcy:cys to creatinine (hcy:cys:cre) partly reflect disturbances in the transsulfuration pathway (hcy:cys), the methionine cycle (hcy:cre), and/or both (hcy:cys:cre). Compared to homocysteine, all three ratios had higher sensitivity and specificity in estimating total B-vitamin status.\textsuperscript{159}

Plasma concentrations of homocysteine have not been observed to be significantly associated with CRC risk,\textsuperscript{132, 160, 161} with one exception: a large study nested in the WHI-OS reported a linear association between homocysteine concentrations and CRC risk.\textsuperscript{162} The authors hypothesized that the results could be due to the larger all-female cohort with overall lower plasma concentration of homocysteine compared to previous studies. In the same study, higher plasma cysteine was associated with a decreased CRC risk. Because vitamin B6 deficiency may impair homocysteine catabolism, low plasma concentrations of cysteine could be an expression of poor vitamin B6-status,\textsuperscript{156} and since plasma concentrations of pyridoxal' 5-phosphate (PLP), the most commonly used marker of vitamin B6-status, is consistently associated with a decreased CRC risk,\textsuperscript{163} the association between cysteine and CRC could reflect a low vitamin B6 status. Alternatively, since the transsulfuration pathway supplies approximately half of the cysteine needed for synthesis of the major redox buffer glutathione,\textsuperscript{95} reactive oxygen species could hypothetically lie behind the increased CRC risk. Another study found that plasma concentrations of cysteine have a similar trend, although statistically nonsignificant; the study’s small sample size (n = 118) may explain the lack of a significant association.\textsuperscript{160}

**B-vitamins Involved in One-carbon Metabolism and CRC Risk**

One-carbon metabolism depends on B-vitamins as essential co-factors. Vitamin B2 is a cofactor for MTHFR, vitamin B12 and m-THF (as a co-substrate) is a cofactor for MS, and vitamin B6 is a cofactor for SHMT, CBS, and CSE.\textsuperscript{84, 164} Thus, vitamin B6 modulates the influx of methyl groups from serine into the folate cycle by converting THF to me-THF as well as the efflux
of homocysteine through the transsulfuration pathway. Vitamin B2 is a co-factor for the enzymatic reaction catalyzed by MTHFR that regulates the balance between me-THF and m-THF and consequently the balance between the folate cycle (me-THF) and the methionine cycle (m-THF). Finally, vitamin B12 is necessary for the only reaction m-THF can take part in, remethylation of homocysteine to methionine transferring the methyl groups donated from serine to the universal methyl donor SAM. Poor vitamin B12 availability can result in an accumulation of me-THF, a phenomenon known as the folate trap and inhibition of thymidine synthesis and increased genome instability. Excess homocysteine can be transformed to the amino acid cysteine by the vitamin B6 dependent enzymes of the transsulfuration pathway.

Overall, associations between dietary intake of these B-vitamins and CRC risk are inconsistent. Presently, there are no reports on statistically significant associations to CRC risk with either micronutrient.

The most commonly used plasma marker of vitamin B6 status is pyridoxal' 5'-phosphate (PLP). In contrast to the studies on estimated dietary intake of vitamin B6, high plasma concentrations of PLP are consistently associated with a lower CRC risk.

Vitamin B2 status in plasma can be measured either as riboflavin alone or a combination of riboflavin and flavin mononucleotide (FMN). Riboflavin is the precursor of FMN and both vitamers are useful indicators of vitamin B2 status in population studies. Two previous studies have been conducted on plasma markers of vitamin B2: in a study from 2008, no association to CRC risk was observed for plasma concentrations of riboflavin, and in a study nested in the EPIC-cohort (1300 case participants) total plasma vitamin B2 concentrations were associated with a decreased CRC risk.

Vitamin B12 status is generally measured in plasma as total cobalamin – all forms of vitamin B12 bound to different binding proteins. The serum marker methylmalonic acid (MMA) and total homocysteine are also routinely used as functional vitamin B12 markers; in cancer patients, a combination of plasma cobalamin, MMA, and homocysteine has been proposed to be better for a total assessment of vitamin B12 status. Four prospective studies on plasma markers of vitamin B12 status and CRC risk have been conducted so far and no significant associations have been found. One of these studies, nested in the NSHDS and based on a portion of the CRC cases and controls in this thesis, found that plasma concentrations of cobalamin were associated with a lower risk of rectal cancer, but not CRC. It was suggested that this was due to fewer rectal tumors with microsatellite instability (MSI),
which is associated with hypermethylation of the mismatch repair gene *MLH1*.\textsuperscript{174} However, similar associations between plasma cobalamin and rectal cancer risk were not observed in the larger EPIC study or in a cohort of male smokers.\textsuperscript{161, 170}

A recently published comprehensive investigation of all one-carbon metabolites using Bayesian network learning found that vitamin B2 and B6 had strong independent associations with lower CRC risk.\textsuperscript{141} Plasma concentrations of vitamin B2 and B6 are also associated with a lower risk of distal colorectal adenoma.\textsuperscript{135}

**Inflammatory Interaction with Vitamin B6 and CRC Risk**

Vitamin B6 is most commonly measured in plasma as PLP.\textsuperscript{164} PLP is the active coenzyme form of vitamin B6 and functions in over 100 different enzymatic reactions in the human body.\textsuperscript{175} PLP is a cofactor for enzymes in the kynurenine pathway,\textsuperscript{176} and in one-carbon metabolism, PLP is involved in the transsulfuration pathway and the folate cycle.\textsuperscript{84} Free PLP in plasma can be converted to pyridoxal (PL) by alkaline phosphatase, an enzyme located on the membrane of all cell types.\textsuperscript{164} PL is dephosphorylated PLP that is capable of crossing the cell membrane and can therefore be categorized as the transport form of vitamin B6.\textsuperscript{164} Excess PL is catabolized to 4-pyridoxic acid (PA) in the liver and excreted by the kidneys.\textsuperscript{164}

Conditions associated with inflammation – e.g., cancer, heart disease, stroke, diabetes, rheumatoid arthritis, and inflammatory bowel diseases – are associated with low plasma PLP.\textsuperscript{164} Furthermore, Plasma PLP is inversely associated with inflammatory biomarkers, including C-reactive protein (CRP) and neopterin.\textsuperscript{177} The decrease of plasma PLP during inflammation is due to both increased catabolism and increased tissue uptake at the site of inflammation.\textsuperscript{177}

To further study the inflammatory aspects of vitamin B6, a ratio of the different species is used. PAr is the ratio of PA/(PLP+PL) – i.e., the catabolite divided by the sum of the active and the transport forms of vitamin B6.\textsuperscript{164, 177} This ratio mirrors the increased catabolism observed in inflammation as well as the redistribution of plasma PLP and PL into tissues with inflammatory activity. Consequently, variance in PAr was observed in multiple linear regression models to be almost entirely determined by a combination of four other inflammatory markers, although the common biomarker confounders smoking and kidney function did not influence PAr. PAr, unlike PLP, was not influenced by vitamin B6 supplementation.\textsuperscript{177} Taken together, PAr shows
promise as a sensitive marker of vitamin B6 mediated systemic inflammation, and PAr is robust in regards to potential confounders.

