

Folate in Cancer and Cardiovascular Disease

Prospective Studies from the Population-based Northern Sweden Health and Disease Study

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This thesis is dedicated to Steve Adamowicz.
Thank you for teaching me science.

THESIS AT A GLANCE

Folate in Cancer and Cardiovascular Disease Prospective studies from the population-based Northern Sweden Health and Disease Study

| Endpoint | Paper | Reference | Cases | Variables studied | Main findings* |
|-----------------------------------|-------|---|--------------------------|---|---|
| Colorectal cancer (CRC) | I | Van Guelpen, <i>et al</i> , Gut 2006;55(10):1461-66 | 226 | Plasma folate and homocysteine concentrations | Subjects with the highest plasma folate levels had the same risk of CRC as subjects with the lowest levels. Subjects with levels mid-range had nearly double the risk. |
| | - | Thesis | 180 | MTHFR 677C>T and 1298A>C polymorphisms Promoter hypermethylation of p16 and hMLH1 in CRC tissue | The MTHFR 677C>T polymorphism was associated with a 60% reduction in CRC risk that was independent of folate status. Prediagnostic plasma folate levels were higher in cases with at least one gene hypermethylated. |
| Prostate cancer (PCa) | II | Hultdin, Van Guelpen, <i>et al</i> , Int J Cancer 2005;113:819-24 | 254 | Plasma folate, vitamin B12, and homocysteine concentrations | Subjects with the highest vitamin B12 levels had nearly three times the risk of PCa compared to subjects with the lowest levels. |
| Stroke (ischemic and hemorrhagic) | III | Van Guelpen <i>et al</i> , Stroke 2005;36:1426-31 | 334 isch. 62 hem. | Plasma folate and vitamin B12 concentrations Dietary intake of folate and vitamin B12 | Subjects with the highest levels of plasma or dietary folate had one fifth the risk of hemorrhagic stroke compared to subjects with the lowest levels. Results were attenuated somewhat and no longer statistically significant after adjusting for homocysteine. |
| Acute myocardial infarction (AMI) | IV | Van Guelpen <i>et al</i> , Submitted, 2006 | 532 | Plasma folate and vitamin B12 concentrations Dietary intake of folate, vitamin B12, vitamin B2, and vitamin B2 | Subjects with the highest plasma folate levels had half the risk of AMI compared to subjects with the lowest levels. The association was independent of homocysteine. |

*Only statistically significant findings are noted.

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ABBREVIATIONS

| | |
|-----------------|---|
| AR | Average daily requirement |
| AMI | Acute myocardial infarction |
| BH ₄ | Tetrahydrobiopterin |
| BMI | Body mass index |
| CI | Confidence interval |
| CT | Computerized tomography |
| CRC | Colorectal cancer |
| CV | Coefficient of variation |
| CVD | Cardiovascular disease |
| dTMP | Deoxythymidylate monophosphate (thymine) |
| dUMP | Deoxyuridylate monophosphate (uracil) |
| eNOS | Endothelial nitric oxide synthase |
| EPIC | European Prospective Investigation into Cancer and Nutrition |
| FAD | Flavin adenine dinucleotide (coenzyme form of vitamin B2) |
| FMN | Flavin adenine mononucleotide (coenzyme form of vitamin B2) |
| IARC | International Agency for Research on Cancer |
| LDL | Low-density lipoprotein |
| LI | Lowest daily intake |
| MMA | Methylmalonic acid |
| MONICA | Multinational Monitoring of Trends and Determinants in Cardiovascular Disease |
| MSP | Mammography Screening Project |
| MTHFR | Methylenetetrahydrofolate reductase |
| NO | Nitric oxide |
| NSAID | Non-steroidal anti-inflammatory drug |
| OR | Odds ratio |
| PCa | Prostate cancer |
| PCR | Polymerase chain reaction |
| PLP | Pyridoxal phosphate (metabolically active form of vitamin B6) |
| P-tHcy | Plasma total homocysteine concentration |
| P-Folate | Plasma folate concentration |
| P-B12 | Plasma vitamin B12 (cobalamin) concentration |
| RI | Recommended daily intake |
| SAH | S-adenosylhomocysteine |
| SAM | S-adenosylmethionine |
| SNP | Single nucleotide polymorphism |
| THF | Tetrahydrofolate |
| TCN | Transcobalamin |
| VIP | Västerbotten Intervention Project |
| Vitamin B2 | Riboflavin |
| Vitamin B6 | Pyridoxine |
| Vitamin B9 | A previously used term for folate |
| Vitamin B12 | Cobalamin |
| WHO | World Health Organization |

ABSTRACT

BACKGROUND: Folate, a B-vitamin found primarily in fruits and vegetables, especially leafy greens, and other B-vitamins involved in folate metabolism are believed to protect against cancer and cardiovascular disease. Maintaining an adequate folate status ensures availability of methyl groups for DNA synthesis and for all methylation reactions in the body, and prevents the accumulation of homocysteine, a sulphur-containing amino acid that has been linked to cardiovascular disease. The aim of this thesis was to relate factors involved in folate metabolism to the risk of developing colorectal cancer (CRC), prostate cancer (PCa), stroke (ischemic and hemorrhagic), and acute myocardial infarction (AMI).

SUBJECTS AND METHODS: These were nested case-referent studies, with 226 CRC, 254 PCa, 396 stroke (334 ischemic and 62 hemorrhagic), and 571 AMI cases, and double, matched referents from the population-based Northern Sweden Health and Disease Study.

CRC RESULTS: A bell-shaped association was observed between plasma folate concentrations and the risk of CRC [multivariate odds ratio (OR) for the middle versus lowest quintile, 2.00 (95% CI 1.13-3.56)]. Homocysteine was not associated with CRC risk. A reduced risk was observed for the MTHFR 677C>T polymorphism [OR for TT versus CC, 0.41 (95% CI 0.19-0.85), $P_{\text{trend}}=0.062$] that was independent of plasma folate status. Prediagnostic plasma folate concentrations were higher in cases with promoter hypermethylation in the p16 and/or hMLH1 tumor suppressor genes in CRC tissue compared to cases without promoter hypermethylation in these genes ($P=0.025$).

PCa RESULTS: Increasing plasma levels of folate and vitamin B12 were associated with increased risk of PCa [OR for the highest versus lowest quartile, 1.60 (95% CI 1.03-2.49), $P_{\text{trend}}=0.02$ for folate, and 2.63 (95% CI 1.61-4.29), $P_{\text{trend}}<0.001$ for vitamin B12]. Increasing plasma homocysteine levels were associated with a reduced risk of borderline significance. In multivariate analyses, the risk estimate remained statistically significant only for vitamin B12.

STROKE RESULTS: Plasma folate concentrations were associated with the risk of hemorrhagic stroke in an inverse linear manner after adjustment for conventional risk factors including hypertension [multivariate OR for the highest versus lowest quartile, 0.21 (95% CI 0.06-0.71), $P_{\text{trend}}=0.008$]. Risk estimates were attenuated by the inclusion of homocysteine in the model [OR 0.34 (95% CI 0.08-1.40), $P_{\text{trend}}=0.088$]. Similar results were obtained for folate intake. Neither plasma folate levels nor folate intake demonstrated a clear association with the risk of ischemic stroke, and neither plasma nor dietary vitamin B12 was associated with the risk of either type of stroke.

AMI RESULTS: Plasma folate concentrations demonstrated an inverse association with risk of AMI that was independent of other risk factors, including homocysteine [multivariate OR for the highest versus lowest quintile, 0.56 (95% CI 0.34 – 0.90), $P_{\text{trend}}=0.080$]. For vitamin B12, no clear risk relationships were apparent. None of the risk estimates for dietary intake of folate, vitamin B12, vitamin B6, or vitamin B2 were statistically significant, although the results for folate and vitamin B12 intake were in line with those for the plasma variables.

CONCLUSIONS: The results of these population-based, prospective studies suggest that although a high folate status may be associated with a reduced risk of cardiovascular diseases, the relationship with cancer risk seems to be more complicated. The possibility of a detrimental component to the role of folate and vitamin B12 in carcinogenesis may have implications in the ongoing debate concerning mandatory folate fortification of foods.

POPULAR SCIENCE SUMMARY

The importance of diet in health is currently a topic of great interest, both in the media and in the research world. The obesity epidemic in industrialized countries, and increasingly in the developing countries, is arguably the most pressing issue, but there is also much interest in specific dietary components, such as vitamins, and the role they may play in promoting health. The aim of this thesis was to examine how a person's folate status (often called folic acid, which is actually the pure, synthetic form of folate) affects the risk of developing colorectal cancer (cancer of the large bowel and rectum), prostate cancer, stroke, and acute myocardial infarction (heart attack).

Folate is a B-vitamin found in many foods, but especially in fresh fruits and vegetables. Dark green, leafy vegetables are particularly rich in folate. All of the study subjects in the thesis were residents of northern Sweden population and were therefore not exposed to mandatory fortification of foods with folic acid, as is the case in North America and a number of other countries. The diet in northern Sweden is low in fruits and vegetables, and so the folate status in this population is also relatively low.

Other B-vitamins, most importantly B12, are also involved in folate metabolism and were therefore included in the studies in this thesis. Vitamin B12 is found almost exclusively in foods of animal origin, such as meats and dairy products.

The studies in this thesis were based on blood samples and information collected over the past 20 years in three studies, the largest of which is the Västerbotten Intervention Project (VIP), with approximately 73 500 participants. In the VIP, all residents of the county of Västerbotten are invited to a free health examination upon turning 40, 50, or 60 years old, as part of an effort to reduce the rates of cardiovascular disease in the region. At that time, participants are invited to donate a blood sample and fill out a lifestyle questionnaire (concerning, among other topics, smoking, alcohol, exercise, and dietary habits), for use in future research.

An important detail of the studies in this thesis is that the blood samples and information used were collected before the participants were diagnosed with any of the diseases in question. There is considerable value in knowing, for example, how a person ate before getting cancer, because afterward, he or she might change their

diet, or might “remember” a healthier diet if asked about dietary habits before the diagnosis. Of course, it is impossible to know who will contract a given disease, so it is crucial in this type of study, called a prospective study, to recruit as many participants as possible.

For each disease studied, a group of people who had the diagnosis, referred to as cases, were compared a group of people who did not, referred to as referents, or sometimes controls. Cases and referents were carefully matched for sex, age, and a number of other characteristics. Known risk factors, such as cholesterol levels in cardiovascular disease, were accounted for in the statistical analyses.

The risk of colorectal cancer was lowest for people with the lowest plasma levels of folate. As folate levels increased so did the risk, but only to a certain point, after which it decreased again. In other words, both low and high levels of folate appeared to reduce the risk of colorectal cancer. People with a common mutation that lowers folate levels, *MTHFR 677C>T*, also had a reduced risk of colorectal cancer. These results were surprising because most studies have shown a simple dose-response relationship, with a higher risk of colorectal cancer at lower folate levels. The folate levels of the people in this study were lower than in other studies, so the population in northern Sweden population may be better suited to studying effects at the lower end of the folate spectrum.

It has been suggested that although a high folate status may be able help prevent a tumor from forming, it might also help an established tumor, even a microscopic one, to grow. Since colorectal cancer generally takes years to develop, low levels of folate might appear to reduce the risk by starving a tumor, slowing its growth and thus delaying diagnosis. This might explain the reduced risk of colorectal cancer in people with lower folate levels.

The main job of folate in the body is to provide one-carbon groups, methyl groups, for methylation reactions in the body. An example that is interesting in terms of cancer development is the methylation of DNA. In healthy tissue, methyl groups are attached along the DNA, providing stability. However, when they occur at the region of a gene that controls gene expression, the promoter, they act like a switch, silencing gene expression. In cancer, certain important genes that should be active all the time (so-called tumor suppressor genes) often have methyl groups bound to their promoters, and are thus turned off.

To test whether prediagnostic folate status might be related to promoter methylation of tumor suppressors, two genes, p16 and hMLH1, were analyzed in tumor tissue from the colorectal cancer cases. Folate levels were found to be higher in cases with promoter methylation in at least one of the two tumor suppressor genes analyzed. Although this finding is preliminary and more genes will be examined, it hints at a mechanism by which low folate levels might prevent colorectal cancer. However, any clinical applications of such an effect, even if confirmed, would be limited to certain high-risk patients.

In the prostate cancer study, people with the highest plasma levels of vitamin B12 had an almost three times higher risk of prostate cancer than people with the lowest levels. High levels of folate also seemed to increase the risk of prostate cancer, but when other factors, including vitamin B12, were accounted for the association disappeared. Vitamin B12 helps folate donate methyl groups for methylation reactions, and so the same hypotheses might be considered for prostate cancer as for colorectal cancer, that vitamin B12 aids in tumor growth or contributes to gene-specific hypermethylation. However, since vitamin B12 is only found in foods of animal origin, especially meat, it is also possible that the increased risk might be due a high intake of meat rather than to vitamin B12 specifically.

In cardiovascular disease, high levels of folate are believed to reduce risk by holding a person's homocysteine levels in check. Homocysteine is an amino acid in the blood that is linked to cardiovascular disease. However, whether homocysteine has a causal, biological role in cardiovascular disease or is simply a marker for some other factor is unknown.

Both high levels of folate in plasma and a high dietary intake of folate were associated with a reduced risk of hemorrhagic stroke (a bleeding in the brain), but showed no association with ischemic stroke (a blood clot in the brain). The association with hemorrhagic stroke could not be completely explained by homocysteine, suggesting that folate may have a protective effect beyond its ability to lower homocysteine levels. Hemorrhagic stroke accounts for only about 15% of all strokes, so this study was the first with enough cases to analyze hemorrhagic strokes separately. On the other hand, a number of studies have shown that folate reduces the risk of ischemic stroke, and so the null result for ischemic stroke was unexpected. It is possible that the folate levels of the people in this study were too

low to detect an association. Neither plasma levels nor dietary intake of vitamin B12 was associated with the risk of either type of stroke.

In the acute myocardial infarction study, people with the highest plasma folate levels had the lowest risk, regardless of their homocysteine status. The results for dietary folate intake were consistent with those for the plasma levels, but were not statistically significant. For vitamin B12 (plasma levels and dietary intake) and for dietary intake of vitamins B6 and B2, no clear associations were apparent.

These findings, as well as numerous other epidemiological studies, suggest that folate may protect against cardiovascular disease. However, recent large, placebo-controlled studies of B-vitamin therapy have generally found no effect on either stroke or heart disease, which has led to much controversy and debate. The trials were all based on patients who already had cardiovascular disease, and were thus secondary prevention studies. In addition, the treatment periods were all under five years. Therefore, the potential effects of a diet high in folate consumed over many years has yet to be determined.

The conclusions of this thesis were that while folate may reduce risk of cardiovascular disease, its association with cancer development appears to be more complicated. The possibility of a detrimental component to the role of folate and vitamin B12 in cancer development may have implications on the current debate concerning mandatory folic acid fortification of foods.

ORIGINAL ARTICLES

- I) Van Guelpen B, Hultdin J, Johansson I, Hallmans G, Stenling R, Riboli E, Winkvist A, Palmqvist R. Low folate levels may protect against colorectal cancer. *Gut* 2006;55(10):1461-66.
- II) Hultdin J, Van Guelpen B, Bergh A, Hallmans G, Stattin P. Plasma folate, vitamin B12, and homocysteine and prostate cancer risk: A prospective study. *Int J Cancer* 2005;113:819-24.
- III) Van Guelpen B, Hultdin J, Johansson I, Stegmayr B, Hallmans G, Nilsson TK, Weinehall L, Withhöft C, Palmqvist R, Winkvist A. Folate, vitamin B12 and risk of ischemic and hemorrhagic stroke: a prospective, population-based study of plasma concentrations and dietary intake. *Stroke* 2005;36:1426-1431.
- IV) Van Guelpen B, Hultdin J, Johansson I, Withhöft C, Weinehall L, Eliasson M, Hallmans G, Palmqvist R, Jansson J-H, Winkvist A. Folate and related B-vitamins and risk of acute myocardial infarction: a nested case-referent study of plasma concentrations and dietary intake. Submitted 2006.

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BACKGROUND

INTRODUCTION

“An ounce of prevention is worth a pound of cure.” (Henry de Bracton, 1240)

The most common causes of death in the western world are non-communicable, chronic, diseases. This is the case for many types of cancer, including colorectal (CRC) and prostate cancer (PCa), and for cardiovascular disease (CVD). When people migrate from developing countries to industrialized countries, their incidence of these “western” diseases rapidly approaches levels in the host country. An often-cited, example is the Hawaii Japanese, who emigrated from Japan at the turn of the nineteenth century. The long-term Honolulu Heart Study, initiated in 1940, has provided valuable insight into the changing lifestyle and disease patterns of this group¹. The incidence rates of chronic diseases have traditionally been highest in the industrialized countries (as illustrated for CRC in Figure 1). However, as developing countries adopt a more western lifestyle, including western dietary habits, a major transition is occurring, with increases in obesity and chronic diseases affecting much of the developing world²⁻⁴. These observations all point toward an important role of lifestyle in the etiology of chronic diseases.

In Sweden, cancer is the second leading cause of death, and rates are increasing⁵. Although this is due largely to the aging population, the challenge of identifying risk factors and developing cancer prevention strategies remains important. In contrast to cancer, the incidence and mortality rates for acute myocardial infarction (AMI, heart attack) have declined dramatically in recent decades⁵⁻⁷. This can be attributed primarily to lower smoking rates in men, as well as better treatment and preventative medicine, such as statins for cholesterol and antihypertensive drugs, but it is probably to some extent also a result of changing dietary patterns⁶. However, despite these promising trends, decreases in the incidence and mortality due to stroke have been more modest^{5, 6, 8}, and CVD is still the leading cause of death in both men and women in Sweden⁵.

