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2010

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*Citation for published version (APA):*

Ericson, U. (2010). *Folate - Associations with breast cancer depending on intake, metabolism, genetic variation and estrogen receptor status*. [Doctoral Thesis (compilation), Department of Clinical Sciences, Malmö]. Nutrition Epidemiology Research Group.

*Total number of authors:*

1

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# Folate

Associations with breast cancer depending on intake, metabolism, genetic variation and estrogen receptor status

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Printed by Media-Tryck, Lund, Sweden, 2010  
ISBN 978-91-86443-52-8

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# Folate

Associations with breast cancer depending on intake,  
metabolism, genetic variation and estrogen receptor status

**Ulrika Ericson**



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## **Akademisk avhandling**

som, med vederbörligt tillstånd av medicinska fakulteten vid Lunds universitet, för avläggande av doktorsexamen i medicinsk vetenskap kommer att offentligen försvaras i aulan på Clinical Research Center, ingång 72, Universitetssjukhuset MAS, Malmö, lördagen den 10 april 2010 kl. 09.30

## **Fakultetsopponent**

Professor Alicja Wolk, Karolinska Institutet, Stockholm, Sweden

# SUMMARY

Folate is a B-vitamin that may influence cancer development via its role as methyl donor for DNA synthesis and methylation.

Plant foods contain many bioactive compounds including folate and fiber. Results from the Malmö Diet and Cancer (MDC) cohort indicate lower breast cancer risk at high fiber intake. Folate intake may partly explain this association.

Aims of this thesis were to examine if intakes or plasma concentrations of folate are associated with postmenopausal breast cancer risk, and if associations depend on genetic variation of the folate metabolizing enzyme methylenetetrahydrofolate reductase (MTHFR) or estrogen receptor  $\alpha$  and  $\beta$  expression of tumors.

Food habit information and blood samples were collected 1991-96 from 17 035 women between 45 and 73 years in the MDC study. Until end of 2004, 544 cases of invasive breast cancer were diagnosed.

High folate intake was associated with lower breast cancer risk. The MTHFR 677T allele was associated with increased risk. Among women with this allele, the risk increased with higher intakes and plasma concentrations of folate, and was especially pronounced for folate supplement consumers. Estrogen receptor  $\beta$  negative (ER $\beta$ -) breast cancer increased with higher plasma folate concentrations independently of 677C>T genotype.

The results encourage consumption of folate rich foods. However, since women with disturbed folate metabolism due to genetic predisposition may respond differently to high intakes, and high folate status may promote development of ER $\beta$ - tumors, folate supplements should be cautiously recommended. The results also contribute knowledge to current discussions regarding mandatory folate fortification of foods.

# SAMMANFATTNING PÅ SVENSKA

Folat är ett B-vitamin som kan bidra till minskad risk för en rad sjukdomar, däribland en speciell form av blodbrist, ryggmärgsbråck hos nyfödda, hjärt-kärlsjukdom och vissa cancerformer.

Folat förekommer framför allt i många vegetabiliska livsmedel. Speciellt höga halter har gröna bladgrönsaker, baljväxter (t.ex. kikärter) och lever. Betydande folatkällor i svensk kost är också frukt och grovt bröd.

Dessutom innehåller många kosttillskott den syntetiska formen av folat, som kallas folsyra. Kost rik på frukt, grönsaker och fullkornsprodukter har kopplats till bättre hälsa. Det är dock oklart vilka kostkomponenter som skyddar mot, eller har störst betydelse för utveckling av olika sjukdomar. Kost med stor andel vegetabilier är rik på vitaminer, mineraler och andra bioaktiva ämnen som på olika sätt samverkar med varandra. Det kan därför vara svårt att avgöra vilken roll ett speciellt näringsämne har.

Man uppskattar att var tionde kvinna i Sverige kommer att få bröstcancer under sin livstid. Teoretiskt sett skulle folat kunna påverka insjuknande i bröstcancer. Folat verkar genom att överföra kolfragment till olika kemiska reaktioner i kroppen. Folat har därigenom en viktig roll vid cellbildning, eftersom det kan påverka uppbyggnaden av intakt DNA. Dessutom kan tillgången på folat påverka hur information i våra gener översätts till olika cellstrukturer som kan stimulera respektive hämma tumörutveckling (t.ex. tumorsuppressorer). Studier som undersökt samband mellan intag respektive blodnivåer av folat och bröstcancer är dock inte samstämmiga.