NAD+ synthesis from tryptophan via the kynurenine pathway mainly takes place in the liver.\textsuperscript{176} Two of the enzymes in this pathway are strictly PLP-dependent: the kynurenine aminotransferase (KAT) enzyme that converts 3-hydroxykynurenine (HK) to xanthurenic acid (XA) and kynureninase (KYNU) that converts the same substrate to 3-hydroxyanthranilic acid (HAA).\textsuperscript{176} Consequently, increased concentrations of HK in plasma and urine are observed in vitamin B6-deficiency.\textsuperscript{164, 176} The ratios of HK to XA and HAA (HK:XA and HK:HAA, respectively) are therefore increased in PLP-deficiency and inversely associated with plasma PLP.\textsuperscript{178} HK:XA has a slightly stronger association with plasma PLP than HK alone and is less influenced by confounders such as BMI, kidney function, and inflammation.\textsuperscript{178} Therefore, HK:XA is a potential marker of functional intracellular vitamin B6 status. It is currently unknown if this ratio also reflects aberrations in the kynurenine pathway and if these potential aberrations might influence carcinogenesis. However, XA has been observed to be negatively associated with the inflammatory marker CRP and cancer mortality.\textsuperscript{179}

In the Hordaland Health Study cohort, PAr was associated with cancer risk after adjusting for confounders although HK:XA only reached borderline significance.\textsuperscript{180} When stratifying for cancer site, PAr was mainly associated with lung cancer risk, but also weakly associated with an increased CRC risk.\textsuperscript{180}

In summary, vitamin B6 status in plasma is commonly estimated as PLP, but the influence of inflammation on PLP concentrations makes the association between decreased CRC risk and plasma PLP observed across many studies difficult to interpret.
Summary and Overall Perspectives

CRC is one of the most commonly diagnosed cancers and has a high societal and economic cost as many people will either be diagnosed or have a friend or family member diagnosed with CRC. Although treatment is slowly improving, CRC is still one of the most common causes of cancer death. Epidemiological studies on emigrated populations and changes in populations over time suggest that there is a strong environmental aspect in CRC incidence. Many of the suggested environmental risk factors are grouped together as factors associated with a Western lifestyle (i.e., stress, circadian rhythm disturbances, smoking, an energy dense diet lacking in micronutrients, and lack of physical activity). Many of the environmental risk factors are still unknown or not sufficiently researched.

In the NSHDS cohort, low plasma folate status is associated with a lower CRC risk. In 2007, these results were taken into account when the Swedish Agency for Health and Disease assessed the potential risks and benefits of folic acid fortification. The complexity of one-carbon metabolism is mirrored in the differing opinions researchers have on mandatory folic acid fortification of grains. While many researchers stress the importance of lowering the incidence of birth defects, others stress the need for more research, especially on cohorts with an overall low folate status since folic acid fortification and supplementation often results in supraphysiological folate concentrations. The population-based NSHDS cohort provides an overall low folate status and is sufficiently large to allow for subgroup analysis.

One-carbon metabolism influences both nucleotide synthesis and methylation reactions, not only providing fodder for rapidly dividing cells but also increasing genome stability. Folate acts as a carrier of the methyl groups needed for both methylation and nucleotide synthesis, while other one-carbon metabolites are only involved in methylation. A comparison of these one-carbon metabolites and folate in relation to CRC risk makes it possible to distinguish between the two main outputs of one-carbon metabolism and relate these outputs to carcinogenesis.

Previous research into one-carbon metabolism has treated the many factors as independent entities when relating them to risk estimates, despite the interdependence of the metabolites and enzymes involved. This can be mitigated by calculating interactions between metabolites, or by estimating the total status of the major pathways of one-carbon metabolism, and relating these estimates to CRC risk. Interactions between folate and cobalamin have been observed in cancer, cognitive decline, and anemia. Could a possible deleterious role for folic acid be attenuated by fortifying foodstuffs with both
folic acid and vitamin B12?\textsuperscript{184} Moreover, metabolites in the choline oxidation pathway (mainly betaine and choline) are particularly important in individuals with low folate status, and has, similarly to folate, implications for epigenetic alterations and plasma homocysteine concentrations.\textsuperscript{86, 185, 186} Should these metabolites also be considered to be included in a fortification program? A broader understanding of the impact of one-carbon metabolism as a whole could have implications for public health initiatives.

While the rationale and motives for multi-metabolite fortification can be well-meaning and scientifically sound, there is always a risk of negative unforeseen public health outcomes. In 1994 evidence of harmful effects of vitamin supplements started to emerge in the Alpha-Tocopherol Beta-Carotene Cancer Study. Almost 30000 male Finnish smokers took part in the randomized controlled trial with lung cancer as the primary outcome. Contrary to expectations, beta-carotene increased deaths from lung cancer and ischemic heart disease. Similarly, the Selenium and Vitamin E Cancer Prevention Trial was a randomized controlled trial involving over 35000 men initiated to investigate if vitamin E and selenium could influence the risk of prostate cancer. The trial had to be stopped prematurely in 2011 when a significant increase in prostate cancer incidence was observed in the treatment arm.\textsuperscript{187} In the Norwegian Aspirin/Folate Polyp Prevention Study, over 1000 participants with a previous history of colorectal adenoma took part in a randomized controlled trial with folic acid and aspirin as the intervention and recurrence of colorectal adenoma as the primary outcome. Folic acid supplementation resulted in an increased risk of three or more colorectal adenomas and non-significant increases in overall colorectal adenoma recurrence.\textsuperscript{188}

A thorough biochemical knowledge of one-carbon metabolism is necessary in order to understand the relation to CRC risk. The interdependence and interaction between the many factors involved calls for improved methods to validate the previous results based on single factors in relation to risk, and to gain new insights. Studies carried out in populations with a high portion of total folate intake derived from naturally occurring folates gives the opportunity to study one-carbon metabolism in a setting where the lower spectrum of physiological folate status is represented.
Aims of the Thesis

This thesis investigates the associations of one-carbon metabolites to CRC risk in a mature, relatively large population-based cohort. We used prospective blood samples collected from over 600 case participants and approximately 1200 matched control participants in prospective case-control studies nested in the NSHDS.

This thesis consists of four papers with the following specific aims:

**Paper I**

- To investigate plasma concentrations of folate, vitamin B12, and homocysteine in relation to CRC risk.
- To stratify the study cohort according to tumor and case participant characteristics and to investigate further the associations of the included plasma markers to CRC risk.

**Paper II**

- To investigate plasma concentrations of one-carbon metabolites predominantly involved in remethylation of methionine from homocysteine in relation to CRC risk.
- To divide the full study cohort according to tumor and case participant characteristics and to investigate the interactions between one-carbon metabolism factors in relation to CRC risk.
- To calculate marginal absolute risk estimates and risk difference, expressed as changes in CRC incidence per 100,000 individuals.

**Paper III**

- To investigate different aspects of vitamin B6 status in relation to CRC risk using three different markers.
- To specifically investigate timing to exposure using the long follow-up from screening to diagnosis.