Background

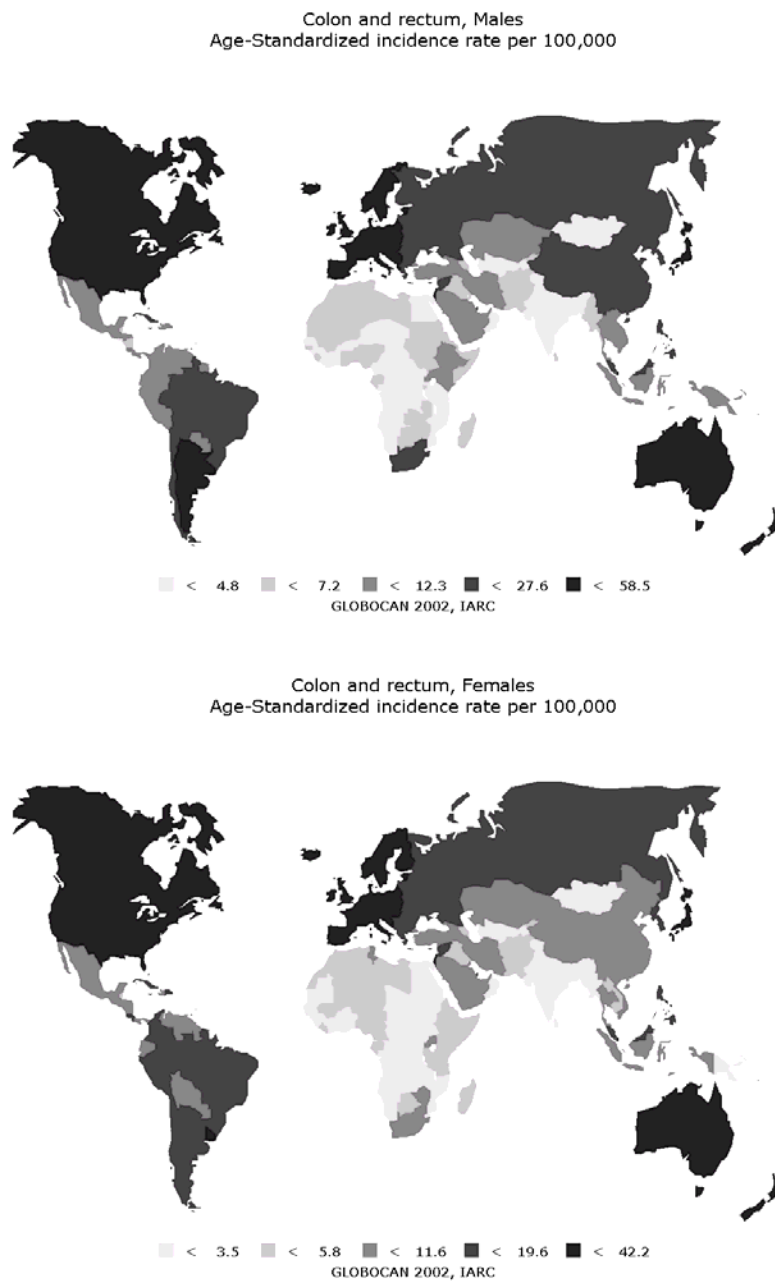


Figure 1. Age-standardized incidence rates (per 100 000) for colorectal cancer in men and women. Incidence rates are highest in western Europe, North America, and Australia. Source: J. Ferlay, F. Bray, P. Pisani and D.M. Parkin. GLOBOCAN 2002: Cancer Incidence, Mortality and Prevalence Worldwide. IARC CancerBase No. 5. version 2.0, IARC Press, Lyon, 2004. <http://www-dep.iarc.fr/>

In efforts to identify potential risk factors for a disease, the field of epidemiology has a central role. Epidemiology is the study of the causes, distribution (for example, geographical or socioeconomic), prevention and control of disease, and is based on observation rather than experimentation. There have been a number of major epidemiological successes, such as the identification of the role of smoking in lung cancer, hepatitis B infection and aflatoxin exposure in liver cancer, and human papillomavirus infection in cervical cancer. The effects of individual dietary factors in cancer and CVD are likely to be considerably more modest, but for such major public health challenges, even a small reduction in risk can translate into substantial numbers of lives, not to mention financial resources, saved. A drawback of epidemiology is, of course, that it is difficult to establish true biological, cause-effect relationships between risk factors and disease. In order to confirm the results of epidemiological studies, clinical trials, which are considered the gold standard, are usually necessary.

CANCER DEVELOPMENT

According to the general model of carcinogenesis, most tumors originate from a single cell, as the result of the successive acquisition of a handful of somatic mutations^{9, 10}. The mutations are fairly specific to each cancer type and generally occur in genes responsible for cell growth, differentiation, and apoptosis, and in genes that preserve the integrity of genome. Although some events are more common early in carcinogenesis, the total accumulation, rather than order of occurrence, is believed to be most important⁹, providing the cell with a selective advantage that eventually allows it to take on a malignant phenotype.

The genes that are involved in tumorigenesis can be broadly grouped into proto-oncogenes and tumor suppressor genes (including DNA repair genes). Proto-oncogenes have the potential to become oncogenes, that is, to promote cancer development. They can be developmental genes, such as growth factors, that should not be expressed in the cells of an adult human, or a mutant form of a gene normally expressed. Some genes act as oncogenes only when overexpressed. In contrast to proto-oncogenes, tumor suppressors prevent tumorigenesis. That is, they become pro-cancer when silenced or defective, or when their regulation is defective. According to Knudson's "two-hit" hypothesis, the inactivation of both alleles of a tumor suppressor gene is required in order for it to contribute to cancer development¹¹. For example, in a type of familial CRC, hereditary non-polyposis

colorectal cancer (HNPCC), patients have germ-line mutations in one allele of a mismatch repair gene. Any defect silencing the expression of the healthy allele is thus a “second hit”. People with HNPCC mutations have a very high risk of cancer, generally characterized by cancer diagnosis at a young age (especially CRC but also some other locations) and by multiple primary tumors.

Not all genetic alterations in tumorigenesis are due to mutations in the DNA. The field of study of reversible heritable changes in gene function not caused by alterations in the genetic sequence is called epigenetics, and includes DNA methylation and histone modifications, such as acetylation. Histone modifications will not be addressed in this thesis but are described a review by Jones and Baylin¹², in which a comprehensive summary of the putative role of epigenetics in cancer is presented.

DNA methylation occurs at CG-dinucleotides across the genome and is believed to aid in maintaining DNA and chromosome stability¹³. Most tumors demonstrate some degree of global genomic hypomethylation, up to 60% in some cases¹⁴. However, regions dense in CG-dinucleotides, called CpG islands, occur particularly frequently in the promoter region of genes, where they act as a molecular switch, blocking the binding of transcription factors to the promoter when methylated and, consequently, silencing gene expression. Promoter hypermethylation of tumor suppressor genes is believed to have a crucial role in tumorigenesis^{12, 15, 16}, and has been reported to provide the “second hit” in hereditary CRC¹⁷. Promoter hypomethylation, and consequent aberrant expression, of proto-oncogenes has also been suggested to contribute to tumorigenesis^{14, 18}.

In spontaneous CRC, a “CpG methylator phenotype” (CIMP) has been described¹⁹, characterized by a unique pattern of tumor suppressor hypermethylation, that may be a distinct pathway of colorectal tumorigenesis. Although the CIMP hypothesis has been a topic of much controversy²⁰, it has been supported by recent studies²¹⁻²³. CpG hypermethylation is also believed to have a critical role in PCa development. In particular, the glutathione-S-peroxidase P1 (GSTP1) gene is silenced by methylation in more than 90% of all PCa cases and is the most common epigenetic alteration in human cancer^{24, 25}.

Tumors generally demonstrate both uncontrolled proliferation, through faster cell division and/or an increased number of cells dividing, as well as reduced apoptosis.

This creates an imbalance, with more cells dividing than dying, that allows the tumor to grow. The cells of a tumor also have an unlimited replicative potential and are generally resistant to differentiation. The aggressiveness of a tumor can be approximated by its grade and stage. The grade describes a tumor in terms of cellular and morphological traits (differentiation), whereas staging provides a measure of invasiveness and metastasis. In order to be considered malignant, a tumor must generally display the ability to invade and metastasize, which is achieved by infiltration of the basement membrane and entrance into the lymphatic or vascular circulation. The metastasis precursor, which can consist of one or more cells, exits the circulation by penetrating or rupturing the vessel wall and establishes a metastasis at a location distant to the primary tumor. The location of a metastasis is determined primarily by the anatomy of the circulatory system and by the microenvironment at the site of metastasis. In CRC, metastasis is most often to the liver or lung, whereas in PCa, bone metastasis is most common. Most cancer deaths are due to metastasis rather than the primary tumor²⁶.

COLORECTAL CANCER

In Sweden, CRC is the second most common cancer in both women (after breast cancer) and men (after PCa). In 2003, approximately 5600 people in Sweden were diagnosed with CRC⁵. In the majority of patients the tumor is removed by surgical resection, but approximately half of all patients with CRC die of their disease⁵.

CRC can be quite slow growing, taking up to 10 years to develop. According to the general model for CRC, most malignant tumors develop from hyperproliferation in the epithelium that progresses to an adenoma, which is a benign tumor of the glandular colorectal epithelium, and finally to an adenocarcinoma^{9, 10}. Virtually all colorectal cancer is located in the epithelium, and tumors occur with approximately equal frequency in the right colon (caecum, ascending colon, and transverse colon), left colon (descending and sigmoid colon), and rectum. Colon cancer is approximately equally common men and women, whereas rectal cancer is slightly more common in men⁵. Most cases of CRC are sporadic, with hereditary CRC accounting for 10-25% of cases, depending on the definition used²⁷. However, hereditary factors have been reported to contribute to up 35% of all cases²⁸. The best characterized types of hereditary CRC are HNPCC, described above, and familial adenomatous polyposis (FAP), which together make up approximately 5% of hereditary CRC²⁷. FAP is an autosomal dominant disease, characterized by the

growth of hundreds of potentially precancerous polyps. Prophylactic colectomy is common in FAP patients, and so the vast majority of hereditary CRC cases are due to HNPCC and other genetic mutations.

Established risk factors for CRC include low physical activity²⁹, obesity (especially abdominal obesity)³⁰, and a high-energy diet rich in red meats and processed or charred meats³¹⁻³³. A diet high in fruits and vegetables may also reduce risk, although the evidence from prospective cohort studies has been weaker than from retrospective case-referent studies³⁴. Smoking³⁵ and alcohol consumption³⁶ are also associated with small increases in risk, whereas the use of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) may reduce risk³⁷. Metabolic factors, particularly high levels of insulin and insulin-like growth factors are believed to contribute to CRC carcinogenesis³⁸⁻⁴⁰ and may explain the association between obesity and to some extent also low physical activity with CRC⁴¹. However, the mechanism for physical activity has traditionally been thought to be reduced bowel transit time, minimizing exposure of the intestinal wall to mutagens in the stool. For obesity, the potential role of chronic inflammation is also gaining interest⁴².

The role of fruits and vegetables in CRC carcinogenesis has been a topic of considerable research and discussion for many years. The inverse association between fruit and vegetable consumption and CRC risk has generally been explained by dietary fiber, which increases stool bulk and, like physical activity, reduces bowel transit time. Although a recent large prospective study found an inverse association between fiber intake and CRC risk⁴³, the results of a meta-analysis were less convincing⁴⁴. Furthermore, a number of prevention trials have also failed to support a role of dietary fiber in preventing CRC⁴⁵⁻⁴⁹. As a consequence, the fiber hypothesis has been called into question, and much attention has shifted to other components of fruits and vegetables, such as folate, that might explain their association with CRC risk.

PROSTATE CANCER

In Sweden, PCa is the most common cancer in men, accounting for just over a third of all male cancer cases. 9000 new PCa cases were diagnosed in Sweden in 2003⁵. The incidence of PCa is increasing at a rate of 4.0% per year⁵, due largely to opportunistic screening for prostate-specific antigen (PSA), a protein marker for PCa.

Like CRC, virtually all prostatic tumors are adenocarcinomas. They are usually peripherally located and multi-centric, and are believed to develop from premalignant lesions called prostatic intraepithelial neoplasia. PCa is a slow-growing cancer with diagnosis most often occurring after the age of 60 years, generally around 75 years⁵, and many cases of PCa are first detected at autopsy. The five year survival for PCa ranges from about 90% for localized tumors (stage T1 and T2) to 10% if there is growth outside the prostate (T4)²⁶. One of the key challenges in prostate cancer research is therefore to identify methods to distinguish between tumors that will remain latent and those that will have an aggressive phenotype. Treatment of PCa depends on stage, and can range from “watchful waiting” to radical prostatectomy, radiation therapy, and in advanced cases, castration therapy. Androgen-dependent tumors become resistant to androgen deprivation after a few years, however, and so castration therapy is essentially palliative.

The etiology of PCa is largely unknown⁵⁰. Like CRC, most cases of PCa are sporadic. Hereditary PCa accounts for 5-10% of cases (or up to 20%, depending on the definition used)⁵¹. However, genetic factors are believed to contribute to up to 40% of cases²⁸. Few modifiable risk factors for PCa have been established. Androgens have been implicated in PCa development in experimental studies⁵⁰, but not in prospective cohort studies⁵². Recent reports have suggested a positive association between androgens and the risk of low-grade or non-aggressive PCa, but an inverse association with the risk of high-grade or aggressive PCa⁵³⁻⁵⁵. Such a differential effect has also been proposed for the role of body size, that excess body weight might reduce the risk of low-grade PCa and increase the risk of high-grade PCa, as a possible explanation for the conflicting results of epidemiological studies⁵⁶. Tobacco smoking⁵⁷ and alcohol consumption^{58, 59} are not believed to be associated with PCA risk. Sexually transmitted diseases⁵⁰ and frequency of ejaculation have also been speculated to influence the risk of developing PCA (a high frequency has been associated with both an increased⁶⁰ and a decreased risk⁶¹). Use of aspirin and other NSAIDs may reduce the risk of PCa⁶²

Some dietary factors have also been associated with a reduced risk of PCa, especially lycopene (found in high amounts in tomatoes), but also other carotenoids, vitamin E, omega-3 fatty acids, and selenium⁶³. Epidemiological studies have suggested an inverse association between consumption of cruciferous vegetables, such as cabbage, broccoli, and Brussels sprouts, and the risk of PCa⁶³.

Cruciferous vegetables are rich in the phytochemicals sulforaphane and indole-3 carbinol, the antitumorigenic properties of which are well-documented⁶⁴. High consumptions of meats, especially charred meats, saturated fats, alpha-linolenic acid, and calcium and dairy products, have been associated with an increased risk of developing PCa⁶³.

CARDIOVASCULAR DISEASE

CVD refers to diseases of the heart and blood vessels, of which stroke and AMI are two of the major forms. CVD is the leading cause of death in Sweden, accounting for approximately 45% of the total mortality⁵. In men, AMI accounts for 30% and stroke for 20% of the CVD mortality, whereas in women the corresponding figures are 23% and 27%, respectively⁵. In both sexes, the remainder of the CVD mortality is predominantly coronary heart disease. CVD rates have decreased dramatically (by approximately 50%) since 1970⁵, particularly in northern Sweden⁶. This is due primarily to reductions in AMI incidence and mortality (Figure 2). Although the incidence of stroke has been relatively stable in recent decades, rates have begun to decrease over the past few years⁶, and stroke mortality has been decreasing slowly but steadily since around 1990⁵.

The major biological process leading to both ischemic stroke and AMI is atherosclerosis and the formation of a thrombus. Atherosclerosis is believed to begin early in life as a “reaction to injury” at vulnerable, high-stress sites such as branch points in the vascular system. The initial atherosclerotic lesion is the fatty streak, characterized by the accumulation of lipid-filled macrophages called foam cells in the vascular endothelium, the innermost layer of the vessel wall. Oxidative stress, such as that produced by the oxidation of low-density lipoproteins (LDL), and resulting damage to endothelium, lead to further accumulation of macrophages and debris in the developing plaque. Smooth muscle cell proliferation and, consequently, the production of fibrous connective tissue components increase. Mural thrombosis occurs and is incorporated into the increasingly organized fibroinflammatory lipid plaque, which is vascularized and contains a necrotic center. Complications that can follow include ulceration, fissure, and calcification of the plaque, and aneurysm, a localized, sac-like bulge caused by a weakening of the vessel wall. Continued growth of the plaque can lead to stenosis, a narrowing of the vessel lumen. The plaque can also rupture, causing thrombosis and occlusion, and ultimately an acute ischemic event⁶⁵.

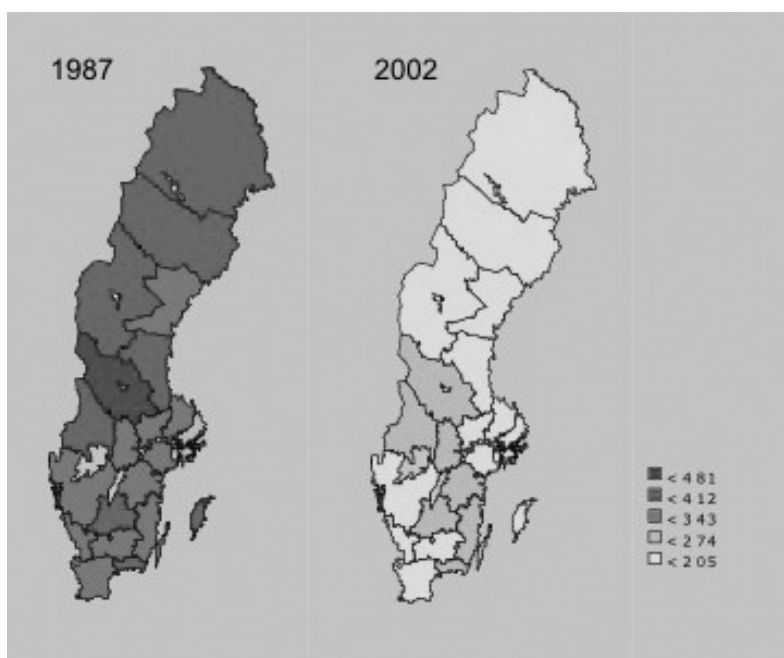


Figure 2. Age-adjusted mortality (per 100 000) due to acute myocardial infarction, Sweden, all ages (Data from the National Board of Health and Welfare, www.sos.se)

An acute ischemic event is often the result of a plaque from the arterial wall that ruptures, expands, lodges in the lumen of an artery, and forms a thrombus (or a thromboembolus if it lodges at a location distant to its origin). This cuts off circulation to the tissue supplied by that particular artery, which becomes ischemic, that is, starved of oxygen, and eventually necrotic. When an ischemic event occurs in a coronary artery, an AMI results. The majority of strokes in Sweden^{5, 8}, as in most western countries, are also ischemic in nature (approximately 80%), occurring when a thrombus or thromboembolus blocks cerebral circulation.

Established risk factors for atherosclerosis and ischemic events include increasing age, male sex, hypertension (both systolic and diastolic blood pressure), hyperlipidemia (especially elevated levels of LDL cholesterol), smoking, diabetes mellitus, obesity, physical inactivity, and a diet rich in saturated animal fats and low in fruits and vegetables^{65, 66}. In contrast to the risk increase associated with saturated fats, long-chain omega-3 fatty acids found in oily fish may reduce risk⁶⁷. Other factors including psychosocial stress⁶⁸ and especially inflammation⁶⁹ have also been

implicated in atherogenesis. Cholesterol is believed to be more important in AMI than in ischemic stroke, and the use of statins to lower cholesterol levels has had a major impact on the incidence of AMI in industrialized countries. Hypertension on the other hand is somewhat more important in ischemic stroke.

Strokes not due to ischemic events include two types of hemorrhagic stroke, intracerebral and subarachnoid hemorrhage, which account for approximately 15% and 5% of all stroke cases, respectively. In contrast to the atherosclerotic origin of ischemic stroke, intracerebral hemorrhages are usually primarily the result of chronic hypertension. Consequent weakening of the wall causes aneurysm, which, upon rupturing, results in hemorrhagic stroke. In this thesis, hemorrhagic stroke refers to intracerebral hemorrhage.