I undersökningen Malmö Kost Cancer har man sett att kvinnor med högt fiberintag har lägre risk för bröstcancer. Eftersom kostfibrer kommer från vegetabiliska livsmedel, skulle detta samband delvis kunna bero på andra verksamma ämnen i vegetabilier, t.ex. folat. Syftet med denna avhandling har varit att bland kvinnor som deltog i Malmö Kost Cancer undersöka sambandet mellan kostens innehåll av folat och risken att insjukna i bröstcancer efter klimakteriet.

I Malmö Kost Cancer samlade man i början på 1990-talet in information om 28 098 malmöbors matvanor och livsstil. Dessutom samlade man in blodprover samt mätte och vägde deltagarna. Fyrtio procent av alla

malmöbor mellan 45 och 73 år deltog i studien. Totalt deltog 17 035 kvinnor. Det är få studier i världen som samlat in så detaljerad information om matvanor. Deltagarna fick föra dagbok över lagade måltider och sin konsumtion av vitamintabletter under en vecka, samt fylla i omfattande formulär om sina matvanor. Därefter fick de svara på frågor om sina matvanor i en 45 minuter lång intervju. Vi följde kvinnorna till slutet av 2004, då 544 hade insjuknat i bröstcancer.

Förutsättningarna för att undersöka sambandet mellan folatintag och bröstcancer bland kvinnor som deltog i Malmö Kost Cancer är mycket goda. Först och främst är informationen om kvinnornas folatintag från mat av hög kvalitet och det finns även information om folatintag från vitamintabletter samt om intag av andra B-vitaminer som kan påverka hur folat utnyttjas i kroppen. Dessutom är de insamlade blodproverna värdefulla som komplement till uppgifterna om matvanor, då det är svårt att i detalj lämna uppgifter om kostvanor. Folatnivån i blod är en mer objektiv markör, även om den inte exakt speglar folatintaget. En annan fördel är att det funnits möjlighet att analysera DNA. I stället för att bara undersöka sjukdomsrisk hos personer som får i sig olika mycket folat från kosten, kan man studera sjukdomsrisk hos personer med förändringar i generna som påverkar hur folat utnyttjas i kroppen. På så sätt kommer man ifrån problem med att de som äter mycket frukt och grönsaker också får i sig andra ämnen, som kan ha betydelse för cancerutveckling och göra det svårt att avgöra om folat i sig har någon effekt. Det finns genetiska varianter av det folatomvandlande enzymet metylentetrahydrofolatreduktas (MTHFR), som är kopplade till nedsatt enzymfunktion. Dessa genetiska varianter kan därmed påverka hur folat från kosten kan användas i kroppen. Folatintagets betydelse för bröstcanceruppkomst skulle därför kunna skilja sig mellan kvinnor med eller utan de genetiska varianterna. Slutligen finns det många olika typer av bröstcancer, vars uppkomst kan påverkas av olika kostfaktorer. Ett exempel är bröstcancer med höga eller låga halter av receptorer som tar emot signaler från det kvinnliga könshormonet östrogen. Det finns två typer av östrogenreceptorer, alfa och beta. Tumörer med höga halter av alfareceptorn stimuleras av östrogen och behandlas därför med ämnen som konkurrerar med östrogen om att binda till alfareceptorn. Betareceptorn är mindre utforskad, men verkar vanlig i normala bröstceller, medan förekomsten är lägre i tumörer. Betareceptorn har föreslagits verka som en tumörsuppressor och därmed hämma tumörutveckling. Vi hade information om förekomsten av dessa receptorer i brösttumörer och kunde

därmed undersöka om folat var starkare kopplat till någon av dessa tumörtyper.

Vi fann att de kvinnor vars intag av folat motsvarade det rekommenderade intaget i Sverige endast hade hälften så stor risk att drabbas av bröstcancer jämfört med dem som hade lägst intag av folat, men vars intag av andra B-vitaminer och fibrer inte skilde sig.