**Paper IV**

- To investigate the performance of three different ratio-based B-vitamin markers in relation to CRC risk.
- To investigate the ability of the ratios to determine low total B-vitamin score in both case and control participants.
Materials and Methods

Study Population

The studies in this thesis are based on the inhabitants of Västerbotten County. Västerbotten lies in the northern part of Sweden stretching from the Baltic Sea to the Norwegian border. Västerbotten is a large county, slightly larger than Denmark, but sparsely populated, with a total population of 260,000, of which 120,000 live in the coastal county capital, Umeå. Umeå is an expanding university city with a large proportion of students, so the populations have a relatively low mean age. The rural western areas of the county have a low population density and a higher median age.

Study Cohort

The studies in this thesis are nested in the Northern Sweden Health and Disease Study (NSHDS). The NSHDS consists of three subcohorts, the Northern Sweden Monitoring of Trends and Determinants in Cardiovascular Disease cohort (MONICA), the Västerbotten Intervention Programme cohort (VIP), and the Mammography Screening Project cohort (MSP). The VIP and MSP cohorts are described in further detail below. Because the MONICA cohort is mainly used for cardiovascular disease research, it is not part of the investigations in this thesis. As the NSHDS is a population-based study cohort, the CRC incidence and mortality in the cohort is essentially identical as in Sweden as a whole (Figure 1).

The Västerbotten Intervention Programme

The VIP was initiated in 1985 as the Norsjö Project, a response to the high mortality in cardiovascular disease in Västerbotten County and particularly in the Norsjö Municipality. This population-based cohort aimed to include all residents in Västerbotten County. Residents are invited to take part in a health examination when turning 30 (1985-1996 only), 40, 50, and 60 years old. The health examination consists of a medical examination and laboratory tests, collection of a fasting blood sample, and an extensive diet and lifestyle questionnaire.

The participation rate in the VIP is approximately 60% of the total invited population and in the most recent survey had increased to 66% between 2005 and 2010. A comparison of the rate of cancer incidence in the VIP cohort to the expected incidence supports the population-based nature of the cohort, and selection bias has been observed to be low. As of December 31, 2009,
the stop date for case identification for the studies in this thesis, the VIP included 85,877 individuals and 115,147 blood samples.

**The Mammography Screening Project**

From 1996 until 2006, all women in Västerbotten County between the ages of 50 and 70 years old who underwent mammography screening were invited to participate in the MSP. In addition to the screening, the women were invited to donate a blood sample for future research and to complete a questionnaire on reproductive history, diet, and lifestyle factors. The participation in the screening program was 85% and participation both the screening and the donation of a blood sample was 33%. The included women had a higher incidence of breast cancer than expected one year after mammography, but this was compensated by a lower incidence in the following years. These women also had a slightly lower risk of lung cancer. At its conclusion, the MSP included 54,401 blood samples from 28,802 women.

**Blood Sampling and Analysis**

In the NSHDS, plasma from venous blood samples is collected in sample tubes that are separated into buffy coat, plasma, and erythrocyte fractions. The plasma samples are then aliquoted and frozen within one hour of collection either at -80°C or -20°C for up to one week before transfer to a -80°C freezer at a central location at Umeå University Hospital for long-term storage. The VIP blood samples are fasting samples collected in the morning. The MSP blood samples were collected throughout the day, and are almost exclusively non-fasting blood samples.

The concentration of one-carbon metabolites and polymorphisms involved in one-carbon metabolism were analyzed in EDTA plasma at Bevital AS (Bergen, Norway, www.bevital.no). Plasma concentrations of tHcy and cysteine were measured using an isotope dilution gas chromatography–mass spectrometry method (between-day CV: 2–8%). Vitamin B12 (cobalamin) and folate concentrations were determined with a microbiological method using *Lactobacillus leichmannii* and *Lactobacillus casei*, respectively, which were adapted to a microtiter plate format and carried out by a robotic workstation (between-day CV: 5%). Plasma concentrations of methionine, methionine sulfoxide, choline, betaine, dimethylglycine, creatinine, neopterin, kynurenines (kynurenin, HK, and XA), vitamin B2 (riboflavin), symmetric dimethylarginine (SDMA), and vitamin B6–species (PLP, PA, and PL) were measured with liquid chromatography–mass spectrometry methods (between-day coefficient of variation (CV): 3–13%). Single nucleotide polymorphisms (SNPs) were determined using MALDI-TOF mass
spectrometry (average error rate of <0.1% estimated by analysis of sample duplicates). The samples were analyzed in matched case-control sets with the position of the case in each set assigned randomly. The laboratory staff and investigators were blinded to case and control status.

**Variables**

Plasma concentrations of metabolites and SNPs involved in or related to one-carbon metabolism were analyzed in the articles presented in this thesis. The metabolites and inflammatory markers were folate, vitamin B6-species (PLP, PA, and PL), vitamin B2 (riboflavin), vitamin B12 (cobalamin), homocysteine (tHcy), cysteine, methionine, methionine sulfoxide, choline, betaine, dimethylglycine, kynurenine, and sarcosine. In Paper III, two ratios were calculated from metabolite concentrations: HK:XA (HK/XA) and PAr (PA/(PL+PLP)). In Paper IV, a B-vitamin score and three homocysteine ratios were calculated from plasma concentrations. The B-vitamin score was calculated as the sum of the standardized concentrations of riboflavin, PLP, folate, cobalamin, and betaine. Standardization of the plasma concentrations was performed by subtracting the mean and dividing by the standard deviation. Furthermore, the ratio-based B-vitamin markers hcy:cys (tHcy/cysteine), hcy:cre (tHcy/creatinine), and hcy:cys:cre (tHcy/cysteine/creatinine) were also calculated. Lastly, the sensitive kidney function marker SDMA, and the inflammatory markers neopterin and kynurenine-to-tryptophan-ratio (KTR, kynurenine/tryptophan) were calculated.

Included SNPs were MTHFR 677 C>T, MTR 2756 A>G, and BHMT 742 G>A. Other factors considered were smoking status (current, ex-smoker, and never smoker), body mass index (BMI) (< 25, 25–30, ≥ 30 kg/m2), alcohol intake (zero intake, above/below sex-specific median of self-reported grams of alcohol/day), dietary fiber intake (above/below sex and age-specific (10-year groups) median of self-reported intake in grams/2000 kcal), recreational- and occupational physical activity (self-reported on a scale from 1-5, ranging from 1 for sedentary and 5 for highly active), and estimated glomerular filtration rate (eGFR) calculated by the Cockcroft-Gault formula (based on age, plasma creatinine levels, sex, and body weight).

**Study Design**

As all the included studies have a prospective design, ideally the blood samples were collected from the case participants when they were still healthy. However, CRC is a slowly developing disease and could take up to 20 years to develop from the period from when the tumor first could be detected by
screening until diagnosis.\textsuperscript{46–48} One-carbon metabolism and inflammation may be involved in the promotion of CRC but not necessarily in the initiation of CRC.\textsuperscript{138} The timing of the exposure of folate and other one-carbon metabolites is therefore important to consider.

There are also several opportunities and pitfalls when assessing associations between biomarkers of inflammation and cancer. Associations between inflammatory biomarkers and cancer may be explained by other factors that increase both biomarker concentrations and the risk of cancer (i.e., confounding) or by the tumor itself increasing biomarker concentrations (i.e., reverse causality). To address this issue, several procedures has been used: a sensitivity analysis where samples collected close to diagnosis are censored;\textsuperscript{200} Mendelian randomization studies where random polymorphisms in genes associated with increased biomarker concentrations are a proxy for increased biomarker concentration enabling a randomized trial like set-up;\textsuperscript{201} and repeat sampling where biomarker concentrations are followed over time.\textsuperscript{202} Ideally, studies should have samples collected long before diagnosis.\textsuperscript{203} In our studies, we have a long median follow-up time from sampling to diagnosis and a sufficiently large number of case participants - conditions that allowed us to stratify the cohort according to follow-up time.