FOLATE METABOLISM

Folate Biochemistry

Folate metabolism is the metabolism of methyl, or one-carbon, groups, as illustrated in Figure 3. Methyl groups are obtained from dietary serine, choline (and the choline metabolite, betaine), and methionine, and transferred to folate in transmethylation reactions. Vitamin B6 (pyridoxine) acts as a cofactor for the harvesting of methyl groups from serine. The methyl groups can then be used in the two main pathways of folate metabolism, nucleotide synthesis and methylation. These two pathways are separated by an irreversible reaction mediated by methylenetetrahydrofolate reductase (MTHFR), for which vitamin B2 (riboflavin) in the form of flavin adenine dinucleotide (FAD) is a cofactor. The substrate of the reaction, 5,10-methylenetetrahydrofolate (5,10-methyleneTHF), is a cofactor for purine and thymidylate (thymine) synthesis, and the product of the reaction (5-methylTHF) donates methyl groups for the remethylation of homocysteine to methionine. Vitamin B12 (cobalamin) acts as a cofactor for methionine synthase. Methionine is converted to S-adenosylmethionine (SAM), which is the universal methyl donor for all methylation reactions in the body, such as DNA, protein, and phospholipid methylation, and is an allosteric inhibitor of MTHFR. Loss of the methyl group from SAM yields S-adenosylhomocysteine (SAH), which, by

competing with SAM at the regulatory region of MTHFR, prevents enzyme inhibition. Homocysteine, the product of SAH, can be metabolized by remethylation to methionine, as noted above, or via cystathionine to cysteine, by reactions for which vitamin B6 is a cofactor. This second metabolic pathway is important primarily in the postprandial state. Remethylation via betaine can also contribute to homocysteine metabolism, and dietary betaine and choline (a precursor to betaine) have been found to be negatively associated with homocysteine status⁷⁰.

Since the MTHFR reaction is essentially irreversible *in vivo*, vitamin B12 deficiency can cause a build-up of 5-methylTHF, thus inducing a secondary, functional folate deficiency. This phenomenon has been dubbed the “folate trap” and has been described in a human patient⁷¹. Since folic acid can enter the methylation cycle as dihydrofolate, it can bypass the vitamin-B12-dependent remethylation of homocysteine. The use of folic acid supplements can thus mask the hematological characteristics of vitamin B12 deficiency, megaloblastic anemia, which is due to aberrations in nucleotide synthesis, while allowing irreversible neurological complications to progress.

Homocysteine

Homocysteine is a sulfur-containing amino acid not found in foods. It is a marker of biological folate status, and an established risk factor for CVD. The idea that moderately elevated homocysteine levels might be related to CVD was first suggested by McCully, in 1969⁷², based on a comparison of a patient with a severe abnormality in vitamin B12 metabolism and a patient with cystathione β -synthetase (CBS) deficiency. Vitamin B12 and CBS mediate separate pathways of homocysteine metabolism (Figure 3), leading to the conclusion that elevated homocysteine, the common link between the two patients, was responsible for their very similar vascular pathology⁷². Animal studies and other experimental studies^{73, 74}, as well as numerous epidemiological studies⁷⁵⁻⁷⁷, have supported McCully’s hypothesis. However, whether homocysteine has a true biological role in the development of CVD, or is simply a marker of some other causal factor, remains to be established.

The suggested mechanisms for the putative role of homocysteine in cardiovascular disease include pro-oxidative and inflammatory effects that promote thrombosis,

effects on blood pressure through interactions with endothelial nitric oxide synthase (eNOS) and/or asymmetric dimethylarginine (ADMA, an endogenous inhibitor of eNOS), and altered DNA methylation patterns leading to aberrant gene expression patterns⁷³. Since some of these mechanisms are also believed to contribute to cancer development, it has been proposed that elevated homocysteine levels might increase the risk of cancer⁷⁸, but evidence to date is inconclusive⁷⁹⁻⁸².

Most homocysteine in plasma is bound to albumin, although up to one third is bound by disulphide bonds to either a cysteine or another homocysteine (forming a homocysteine dimer), and small amounts exist as free homocysteine⁸³. Blood measurements of homocysteine generally refer to total homocysteine (tHcy, sometimes written homocyst(e)ine), because analyzing the different forms of homocysteine is difficult. The main determinants of homocysteine include folate and vitamin B12 status (which depend on dietary intake, absorption, and metabolism), renal function, polymorphisms in the MTHFR gene, and to a lesser extent, vitamin B6 and B2 deficiency, smoking, high coffee consumption, and chronic high alcohol consumption⁸⁴⁻⁸⁷. Supplementation with folic acid can reduce homocysteine levels by up to approximately 25%, and supplementation with vitamin B12 can provide a further 7% reduction^{88, 89}.

MTHFR

A thermolabile variant of the MTHFR protein was first noted in 1977⁹⁰, and was later shown to be due to a C>T single nucleotide polymorphism (SNP, a mutation occurring in >1% of the population) at base 677 in the MTHFR gene⁹¹. The presence of the T-allele results in an amino acid substitution, alanine to valine, at position 222 in the polypeptide, and reduces enzyme activity by approximately two thirds in TT-homozygotes^{91, 92}. Though not located in the vicinity of the N-terminal catalytic domain, the 677C>T SNP is believed to increase susceptibility to FAD dissociation by altering the local protein structure^{93, 94}. The effect of the T-allele on folate and thus homocysteine status is detrimental^{95, 96}, but appears to depend on the consumption of folate and vitamin B2. A sufficiently high folate intake can stabilize the enzyme, both by its presence at the active site and by increasing the SAM/SAH ratio and thereby upregulating MTHFR activity^{93, 97}. A homocysteine-lowering effect of vitamin B2 supplementation has also been demonstrated and may or may not be specific to TT-homozygotes^{98, 99}.

The prevalence of the MTHFR 677C>T SNP varies widely by geography and ethnicity, ranging from 3% to 32% worldwide and increasing along a north to south gradient in Europe¹⁰⁰. The T-allele appears to be more common in regions with a higher dietary folate consumption¹⁰¹. Some evidence suggests that it may increase the risk of spontaneous abortions^{102, 103}, and that a higher folate intake may protect a developing fetus carrying the T-allele^{104, 105}. Concerns have therefore been raised that increasing the folate intake of a population, via folic acid fortification of foods for example, might lead to a higher prevalence of the MTHFR 677C>T SNP, increasing the folate requirements of the population and possibly also the risk of some diseases¹⁰⁶.

Another SNP in the MTHFR gene, 1298A>C, has also been suggested to affect folate status^{92, 107}. However, it is in almost complete linkage disequilibrium with the 677C>T SNP, with two variant alleles very rarely occurring on the same strand¹⁰⁸. It is thus difficult to distinguish possible effects of the 1298C allele from those of the 677T allele.

MTHFR and Mendelian Randomization

In the case of the MTHFR 677C>T SNP, the rare allele is associated with a phenotype similar to one obtainable by an environmental exposure, that is, a low dietary consumption of folate. A study comparing disease incidence in people with and without the rare allele can therefore be likened to a randomized controlled trial extending over a lifetime, as illustrated in Figure 4. According to Mendel's principle of the random segregation of alleles, inheritance of traits occurs independently of all other traits. Furthermore, the 677C>T SNP would not be expected to be associated with any environmental risk factor. Thus, the risk of confounding by other genetic traits or by environmental exposures is minimal. This potentially valuable tool of genetic epidemiology has been dubbed Mendelian randomization.

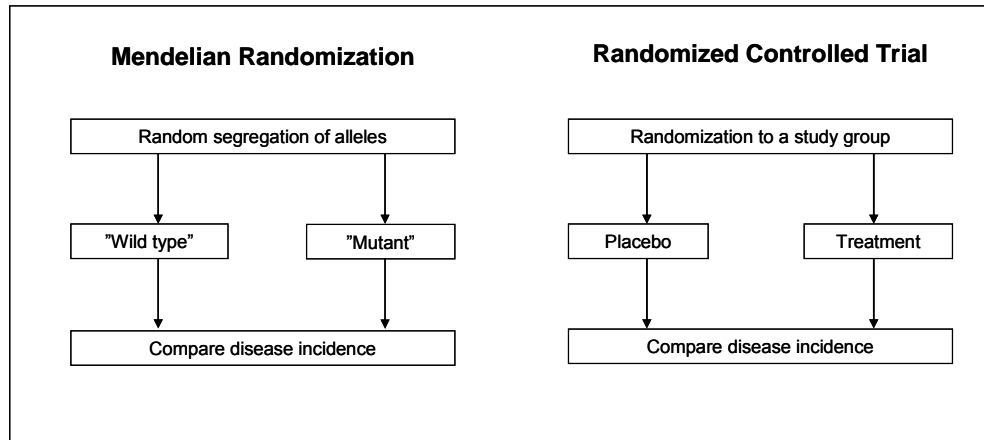


Figure 4. Adapted from Davey-Smith and Ebrahim¹⁰⁹. According to Mendel's principle of the random segregation of alleles, the inheritance of genetic traits occurs independently of all other traits. Single nucleotide polymorphisms that have a phenotype similar to one obtainable by an environmental exposure can thus be likened to a randomized clinical trial, since potential confounders should be distributed equally in the wild-type "placebo" group and the mutant "treatment" group. This potentially valuable tool of epidemiology had been dubbed Mendelian randomization.

THE B-VITAMINS IN FOLATE METABOLISM

The B-vitamins are a collection of water-soluble vitamins, often found in the same foods, that were once thought to be a single substance. There are eight B-vitamins, thiamine (B1), riboflavin (B2), niacin (B3), pyridoxine (B6), folate (also previously referred to as B9), cobalamin (B12), pantothenic acid, and biotin. Only the B-vitamins directly involved in folate metabolism are considered in this thesis. These include folate and vitamins B12, and to a lesser extent vitamins B6 and B2.

Folate

Folate, so named because it is found in high amounts in plant foliage, is actually found in a wide range of foods of both plant and animal origin, such as leafy green vegetables, beans, whole grains, mushrooms, potatoes, liver, and beef. Folate refers to pteroylmonoglutamate (composed of a pteridine ring, PABA, and glutamate) and related molecules. Pteroylmonoglutamic acid, folic acid, does not occur naturally in foods. It is the pure, synthetic form of folate commonly used in supplements and in the fortification of food stuffs. Naturally-occurring folate is a variable mixture of different forms of mono- and polyglutamates, although polyglutamates are generally dominant. Polyglutamates must be hydrolyzed to monoglutamates,

including 5-methylTHF and 5-formylTHF, for absorption in the intestinal mucosa, primarily by active transport but also to a lesser extent by passive diffusion. Within the cell, folate is reduced to tetrahydrofolate for reconjugation to polyglutamates, which make up the storage depot of the cell.

Folate in blood is contained in both erythrocytes (red blood cells) and plasma. Erythrocyte folate levels are indicative of a person's folate consumption in recent months, whereas plasma or serum folate levels reflect the few days prior to blood sampling. The question of which provides a better measure of folate status is much discussed. Although erythrocyte folate may be the better choice in theory, the laboratory analysis is more complicated, with more steps that must be performed manually. This can result in greater variation in results. Plasma or serum levels can be analyzed quickly and cheaply, and are considered to be acceptable for use in large epidemiologic studies¹¹⁰ and in clinical practice¹¹¹. Both erythrocyte and plasma or serum folate concentrations are sensitive to seasonal variation in folate consumption.

The dominant form of folate in erythrocytes¹¹² and plasma/serum¹¹³ is 5-methylTHF. Both measures of circulating folate concentrations are correlated with levels in the colonic mucosa^{114, 115}. Whether the same is true for the prostate has apparently not been studied. In one large study, both erythrocyte and plasma concentrations of 5-methylTHF were correlated to lymphocyte total folate levels¹¹⁶.

The bioavailability of natural folate from foods has been estimated to be low, roughly 50% of that of synthetic folic acid, depending on the composition of folates, losses due to storage, processing, and heat, and variation in nutritional status and the ability to deconjugate and bind folate¹¹⁷. Some drugs, such as anticonvulsants and the anticancer drug methotrexate, are detrimental to a person's folate status.

The diet in Sweden is low in folate, with estimates suggesting that the average daily intake is below the recommended intake (RI) of 300 µg/day for adults (400 µg/day for women of childbearing age) (Tables 1 and 2). The Swedish recommendations for the general population are lower than the 400 µg/day of folate equivalents recommended in the United States¹¹⁸. Due to the lower bioavailability of dietary folate compared to folic acid, 1 µg of dietary folate is considered to provide 0.5 µg of folate equivalent. A person not taking folic acid supplements would therefore,

according to the American guidelines, need to consume 800 µg of dietary folate per day in order to meet recommendations.

Vitamin B12

All vitamin B12 is produced by bacteria in soil and water or in the rumen and intestine of animals. Since vitamin B12 in the human colon cannot be absorbed, dietary vitamin B12 is obtained essentially only from animal products, such as organ meats, muscle meats, fish, milk, cheese, and eggs. Some land plants, primarily in tropical environments, contain trace amounts of vitamin B12 due to contamination. Some sea plants have also been found to be rich in vitamin B12¹¹⁹, but concerns have been raised that during the drying process a considerable amount may be converted to vitamin B12 analogues that could actually impair cobalamin metabolism¹²⁰.

Vitamin B12 includes a number of related compounds containing a central cobalt atom in a porphyrin-like planar corrin ring. Four natural forms of vitamin B12 have been isolated, hydroxycobalamin, glutathionylcobalamin, methylcobalamin, and adenosylcobalamin. A fifth form, cyanocobalamin, does not occur naturally, but is the most common commercial form of vitamin B12. Cyanocobalamin and hydroxycobalamin are the most metabolically active forms, and methylcobalamin and adenosylcobalamin are the only two with coenzyme functions. Adenosylcobalamin mediates the conversion of methylmalonyl-coenzyme A (methylmalonyl-CoA) to succinyl-CoA, but if vitamin B12 levels are low, methylmalonic acid (MMA) is produced instead. MMA status can therefore be used in clinical practice as a marker for vitamin B12 deficiency, although impaired renal function also contributes to elevated levels¹²¹.

Although small amounts of vitamin B12 are absorbed by passive diffusion, the general mechanism of vitamin B12 uptake is complicated. Acidic conditions in the stomach free the various forms of cobalamin from dietary proteins, allowing them to bind to haptocorrins (also called R-binders), glycoproteins produced in the mouth and stomach. The haptocorrins are degraded by pancreatic enzymes in the ileum, freeing the cobalamin to bind to intrinsic factor. Intrinsic factor, a binding protein produced by the gastric parietal cells, carries the cobalamin to an ileal receptor, cubilin, to which it binds in a calcium-dependent reaction. The complex of intrinsic

Background

factor, cobalamin, and cubilin can then be recognized and endocytosed by a general transporter protein called megalin.

In the blood, vitamin B12, 60-80% of which is methylcobalamin, is bound to the haptocorrins transcobalamin (TCN) I-III. Although approximately 80% is bound to TCN I¹²², both TCN I and III exist in a steady state, whereas TCN II has a high turnover, providing vitamin B12 for cellular uptake.

With the exception of considerable losses from milk during pasteurization, vitamin B12 is resistant to degradation due to heat and storage. However, despite its relative stability, the bioavailability of vitamin B12 is highly variable, depending on a number of conditions in the alimentary tract. If the pH in the stomach is not sufficiently low, then vitamin B12 cannot be freed from dietary proteins efficiently. This can occur as a consequence of decreased secretion of gastric acid, usually due to chronic gastritis, or by excessive use of antacids. Such food-cobalamin malabsorption is the most common cause of vitamin B12 deficiency¹²³. The absorption of supplemental vitamin B12, which is not protein bound, is not influenced by stomach acidity and can thus alleviate food-cobalamin deficiency. Lack of intrinsic factor, however, affects the absorption of both naturally-occurring and supplemental vitamin B12, and causes pernicious anemia. Small amounts of vitamin B12 consumed will still be absorbed by passive diffusion, and so an adequate vitamin B12 status can be maintained by supplementation at high doses¹²³.

Although the diet in Sweden exceeds the RI for vitamin B12 (Tables 1 and 2), deficiency, which can lead to megaloblastic anemia and irreversible neurological damage, is a common problem, particularly in the elderly. The prevalence of vitamin B12 deficiency is believed to be between 5-15% in the elderly, and in 2000, 13% of the residents of Sweden over 70 years of age received vitamin B12 treatment by prescription, compared to only 1% in residents under 70 years¹²⁴. Sweden is the only country in which high oral doses are used to treat vitamin B12 deficiency, accounting for approximately two thirds of all vitamin B12 prescriptions in 2000¹²⁴. Although enough vitamin B12 is stored in the liver of a well-nourished person to prevent deficiency symptoms for several years, vegans, who consume no animal products, eventually become dependent on vitamin B12 supplements.

Vitamins B6 and B2

Vitamin B6 is found in a variety of foods, including meat, fish, whole grains, vegetables, nuts and seeds. The bioavailability of vitamin B6 from animal sources is higher than that of plant sources. In the blood, vitamin B6 exists predominantly in the metabolically active coenzyme form, pyridoxal phosphate (PLP). Vitamin B2 occurs in high amounts in vegetables, such as leafy green vegetables, and nuts, but in the western diet animal products are important dietary sources. Vitamin B2 is the precursor to two coenzymes, FAD, as noted above, and flavin adenine mononucleotide (FMN). The average consumptions of vitamins B6 and B2 in Sweden are roughly in line with national recommendations (Tables 1 and 2).

Table 1. Swedish dietary intake guidelines¹²⁵ for folate, vitamin B12, vitamin B6, and vitamin B2 in adults.

| | LI | | AR | | RI | |
|-----------------|-----|-------|-----|-------|------------------|------------------|
| | Men | Women | Men | Women | Men | Women |
| Folate, µg | 100 | 100 | 200 | 200 | 300 | 300* |
| Vitamin B12, µg | 1.0 | 1.0 | 1.4 | 1.4 | 2.0 | 2.0 |
| Vitamin B6, mg | 1.0 | 0.8 | 1.3 | 1.0 | 1.6 | 1.2 |
| Vitamin B2, mg | 0.8 | 0.8 | 1.4 | 1.1 | 1.7 [†] | 1.3 [†] |

LI, lowest daily intake; AR, average daily requirement; RI, recommended daily intake

* 400 for women of childbearing age

[†] 1.5 for men and 1.2 for women aged 60-75 years

Table 2. Average daily intake of folate, vitamin B12, vitamin B6, and vitamin B2 in participants aged 18-74 years in two Swedish national nutrition surveys.

| | Riksmaten ^{126, 127} 1997/98 | | HULK ¹²⁷ 1989 | |
|-----------------|---------------------------------------|------------------|--------------------------|------------------|
| | Men (N=589) | Women (N=626) | Men (N=769) | Women (N=804) |
| Folate, µg | 232 | 217 | 226 | 194 |
| Vitamin B12, µg | 6.9 | 6.0 | 8.6 | 6.6 |
| Vitamin B6, mg | 2.2 | 1.9 | 2.1 | 1.7 |
| Vitamin B2, mg | 1.9 | 1.6 | 2.1 | 1.7 |

FOLATE AND CANCER DEVELOPMENT

Nucleotide Synthesis

Sufficient nucleotide synthesis is critical to genome stability. This is particularly true for the synthesis of thymine (deoxythymidylate monophosphate, dTMP) from uracil (deoxyuridylate monophosphate, dUMP), which, if impaired by low folate availability, can lead to an overrepresentation of uracil in the nucleotide pool and increased misincorporation of uracil into DNA. The mismatch repair process that follows requires strand cleavage in order to excise the uracil. Under conditions of folate deprivation, this can lead to a cycle of repeated strand breaks and ineffective DNA repair, thus threatening DNA stability. Studies to date have tended to report observations in line with this idea¹²⁸⁻¹³⁰, though some notable exceptions have also been published^{131, 132}.