Över hälften av kvinnorna var bärare av den genetiska varianten MTHFR 677T, som påverkar hur folat kan användas i kroppen. Dessa kvinnor hade ökad risk för bröstcancer. De kvinnor som hade den genetiska varianten, och samtidigt hade en folatrik kost eller höga folatnivåer i blodet, löpte ännu större risk att drabbas av bröstcancer. Denna riskökning var speciellt uttalad i samband med intag av vitamintabletter med folsyra.

Höga blodnivåer av folat var också kopplade till ökad risk för brösttumörer med låg koncentration av östrogenreceptor beta. Detta samband sågs hos alla kvinnor och verkade inte påverkas av de undersökta varianterna i MTHFR-genen. Det är möjligt att höga folatnivåer medverkar till att stänga av genen som kodar för östrogenreceptor beta. Färre receptorer tar då emot signaler som kanske skulle kunna bromsa tumörutvecklingen.

Sammanfattningsvis stödjer resultaten från denna avhandling rekommendationer om folatrik kost med mycket frukt, grönsaker, baljväxter och fullkornsprodukter. Folat verkar dock ha en komplicerad roll i samband med bröstcancerutveckling. På grund av ärftlighet kan till exempel vissa individer ha försämrad förmåga att på bästa sätt utnyttja stora mängder folat. Dessutom verkar höga blodnivåer vara kopplade till ökad risk att utveckla brösttumörer med låg förekomst av östrogenreceptor beta. Dessa resultat kan bidra som underlag i den diskussion som pågår i många länder angående obligatorisk folatberikning av livsmedel. I nuvarande läge bör högre doser av folat, i form av vitamintabletter, bara rekommenderas till grupper eller individer med ökat folatbehov.





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<b>ORIGINAL PAPERS</b>	
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# LIST OF PAPERS

This thesis is based on the following original papers, which in the text will be referred to by their roman numerals.

- I. Ericson, U., Sonestedt, E., Gullberg, B., Olsson, H., and Wirfalt, E. High folate intake is associated with lower breast cancer incidence in postmenopausal women in the Malmo Diet and Cancer cohort. *Am J Clin Nutr*, 86: 434-43, 2007.
- II. Ericson, U., Sonestedt, E., Ivarsson, M. I., Gullberg, B., Carlson, J., Olsson, H., and Wirfalt, E. Folate intake, methylenetetrahydrofolate reductase polymorphisms, and breast cancer risk in women from the Malmo Diet and Cancer cohort. *Cancer Epidemiol Biomarkers Prev*, 18: 1101-10, 2009.
- III. Ericson, U. C., Ivarsson, M. I., Sonestedt, E., Gullberg, B., Carlson, J., Olsson, H., and Wirfalt, E. Increased breast cancer risk at high plasma folate concentrations among women with the MTHFR 677T allele. *Am J Clin Nutr*, 90: 1380-9, 2009.
- IV. Ericson U, Borgquist S, Ivarsson MI, Sonestedt E, Gullberg B, Carlson J, Olsson H, Jirström K, and Wirfalt E. High plasma folate concentrations are associated with increased risk of oestrogen receptor  $\beta$  negative breast cancer in a Swedish nested case control study. Submitted.

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# ABBREVIATIONS

CI	Confidence interval
CS	Cystathionine $\beta$ -synthase
CV	Coefficient of variation
DFE	Dietary folate equivalents
DHF	Dihydrofolate
EPIC	European Prospective Investigation into Cancer and Nutrition
ER $\alpha$ -	Estrogen receptor alpha negative
ER $\alpha$ +	Estrogen receptor alpha positive
ER $\beta$ -	Estrogen receptor beta negative
ER $\beta$ +	Estrogen receptor beta positive
HR	Hazard ratio
IARC	International Agency for Research on Cancer
ICC	Intraclass correlation coefficient
MDC	Malmö Diet and Cancer
MHT	Menopausal hormone therapy
MS	Methionine synthase
MTHFR	Methylenetetrahydrofolate reductase
OR	Odds ratio
PGA	Pteroylmonoglutamic acid
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SHMT	Serinehydroxymethyl transferase
SNP	Single nucleotide polymorphism
THF	Tetrahydrofolate



# INTRODUCTION

Breast cancer is the most common cancer in women world-wide and it is estimated that 10 % of all Swedish women will develop breast cancer during their lifetime. Folate is a B-vitamin, abundant in green leafy vegetables and legumes, which may affect breast cancer incidence via its influence on synthesis and methylation of DNA.