The studies in this thesis are nested in the NSHDS, and the case participants are matched with control participants taken from the full study cohort depending on matching criteria, such as sex and age. In biobank research, a nested case-control design also allows for matching according to when a sample is collected, effectively matching for storage time.\textsuperscript{204} Matching for storage time is important because biological samples may degrade over time. However, matching on several criteria means that the control participants do not represent the whole cohort, but rather a cohort with the same distribution of the matching criteria variables in the case and control participants. For example, our studies are skewed towards women because of the inclusion of the all-female MSP cohort. This sex difference is not seen in the NSHDS.

\textit{Selection of CRC Case and Control Participants}

CRC case participants diagnosed between October 17, 1986, and March 31, 2009, were identified by linkage with the essentially complete Cancer Registry of Northern Sweden (ICD-10 C18.0 and C18.2–C18.9 for colon and C19.9 and C20.9 for rectum). All cases and tumor data were verified by a single pathologist who specialized in gastrointestinal pathology. Patient records were used to verify the tumor site. Exclusion criteria were previous cancer diagnosis (other than non-melanoma skin cancer), primary tumor location outside of the colorectum, prioritization to other studies, insufficient volume
of stored plasma sample, serious infectious disease (for the protection of the laboratory staff), or no matching control available. For each case participant in our studies, two controls were selected, matched by age (±6 months), sex, cohort, fasting status, and year of blood sampling and data collection. The exclusion criteria for the control participants were the same as for the case participants. Furthermore, five case-control sets had a high proportion of methionine sulfoxide, indicating the full set had been thawed for a prolonged time. See Paper I for further discussion. After exclusions (81 cases and 41 controls), 613 cases and 1190 controls were included for data analyses, with the exception of Paper I where 331 case and 662 control participants were included. In Paper I the analyzed metabolites have previously been investigated in two studies nested within the same cohort.

The selection of case and control participants is depicted in Figure 8.

Figure 8. Cases and control participants in Papers II, III, and IV. In Paper I, 16 cases were excluded (11 cases because of tumor outside of the colorectum or not verifiable and five cases and their matched controls because of high methionine sulfoxide). a excluding non-melanoma skin cancer. b high methionine sulfoxide indicates degradation of samples.

**Statistical Analyses**

We used Mann-Whitney U and Chi-square tests to test for differences in baseline characteristics between the case and control participants. Linear regression was used to evaluate associations between exposure variables and baseline characteristics. Multivariable-adjusted odds ratios (ORs) for CRC risk were calculated using conditional logistic regression models stratified on the matched case sets. The exposure variables were divided into ordinal variables using percentile cut-offs (tertiles or quartiles) depending on the
distribution in the control participants. In Paper III, we also divided the study cohort as vitamin B6 deficient or sufficient depending on plasma PLP concentrations. Trends across the ordinal variables were tested by modeling the variables as continuous variables. Interactions between exposure variables were tested with likelihood ratio tests comparing an interaction model to an additive model. We estimated subgroup-specific ORs for CRC depending on tumor and case participant characteristics (with controls given the same value as their index case to retain matching) by conditional logistic regression. In Paper I, heterogeneity between results of the subgroup analysis was investigated by a Chi-squared based test. In Paper III and IV, heterogeneity was tested with likelihood ratio tests, comparing a model where risk associations varied across tumor subtypes to a model where all associations were held constant.

In order to improve the interpretability and validity of the analyses, in Paper II and III we also computed absolute risk estimates in the form of marginal risk differences (presented as estimated changes in the number of cases per 100 000 cohort participants). We used a weighted maximum likelihood estimator using cumulative incidence data from the whole NSHDS and within groups defined by age, cohort, sex, and year of sampling. Cross-validation of 95% CIs for the risk differences were calculated by normal approximation based on 1000 nonparametric bootstrap replications resampled from within the matched case set.

In Paper III, we evaluated dose-response and potential nonlinear relations by modeling continuous log-transformed plasma biomarkers in relation to CRC risk with the use of restricted cubic splines in logistic regression models (with 5 knots at the 5th, 25th, 50th, 75th, and 95th percentiles of the plasma biomarker distributions). We tested for nonlinearity with the Wald test, which compared the model with spline terms to a linear model.

In Paper IV, the influence of the B-vitamin score and other variables on tHcy and B-vitamin markers were evaluated in multiple linear regression models. We also evaluated the contribution of individual B-vitamins by estimating the same models but with log-transformed standardized B-vitamins included as separate variables. To compare associations between B-vitamin score and the ratio-based B-vitamin markers, we evaluated regression-based sensitivity (B-vitamin marker variance explained by total B-vitamin score), regression-based specificity (B-vitamin marker variance explained by total B-vitamin score divided by total explained variance), and performance (defined as sensitivity multiplied by specificity). Furthermore, we complemented these analyses with conventional receiver operating characteristics (ROC) analysis to estimate sensitivity, specificity, and area under the ROC curve (AUC) for
the markers in identifying individuals with a B-vitamin score lower than the 5th percentile in controls.

In Paper I, all calculations were made in IBM SPSS Statistics version 21.0 (IBM Corporation). In Papers II-IV, calculations were made in R v.3.3.2 (R Foundation for Statistical Computing, Vienna, Austria). Statistical tests and corresponding $P$ values were two-sided and $P$ values <0.05 were considered statistically significant.

**Ethical Approval**

Written informed consent was obtained from all study participants at the time of recruitment to the NSHDS. The study protocol and data handling procedures were approved by the Umeå University Research Ethics Committee (Dnr 03-186 and 2015/167-32).
Main Results and Discussion

Paper I

Main Results

Paper I includes cases diagnosed between 2003 and 2009 and is a follow-up study to two previous studies in the same cohort containing cases diagnosed between 1986 and 2002. The previous studies analyzed plasma concentrations of folate and homocysteine and vitamin B12, respectively. The results of the follow-up study on the same metabolites are strikingly similar to the two previous studies.

The study contains participants from both the VIP (85% of the participants) and the all-female MSP (15% of the participants). This composition is reflected in the percentage of women in the study (59.2 % women). There were no statistically significant differences in baseline characteristics between the case and control participants. The multivariable ORs for CRC according to tertiles of plasma concentrations of folate, homocysteine, and vitamin B12 are shown in Figure 9. We found the lowest CRC risk in the first tertile of folate concentrations, with ORs of 1.62 (95% CI, 1.08-2.42) and 1.42 (95% CI, 0.94-2.21) for the second and third tertile versus the first tertile of plasma concentrations of folate, respectively. Plasma concentrations of homocysteine or vitamin B12 were not significantly associated with CRC risk. We also stratified the full study cohort into subgroups based on sex, age, follow-up time between screening and diagnosis, tumor site and stage, and cohort. The plasma concentrations of folate were positively associated with CRC risk in most subgroups except for follow-up time above the median of 10.8 years, lower tumor stage, and rectal cancers. For the second versus the first tertile of plasma folate concentrations, significant associations were observed in subgroups based on female sex, age over the median, follow-up time under the median, tumors with higher stage or localization in the right colon, and the MSP cohort. Furthermore, for vitamin B12 we observed a decreased risk of rectal cancer risk for the second and the third tertile versus the first. The third tertile of vitamin B12 versus the first was also associated with a decreased CRC risk in men, but not in women.
Figure 9. Odds ratios (ORs) of CRC risk for tertiles of plasma concentrations of folate, vitamin B12 (cobalamin), and homocysteine. Adjusted OR adjusted for BMI, current smoking, recreational and occupational physical activity, and alcohol intake.