DNA Methylation

Folate status determines the availability of one-carbon groups for all methylation reactions in the body, including DNA methylation. One of the mechanisms by which folate has been hypothesized to reduce the risk of cancer is thus by ensuring proper global DNA methylation¹²⁸. Some¹³³⁻¹³⁵, but not all^{131, 132}, human studies have supported an influence of folate status on such genomic methylation.

The importance of folate in gene-specific promoter hypermethylation is unclear¹³⁶. In human CRC, intracellular folate concentrations have been found to be positively related to gene-specific hypermethylation¹³⁷. Furthermore, an altered phenotype as a result of promoter methylation and consequent gene silencing has been reported in the offspring of mice fed a methyl-supplemented diet during pregnancy¹³⁸⁻¹⁴⁰. These findings seem to contradict the proposed cancer-preventative role of folate in cancer. However, a low folate intake (in combination with a high alcohol intake) has also been associated with increased promoter methylation in tumor suppressor genes in CRC patients, although the results were not statistically significant¹⁴¹. Hypermethylation and silencing of the p53 gene has been observed during tumor progression in a folate-depletion model of rodent hepatocarcinoma¹⁴². In the same animal model, the incidence and extent of hypermethylation of the cell-cycle control gene p16, increased during tumor progression¹⁴³. Folate has also been

suggested to prevent cancer by preventing the demethylation and aberrant expression of proto-oncogenes¹²⁸.

Inverse associations have been reported between the MTHFR 677C>T SNP and both global genomic methylation^{133, 144-146} (though only combination with low folate status in one of the studies¹³³), and gene-specific promoter hypermethylation¹³⁷. The T-allele has also been reported to reduce the risk of C→T transitions in the p53 gene¹⁴⁷. These mutations occur particularly often at methylated CpG island “hotspots” and are a common event in colorectal tumorigenesis. The findings for the MTHFR 677C>T SNP in mechanisms of cancer development are thus largely consistent with those noted above for folate status. However, as for folate, a number of null^{128, 131, 132} and opposite¹⁴⁸ results have also been published.

Possible Cancer-promoting Effect of Folate

Concerns have been raised that the role of folate in tumorigenesis may not only be protective¹⁴⁹. As noted above, one might speculate that excess folate could induce hypermethylation of DNA, possibly increasing the risk of improper silencing of tumor suppressors, contributing to loss of heterozygosity and tumor initiation. In fact, supraphysiological doses of folate have been reported to have a tumor-promoting effect in animal studies¹⁵⁰. In one study that has caused much debate, folate supplementation during pregnancy was associated with an increased risk of breast cancer later in life¹⁵¹. However, due to inherent weaknesses in the study, the implications of the findings are uncertain. Once cellular transformation has occurred and a proliferating neoplasm is established, folate becomes essential for tumor growth. This has been exploited in cancer treatment, in the form of anti-folates such as methotrexate. The potential of folate to promote tumor growth may be of clinical relevance given the slow progression of many cancer types, including CRC and PCa. A high folate status could increase the aggressiveness of a tumor that might otherwise never have progressed to a diagnosis. Thus, in considering potential chemopreventative strategies, the dosage and timing of folate administration appear to be critical.

VITAMIN B12 AND CANCER DEVELOPMENT

Any role for vitamin B12 in cancer development is likely to be similar to that of folate, but it has been a topic of considerably less research. Vitamin B12

concentrations have been reported to be negatively associated with DNA damage¹⁵²⁻¹⁵⁴. In these studies, supplementation with vitamin B12 also reduced DNA damage in younger¹⁵², but not older¹⁵³, subjects. Vitamin B12 deprivation has been found to increase both uracil misincorporation and global DNA hypomethylation in the colonic mucosa of rats¹⁵⁵. In contrast, a positive correlation between vitamin B12 concentrations and markers of genotoxicity has been noted in smokers¹⁵⁶.

FOLATE AND COLORECTAL CANCER

Dietary folate intake is inversely associated with the risk of developing CRC¹⁵⁷⁻¹⁶³, and the association is stronger for intake from foods alone than for total intake including folate from dietary supplements^{157, 158}. Studies of animal models and some small human studies of surrogate endpoints and provisional markers of CRC have supported the results of the epidemiological studies^{150, 164, 165}. For example, folic acid supplementation has been found to reduce cell proliferation in the rectal mucosa of patients with recurrent adenomatous polyps¹⁶⁴. However, in the one large clinical trial to date, the Aspirin-Folate Polyp Prevention Trial, folic acid supplementation was associated with a statistically significant 44% increase in the number of recurrent adenomas¹⁶⁶.

To date, only four other prospective studies have related circulating folate levels to the risk of CRC (Table 3). The Physicians' Health Study of men in the United States reported an increased risk of borderline statistical significance in subjects with low plasma folate concentrations¹⁶⁷, whereas the Alpha-Tocopherol Beta-Carotene Study of male smokers in Finland found no association between serum folate concentrations and risk¹⁶⁸. A statistically significant reduced CRC risk for the highest versus lowest quartile of serum folate was noted in the New York Women's Health Study⁸¹. In the same study, results suggestive of an increased risk of CRC at higher homocysteine levels was also noted⁸¹, a topic that has not been addressed in other studies. A null association between serum folate levels and CRC risk was noted in a cohort from Australia with 45 CRC cases¹⁶⁹.

FOLATE AND PROSTATE CANCER

Evidence for a role of folate in prostate cancer development is limited. Only two other prospective studies have related circulating folate levels to risk of prostate cancer (Table 4). A null association between serum folate concentrations and risk

Background

Table 3. Prospective studies of plasma or serum folate concentrations and risk of colorectal cancer.

| First author and year | Sex | No. cases | Association between folate levels and risk |
|---|-------|-----------|--|
| Ma, <i>et al</i> , 1997 ¹⁶⁷ | M | 202 | ↓ (ns) |
| Glynn, <i>et al</i> , 1996 ¹⁶⁸ | M | 144 | Null |
| Kato, <i>et al</i> , 1999 ⁸¹ | F | 105 | ↓ |
| Rossi, <i>et al</i> , 2006 ¹⁶⁹ | M + F | 45 | Null |

ns, not statistically significant

was reported in the Alpha-Tocopherol Beta-Carotene Study of male smokers in Finland¹⁷⁰, and an inverse association was found in a cohort from Australia with 31 PCa cases¹⁶⁹. Inverse associations between folate intake and risk of PCa¹⁷¹ and advanced PCa¹⁷² have also been reported, although only the latter, with 31 PCa cases, was statistically significant. Neither serum vitamin B12 nor homocysteine concentrations were related to the risk of developing PCa in the Finnish study¹⁷⁰, and a positive association between vitamin B12 intake and PCa risk was found in one small case-control study¹⁷³.

Table 4. Prospective studies of plasma or serum folate concentrations and risk of prostate cancer.

| First author and year | No. cases | Association between folate levels and risk |
|---|-----------|--|
| Weinstein, <i>et al</i> , 2003 ¹⁷⁰ | 232 | Null |
| Rossi, <i>et al</i> , 2006 ¹⁶⁹ | 31 | ↓ (non-linear) |

MTHFR AND CANCER

The MTHFR 677C>T SNP illustrates the complicated role of folate metabolism in cancer. For many cancer sites, including PCa¹⁷⁴⁻¹⁷⁸, results have tended to suggest an increased risk associated with the T-allele. However, in CRC, for which considerably more studies have been published, the totality of evidence suggests that the T-allele may reduce risk^{179, 180}. The role of the MTHFR 677C>T SNP may depend on folate intake. According to this hypothesis, a low folate intake in combination with the T-allele might render the availability of one-carbon groups insufficient to ensure proper DNA methylation. In contrast, in people with a high

folate intake, the T-allele might provide extra folate for DNA synthesis and repair while yielding sufficient 5-methylTHF for methylation reactions. Thus, the presence of T-allele has been proposed to reduce CRC risk in combination with high folate levels, but increase risk under conditions of low folate¹⁶⁷. This putative differential effect of the MTHFR 677C>T SNP in CRC was first suggested in a report from the Physicians' Health Study¹⁶⁷, although the findings were not statistically significant. Further support has come from prospective dietary studies¹⁸¹⁻¹⁸³. However, other reports have demonstrated a risk reduction in carriers of the T-allele that was independent of folate^{184, 185} and homocysteine status¹⁸⁰.

FOLATE AND CARDIOVASCULAR DISEASE

Few prospective studies have addressed the role of folate in cerebrovascular diseases, but especially for folate intake, findings have generally supported an inverse association between folate status and risk¹⁸⁶⁻¹⁹¹. Prospective studies of coronary endpoints have also tended to yield results consistent with a protective effect for folate^{187, 192-203}. The prospective studies of plasma or serum folate concentrations and risk of stroke and coronary outcomes are shown in Tables 5 and 6, respectively. Prospective studies of vitamin B12, vitamin B6, and/or vitamin B2 and the risk of CVD have yielded inverse or null associations^{187, 190, 192, 194, 197, 199-201, 204, 205}, with one exception of a positive association for vitamin B12 and CVD mortality¹⁸⁷.

Table 5. Prospective studies of plasma or serum folate concentrations and risk of cerebrovascular outcomes

| First author and year | Endpoint | Sex | No. cases | Association between folate levels and risk |
|---|------------------------------------|-------|------------------------|--|
| Giles, <i>et al</i> , 1995 ¹⁸⁶ | Ischemic stroke | M + F | 98 | ↓ (ns) |
| | | M | 58 | ↓ (ns) |
| | | F | 40 | ↓ (ns) |
| Zeitlin, <i>et al</i> , 1997 ¹⁸⁷ | Stroke | M + F | 31 | Null |
| Maxwell, <i>et al</i> , 2002 ¹⁸⁹ | Adverse cerebro-vascular outcomes* | M + F | Not given [†] | ↓ |
| | | M | | Null |
| | | F | | ↓ |

ns, not statistically significant

* Including vascular dementia, vascular cognitive impairment, or fatal stroke

[†] In total, 171 men and 198 women were included in the study.

The primary mechanism suggested for an effect of folate, as well as the other B-vitamins in folate metabolism, in reducing the risk of CVD is through the regulation of homocysteine status. However, some evidence from experimental studies suggests that folate may also act independently of homocysteine²⁰⁶⁻²¹³. Few of the epidemiological studies noted above accounted for homocysteine status in the analyses, but those that did found little effect on the risk estimates^{192, 200, 202}, which supports an independent role for folate.

The putative mechanisms for an independent role of folate in CVD include direct superoxide scavenging and interactions with eNOS²⁰⁶. The latter is especially interesting. eNOS, with the aid of an essential cofactor, tetrahydrobiopterin (BH₄), produces the vasodilator nitric oxide (NO) through the conversion of L-arginine to citrulline. If endothelial BH₄ is insufficient, which can result from excessive oxidation, then eNOS not only produces less NO, but can also undergo a steric change and begin to produce superoxide radicals instead. Folate, in the form of 5-methylTHF, can stabilize the BH₄-deficient eNOS and restore its function, thus counteracting a vicious cycle of oxidation and superoxide production^{214, 215}. Folate has also been implicated in NO-independent vasodilation, but the mechanisms are unclear²¹⁶.

A number of meta-analyses concerning the MTHFR 677C>T SNP and CVD risk have been published, and results have tended to support a modest risk increase associated with the presence of the T-allele in both cerebrovascular^{77, 95, 217-219} and coronary endpoints^{77, 219, 220}. However, a recent meta-analysis of coronary heart disease described a substantial heterogeneity in the associations reported for Europe, North America, and Australia (null), compared to the Middle East (increased risk), and Asia (excluding Japan due to heterogeneity of risk estimates, non-significant reduced risk)⁹⁶. In addition, the meta-analysis by Kim *et al*²¹⁹ noted an association in stroke but not AMI.

Studies of percutaneous intervention, including balloon angioplasty and coronary stenting, provided some of the first experimental evidence for a biological role of folate metabolism in CVD^{221, 222}. A reduced risk of coronary restenosis was found in patients taking B-vitamin supplements, primarily in those treated with angioplasty. In contrast, an opposite effect was reported in another, larger study²²³, though not in women, diabetics, or patients with homocysteine levels over 15 $\mu\text{mol/L}$ at baseline.

Background

Table 6. Prospective studies of plasma or serum folate concentrations and risk of coronary outcomes

| First author and year | Endpoint | Sex | No. cases | Association between folate levels and risk |
|--|------------------------|------------|-----------------|--|
| Chasan-Taber, <i>et al</i> , 1996 ¹⁹² | AMI and fatal CHD | M | 333 | ↓ (ns) |
| Morrison, <i>et al</i> , 1996 ¹⁹³ | CHD mortality | M + F | 165 | ↓ |
| | | M | 112 | ↓ (ns) |
| | | F | 53 | ↓ |
| Zeitlin, <i>et al</i> , 1997 ¹⁸⁷ | AMI | M + F | 56 | Null |
| | CVD | M + F | 99 | Null |
| Folsom, <i>et al</i> , 1998 ¹⁹⁴ | CHD | M + F | 232 | ↓ (ns) |
| | | M | 174 | Null (univariate analysis) |
| | | F | 58 | ↓ (univariate analysis) |
| Ford, <i>et al</i> , 1998 ¹⁹⁵ | CVD | M + F | 873 | Null |
| | CVD mortality | M + F | 215 | ↓ (ns, non-linear) |
| Giles, <i>et al</i> , 1998 ¹⁹⁶ | CHD | M + F | 284 | Null |
| | | M | 155 | ↓ (ns) |
| | | F | 129 | Null |
| | | | 88 < 55 y | ↓ |
| | | 196 ≥ 55 y | ↑ | |
| Loria, <i>et al</i> , 2000 ¹⁹⁸ | CVD mortality | M + F | 12 diabetic | ↓ (data not shown) |
| | | | 49 non-diabetic | ↓ (ns in multivariate analysis) |
| De Bree, <i>et al</i> , 2003 ²⁰⁰ | CHD mortality | M | 78 | Null |
| | | F | 25 | ↓ |
| Hung, <i>et al</i> , 2003 ²⁰¹ | CHD mortality | M | 213 | Null (bell-shaped) |
| | | F | 159 | Null |
| | CVD mortality | M | 342 | Null |
| | | F | 302 | Null |
| Voutilainen, <i>et al</i> , 2004 ²⁰² | Acute coronary events* | M | 51 | ↓ |

CVD, cardiovascular disease; CHD, coronary heart disease; ns, not statistically significant

* Including definite and possible AMI and typical prolonged chest pain episodes.

A recent study reported an improvement in stroke mortality rates in the United States and Canada immediately following the implementation of mandatory folic acid fortification of foods in 1998²²⁴. No such trend was apparent in England and Wales, where any folic acid fortification is voluntary²²⁴.

Despite considerable epidemiological and experimental evidence prior to randomized clinical trials of homocysteine-lowering B-vitamin therapy, recent large trials have all failed to demonstrate a reduction in cardiovascular endpoints²²⁵⁻²²⁹. In one of the studies, HOPE-2, the treatment group had a statistically significantly lower incidence of stroke and a higher incidence of hospitalization for unstable angina than the placebo group²²⁸, whereas a tendency toward an increased risk of the composite CVD outcome was reported in the NORVIT trial²²⁵. The question of whether the implementation of mandatory folic acid fortification of foods in North America might have reduced the power to detect effects of B-vitamin supplementation has been raised²³⁰, but the NORVIT trial, was from Norway, where there is no mandatory fortification²²⁵. The results of the clinical trials thus raise concerns about the B-vitamin/homocysteine hypothesis. However, they were all secondary prevention studies with treatment periods of at most 5 years²²⁵⁻²²⁹, and so the potential role of longer term exposure to B-vitamins in the primary prevention of CVD remains to be established.

FOLATE AND OTHER DISEASES

Folate metabolism has been implicated in diseases other than cancer and CVD. The most prominent example is neural tube defects; an adequate periconceptual consumption of folate has a strong preventative effect against neural tube defects in the developing fetus²³¹. Other conditions include various adverse pregnancy outcomes^{102, 103, 232, 233}, depression^{234, 235}, schizophrenia²³⁶, and bone health^{237, 238}. Although the potential role of folate metabolism in Alzheimer's disease and other cognitive/neurological disorders is a topic of much interest, and although some evidence suggests an involvement, primarily for homocysteine²³⁹, results to date have generally not been convincing²³⁹⁻²⁴¹.

FOLIC ACID FORTIFICATION

In 1998, the American and Canadian governments implemented mandatory fortification of flour and cereal grain foods with folic acid. Since then, the folate and homocysteine status of the populations have improved²⁴², and the rate of neural tube defects has decreased substantially²⁴³⁻²⁴⁶. Several other countries have initiated or, like Sweden, are considering mandatory fortification of foods with folate. In the UK, a recent draft report from the Scientific Advisory Committee on Nutrition²⁴⁷, which will provide a recommendation to the Food Standards Agency, concluded that, “Mandatory fortification of flour with folate should be introduced in the UK.” Although the prevention of neural tube defects has been the primary motivation for fortification, other potential health benefits have been taken into consideration, as noted in the British report²⁴⁷. The two main concerns with respect to fortification have been the potential risk of masking B12 deficiency in the elderly and a possible increase in multiple-birth pregnancies. Evidence for multiple births, however, is weak²³³. Fortification with 5-methylTHF instead of folic acid has been suggested²⁴⁸. Unlike folic acid, which can enter the methyl cycle via dihydrofolate, 5-methylTHF enters the folate pool of the cell via the vitamin B12-mediated remethylation of homocysteine to methionine (Figure 3). Fortification with 5-methylTHF would therefore not mask the haematological symptoms of vitamin B12 deficiency. Other factors that may warrant consideration are the potential cancer-promoting role of folate under certain conditions¹⁴⁹ and the possible effects on the MTHFR 677C>T genotype frequencies on the population level¹⁰⁶.

AIMS

GENERAL AIM

The aim of this thesis was to assess the relationship between factors involved in folate metabolism and the risk of developing colorectal cancer, prostate cancer, stroke, and acute myocardial infarction

SPECIFIC AIMS

- To relate prospective plasma folate and homocysteine concentrations and the MTHFR 677C>T and 1298A>C polymorphisms to the risk of developing colorectal cancer
- To relate promoter hypermethylation of the tumor suppressor genes p16 and hMLH1 in tumor tissue to prospective plasma concentrations of folate, homocysteine, and vitamin B12 and the MTHFR 677C>T and 1298A>C polymorphism in CRC patients
- To relate prospective plasma folate, vitamin B12, and homocysteine concentrations to the risk of developing prostate cancer
- To relate prospective plasma folate and vitamin B12 concentrations and dietary intake of folate and vitamin B12 to the risk of developing a first ischemic or hemorrhagic stroke
- To relate prospective plasma folate and vitamin B12 concentrations and dietary intake of folate, vitamin B12, vitamin B6, and vitamin B2 to the risk of developing a first acute myocardial infarction

MATERIAL AND METHODS

STUDY POPULATION

The studies in this thesis were based on the population in the two northernmost counties in Sweden, Norrbotten and Västerbotten (shown in Figure 2). Norrbotten is the largest county in Sweden, with an area one quarter that of the entire country but only 2% of the population²⁴⁹. Västerbotten is the second largest county, with an area one eighth that of Sweden and just over 2% of the population²⁵⁰. Together, these two counties have a population of just over 500 000 spread out over area of 154 000 km². The largest city in the region is Umeå, located in Västerbotten, with approximately 110 000 inhabitants. The average age in Umeå is relatively low, 37 years, due primarily to the 29 000 students attending Umeå University²⁵¹.