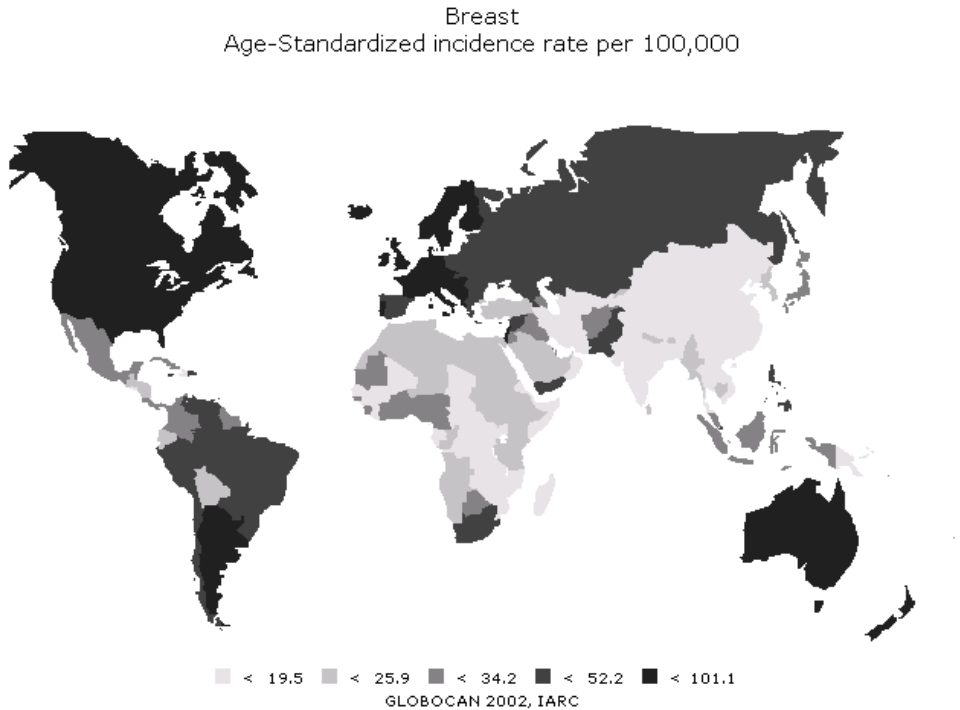
There are several reasons for investigating the relation between folate and breast cancer in women from the Malmö Diet and Cancer (MDC) study: An association between dietary fiber and breast cancer has previously been observed in the MDC cohort, but folate and other compounds co-existing with fiber in plant foods may partly account for this protective association. Detailed information on dietary habits was collected in the MDC study, as well as extensive information about other lifestyle factors and anthropometric measurements, that may confound associations between diet and disease. Blood samples are stored, which will allow the use of plasma folate as a more objective marker of folate intake. DNA is available, which allows genotyping of polymorphisms that via effects on folate metabolism may influence breast cancer development. Since estrogen receptor status of tumors has been analyzed, it is also possible to explore if there are subgroups of women, characterized by receptor status, to whom folate status seem to be of greater importance. Finally, the high breast cancer incidence in Malmö compared with the national incidence, makes the population especially suitable for this study.



# BACKGROUND

Diet is involved in the etiology of cancer in 10 to 70 percent of all cases, according to an estimate by Doll and Peto in 1981 [1]. More precise estimates of dietary involvement in cancer development are complicated, and the importance of specific dietary components is difficult to approximate. We consume foods in different contexts, mixings and preparations, and nutrients interact with each other. In addition heredity may affect the impact of dietary influence. Subsequently, when examining a nutrient in relation to risk of cancer and other chronic diseases, it is necessary to consider other nutritional aspects and life-style factors, as well as genetic predisposition.