**Interpretation**

Folate and vitamin B12 are part of one-carbon metabolism, an intracellular network of enzymatic reactions involving the transfer of methyl groups (Figure 3). The main outputs of one-carbon metabolism are nucleotides for DNA and the labile methyl groups for the universal methylator SAM. Folate in different forms acts as a carrier of one-carbon groups and vitamin B12 is an essential co-factor for MS, an enzyme that takes a methyl group from folate and remethylates homocysteine to form methionine. Methionine, in turn, donates the methyl group to form SAM and reverts to homocysteine. Therefore, low availability of either vitamin B12 or folate can increase the concentration of homocysteine. Homocysteine exists only as a byproduct of one-carbon metabolism and cannot be supplied from dietary sources, although plasma concentrations increase after ingestion of a methionine load.

Intake of folate and vitamin B12 differ in that folate is often found naturally in foods that are considered healthy (e.g., fruits, leafy vegetables, and legumes) and vitamin B12 is found in meat, processed meat, and organ meats. In many countries, the main source of folate is the synthetic form, folic acid, found in supplements or added to grains to decrease the incidence of NTDs (Figure 5). Sweden has not implemented mandatory fortification of grains and the folate status in the NSHDS cohort is low. This situation gives us the opportunity to evaluate the lower spectrum of folate concentrations, whereas in many other populations, folate concentrations are not easily studied since even the groups with the lowest folate concentrations are higher than what would be plausibly achievable by consuming naturally
occurring folates.\textsuperscript{213} A special case is a study of almost 1000 case participants nested in the WHI-OS in which screening was performed before mandatory fortification and diagnosis several years after fortification, essentially ensuring that the blood samples did not reflect the folic acid exposure the participants had been subjected to.\textsuperscript{131} In another study in the same cohort, plasma concentrations of homocysteine were associated with CRC risk.\textsuperscript{162} As folic acid is known to decrease homocysteine concentrations,\textsuperscript{99} this study shares the same issues with blood samples not reflecting exposure over time. Other studies on the associations of homocysteine to CRC risk,\textsuperscript{160, 161} including two from the NSHDS,\textsuperscript{132, 205} did not find any significant associations. Increases in homocysteine concentrations can depend on factors that have opposing associations to CRC risk,\textsuperscript{97, 99, 214} such as the increased risk associated with low vitamin B6 and methionine status,\textsuperscript{163} the lower risk associated with the \textit{MTHFR} 677TT polymorphism,\textsuperscript{107, 133} and the lower risk for CRC associated with low folate status in the NSHDS.\textsuperscript{132, 205} Therefore, we believe that the null association between homocysteine and CRC risk in the NSHDS is biologically plausible.

Many studies on plasma concentrations of folate in relation to CRC have been published observing no significant association,\textsuperscript{126, 129-131, 134, 161} with the exception of one small study where plasma concentrations of folate were associated with a lower CRC risk.\textsuperscript{127} However, several studies in populations with a low folate status and no mandatory folic acid fortification have found an association between folate and a higher risk of either CRC or colon cancer.\textsuperscript{132, 133, 161, 205} As with the studies nested in the NSHDS, Takata et al. found an association between higher CRC risk in the middle quartile versus the lowest quartile of plasma folate concentrations in subjects with a follow-up time closer to diagnosis.\textsuperscript{130} Lee et al. found an association between lower plasma folate concentrations and a lower CRC risk in a study of over 600 case participants in a study on pre-fortification blood samples nested in the Nurses’ Health Study, the Health Professionals Follow-up Study, and the Physicians’ Health Study.\textsuperscript{133} However, the largest study to date, comprising over 1300 case participants in the EPIC cohort, found no association with CRC risk.\textsuperscript{134} In that study, mean folate concentrations were relatively high, which could be a result of differences in diet and voluntary folic acid fortification in different parts of Europe.\textsuperscript{212}

In accordance with our previous study on vitamin B12 in relation to CRC, we found an association between plasma concentrations of vitamin B12 and a lower risk for rectal cancer, but not CRC risk. We also observed a linear decrease in CRC risk in men, but not in women. In a study comprising over 1300 case participants nested in the EPIC cohort, Eussen et al. observed no association between plasma concentrations of vitamin B12 and CRC
irrespective of sex or subsite in the colon. The reason for the discrepancy between the studies is currently unknown. The southern and central European parts of the EPIC cohort have considerably higher plasma concentrations of folate than in the northern region, where the NSHDS is situated. In the NSHDS and other populations with low folate, the combination of low vitamin B12 and the overall low folate status could lead to lower activity of methionine synthase (MS), an enzyme that remethylates homocysteine to methionine for the universal methylator SAM in a reaction where vitamin B12 is a cofactor and me-THF donates methyl groups. Lower MS activity leads to aberrations in DNA and histone methylation, processes that influence tumorigenesis. To some extent, women are protected from the deleterious effect of decreased MS activity since the alternative path of remethylation of homocysteine through BHMT is more active in premenopausal women. Furthermore, compared to women, men are almost twice as likely to develop rectal cancer. Consequently, the association between vitamin B12 and a lower rectal cancer risk may partly be explained by the association between plasma concentrations of vitamin B12 and a lower CRC risk overall found in men.

When examining causation in prospective nested case-control studies, it is necessary that both the prospective case and control participants are healthy at baseline. That is, the exposure must precede events related to the outcome. However, this is difficult to attain in slowly developing diseases and chronic disease such as CRC. The time period from when a CRC tumor first could be detected by screening until diagnosis has been estimated to between five and 20 years. Most nested case-control studies on folate have a median follow-up time from screening to diagnosis of well below ten years. This short follow-up time indicates that some of the prospective case participants have established pre-cancerous lesions when their blood sample is collected. In both our study and in the previously mentioned study by Takata et al., the associations were significant in samples collected closer to diagnosis. In addition, plasma concentrations of folate are not associated with the precursor to CRC, colorectal adenoma. Consistent with animal model research, these prospective studies suggest that folic acid supplementation is protective in the normal mucosa, but may accelerate the growth of established pre-cancerous lesions. A considerable part of the population will at some point develop CRC (the cumulative incidence of CRC is over 5% at 80 years of age (Figure 1)), and many people with precancerous lesions would be affected by mandatory folic acid fortification. In comparison, NTDs are rare, but similar to CRC represents a considerable public health issue. Different measures have been proposed to mitigate this situation. Public health efforts to increase use of folic acid supplements during pregnancy have largely been unsuccessful. Some alternatives have been proposed instead of mandatory folic acid fortification, such as packaging folic
acid pills with feminine hygiene products. One could also argue that a comprehensive CRC screening program may attenuate a potential increase of CRC progression from folic acid fortification. In the US where mandatory folic acid fortification was implemented in 1998, the CRC incidence and mortality are steadily decreasing, which could be a result of widespread screening with colonoscopy. While the pros and cons of folic acid fortification are still debated, it is important to stress the importance of folate in preventing NTDs. Since CRC is rarely present in the young (Figure 1), temporary folic acid supplementation is unlikely to influence CRC development in this population. We, the authors of Paper I, strongly recommend folic acid supplementation for women of childbearing age.
Paper II

Main Results

This prospective case-control study nested in the NSHDS Paper II contains all case participants diagnosed between 1985 and 2009 and their matched control participants. The selection of case and control participants is depicted in Figure 8.