STUDY COHORT

The study base for this thesis was the Northern Sweden Health and Disease Study (NSHDS). The name NSHDS was adopted to represent three separate cohorts, the Northern Sweden MONICA Project, the Västerbotten Intervention Project (VIP), and the local Mammography Screening Project (MSP), which have been merged for research purposes. Each cohort is described in detail below. All samples and data are managed and stored at a central location, the Northern Sweden Medical Biobank, at Umeå University Hospital.

The cumulative numbers of study participants and blood sampling occasions each year since the inception of the NSHDS are presented in Figures 5-7. As of December, 2005, the NSHDS included approximately 90 000 participants and 160 000 sampling occasions.

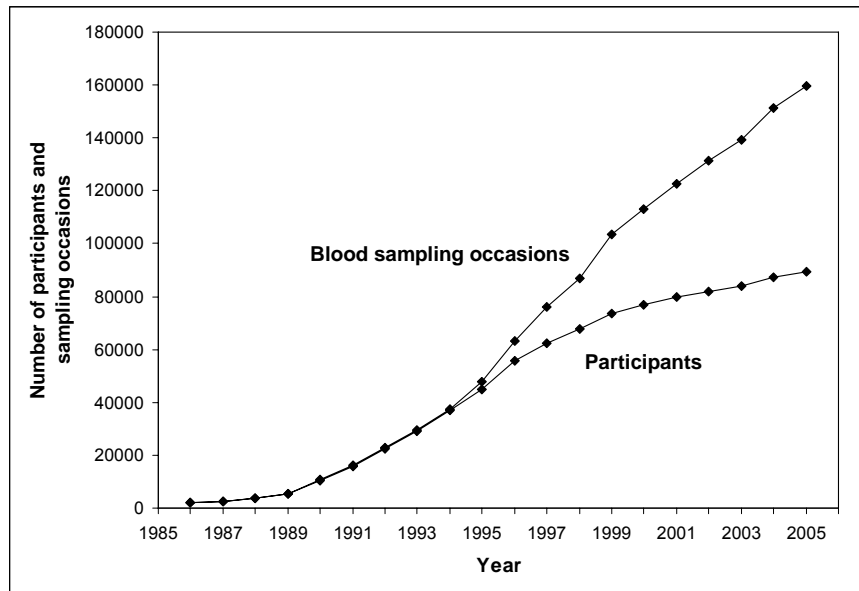


Figure 5. Cumulative number of participants and blood sampling occasions in the Northern Sweden Health and Disease Study. Data provided by Hubert Sjödin and Jie-Xian Zhang, Northern Sweden Medical Biobank, 2006.

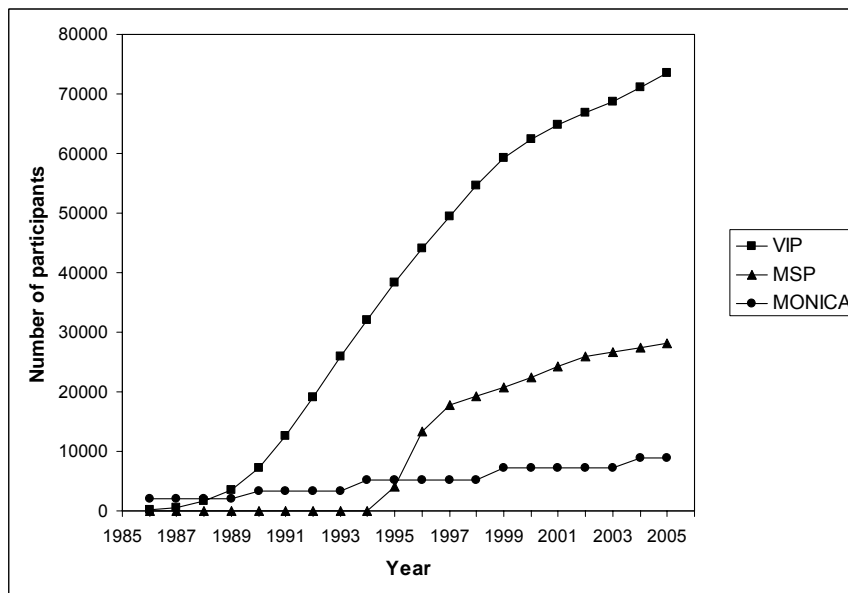


Figure 6. Cumulative number of participants in the cohorts of the Northern Sweden Health and Disease Study. Data provided by Hubert Sjödin and Jie-Xian Zhang, Northern Sweden Medical Biobank, 2006. VIP, Västerbotten Intervention Project; MSP, Mammography Screening Project; MONICA, the northern Sweden contribution to the World Health Organization Monitoring of Trends and Determinants in Cardiovascular Disease.

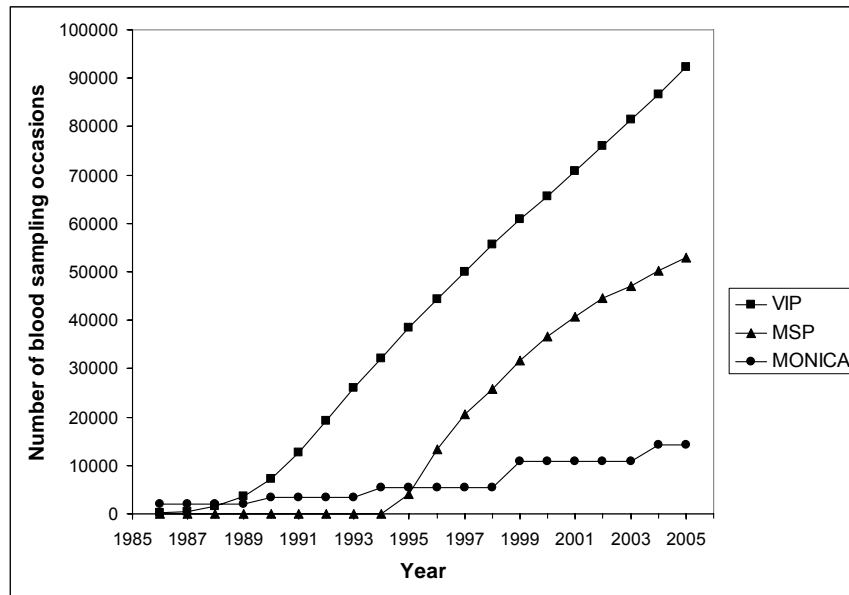


Figure 7. Cumulative number of blood sampling occasions in the cohorts of the Northern Sweden Health and Disease Study. Data provided by Hubert Sjödin and Jie-Xian Zhang, Northern Sweden Medical Biobank, 2006. VIP, Västerbotten Intervention Project; MSP, Mammography Screening Project; MONICA, the northern Sweden contribution to the World Health Organization Multinational Monitoring of Trends and Determinants in Cardiovascular Disease.

MONICA

In 1985, a multi-center World Health Organization (WHO) study, Multinational Monitoring of Trends and Determinants in Cardiovascular Diseases (MONICA), was initiated with the aim of charting trends in CVD incidence rates and risk factors associated with CVD. In total, 39 populations from 26 countries are represented. The counties of Västerbotten and Norrbotten make up one of the two Swedish MONICA centers, the other being Gothenburg. In the Northern Sweden MONICA Study, 2000 or 2500 randomly selected 25-74 year olds (stratified for age and sex) were invited to participate in a health survey in 1986, 1990, 1994, 1999, and 2004. In 1999, all participants from the previous screenings were invited to return for a follow-up survey. The mean participation rate in the northern Sweden MONICA screenings has been 77%.

VIP

In the mid-1980's, CVD prevalence and incidence rates in northern Sweden were among the highest not only in Sweden, but also among all the MONICA centers, particularly for men⁶. Cholesterol was identified as a major contributing factor, as well as diabetes and pre-stages of diabetes, and hypertension. In an attempt to reduce the incidence of CVD, a major public health strategy, the Västerbotten Intervention Project (VIP), was initiated. The VIP began in the community of Norsjö and now encompasses the entire county of Västerbotten. It has included measures on the population level, such as the marking of food products with a green "healthy choice" key hole symbol that is now established nationally. Efforts on the individual level are based on a free health examination, to which all residents of Västerbotten are invited upon turning 30 (1985-1996 only), 40, 50, and 60 years old. At the examination, which is held at a person's local health center, cardiovascular risk profile is assessed and advice is given. The mean participation rate in the VIP has been 59%.

Selection bias has been assessed in the VIP²⁵². In that study, participants were compared to non-participants, using data from the 1990 population and housing census. Only marginal differences were observed with respect to education level and socio-economic group, but participation rates were somewhat lower in the unemployed and in those with incomes in the lowest quartile.

MONICA and VIP Protocol

The VIP health examination was designed to be as similar as possible to the MONICA survey. As part of both programs, participants are invited to complete an extensive lifestyles questionnaire, including a food frequency questionnaire, and to donate a fasting blood sample to the Northern Sweden Medical Biobank for use in future research. The questionnaire and blood sampling procedures are essentially the same in both MONICA and VIP. Details of the collection and definition of some key variables that are relevant to this thesis are summarized in Tables 7 and 8.

Blood samples are collected in the morning after 5 minutes rest in a seated position (preferably after an overnight fast or at least 4 hrs of fasting). 20 mL of peripheral venous blood are drawn without stasis into one heparin Venoject tube (10 mL), and one EDTA Venoject tube (10 mL). The tubes are inverted several times, left to

stand in an upright position for 15 minutes, centrifuged for 15 min at 1500g, then aliquoted for storage. Each 10 mL sample is aliquoted into five cryotubes: three of plasma, one of buffy coat, and one of erythrocytes. Samples are frozen within one hour of collection, either at -80°C or at -20°C for up to one week before transfer to a -80°C freezer at the Northern Sweden Medical Biobank for long-term storage.

Mammography Screening Project

In the MSP, founded in 1995, all women in Västerbotten within the age range of approximately 40-70 years are invited to undergo mammography every two or three years. Age limits and frequency of examination have varied over the years depending on the availability of public funds. At the time of screening, the women are invited to complete a questionnaire concerning reproductive history, and to donate a blood sample for use in future research (participation in screening 85%, participation in screening and donation of blood sample 33%). The sampling protocol for the MSP is the same as for the VIP and MONICA cohorts. However, blood samples are collected throughout the day. All MSP subjects with unknown fasting status are thus considered to have fasted 0-4 hours.

STUDY DESIGN

The studies in this thesis had a prospective design. The term “prospective” means that exposure information and blood samples were collected from the study subjects while they were all still healthy, that is, before being diagnosed with the disease to be studied. There is considerable value in knowing that a person had a given exposure, or potential risk factor, before contracting the disease in question. For example, one might want to compare a number of people who had had an AMI to a number of people who had not. However, if the AMI patients had undergone lifestyle changes since having an AMI – perhaps they stopped smoking, began exercising, or improved their diet – then the results could be misleading. The prospective study design thus eliminates, or at least minimizes, the possibility that the disease influenced a given risk factor, a type of bias called reverse causation. The problems of selection bias and recall bias are also minimized. For example, fatal and not just non-fatal cases can be studied, which reduces selection bias. With respect to recall bias, people with a given disease are no more likely than people without the disease to have remembered or described a healthier diet than they actually consumed, because all subjects were free from the disease when they

Subjects and Methods

Table 7. Details of collection of some key variables considered in the studies in this thesis.

| Source | Variable | Details of collection |
|---------------|-------------------|---|
| Health exam | Height | Without shoes, to the nearest cm |
| | Weight | In light clothing (without shoes), to the nearest kg in the VIP and 0.2 kg in MONICA. Self-reported in the MSP until 1997. |
| | BMI | Weight / height ² (kg/m ²) |
| | Blood pressure | Measured twice after 5 minutes rest (mean recorded) Measurements were corrected for body position (sitting or supine/recombant in VIP, sitting in MONICA) |
| | Cholesterol | Measured on Reflotron benchtop analyzers at the primary health centers in the VIP and by an enzymatic method at a central hospital laboratory in MONICA (both methods from Boehringer Mannheim GmbH Diagnostica, Germany). Measurements from the VIP were corrected using the enzymatic method as the standard. |
| | Glucose tolerance | Two-hour oral glucose tolerance test using 75 g glucose dissolved in 300 mL water |
| Questionnaire | Medical history | CVD emphasized |
| | Medications | Specific questions (yes or yes/no) for use during the past year, eg. "medication to lower blood pressure" |
| | Social factors | Employment, education, civil status |
| | Tobacco habits | Smoking (cigarettes, cigars, pipe) and snuff |
| | Physical activity | Several detailed questions |
| | Nutrient intake | Validated 82- or 84-item semi-quantitative food frequency questionnaire, or a shortened 65- or 66-item version of the same questionnaire |
| | Alcohol intake | Separate questions for light beer (0.5%, only some versions of the FFQ), medium beer (3.5%), strong beer (≥5.5%), wine, dessert wine (only some versions of the FFQ), and spirits |

BMI, body mass index; CVD, cardiovascular disease; FFQ, food frequency questionnaire; MONICA, the northern Sweden contribution to the World Health Organization Monitoring of Trends and Determinants in Cardiovascular Disease; MSP, Mammography Screening Project; VIP, Västerbotten Intervention Project.

Subjects and Methods

Table 8. Definition of some key variables considered in the studies in this thesis.

| Variable | Definition |
|-------------------|---|
| Smoking status | <p>Papers I, and IV (VIP, MONICA, and MSP cohorts) <i>Yes:</i> current smoker (daily use) <i>No:</i> Never-smoker / occasional smoker (for example, party smoker) / former smoker</p> <p>Papers II and III (VIP and MONICA cohorts): <i>Current smoker:</i> daily use <i>Former smoker:</i> previous daily use but no current use <i>Non-smoker:</i> never-smoker or occasional smoker (for example, party smoker)</p> |
| Hypertension | <p><i>Hypertensive:</i> Systolic blood pressure \geq 160 mm Hg and/or diastolic blood pressure \geq 95 mm Hg and/or reported use of medication to lower blood pressure during the 14 days prior to the health survey <i>Normotensive:</i> Systolic blood pressure $<$ 160 mm Hg and diastolic blood pressure $<$ 95 mm Hg and no reported use of medication to lower blood pressure during the 14 days prior to the health survey <i>Missing:</i> No blood pressure measurement and no reported use (no or missing) of medication to lower blood pressure</p> |
| Diabetes | <p><i>Diabetic:</i> Fasting plasma glucose \geq 7.0 mmol/L and/or postload plasma glucose \geq 11.0 mmol/L (12.2 mmol/L in VIP subjects, for whom capillary plasma used) and/or self-reported diabetes <i>Non-diabetic:</i> Fasting plasma glucose $<$ 7.0 mmol/L and postload plasma glucose $<$ 11.0 mmol/L (12.2 mmol/L in VIP subjects, for whom capillary plasma used) and no self-reported diabetes <i>Missing:</i> No glucose tolerance test and no self-reported (no or missing) diabetes</p> |
| BMI | <p>Paper I: <i>Normal:</i> BMI $<$ 25 kg/m² <i>Overweight:</i> BMI 25-30 kg/m² <i>Obese:</i> $>$30 kg/m²</p> <p>Paper II: quartiles based on the distribution of the full study group</p> <p>Paper III and IV: continuous variable, missing values given the median value of the referent subjects in multivariate analyses so as to ensure a conservative effect on results</p> |
| Cholesterol | <p>Continuous variable, missing values given the median values of the referent subjects in multivariate analyses so as to ensure a conservative effect on results</p> |
| Physical activity | <p>Separate categorical variables for recreational and occupational physical activity</p> |

BMI, body mass index; MONICA, the northern Sweden contribution to the World Health Organization Monitoring of Trends and Determinants in Cardiovascular Disease; MSP, Mammography Screening Project; VIP, Västerbotten Intervention Project.

completed the lifestyle questionnaire. At the time of collection of blood samples and other information it is impossible to know who will contract a given disease. It is thus crucial in prospective cohorts such as the NSHDS to recruit as many participants as possible.

A more specific name for the study design used in this thesis is “nested case-referent study”. The term “nested” refers to the fact that subjects were part of a larger study, the NSHDS. In a cohort study, participants with a diagnosis of interest are compared to the rest of the cohort. For factors requiring laboratory analyses, however, this is unnecessarily expensive and time consuming. Instead, cases can be compared to a smaller number of healthy referents (sometimes called controls) who are randomly selected from the cohort, thus the term “case-referent study”. For each case, one or two referents are generally selected.

Referents can be chosen so that they have, for example, the same sex, age, and sample storage time as their matched case. The selection of matching criteria can have important implications for a study because while matching does reduce bias, it can also reduce the variation in exposure. For example, in this thesis, subjects in the CVD studies were matched for geographical region; cases and their referents had donated blood samples at the same health center or hospital. This was to ensure that the handling of samples did not differ substantially between cases and their matched referents. However, if subjects from the same geographical region also have similar diet or genetics, for example, then it might be difficult to identify risk factors for the disease in question.

STUDY SUBJECTS

Selection of Colorectal Cancer and Prostate Cancer Cases

Adenocarcinoma cases of CRC (ICD-10 18.0 and 18.2-18.9 for colon, 19.9 and 20.9) for rectum) and PCa (ICD-10 C61) were identified by linking the NSHDS with the National Cancer Registry using Swedish personal numbers as the linkage variable. CRC cases diagnosed between 1985 and 2002, and PCa cases diagnosed between 1985 and 2001, were included (Papers I and II). Exclusion criteria for the CRC and PCa studies were previous cancer diagnosis, previous stroke or AMI (due to prioritization to other studies), and lack of sufficient blood sample.

For CRC, verification of the diagnosis by histopathology was an inclusion criterion (Paper I), and questionable diagnoses were reviewed by a pathologist. Tumor site and clinical stage (Dukes) were extracted from pathology reports and patient charts.

For prostate cancer, tumor characteristics were obtained from the Primary Prostate Cancer Registry of Northern Sweden at Umeå University Hospital, which has been in operation since 1992. Information is extracted from medical records by a research nurse, verified by the treating physician, and then entered into the registry database. For data prior to 1992, data were extracted directly from patient records and entered into the study file. Grading was according to the WHO system until the year 2000. Since then, the Gleason score has been used almost exclusively. In order to standardize the data, WHO G1 and Gleason scores 2-5 were considered highly differentiated, WHO G2 and Gleason scores 6-7 were considered moderately differentiated, and WHO G3 and Gleason scores 8-10 were considered poorly differentiated.

Selection of Stroke and AMI Cases

Stroke and AMI cases occurring between 1985 and 2000 were identified by linking the NSHDS with the Northern Sweden MONICA registry using Swedish personal numbers as the linkage variable. The MONICA registry, described in Stegmayr et al²⁵³, includes strokes occurring in persons between the ages of 25 and 74 years, and AMI occurring in persons between the ages of 25 and 64 years, in the counties of Västerbotten and Norrbotten. Since the year 2000, AMI cases in persons up to age 75 years have also been registered. Hospital records, general practitioner's reports, death certificates, and, when available, autopsy reports are screened for suspected events and then validated according to WHO MONICA criteria. Events occurring within 27 days of each other are considered a single event, and death within 27 days of an event is considered a fatal event.

Exclusion criteria for the stroke and AMI studies in this thesis (Papers III and IV) were previous stroke or AMI, or cancer diagnosis in the 5 years prior to or 1 year after diagnosis with stroke or AMI, either according to the MONICA registry and National Cancer Registry, or after validation if suggested by questionnaire data or patient records. Cases with insufficient blood samples were also excluded from all studies except the dietary portion of the AMI study (Paper IV).

In the years 1985 to 1997, 7.8% of fatal stroke cases, 1.5% of all stroke cases, 4.3% of fatal AMI cases, and 1.5% of all AMI cases were unclassifiable with respect to whether the event was incident or recurrent incident²⁵³. These figures have increased somewhat in the years since²⁵³. Such unclassifiable cases were excluded from the studies in this thesis.

In the MONICA database, strokes are classified as subarachnoid hemorrhage (ICD-9 430, blood-stained cerebrospinal fluid and aneurysm or arteriovenous malformation found by angiography or positive CT or autopsy), intracerebral hemorrhage (ICD-9 431, positive CT or autopsy), ischemic stroke (ICD-9 434, no hemorrhage on CT or autopsy), and unspecified stroke (ICD-9 436, no CT or autopsy performed). ICD-9 codes 430–438 were considered to correspond to ICD-10 codes I60–69. The MONICA exclusion criteria for stroke cases are TIA, lesions detected by CT but with no acute focal signs, subdural and extradural hemorrhage, stroke due to trauma, and acute stroke with concomitant brain tumor or severe blood disease. Only in patients with subarachnoid hemorrhage or in deep coma are global clinical signs accepted. Subarachnoid hemorrhages and unspecified stroke were excluded from the study in this thesis (paper III).

AMI cases (ICD-9 410, ICD-10 I21-3) are identified for the MONICA registry based on medical history, symptoms, and ECG results. In fatal cases, death certificates and autopsy reports are consulted. Criteria for definite AMI include definite serial ECG progression, at least one cardiac enzyme level of at least twice the upper limit for normal plus either an ECG progression labeled as “probable AMI” together with atypical symptoms or an abnormal ECG together with typical symptoms, or visible evidence of fresh AMI or coronary thrombosis on autopsy. Possible fatal AMI events are also included in the MONICA registry and were included in the study in this thesis (Paper IV), defined as history of ischemic heart disease but autopsy not performed, evidence of chronic occlusive ischemic heart disease or old infarction on autopsy, or symptoms before death and no evidence of other cause of death.

Selection of Referent Subjects

Five potential referents were matched to each index case (by the research working group for each endpoint) according to the matching criteria in Table 9. For CRC, PCa, and stroke (Papers I-III), the first two referents for whom heparin plasma and

samples were available and who had consented to genotyping were used in the analyses. In the AMI study (paper IV), as many referents as possible (i.e. up to five per case) were included in the analyses of dietary data, and two referents were selected for the plasma analyses. Exclusion criteria included death or diagnosis with cancer, stroke, or AMI prior to the diagnosis of the index case and, for all studies except the dietary portion of Paper IV, insufficient blood samples.

Table 9 – Matching criteria for the cases and referents.

| Endpoint | Sex | Age | Recruitment Date | Cohort | Fasting Status | Geographical Region |
|----------|-----|------------------------|------------------|--------|----------------|---------------------|
| CRC | Yes | ± 1 year | ± 6 months* | Yes | Yes | No |
| PCa | Yes | ± 6 months | ± 2 months | Yes | No | No |
| Stroke | Yes | ± 2 years | ± 1 year | Yes | No | Yes |
| AMI | Yes | ± 2 years [†] | ± 4 months | Yes | No | Yes |

* Generally to within one or two months

[†] Generally ± 1 year

For all four endpoints, identification of cases and selection of referents has been performed on more than one occasion. For CRC and PCa, the original study file has been updated, whereas in the stroke study, two separate study files, covering different time periods were combined. For AMI, like stroke, two study files exist, but since the first study included only 78 cases of definite AMI, and the remaining amounts of plasma were insufficient for analysis of folate and vitamin B12 concentrations, only the second, much larger, study was included in Paper IV. When new cases are identified, the possibility arises that some cohort participants who were selected as referents in the previous study will have since been diagnosed with the endpoint in question and thus become cases in the most recent study. In this thesis, such subjects were allowed to remain as both referents and cases. Plasma analyses were performed twice, once in each case-referent triplet. There were two doubles occurred in the CRC study, eleven in the PCa study, and none in the stroke study. For the AMI study the problem of doubles was not applicable.

PLASMA ANALYSES

Plasma specimens were analyzed in triplets of 1 case and 2 referents, with the position of the cases varied at random within each case-referent triplet to avoid systemic bias and interassay variability. The investigators and laboratory staff were blinded to case and referent status.

Folate and Vitamin B12

In the studies of CRC, PCa, and stroke (papers I-III), plasma folate and vitamin B12 concentrations (P-Folate and P-B12, respectively) were analyzed by Quantaphase II[®] radioassay (BioRad Diagnostic Group, CA, USA). Intra-assay coefficients of variation for two control samples with P-Folate and P-B12 representing a normal physiological range (approximately 4-19 nM for folate and approximately 300-700 pM for vitamin B12) were all under 7.5%.

In the AMI study (paper IV), P-Folate and P-B12 were analyzed by DPC[®] Solid Phase No Boil Dualcount radioassay (DPC Diagnostic Products Corporation, CA, USA). The standard curve for P-B12 appeared to follow a quadratic scale rather than the linear scale noted in the kit instructions, and concentrations were therefore recalculated in Excel. Intra-assay coefficients of variation for P-Folate were 15.8% and 12.1% for control samples with mean concentrations of 7.3 nmol/L and 30.4 nmol/L, respectively. Intra-assay coefficients of variation for P-B12 were 16.5% and 10.4% for control samples with mean concentrations of 263 pmol/L and 1001 pmol/L, respectively. In order to conserve plasma, both the BioRad Quantaphase II[®] and DPC[®] methods were optimized to allow a 1:2 dilution, thus reducing the amount of plasma required from 200 μ L to 100 μ L.

Homocysteine

Total plasma homocysteine (P-tHcy) was measured by a fluorescence polarization immunoassay on an IMx[®] unit (Abbott Laboratories, IL, USA). In the stroke study (paper III), the first 190 subjects were analyzed several years earlier by high performance liquid chromatography (HPLC) with electrochemical detection, and results were calibrated according to the immunoassay. Intra-assay coefficients of variation for two sets of two control samples per batch (with P-tHcy representing a medium to high normal physiological range (approximately 13-26 μ M) were all under 3.0%.

GENOTYPING ANALYSES

MTHFR 677C>T and 1298A>C genotypes were generated using the TaqMan allelic discrimination method at the Center for Genome Research, Department of

Medical Biosciences, Umeå University, Sweden. TaqMan assays and reagents were from Applied Biosystems (Foster City, CA, USA). PCR reactions were performed on GeneAmp PCR system 9700, and PCR programs were according to the manufacturer (ABI). PCR products were analyzed on the ABI PRISM 7900HT Sequence Detection System. For the first 190 subjects in the stroke study (Paper III), restriction fragment length polymorphism (RFLP) analysis was employed according to Frosst *et al*⁹¹ for 677C>T and Weisberg *et al*¹⁰⁷ for 1298>A/C.

METHYLATION ANALYSES

Promoter methylation in two genes common to CIMP gene panels^{20, 22, 23}, p16 and hMLH1, was assessed by methylation-specific PCR. Formalin-fixed, paraffin-embedded, archival CRC tissue samples were available for 187 of the cases in Paper I.

DNA was extracted from 10µm tissue slices (Nucleon BACC2, RPN 8502, GE Healthcare, Uppsala), and bisulphite modification was performed using the CpGenome™ Universal DNA Modification Kit (Chemicon, Temecula, CA). The protocol provided with the kit was followed exactly, using a starting amount of 1 µg DNA, and eluting into 25 µl TE buffer in the final step. CpGenome™ Universal Methylated DNA (Chemicon, Temecula, CA) was used as a positive control for each pair of methylated-specific primers, and as a negative control for each corresponding pair of unmethylated-specific primers. CpGenome™ Universal Unmethylated DNA (Chemicon, Temecula, CA) was used as a positive control with each pair of unmethylated-specific primers, and as a negative control with each corresponding pair of methylated-specific primers.

PCR was performed in 25 µL reaction mixtures, containing 1× Gold Buffer (supplied with Taq polymerase), 200 µmol/L dNTPs, 1.25 U AmpliTaq Gold® (ABI, Foster City, CA), and 1 µL bisulphite modified DNA. Details of the primers used are given in Table 10. The cycling conditions were: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 65 °C for 30 s, and 72 °C for 30 s, and ending with a final extension of 72 °C for 10 min.

The products were analysed by electrophoresis on 2% MetaPhor® Agarose gels (Cambrex, Rockland, ME), and visualized with ethidium bromide under ultraviolet light.

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Table 10. Details of the primers used in the PCR for p16 and hMLH1 promoter methylation in CRC tissue.

| Primer | Specificity | Sequences (5'-3') | MgCl ₂ , mmol/L | Primer, µmol/L |
|-----------|--------------|-------------------------------|-------------------------------|-------------------|
| P16-M-F | Methylated | TTATTAGAGGGTGGGGCGGATCGC | 1.5 | 0.25 |
| P16-M-R | Methylated | GACCCCGAACCGCGACCGTAA | | |
| P16-U-F | Unmethylated | TTATTAGAGGGTGGGGTGGATTGT | 1.5 | 0.5 |
| P16-U-R | Unmethylated | CAACCCCAAACCACAACCATAA | | |
| hMLH1-M-F | Methylated | ACGTAGACGTTTTATTAGGGTCGC | 2.0 | 0.5 |
| hMLH1-M-R | Methylated | CCTCATCGTAACTACCCGCG | | |
| hMLH1-U-F | Unmethylated | TTTTGATGTAGATGTTTTATTAGGGTTGT | 3.0 | 0.125 |
| hMLH1-U-R | Unmethylated | ACCACCTCATCATAACTACCCACA | | |

F, forward; R, reverse; U, unmethylated; M, methylated.

DIETARY ASSESSMENT

Dietary intake data for all MONICA surveys and for the years from 1992 to present in the VIP have been compiled in electronic databases. In the early years of the VIP (1985-1992), each municipality constructed its own lifestyles questionnaire. Although the questionnaires were not entirely incompatible, they were not optically readable and so in general, only some key non-dietary variables, such as smoking status, that could be standardized among the various versions of the questionnaire have been entered into the NSHDS database. In 1992, a single optically readable version of the questionnaire was adopted for the entire county, but in the years since, the number of items in the FFQ has varied due to financial constraints. Approximately 85% of the study subjects in Papers III and IV with optically readable questionnaires had completed a validated 82- or 84-item FFQ²⁵⁴, and nearly 15% had completed a shortened 65- or 66-item version of the same FFQ.

The FFQ estimates frequency of intake on a 9-level scale including never, up to once per month, 1-3 times per month, once per week, 2-3 times per week, 4-6 times per week, once per day, 2-3 times per day, and 4 or more times per day. In order to estimate portion sizes, photographs of four dinner plates showing increasing amounts of potato/pasta/rice, vegetables, and meat/fish/poultry were included in the FFQ. For other foods, age- and sex-standardized and Swedish standard portion sizes or natural sizes, such as an apple, are used²⁵⁵.

Exclusion criteria for the FFQ were described in the validation study and are, in brief, missing values for more than 10% of the FFQ items or any missing values in the section concerning portion sizes²⁵⁴. In order to calculate daily intake of energy and nutrients, the consumption frequencies are converted to frequency per day, multiplied by portion size, and then multiplied by the energy or nutrient content given in the Swedish Food Tables and the internet-based Swedish Food Database^{256, 257}. The folate content of various foods was updated according to recent analyses. For multivitamin users, 200 µg of folate, 2.5 µg of vitamin B12, 1.5 mg of vitamin B6, 1.5 mg of vitamin B2, and 10mg of vitamin E (estimates based on supplements available in Sweden during the relevant time period) were added to the dietary intakes from foods to estimate total intake.

STATISTICAL ANALYSES

Baseline characteristics and study variables for cases and referents were compared by Mann-Whitney or Student's t-test (continuous variables) and Chi-squared tests (categorical variables). Relationships between variables were assessed using Spearman rank correlations. Odds ratios (OR) for disease and 95% confidence intervals (CI) were calculated by conditional logistic regression for quartiles or quintiles based on the variable distributions of the referent subjects (or in the PCa study, Paper II, all subjects). Quartile and quintile cut-offs for the plasma variables were calculated separately by sex, and for the dietary intake variables by sex and version of the FFQ. Tests for trend were performed by including the quartiles or quintiles as continuous variables in the regression analyses. In multivariate analyses, missing values were treated as a separate category (categorical variables) or given the median-value of the referent subjects (continuous variables). P-values and confidence intervals were not corrected for multiple testing. Statistical tests and corresponding P-values were two-sided, and SPSS version 13.0 (Chicago, IL, USA) was used for all statistical analyses.

ETHICAL APPROVAL

The study protocol for all published papers and unpublished data presented in this thesis were approved by the Research Ethics Committee of Umeå University, Umeå, Sweden. The data handling procedures were approved by the Swedish

Subjects and Methods

National Computer Data Inspection Board. All subjects provided written informed consent.

RESULTS AND DISCUSSION

FOLATE STATUS IN THE STUDY POPULATION

Folate, Vitamin B12, and Homocysteine

In order to provide a description of the folate status in the study population, the referent subjects from Papers I-IV were pooled. Although they were selected to match disease cases and were therefore not a random selection, they all came from the same population-based cohorts and should be reasonably representative of healthy men and women in northern Sweden, in the age range studied. Approximately 50% of the subjects were 59-61 years old at recruitment, and approximately 80% were 49 to 61 years old. P-Folate, P-B12, and P-tHcy data were available for approximately 1800 men and 850 women. Dietary folate and vitamin B12 intake obtained from the validated 82- or 84-item FFQ were available for 953 men and 355 women.

P-Folate, P-B12, and P-tHcy status are each presented separately below, but median levels in men and women and in younger and older subjects are summarized in Table 11. Men had lower P-Folate and P-B12, and higher P-tHcy, than women. P-Folate and P-B12 were also lower, and P-tHcy higher, in older men compared to younger men, whereas in women, only P-tHcy varied by age.

Table 11. Plasma concentrations of folate (P-Folate), vitamin B12 (P-B12), and homocysteine (P-tHcy) in a pooled analysis of the referents subjects from Papers I – IV, median and 25-75th percentiles.

| | All ages | ≤ 59 years | > 59 years |
|------------------|-------------------|-------------------|--------------------|
| Men | N≥1791 | N≥729 | N≥1055 |
| P-Folate, nmol/L | 8.0 (5.9 – 10.9) | 8.3 (6.3 – 11.5) | 7.6 (5.8 – 10.3) |
| P-B12, pmol/L | 299 (235 – 380) | 316 (252 – 400) | 288 (222 – 367) |
| P-tHcy, μmol/L | 11.3 (9.7 – 13.4) | 10.6 (9.1 – 12.5) | 11.8 (10.1 – 13.9) |
| Women | N≥826 | N≥329 | N≥497 |
| P-Folate, nmol/L | 8.8 (6.3 – 12.2) | 8.8 (6.6 – 11.7) | 8.8 (6.2 – 13.0) |
| P-B12, pmol/L | 318 (248 – 406) | 315 (257 – 402) | 320 (242 – 409) |
| P-tHcy, μmol/L | 10.1 (8.9 – 12.4) | 9.4 (8.2 – 11.3) | 11.0 (9.0 – 12.9) |

The differences between men and women were statistically significant for all three variables. The same was true for the differences between the younger and older men, whereas in women, only P-tHcy differed significantly. (Mann-Whitney test, all significant P-values were <0.001.)

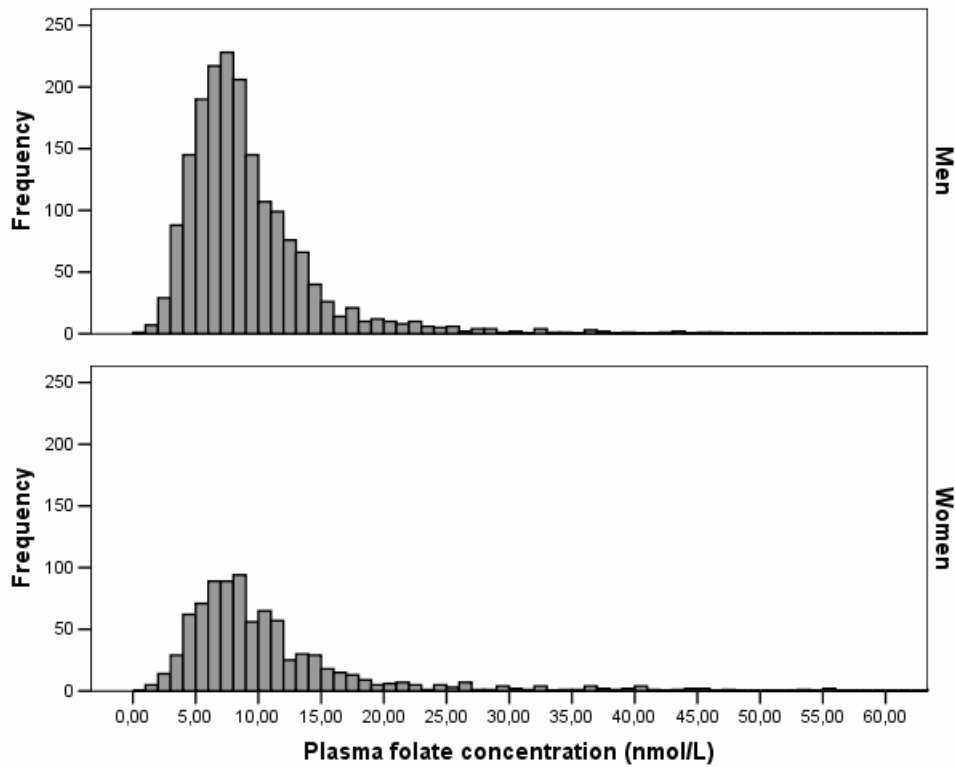


Figure 8. Histogram of plasma folate concentrations in men and women from the population-based cohorts of the Northern Sweden Health and Disease Study who were selected as referents for the studies in this thesis.