The study contains participants from both the VIP (78% of the participants) and the all-female MSP (22% of the participants). Consequently, the study has a higher percentage of women than men (59% women). Figure 10 depicts the multivariable ORs for CRC according to tertiles of plasma concentrations of betaine, choline, dimethylglycine, sarcosine, and methionine. We found significant associations between a lower CRC risk and the third versus the first tertile of betaine and methionine concentrations, with ORs of 0.76 (95% CI, 0.59-0.99) and 0.72 (95% CI, 0.55-0.94), respectively. We calculated absolute risk difference as incidence per 100 000 individuals for both betaine and methionine. When comparing the third and the first tertiles of plasma concentrations, the risk difference for both metabolites was approximately 200 fewer diagnosed CRC cases per 100 000 participants. Plasma concentrations of choline, dimethylglycine, or sarcosine were not significantly associated with CRC risk. We also stratified the full study group based on the median follow-up time between screening and diagnosis. The plasma concentrations of betaine were inversely linearly associated with CRC trend in the subgroup above the median follow-up time, whereas for methionine a similar risk distribution was observed in the subgroup below the median follow-up-time.

We explored biologically plausible interactions between one-carbon factors. Low folate combined with high methionine status was associated with a low CRC risk, OR 0.39 (95% CI, 0.24-0.64). Methionine was mainly associated with a lower CRC risk in study participants with the variant MTR 2756 AG/GG genotypes.
Figure 10. Odds ratios (ORs) and Risk differences (RDs) of CRC risk for plasma concentrations of choline, betaine, dimethylglycine, sarcosine, and methionine. ORs are unadjusted since no potential confounder substantially changed the ORs. RDs are adjusted for the matching variables, BMI, smoking status, occupational physical activity, and plasma folate, riboflavin (vitamin B2), cobalamin (vitamin B12), and methionine concentrations.

**Interpretation**

The one-carbon metabolism factors included in Paper II are all involved in the methionine cycle and the transfer of methyl groups carried by folates or from the choline oxidation pathway for the universal methylator SAM. In the choline oxidation pathway, BHMT catalyzes the transfer of a methyl group from betaine to homocysteine to produce methionine and dimethylglycine. Betaine can be supplied either through diet or through oxidation of choline. Dimethylglycine, in turn, can be oxidized to sarcosine.

Methionine in one-carbon metabolism is the product of the vitamin B12 dependent enzyme MS and used for remethylation of S-adenosylhomocysteine (SAH) to S-adenosylmethionine (SAM), simultaneously reverting methionine back to homocysteine (Figure 3). Excess homocysteine can also be diverted through the PLP-dependent transsulfuration pathway where homocysteine provides sulfur for cysteine formation. Methyl groups for MS are provided by me-THF, a form of folate formed by MTHFR that has no other use than donating methyl groups for MS. Consequently, vitamin B12 deficiency decreases the activity of MS and
increases the accumulation of me-THF. This phenomenon is known as the folate trap.

The metabolites in Paper II have only been analyzed in relation to CRC in two cohorts, EPIC and WHI-OS. Both are large cohorts consisting of approximately 1300 and 800 case participants, respectively, and their matched controls. In EPIC, plasma concentrations of betaine, choline, and methionine were associated with a lower CRC risk, although choline predominantly in women and betaine only in a subgroup with a low folate status (similar to the folate status in the NSHDS). The WHI-OS is an all-female cohort in which mandatory folic acid fortification of grains was introduced after sample collection but before CRC diagnosis, as previously described. Conceivably, this should not be as important regarding the metabolites studied in Paper II since no direct influence on metabolite concentrations takes place. In the WHI-OS, plasma concentrations of betaine were associated with a lower CRC risk, while choline and dimethylglycine were not associated with CRC risk. Plasma concentrations of sarcosine have not previously been studied in relation to CRC risk.

Taken together, the investigations in the EPIC, WHI-OS, and NSHDS indicate weak inverse associations between betaine, choline, and methionine and CRC risk. However, specific conditions that could influence the associations include a possible interaction with plasma folate for methionine and choline only being associated with lower CRC risk in women in EPIC but not at all in the all-female WHI-OS or in the NSHDS.

Odds ratios and other relative risk estimates can be difficult to interpret and do not consider the incidence rate of the disease. As an example, each year in Sweden approximately 100,000 children are born and 20-25 of these have NTDs, and the yearly CRC incidence is approximately 6000. A hypothetical added exposure with an estimated risk association of OR 2.0 would result in 6000 more diagnosed cases of CRC and 25 more cases of NTDs. An absolute risk estimate takes the incidence of the outcome in the studied population into account. In the NSHDS, the cumulative incidence of CRC from 1987 to 2009 was approximately 830 per 100,000 participants. We used the incidence rate in each matching group defined by age, cohort, sex, and sampling year to calculate the average cumulative incidence in our nested study cohort (approximately 660 per 100,000 participants). The total risk difference of 200 fewer diagnosed cases of CRC per 100,000 participants for high plasma concentrations of betaine and methionine versus low plasma concentrations represents a considerable number of CRC case participants.
One of the more interesting findings of the study is the interaction between low plasma concentrations of folate and high plasma concentrations of methionine, resulting in a substantial decrease of CRC risk (>60%). As previously mentioned, folate is active in both the methionine and the folate cycle, whereas methionine and betaine are only active in the methionine cycle. Therefore, in the NSHDS where low folate status is associated with a lower CRC risk, the association between the methyl donors methionine and betaine with respect to lower CRC risk suggests that the output of the folate cycle, and not the methionine cycle, accounts for the increase in CRC risk. The folate cycle involves the synthesis of nucleotides for DNA, essential for cancer cells, and folate deficiency causes misincorporation of uracil into DNA similar to the commonly used chemotherapy 5-FU. The interaction with methionine also suggests a protective association to CRC risk only when the methionine cycle is functioning well.