The distribution of P-Folate in men and women is presented in Figure 8. No men and two women had concentrations over 60 nmol/L, the highest level shown in Figure 8. In total, 35.7% of men and 29.2% of women had concentrations under 6.8 nmol/L, an often-used decision limit for folate deficiency, and 87.6% of men and 79.3% of women were under 13.6 nmol/L, a level sometimes considered the lower limit for acceptable folate status.

The median and 25-75th percentiles for dietary folate intake were 244 (198-306) µg/day in men and 226 (179-274) µg/day in women (Mann-Whitney $p < 0.001$). These findings are consistent with the two nation-wide surveys, Riksmaten and HULK (Table 2). 26.0% of men and 35.8% of women were under the average daily requirement (AR) of 200 µg/day, and only 26.2% of men and 16.6% of women met the current RI of 300 µg/day.

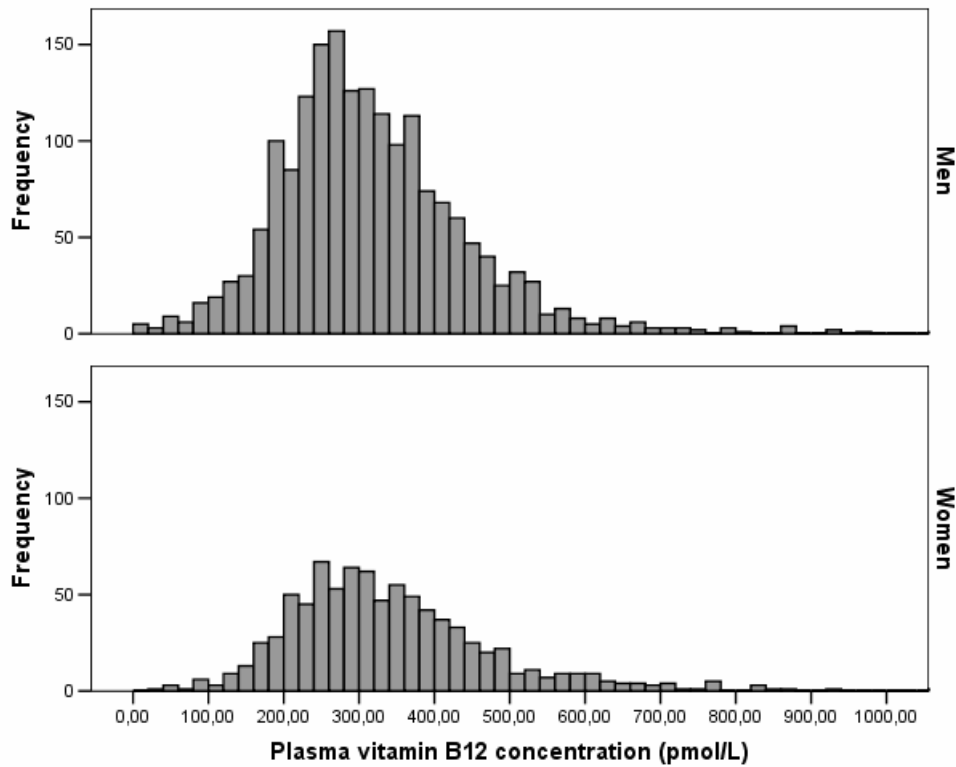


Figure 9. Histogram of plasma vitamin B12 concentrations in men and women from the population-based cohorts of the Northern Sweden Health and Disease Study who were selected as referents for the studies in this thesis.

The distribution of P-B12 in men and women is presented in Figure 9. Two men and three women had concentrations over 1000 pmol/L, the highest level shown in Figure 9. In total, 5.1% of men and 3.3% of women had concentrations under 148 pmol/L, the most commonly used limit for deficiency. A decision limit of 221 pmol/L is also employed sometimes, and 20.5% of men and 16.5% of women were under this level.

The median and 25-75th percentiles of dietary vitamin B12 intake were 5.9 (4.5-7.7) µg/d in men, 4.7 (3.7-6.3) µg/d in women (Mann-Whitney $p < 0.001$). Although these levels are somewhat lower than those noted in Riksmaten and HULK (Table 2), virtually no subjects were under the AR of 1.4 µg/d (0.4% of men and no women). 99.4% of men and 97.2% of women met the RI of 2.0 µg/d.

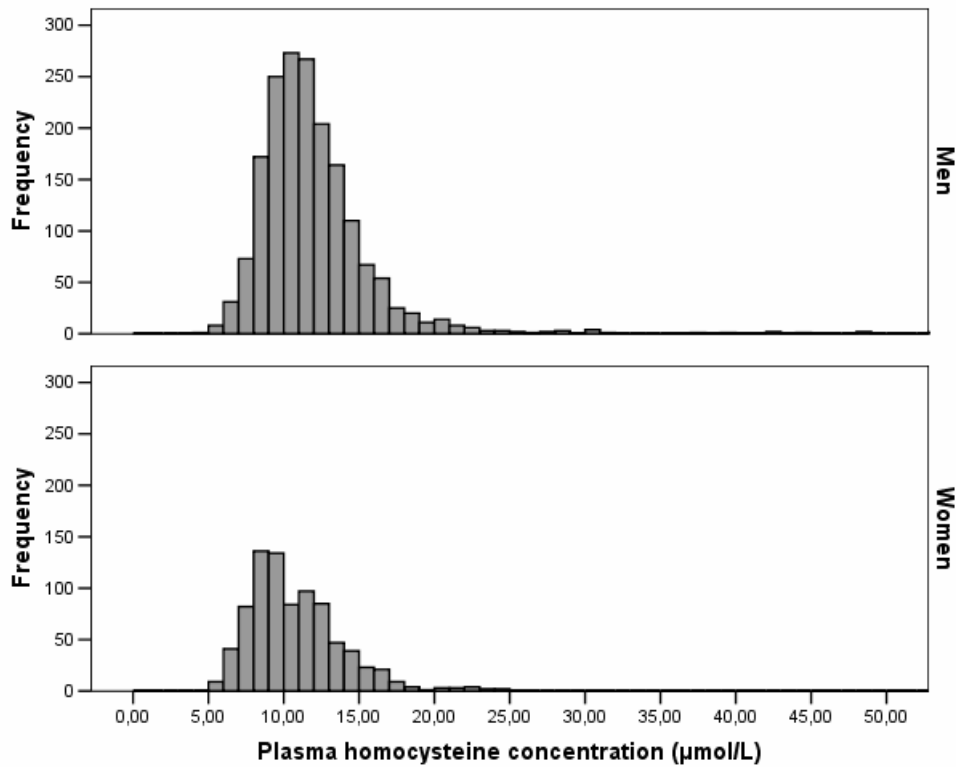


Figure 10. Histogram of plasma total homocysteine concentrations in men and women from the population-based cohorts of the Northern Sweden Health and Disease Study who were selected as referents for the studies in this thesis.

The distribution of P-tHcy in men and women is presented in Figure 10. Seven men and no women had concentrations over 50 µmol/L, the highest level shown in Figure 10. The limits employed for hyperhomocysteinemia generally range from about 9 to 15 µmol/L. In the present analyses, 83.9% of men 67.3% of women had concentrations over 9 µmol/L, and 13.0% of men and 8.7% of women had concentrations over 15 µmol/L.

These findings suggest that the middle-aged and younger elderly population in northern Sweden has a low folate status and elevated homocysteine levels, which is consistent with the diet in northern Sweden, high in meat and potatoes and low in fruits and vegetables, as described in the introduction. Use of multivitamin supplements in the 14 days and/or year prior to recruitment to the cohort was more common in women than men (22.8% and 14.9%, respectively, chi-squared test, $p=0.001$), which may have contributed to their higher P-Folate and P-B12 and

lower P-tHcy levels. Although 30% of the women with plasma samples were from the MSP, and were therefore essentially all non-fasting at blood sample collection, excluding these subjects did not affect the results.

MTHFR Genotype Frequencies

Genotype frequencies for the MTHFR 677C>T and 1298A>C SNPs, and combinations of the two, are shown in Table 12. Hardy-Weinberg equilibrium was noted for both SNPs. 8.4% of subjects were homozygous for the 677T-allele, which is consistent with the idea of an increasing frequency of the T-allele along a north-south gradient in Europe¹⁰⁰. Furthermore, no subjects had more than two rare alleles, suggesting complete linkage disequilibrium between the 677C>T and 1298A>C SNPs in the northern Swedish population. This is not surprising; the 677T/1298C haplotype is extremely rare and may be limited to certain populations in the united Kingdom and North America¹⁰⁸. The MTHFR 677T-allele was associated with lower P-Folate and higher P-tHcy (Table 12).

Table 12. Genotype distributions for the MTHFR 677C>T and 1298A>C polymorphisms in a pooled analysis of the referents subjects from Papers I – III.

| | | | | |
|-------------------|-------------------|-------------------|--------------------|--------|
| MTHFR 677C>T | CC | CT | TT | P* |
| Frequency (%) | 856 (53.0%) | 624 (38.6%) | 136 (8.4%) | |
| P-Folate (nmol/L) | 7.9 (5.8 – 10.8) | 7.4 (5.4 – 10.2) | 6.5 (4.7 – 8.6) | <0.001 |
| P-tHcy (µmol/L) | 11.1 (9.4 – 13.2) | 11.6 (9.9 – 13.5) | 13.1 (11.2 – 17.2) | <0.001 |
| MTHFR 1298A>C | AA | AC | CC | |
| Frequency (%) | 693 (43.0%) | 721 (44.7%) | 198 (12.3) | |
| P-Folate (nmol/L) | 7.6 (5.2 – 10.9) | 7.6 (5.6 – 10.5) | 7.6 (5.6 – 9.6) | 0.851 |
| P-tHcy (µmol/L) | 11.6 (9.7 – 13.9) | 11.4 (9.6 – 13.3) | 11.8 (9.9 – 13.7) | 0.273 |
| | 677CC | 677CT | 677TT | |
| 1298AA | 249 (15.5%) | 309 (19.2%) | 135 (8.4%) | |
| 1298AC | 409 (25.4%) | 312 (19.4%) | - | |
| 1298CC | 197 (12.2%) | - | - | |

* Kruskal-Wallis test

BASELINE CHARACTERISTICS

In total, 226 CRC, 254 PCa, 397 stroke (of which 334 were ischemic and 62 were hemorrhagic), and 571 AMI were included in the studies in this thesis (Table 13). The ratio of men to women reflects the design of the cohorts in the NSHDS to some extent. In both the CRC and AMI studies, women were somewhat overrepresented

due to the inclusion of the all-female MSP. Subjects in the MSP tend also to be older, since women are screened up to the age of 70 years, and to have shorter follow-up times, due to more frequent screening. Follow-up times ranged from less than one year to a maximum of 14 years, with medians of 3.7 to 4.9 years.

Table 13. Sex, age at diagnosis and follow-up times of cases, median and 25-75 percentiles.

| Endpoint | N | Men / Women | Age at diagnosis, y | Follow-up time, y | |
|--------------------|-----|-------------|---------------------|-------------------|---------|
| | | | | Median | Maximum |
| CRC | 226 | 94 / 132 | 62.9 (57.3-66.9) | 4.2 (2.4-6.7) | 12.7 |
| PCa | 254 | 254 men | 63.6 (60.9-66.4) | 4.9 (2.9-7.0) | 13.2 |
| Ischemic stroke | 334 | 185 / 149 | 61.5 (53.9-65.4) | 3.8 (1.9-6.3) | 11.6 |
| Hemorrhagic stroke | 62 | 44 / 18 | 61.5 (54.1-64.6) | 3.9 (1.8-5.7) | 9.5 |
| AMI | 571 | 406 / 165 | 60.6 (53.4-64.5) | 3.7 (2.1-5.7) | 13.0 |

Colorectal Cancer

In the CRC study (Paper I), 31.9% of tumors were located in the right colon, 31.0% in the left colon, and 37.2% in the rectum, and the majority were Dukes B or C (Paper I, Table 1). No statistically significant differences were observed between cases and referents for baseline characteristics including BMI and smoking (Paper I, Table 1). Overweight and obesity are believed to be risk factors for CRC, but BMI may not be the best measure of excess body weight. A recent report from the large, multi-center European Prospective Investigation into Cancer and Nutrition (EPIC) study found stronger associations for the waist-to-hip ratio³⁰. Smoking, particularly many years prior to diagnosis, may also increase risk slightly³⁵. The lack of an association in the present study may therefore have been due to the use of a current smoking variable, or to the low prevalence of smokers in the study population.

Prostate Cancer

In many countries, and particularly in the USA, intensive PSA screening has led to rises in PCa incidence rates. However, no formal screening program has been in place in the catchment area of the NSHDS, and in this study, only 12.2% of the PCa cases were identified through opportunistic screening (Paper II, Table 1). 21.3% of the cases were advanced at diagnosis, defined as locally advanced tumor (T3 or T4), and/or lymph node metastasis (N1), and/or metastasis on bone scan (M1), and/or serum PSA (S-PSA) above 50 ng/mL. No statistically significant differences were found between cases and referents for baseline characteristics including BMI

and smoking (Paper II, Table II). This is not surprising since neither has been established as a risk factor for PCa^{56,57}.

Stroke

Baseline characteristics of the ischemic and hemorrhagic stroke cases and their matched referents are presented in a table that was published on-line, included as an appendix to Paper III. Cases with ischemic stroke had higher baseline BMI and cholesterol levels than their matched referents, although the result for cholesterol was of borderline statistical significance. Ischemic stroke cases also had a higher baseline prevalence of diabetes, hypertension, and smoking than referents. These results are in line with current understanding of ischemic stroke, as having the same risk factors as other ischemic diseases such as AMI. For hemorrhagic stroke, similar tendencies were observed, with the exception of cholesterol, for which levels were virtually identical in cases and referents. However, the differences were statistically significant only for BMI and hypertension, which may in part reflect the lower number of hemorrhagic compared to ischemic stroke cases, but is also consistent with the relative importance of blood pressure compared to other risk factors in hemorrhagic stroke.

Acute Myocardial Infarction

AMI cases had higher baseline BMI and cholesterol levels, as well as a higher prevalence of diabetes, hypertension, and smoking than their matched referents (Paper IV, Table 1). Thus, the traditional risk factors for AMI, and for ischemic events in general, were represented.

RELATIONSHIPS BETWEEN EXPOSURE AND DISEASE

Colorectal Cancer, Paper I

P-Folate was statistically significantly related to risk of CRC in a bell-shaped manner (Paper I, Table 2). Univariate ORs were, for the middle versus lowest quintile, 2.26 (95% CI 1.29-3.95); for the highest versus lowest quintile, 1.55 (95% CI 0.85-2.85); and for concentrations ≥ 15.0 versus < 5 nmol/L, 1.10 (95% CI 0.52-2.32). Risk estimates were attenuated somewhat by adjustment for BMI, current

smoking, recreational and occupational physical activity levels, and alcohol consumption. In contrast to the findings for folate, neither P-tHcy (Paper I, Table 2) nor P-B12 (unpublished data) was statistically significantly associated with CRC risk.

Undiagnosed CRC in the cases at the time of blood sampling might explain the apparent risk reduction at lower P-Folate, since proliferating neoplasms are dependent on folate in order to grow and progress. However, in subjects with longer follow-up times, who would have been less likely to have an established neoplasm at baseline, P-Folate demonstrated a strong, positive, dose-response association with CRC risk [multivariate OR for highest versus lowest quintile 3.87 (95% CI 1.52-9.87), $P_{\text{trend}}=0.007$] (Paper I, Table 3). The possibility that this was a chance finding cannot be excluded, but it is none the less an interesting observation, and may warrant further study.

The 677T-allele was inversely related to CRC risk [OR for TT versus CC 0.44 (95% CI 0.21-0.88), $P_{\text{trend}}=0.048$], a finding that was essentially unchanged in magnitude but attenuated in significance in multivariate analysis [OR 0.41 (95% CI 0.19-0.85), $P_{\text{trend}}=0.062$] (Paper I, Table 4). In contrast to MTHFR 677C>T, a positive association with CRC risk was found for the 1298A>C mutation [OR for CC versus AA 1.52 (95% CI 0.90-2.57), $P_{\text{trend}}=0.063$], the P-trend for which became statistically significant in the multivariate model ($P_{\text{trend}}=0.028$) (Paper I, Table 4). However, the results of a multivariate interaction analysis of genotype combinations, with 677CC-1298AA subjects as the reference group, suggested that the association for 1298A>C may have been largely due to linkage disequilibrium with 677C>T (Paper I, Table 4).

A multivariate interaction analysis of CRC risk by combinations of MTHFR 677C>T genotype and P-Folate is presented in Paper I, Figure 1. The reduced CRC risk in subjects homozygous for the T-allele was essentially independent of folate status. This finding contradicts the current hypothesis that the 677C>T SNP reduces CRC risk when folate status is high but increases risk when folate status is low^{167, 181-183}, but is consistent with some studies suggesting that this may not be the case^{180, 184, 185}.

Taken together, these findings suggest that a low folate status may reduce the risk of CRC, which is in contrast to a wealth of evidence suggesting an increased risk at

lower folate levels^{150, 157}. Folate levels in the present study were considerably lower than those in previous prospective studies reporting an inverse association with CRC risk^{81, 167}. The study population of the NSHDS may thus be better suited to studying the role of folate deficiency in colorectal tumorigenesis. The one previous prospective study with folate levels comparable to those in the present study reported a null association¹⁶⁸.

Colorectal Cancer, Promoter Methylation (Preliminary Results)

The preliminary results presented in this section are from an ongoing study that is the first to relate prediagnostic folate status to promoter hypermethylation in CRC tissue. Of the 187 CRC cases with archival tumor tissue available, p16 was successfully assayed in 156 and hMLH1 in 166. Of these, the percent positive for promoter methylation was 30.1% for p16 and 22.9% for hMLH1. In order to test for associations between promoter hypermethylation and folate status, cases were classified as having 0 or ≥ 1 genes positive for promoter methylation. 62.8% of cases were negative for promoter methylation in both genes, 37.2% were positive for promoter methylation in at least one gene.

Promoter hypermethylation frequencies according to baseline and clinical characteristics are presented in Table 14. Promoter hypermethylation was associated with older age at diagnosis ($P=0.024$) and with right-sided CRC, though the latter was not statistically significant ($P=0.087$). These findings are in line with previous studies of the CIMP pathway of CRC^{21, 23, 258}.

P-Folate was higher in cases with promoter methylation in at least one of the two genes (median 8.8 versus 7.9 nmol/L, $P=0.025$) (Table 15). P-B12, P-tHcy, and MTHFR 677C>T and 1298A>C genotypes did not vary by promoter hypermethylation status. Although these results should be considered preliminary (more genes will be studied), they suggest that folate status may influence promoter hypermethylation in tumor suppressor genes. This supports some^{137-140, 147}, but not all¹⁴¹⁻¹⁴³ previous studies. If the findings presented here are confirmed by further analyses, then they may be of relevance for further study of the CIMP pathway of CRC tumorigenesis. They may also explain the apparent reduced CRC risk at low folate levels reported in Paper I.