Tumor cells require methionine added to cell media to proliferate, but normal cells can proliferate with only the precursor homocysteine, a phenomenon known as the methionine dependency of tumor cells. These in vitro results have been replicated in vivo in animal models fed a low methionine diet. Accordingly, methionine restriction has potential as a dietary intervention in cancer patients. However, dietary methionine restriction does not consistently reduce plasma concentrations of methionine, and degradation of the surrounding tumor stroma can supply sufficient methionine for the tumor. We found that higher plasma concentrations of methionine were more strongly associated with lower CRC risk in patient samples collected closer to diagnosis. This finding indicates that low methionine status did not inhibit tumor growth and that tumor cell methionine dependency did not influence the CRC risk in our cohort.
Paper III

Main Results

The study contains the same participants as in Paper II and case and control participant selection is depicted in Figure 8. The multivariable ORs for CRC according to quartiles of HK:XA, PAr, and PLP are shown in Figure 11. We found significant associations between a higher CRC risk and the fourth versus the first quartile of HK:XA and PAr, with ORs of 1.48 (95% CI, 1.08-2.02) and 1.50 (95% CI, 1.10-2.04), respectively. The third versus the first quartile of plasma concentrations of PLP and sufficient versus deficient vitamin B6 status was significantly associated with CRC risk, with ORs of 0.60 (95% CI, 0.60-0.81) and 0.55 (0.37-0.81), respectively. We stratified the full study group into three groups according to follow-up time between screening and diagnosis. Higher plasma concentrations of HK:XA and PAr were associated with CRC risk more strongly in the subgroups with less than 10.5 years of follow-up time, but no significant association was observed in the subgroup with 10.5 years or more of follow-up time. Plasma concentrations of PLP were not significantly influenced by follow-up time.

![Table of Odds Ratios and Risk Differences](image)

Table 1. Odds ratios (ORs) and Risk differences (RDs) of CRC risk for plasma concentrations of PLP in quartiles and as Vitamin B6 deficiency/sufficiency (cut-off 20 nmol/L), and for quartiles of PAr and HK:XA. ORs and RDs are adjusted for kidney function, BMI, smoking status, alcohol intake, dietary fiber intake, recreational and occupational physical activity, and plasma folate, riboflavin (vitamin B2), and cobalamin (vitamin B12) concentrations. RDs are further adjusted for the matching variables.

Figure 11. Odds ratios (ORs) and Risk differences (RDs) of CRC risk for plasma concentrations of PLP in quartiles and as Vitamin B6 deficiency/sufficiency (cut-off 20 nmol/L), and for quartiles of PAr and HK:XA. ORs and RDs are adjusted for kidney function, BMI, smoking status, alcohol intake, dietary fiber intake, recreational and occupational physical activity, and plasma folate, riboflavin (vitamin B2), and cobalamin (vitamin B12) concentrations. RDs are further adjusted for the matching variables.
**Interpretation**

PLP, the active form of vitamin B6, is the most commonly used marker of vitamin B6 status and is used to determine vitamin B6 deficiency.\(^{164,211}\) PLP is involved in several processes that influence cancer risk and development, such as angiogenesis, inflammation, cell proliferation, and one-carbon metabolism. In one-carbon metabolism, PLP is a co-factor for SHMT, which regulates the influx of labile methyl groups into the folate cycle, and for CBS and CSE, which regulate the degradation of homocysteine through the transsulfuration pathway.

In studies on plasma concentrations of PLP in relation to CRC risk, high PLP concentrations are consistently associated with a lower risk.\(^{163,168}\) In contrast, studies on dietary intake of vitamin B6 have found no significant association with CRC risk.\(^{167,169}\) The discordant results between dietary intake estimates and plasma concentration measurements of vitamin B6 could possibly be explained by confounding as ingestion of high amounts of vitamin B6 may be associated with a generally healthier lifestyle.\(^{163}\) Another possible explanation could be the inverse association between systemic inflammation – a risk factor for CRC\(^ {203,218}\) – and plasma PLP.\(^{163,219}\)

Low plasma concentrations of PLP are associated with several inflammatory disease conditions, including cancer, IBD, cardiovascular disease, rheumatoid arthritis, and type II diabetes.\(^ {220}\) Furthermore, inflammatory biomarkers are inversely associated with plasma PLP status.\(^ {220}\) In IBD patients, PLP concentrations are lower in patients with active disease than in patients with quiescent disease, an observation that could suggest that dietary changes do not decrease PLP concentrations.\(^ {221}\) The association between inflammatory activity and lower plasma concentrations of PLP indicates that either inflammation lowers plasma concentrations of PLP or vitamin B6 protects against inflammation and inflammatory conditions. However, in an intervention study, dietary restriction of vitamin B6 did not increase the inflammatory biomarker CRP and in a study of patients with stable angina pectoris, inverse associations between plasma concentrations of PLP and inflammatory biomarkers were maintained even after vitamin B6 supplementation.\(^ {222}\) In addition, in the population-based NHANES cohort, vitamin B6 deficiency (PLP <20 nmol/L) was inversely associated with CRP independent of vitamin B6 intake.\(^ {223}\) Taken together, these observations indicate that inflammatory processes influence PLP concentrations. Mechanistically, lower PLP concentrations in plasma are a result of the redistribution of PLP into tissue with inflammation and an increase in PLP catabolism of PA during inflammation and oxidative stress.\(^ {220}\)
We examined three estimates of vitamin B6 status to account for inflammatory activity and to allow for a more comprehensive assessment than relying on PLP alone. PAr is a ratio of the catabolite and the sum of the active and transport form of vitamin B6. PAr is less influenced than PLP by common confounders in studies on plasma biomarkers, such as kidney status, supplementation, and smoking.\textsuperscript{177} HK:XA is the ratio of plasma concentrations of the substrate (HK) and product (XA) pair of the vitamin B6 dependent enzyme KAT. HK:XA is negatively associated with plasma concentrations of PLP and is a sensitive marker of vitamin B6 deficiency. Compared to HK, it is not as influenced by inflammation, smoking, kidney status, and BMI.\textsuperscript{178}

We found that vitamin B6 status was associated with CRC risk for all three markers. PLP deficiency was strongly associated with a higher CRC risk. Similarly, HK:XA – the inverse functional vitamin B6 status marker – was associated with a linear increase in CRC risk. PAr, the marker of vitamin B6 flux and catabolism during inflammatory activity, was associated with a higher CRC risk. The results for PAr resemble those of other inflammatory markers, such as CRP and neopterin, and to those observed for PAr in relation to CRC risk in the HUSK cohort. We stratified the full study cohort according to follow-up time between screening and diagnosis and observed that PAr was only associated with CRC risk in study participants with a follow-up time between screening and diagnosis lower than 10.5 years, suggesting a role in tumor progression rather than initiation. HK:XA is a sensitive marker of vitamin B6 deficiency, and accordingly replicates the associations observed for vitamin B6 deficiency defined by PLP. We conclude that inflammation needs to be considered when studying plasma concentrations of vitamin B6 status, particularly in diseases with an inflammatory aspect.
Paper IV

Main Results

The study contains the same participants as in Paper II and III and case and control participant selection are depicted in Figure 8. We validated the sensitivity and specificity of the functional B-vitamin markers total homocysteine (tHcy), and the ratio-based B-vitamin markers hcy:cys, hcy:cre, and hcy:cys:cre as markers of total B-vitamin status (represented by a summary score comprising Z-standardized plasma concentrations of betaine, cobalamin, folate, PLP, and riboflavin). In both case and healthy control participants, the ratio-based B-vitamin markers outperformed tHcy (Table 1). The ratio-based B-vitamin markers and total B-vitamin status had similar associations to CRC risk: approximately a 25% risk decrease associated with estimated higher B-vitamin status versus low B-vitamin status.