Results and Discussion

Table 14. Frequency of promoter methylation of the tumor suppressor genes p16 and hMLH1 in colorectal cancer tissue according to sex, age at diagnosis, tumor site, and Dukes stage

| | Number of genes hypermethylated | | P* |
|-------------|---------------------------------|------------|-------|
| | 0 | ≥ 1 | |
| Men | 50 (65.8%) | 26 (34.2%) | 0.475 |
| Women | 63 (60.6%) | 41 (39.4%) | |
| Age ≤ 65 y | 78 (69.0%) | 35 (31.0%) | 0.024 |
| Age > 65 y | 35 (52.2%) | 32 (47.8%) | |
| Right colon | 30 (51.7%) | 28 (48.3%) | 0.087 |
| Left colon | 33 (64.7%) | 18 (35.3%) | |
| Rectum | 50 (70.4%) | 21 (29.6%) | |
| Dukes A/B | 61 (63.5%) | 35 (36.5%) | 0.725 |
| Dukes C/D | 50 (61.0%) | 32 (39.0%) | |

* χ^2 test

Table 15. Prediagnostic plasma concentrations of folate, vitamin B12, and homocysteine, and MTHFR 677C>T and 1298A>C genotype frequencies according to promoter methylation status of the tumor suppressor genes p16 and hMLH1 in colorectal cancer tissue.

| | Number of genes hypermethylated | | P* |
|--------------|---------------------------------|-------------------|-------|
| | 0 | ≥ 1 | |
| Folate | 7.9 (5.9 – 9.7) | 8.8 (6.9 – 12.1) | 0.025 |
| Vitamin B12 | 283 (228 – 355) | 286 (233 – 359) | 0.601 |
| Homocysteine | 11.7 (10.0 – 13.8) | 11.5 (9.6 – 13.8) | 0.825 |
| MTHFR 677 | | | 0.768 |
| CC | 59 (53.6%) | 39 (59.1%) | |
| CT | 46 (41.8%) | 24 (36.4%) | |
| TT | 5 (4.5%) | 3 (4.5%) | |
| MTHFR 1298 | | | 0.233 |
| AA | 38 (34.5%) | 25 (37.9%) | |
| AC | 59 (53.6%) | 28 (42.4%) | |
| CC | 13 (11.8%) | 13 (19.7%) | |

* Mann-Whitney test for the plasma variables, χ^2 test for the MTHFR variables

Prostate Cancer

The highest quartile of P-Folate was associated with an increased risk of developing PCa [OR 1.60 (95% CI 1.03-2.49), $P_{\text{trend}}=0.02$], and the highest quartile of P-tHcy with a reduced risk of borderline statistical significance [OR 0.67 (95% CI 0.43-1.04), $P_{\text{trend}}=0.08$] (Paper II, Table IIIa,c). For vitamin B12, the risk of PCa increased with each quartile up to an OR of 2.63 (95% CI 1.61-4.29), $P_{\text{trend}}<0.001$, for highest versus lowest quartile (Paper II, table IIIb).

After adjustment for the other two plasma variables, and BMI and smoking, the ORs for the highest versus lowest quartile of folate and homocysteine were attenuated and not statistically significant [OR 1.30 (95% CI = 0.74-2.24), $P_{\text{trend}}=0.17$, for folate, and OR 0.91 (95% CI = 0.51-1.58), $P_{\text{trend}}=0.60$, for homocysteine] (Paper II, Figure 1). In contrast, the corresponding OR for vitamin B12 rose to 2.96 (95% CI = 1.58-5.55), $P_{\text{trend}}=0.001$, in the multivariate model (Paper II, Figure 1).

Based on these findings, one might speculate that an increase in methyl availability could increase susceptibility to promoter hypermethylation and consequent silencing of genes such as GSTP1 and other tumor suppressors in the prostate. However, the positive association between P-Folate and PCa risk described in Paper II was later found to be limited to carriers of the MTHFR 677C>T SNP¹⁷⁸. This suggests that increased methyl availability for DNA synthesis, and thus cell proliferation, in undiagnosed PCa may be a more likely explanation. Given the high prevalence of PCa in the general population⁵⁵, the possibility of a role for folate metabolism in tumor progression warrants further study.

A possible increased risk of PCa in subjects with high vitamin B12 and possibly also folate status contradicts some previous studies¹⁶⁹⁻¹⁷². However, the only other prospective study of comparable size that measured plasma or serum concentrations of folate and vitamin B12 reported a null result¹⁷⁰. A positive association between vitamin B12 intake and PCa risk was found in one small case-control study¹⁷³, and the use of multivitamins has been reported to be associated with an increase in PCa mortality²⁵⁹.

Stroke

P-Folate demonstrated a U-shaped association with the risk of ischemic stroke, both in univariate analysis and after adjustment for other risk factors, including hypertension and P-tHcy [multivariate OR, including P-tHcy, for the third versus lowest quartile, 0.58 (95% CI 0.36-0.92)] (Paper III, Table 2). Folate intake was not related to the risk of ischemic stroke (Paper III, Table 2). A biological explanation for the findings for P-Folate seems unlikely, given the considerable evidence for a linear, inverse relationship between folate and risk of ischemic stroke^{186, 188-191, 228}, and it may therefore be a chance finding.

P-Folate was associated with the risk of hemorrhagic stroke in a linear, inverse manner, both in univariate analysis and after adjustment for conventional risk factors [multivariate OR for the highest versus lowest quartile 0.21 (95% CI 0.06-0.71), $P_{\text{trend}}=0.008$] (Paper III, Table 2). When homocysteine was included in the model, the OR was attenuated somewhat and was no longer statistically significant [OR 0.34 (95% CI 0.08-1.40), $P_{\text{trend}}=0.088$]. Folate intake also demonstrated an inverse association with hemorrhagic stroke risk (Paper III, Table 2). All ORs were under 0.2 and statistically significant in univariate analysis and after adjustment for conventional risk factors including hypertension [multivariate OR for the highest versus lowest quartile 0.07 (95% CI: 0.01-0.55), $P_{\text{trend}}=0.031$], but not after further adjustment for homocysteine [OR 0.16 (95% CI 0.02-1.23), $P_{\text{trend}}=0.118$].

This was the first prospective study to relate circulating folate levels to the risk of hemorrhagic stroke. Two previous prospective studies have addressed the role of folate intake, both reporting null results^{190, 191}. The magnitudes of the risk estimates for both P-Folate and folate intake were largely unchanged by adjustment for P-tHcy, suggesting that other mechanisms may be involved in the association between folate status and risk.

One might speculate that the low folate status of the study population may have precluded detection of a reduced risk of ischemic stroke at higher folate levels. If a high folate status affects the risk of both ischemic and hemorrhagic stroke but the effects become apparent at lower folate levels in hemorrhagic stroke, then it may support an involvement via mechanisms common to both outcomes but to different degrees, such as hypertension.

Neither P-B12 nor vitamin B12 intake (Paper III, Table 3) was statistically significantly associated with either type of stroke, although there was a non-significant positive association between increasing intake and risk of ischemic stroke. This is in contrast to one large prospective study, in which vitamin B12 intake was inversely related to the risk of ischemic stroke¹⁹⁰.

Acute myocardial infarction

Univariate and multivariate ORs for the plasma variables are shown in Paper IV, Table 3. P-Folate was inversely related to the risk of a first AMI [OR for highest versus lowest quintile 0.37 (95% CI 0.25-0.54), $P_{\text{trend}} < 0.001$]. P-B12 concentrations were not related to risk in univariate analysis. Results for both P-Folate and P-B12 were essentially unchanged after adjusting for baseline BMI, cholesterol levels, smoking status, diabetes, hypertension, and alcohol consumption. When P-Folate, P-B12 and total plasma homocysteine concentrations were included in the same multivariate model, together with the covariates noted above, the risk estimates for P-Folate were attenuated somewhat but remained statistically significant [adjusted OR for highest versus lowest quintile 0.56 (95% CI 0.34-0.90), $P_{\text{trend}} = 0.080$]. In contrast, a statistically significant but non-linear risk increase emerged for P-B12 [adjusted OR for highest versus lowest quintile 1.58 (95% CI 1.02-2.44), $P_{\text{trend}} = 0.539$].

None of the B-vitamin intake variables were statistically significantly associated with the risk of a first AMI (Paper IV, Table 5). However, the direction of risk estimates for folate and vitamin B12 intake were fairly consistent with the results for P-Folate and P-B12. The magnitude of the OR's for folate and B-vitamin intake were closer to one than those for P-Folate and P-B12, which likely reflects the less precise nature of dietary intake data, especially as assessed by FFQ. However, the lack of statistical significance may have been at least partly due to the lower number of study subjects with dietary intake data available.

The findings in Paper IV are generally consistent with a number of previous prospective studies of coronary endpoints suggesting an inverse association between circulating folate levels (summarized in Table 6), and especially folate intake^{197, 199, 203} and risk, and an unclear association between vitamin B12 and risk^{187, 192, 194, 197, 199-201, 204, 205}. The few previous studies that have adjusted for homocysteine status have, like the present study, found little effect on the risk

estimates for folate^{192, 200, 202}. This suggests a possible role for folate in coronary heart disease independent of homocysteine.

The apparent discrepancy between epidemiological studies and the clinical trials of B-vitamin therapy in CVD has caused much debate²⁶⁰. However, as noted in the introduction, the intervention studies were all secondary prevention studies with only a few years of follow-up²²⁵⁻²²⁹. Thus, the longer-term effects of a high folate status in the primary prevention of CVD remain to be determined. In the NORVIT trial, results were actually suggestive of an increased risk of the primary endpoint, which was a composite of recurrent AMI, stroke, and sudden death due to coronary artery disease, in the treatment group²²⁵. In the HOPE-2 trial, hospitalization for unstable angina was more common, and stroke less common, in the treatment group²²⁸.

The difference in findings between the epidemiological and intervention studies spawns the question, “Might there be a differential effect of folate in the initiation and progression of CVD?” as seems to be the case for cancer. Hypothetical mechanisms do exist. Folate might prevent homocysteine-induced damage to healthy endothelium⁷³ and/or help prevent hypertension^{206, 214-216}, but also promote the proliferation of endothelial cells in a growing plaque. Results suggesting that folate treatment may increase coronary restenosis after percutaneous intervention were speculated to be due to an involvement of folate in proliferation in the endothelium²²³. In a prospective study from Finland, an inverse association between dietary folate intake and acute coronary events was observed that was limited to subjects without previous coronary heart disease¹⁹⁹, which lends some support to this idea.

VITAMIN B12

Vitamin B12 was not associated with a reduced risk of any of the diseases studied in this thesis. On the contrary, P-B12 was strongly positively related to the risk of PCa, and for both ischemic stroke and AMI, results for vitamin B12 intake were somewhat suggestive of a positive association with risk. These findings were unexpected, given the similar roles of folate and vitamin B12 in one-carbon metabolism. Since dietary vitamin B12 is obtained essentially only from animal products, one might speculate that it may be a marker of meat, and thus also saturated fat, intake. Dietary intake and blood levels of vitamin B12 are poorly

correlated, due to conditions such as gastritis and loss of intrinsic factor that can reduce absorption, but in general, high biological levels reflect a high consumption of vitamin B12. However, despite the established association between a high intake of red and processed meats and an increased risk of CRC, P-B12 was not associated with risk in the CRC study (unpublished results). In the PCa study, the explanation for the association between P-B12 and risk may be an increased availability of methyl groups for tumor growth and progression, as proposed above, and in the CVD studies, the lack of an inverse association with risk may reflect the lesser role of vitamin B12 compared to folate in regulating homocysteine levels.

DIETARY ASSESSMENT

Dietary assessment in large epidemiological studies is generally performed by FFQ. Although other techniques, such as weighed food records, can provide a more accurate measure of dietary intake²⁶¹, FFQs are considerably more economical and less time consuming, and are often the only practical means in large studies. The FFQ completed by the majority of subjects in Papers IV and V in this thesis has been found to have a reliability and reproducibility similar to other FFQs²⁵⁴. Although folate and vitamin B12 were not considered in the validation study, dietary intake and plasma concentrations were correlated for both folate and vitamin B12 in Papers III and IV.

The effects of underreporting of dietary intake, a common problem in dietary assessment^{262, 263}, were minimized by adjustment of the B-vitamin intakes for total energy intake. Energy-adjustment also helps to reduce confounding by total energy intake or specific macronutrients²⁶⁴, which are often correlated to the intake of micronutrients. However, energy-adjustment does not take into account selective underreporting, of foods perceived as being unhealthy for example.

Although much research has focused on the potential of specific dietary components for use in the chemoprevention of chronic diseases, a number of associations well established in epidemiological studies have not been confirmed in randomized controlled trials, such as the role of dietary fiber in CRC⁴⁵⁻⁴⁹. Due to inherent limits on study feasibility, most trials have not been designed to test the effect of a long-term high exposure to a given dietary component in the primary prevention of disease. However, they have certainly led to an increased respect for

the complexity of dietary intake. As a consequence, there is a growing interest in studies of dietary patterns, such as the Mediterranean diet, and disease.

COHORTS AND REGISTRIES

The quality of the study base for this thesis was high for many reasons, including the virtually complete recording of cancer diagnoses in the national cancer registry and of stroke and AMI diagnoses in the MONICA registry, the high participation rates in the three cohorts of the NSHDS, the careful data collection and management in the individual cohorts, at the Northern Sweden Medical Biobank, and in the research working groups for each endpoint, and the Swedish personal numbers that allow linking of the NSHDS to the endpoint registries. Furthermore, the NSHDS cohorts have been established long enough to allow for many years of follow-up times between recruitment to a cohort and diagnosis. In the studies in this thesis, follow-up ranged from less than one year to 13 years.

SAMPLE SIZE AND POWER

For each endpoint, the papers in this thesis are the largest, or among the largest, prospective studies to date, despite the use of hard endpoints. However, sample size was still an issue. Particularly in the methylation study, for the analyses of hemorrhagic stroke (Paper III), and for the dietary intake analyses in the stroke and AMI studies (Papers III and IV), greater power would have been desirable. This was also the case for the analyses of subgroups, such as men and women.

In the cohorts of the NSHDS, with the exception of the MSP, participants were still relatively young. However, all the cohorts are population based, and the VIP, the largest cohort, has been found to be representative of the population²⁵². Given the size of the NSHDS, the numbers of cases in the studies in this thesis may seem rather low. This is due to the cumulative nature of the cohorts, with new participants recruited to the VIP and MSP every year, and every few years in the MONICA study.

HEREDITARY DISEASE

This thesis is a study of environmental influence on disease. However, each of the endpoints studied, CRC, PCa, stroke, and AMI, also has a genetic component. Hereditary cases were not excluded from any of the studies in this thesis and no attempt has been made to identify hereditary disease. Although both genetic and environmental factors can contribute to a person's risk of disease, in some cases, the genetic predisposition is strong enough to render virtually any environmental risk factor inconsequential. For such high-penetrance syndromes, for example HNPCC, any effect on results would likely be an attenuation of risk estimates. However, for mutations with low-penetrance, or for which a gene-environment interaction exists, inclusion in the risk analyses could, hypothetically, lead to a strengthening of associations between exposure and disease.

BIAS

In all of the studies in this thesis, strict criteria concerning previous disease were employed in the selection of the referents. This might result in a "super-healthy referent" bias. In this case, the referents would be characterized by an unusually healthy lifestyle and/or genetic makeup that could amplify observed protective associations between lifestyle/folate and risk. However, some null and positive associations were observed between folate status and risk, suggesting that this type of bias was not likely a major problem.

As noted in the Subjects and Methods section, several other potential sources of bias, including other types of selection bias and both recall bias and reverse causality were eliminated or reduced by the prospective, population-based study design. For example underreporting of dietary intake would be expected to be evenly distributed among cases and referents, since all were free from disease when they completed the FFQ, minimizing the risk of recall bias.

A single blood sample was analyzed for each study subject, which is a weakness of the studies in this thesis. However, given the prospective cohort design and the matching of cases and referents, systematic variation between cases and referents seems unlikely. Any effect of variation in plasma analytes on results would therefore most likely be an attenuation of associations between exposure and

disease. Sample storage time was also accounted for, since referents were matched to cases by date of sample donation.

Although undiagnosed disease in cases at baseline cannot be excluded, limiting analyses to cases with diagnosis at least one year after baseline had little effect on the results. Undiagnosed disease in referents at baseline may also have been present, but this would be expected to dilute rather than strengthen any associations between exposure and risk of disease.

P-values and confidence intervals were not corrected for multiple testing. When the cut-off for statistical significance is set at 0.05, five spurious results can be expected for every 100 statistical tests performed. This cannot be considered negligible in epidemiological studies in which every risk estimate calculated is essentially a separate statistical test. However, clear linear or dose-response trends are less likely to be due to chance.

CONFOUNDING

Sex and age were adjusted for by matching cases to referents. Only in the CRC study (Paper I) were subjects matched for fasting status, but limiting the risk analyses to subjects who had fasted for at least 8 hours prior to blood sampling, or adjusting for fasting status in the risk analyses, had little effect on results.

A number of potential confounders were included in the multivariate analyses. Several other covariates were tested in multivariate analyses but not included in the final model, generally because they were not associated with risk or had no effect on the magnitude of the risk estimates for the variable of interest. Some covariates were included even if they were not associated with risk and did not affect risk estimates, but only if there was a strong theoretical reason, for example, a traditional risk factor such as cholesterol in stroke. Data concerning some other potentially relevant factors, such as the use of aspirin and other NSAIDs, were not available for inclusion in the analyses.

CONCLUSIONS

- In this study, prospective plasma folate concentrations were associated with the risk of developing colorectal cancer in a bell-shaped manner. A reduced risk was also observed for the MTHFR 677C>T polymorphism, independent of folate status. For the MTHFR 1298A>C polymorphism, a positive association with risk was found that may have been largely due to linkage disequilibrium with the 677C>T polymorphism. Homocysteine status was not related to the risk of colorectal cancer.
- Prospective plasma concentrations of folate were positively associated with promoter hypermethylation of the tumor suppressor genes p16 and hMLH1 in colorectal cancer tissue. For vitamin B12, homocysteine, and the MTHFR 677C>T and 1298A>C polymorphisms, associations with promoter hypermethylation were null.
- Increasing plasma vitamin B12 concentrations were associated with an almost three times higher risk of prostate cancer. Tendencies toward a positive risk relationship for plasma folate concentrations and an inverse risk relationship for plasma homocysteine concentrations were also observed, but only in univariate analyses.
- Plasma folate concentrations and dietary intake of folate were associated with a reduced risk of hemorrhagic but not ischemic stroke. The risk estimates were modestly attenuated by adjustment for homocysteine status. Neither plasma concentrations nor dietary intake of vitamin B12 was associated with either type of stroke.
- Increasing plasma folate concentrations were associated with a 50% reduced risk of developing an AMI, independent of homocysteine status. No clear risk relationship was apparent for plasma vitamin B12 concentrations. None of the dietary B-vitamin intakes studied were significantly associated with the risk of AMI, although the risk estimates for folate were in line with those for the plasma concentrations.

IMPLICATIONS

The results of this thesis, which was based on a population with low folate status and no mandatory folic acid fortification, suggest that although folate may reduce the risk of cardiovascular disease, its role in cancer appears to be more complicated. The possibility of a detrimental component to the role of folate and vitamin B12 in cancer development may have implications on the current debate concerning mandatory fortification of foods with folate.

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