Table 1 Performance of total B-vitamin score in predicting plasma tHcy, Hcy:Cys, Hcy:Cre, and Hcy:Cys:Cre status in CRC cases and controls.

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* Defined as variance explained by B-vitamin score.
¤ Defined as variance explained by B-vitamin score divided by total variance explained by the model.
§ Sensitivity multiplied by specificity divided by 100.
Abbreviations: CRC, colorectal cancer; tHcy, total homocysteine; ref, reference category.

Interpretation

Homocysteine is a precursor for both methionine and cysteine synthesis in the methionine cycle and transsulfuration pathway, respectively.\(^{84, 99}\) Methionine provides labile methyl groups for SAM synthesis, and creatine synthesis consumes a major portion of SAM-provided methyl groups in humans.\(^{224}\) Creatine is non-enzymatically degraded to creatinine at a rate of approximately 2% per day and secreted into the urine.\(^{225, 226}\) The ratio of homocysteine to creatinine (Hcy:Cre) is thus an estimate of the flux of homocysteine to creatinine, and disturbances in the involved B-vitamins result in an increase in Hcy:Cre. This includes folate, riboflavin (co-factor for MTHFR when transforming m-THF to me-THF), cobalamin (co-factor for MS when transforming me-THF and homocysteine to methionine), and betaine (co-factor for BHMT when transforming betaine and homocysteine to...
methionine). In the transsulfuration pathway, homocysteine provides sulfur in a series of PLP-dependent enzymatic reactions for cysteine, and consequently PLP deficiency results in increases in the ratio of homocysteine to cysteine (Hcy:Cys) (Figure 3).

In clinical settings, plasma homocysteine is a marker of impaired B-vitamin status, but is not convincingly associated with CRC risk in prospective case-control studies. This result is surprising, considering the evidence of several one-carbon metabolites, including PLP, betaine, and riboflavin, being inversely associated with both homocysteine and CRC risk. Furthermore, the MTHFR 677TT polymorphism is associated with a lower CRC risk and higher plasma concentrations of homocysteine. The similar risk distribution for the ratio-based B-vitamin markers and total B-vitamin score are consistent with the ratio-based B-vitamin markers ability to correctly estimate total B-vitamin score, and the previously reported null association between tHcy and CRC risk is also in line with the lower performance of tHcy as a marker of total B-vitamin status.

While Hcy:Cys:Cre was observed to have the highest sensitivity and specificity as a functional marker of total B-vitamin status, both Hcy:Cys and Hcy:Cre performed markedly better than tHcy. Hcy:Cre is particularly interesting as it consists of two markers that are already routinely analyzed in a clinical setting (tHcy and creatinine). Applying a simple algorithm is a cheap and simple way of drastically increasing sensitivity and specificity when assessing total B-vitamin status. The ratio-based B-vitamin markers performed equally well in participants who later developed CRC and in healthy control participants, further increasing their usefulness. Future research relating the ratio-based B-vitamin markers to disease risk, particularly in anemia, could potentially reveal an easy and cost-efficient tool for doctors to increase specificity and sensitivity in disease diagnosis.
Conclusions

**Paper I**
- Low plasma folate concentrations are associated with a lower CRC risk, whereas plasma concentrations of vitamin B12 and homocysteine are not associated with CRC risk. The association between folate and CRC are predominantly observed in case participants with samples collected closer to diagnosis.
- Plasma concentrations of vitamin B12 are associated with lower rectal cancer risk.

**Paper II**
- Plasma concentrations of betaine and methionine are associated with a lower CRC risk. Plasma concentrations of choline, dimethylglycine, and sarcosine are not associated with CRC risk.
- The estimated marginal risk difference for high plasma concentrations versus low plasma concentrations of betaine and methionine were approximately 200 CRC fewer cases per 100 000 individuals.
- In interaction analyses, the lower CRC risk associated with high plasma concentrations of methionine is modified by folate, and the combination of low folate and high methionine status is associated with a substantial decrease in CRC risk.

**Paper III**
- Sufficient versus deficient vitamin B6 status as defined by plasma concentrations of PLP are associated with a lower CRC risk. The inverse vitamin B6 markers PAr and HK:XA are associated with a higher CRC risk.
- PAr, used to estimate vitamin B6-status in inflammation and oxidative stress, are predominantly associated with CRC risk in case participants with samples collected closer to CRC diagnosis.

**Paper IV**
- Total B-vitamin score is associated with a lower CRC risk and the ratio-based B-vitamin markers have a similar association to CRC risk.
- In both case and control participants, the three ratio-based B-vitamin markers perform better than homocysteine alone at determining deficient B-vitamin status.
Future Considerations

One-carbon metabolism in CRC development has been extensively investigated. Findings have shown some degree of inconsistency, but balanced one-carbon metabolism is important for the stability of the genome and epigenetic regulation and may prevent tumor initiation, whereas an excess of some components, especially folate, may facilitate the progression of precancerous lesions.\textsuperscript{85, 117} CRC develops through distinct pathways resulting in molecular subtypes differing in clinical characteristics such as response to treatment.\textsuperscript{227} These subtypes can be defined by different factors, such as mutations in the mutually exclusive \textit{BRAF} and \textit{KRAS} oncogenes, and/or MSI and CIMP status.\textsuperscript{31} Investigating one-carbon metabolism in relation to these molecularly defined CRC subtypes could give new insights into the heterogeneous nature of CRC tumorigenesis.

A characteristic of CRC is the variation in outcome depending on tumor stage,\textsuperscript{15} but also depending on molecular characteristics such as MSI and CIMP status, and immune cell infiltration into the tumor.\textsuperscript{16} Several components of one-carbon metabolism are related to inflammation, and relating aspects of one-carbon metabolism to immune cell activity can give insights not only into tumorigenesis but also patient outcome. A high density of tumor-infiltrating immune cells is associated with beneficial CRC prognosis,\textsuperscript{16} but why the immune system is responding to certain tumors, and how the immune response is beneficial to patients is not fully understood. A factor especially important in CRC survival is cancer cachexia - the breakdown of muscle mass in cancer patients.\textsuperscript{24} Cachexia is thought to arise from inflammatory cytokines released by the tumor.\textsuperscript{21} By combining biobanks at Umeå University and Västerbotten County Council we can gather information from pre-diagnostic blood samples, from tumor tissue, and on patient prognosis. Tumor progression can thus be longitudinally followed and cancer cachexia can be related to one-carbon metabolism and inflammatory activity at different stages of disease. This is an interesting future field of research in both CRC survival and early CRC detection.
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References


120. Mason, J.B. *et al.* A temporal association between folic acid fortification and an increase in colorectal cancer rates may be illuminating important biological principles: a hypothesis. *Cancer Epidemiol Biomarkers Prev* 16, 1325-1329 (2007).


Takata, Y. et al. Plasma folate concentrations and colorectal cancer risk: a case-control study nested within the Shanghai Men's Health Study. *Int J Cancer* 135, 2191-2198 (2014).


Lee, J.E. et al. Plasma folate, methylenetetrahydrofolate reductase (MTHFR), and colorectal cancer risk in three large nested case-control studies. *Cancer Causes Control* 23, 537-545 (2012).


172. van Kapel, J., Spijkers, L.J., Lindemans, J. & Abels, J. Improved distribution analysis of cobalamins and cobalamin analogues in