Since Doll and Peto made their statement, great efforts have been made in the field of nutritional epidemiology, to further investigate both protective and promoting aspects of diet in cancer development. Biological hypotheses about beneficial effects of constituents in fruits and vegetables are manifold. However, epidemiological evidence is still scarce. Some studies have found inverse associations between total fruit and vegetable consumption and cancer at different sites, but most have not been able to detect any associations. Regarding breast cancer, most case-control studies show inverse associations with fruit and vegetable intake [2], while cohort studies do not [3]. Since the content of important bioactive components vary between different plant foods, aggregations may mask protective effects of specific fruits, vegetables or constituents. The high content of folate in many plant foods, such as green leafy vegetables and legumes, may be of particular importance in the development cancer at different sites, including breast cancer.



**Figure 1.** *Worldwide breast cancer incidence*

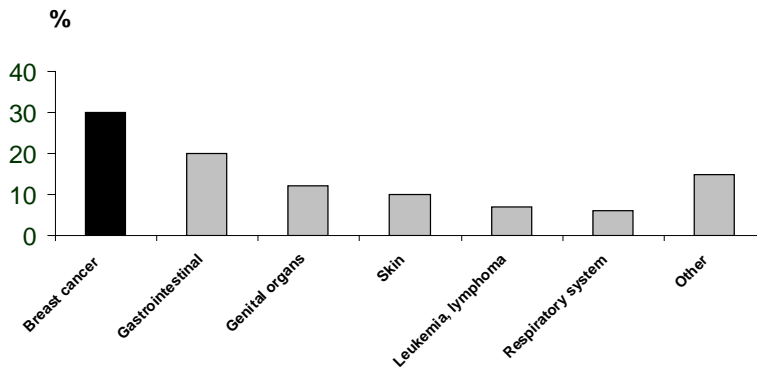
## Breast cancer

### Incidence and mortality

Breast cancer is the most common cancer in women world-wide, and is especially frequent in industrialized areas such as Europe and north America [4]. The highest increase is seen in developing countries, probably reflecting a transition to westernized lifestyle patterns with regard to diet, reproduction and hormone therapy [5]. Ecological studies indicate that environmental factors, rather than genetic, better explain differences between countries: Next-generation Asian immigrants in the USA have higher breast cancer risk than first generation immigrants [6] and similar observations has been made among Polish and Italian immigrants in Australia [7, 8].

In Sweden, one third of all cancers diagnosed in women are breast cancers (**Figure 2**), and it is expected that ten percent of all Swedish women will get breast cancer during their life-time. Breast cancer incidence is higher in urban areas than in rural areas, and higher in Malmö compared with the national incidence [9]. In 2007, 7049 new cases were diagnosed in Sweden. The incidence has increased from below 100 cases per 100 000 inhabitants in the early seventies to over 150 cases today. In contrast, the mortality has decreased, due to early detection and better treatment, and the prognosis is relatively good (The 10-year survival is 79 percent)[10].

**Figure 2.** *Frequent cancers in Swedish women*  
(% of all cancers diagnosed in Swedish women)



Source: National Board of Health and Welfare

## Carcinogenesis

Normal cell proliferation is thoroughly controlled. Carcinogenesis is a multistep process in which normal cells are transformed into malignant cells. This process includes DNA changes that lead to loss of normal cell growth characteristics including: programmed apoptosis (cell death), sensitivity to growth inhibitory signals, dependence on exogenous growth signals, limited cell division, controlled blood vessel growth and ability to invade or metastasize. Together these changes lead to uncontrolled cell growth [11].

DNA changes involved in carcinogenesis affect the expression of specific genes. Proto-oncogenes are genes that contribute to normal cell growth, but the mutant variants of these genes, called oncogenes, stimulate uncontrolled cellular replication. Tumor-suppressor genes regulate cell growth in both normal cells and cancer cells, and mutations in tumor-suppressor genes result in loss of growth inhibitory mechanisms. DNA repair genes are involved in the repair of DNA damage. Mutations in these genes lead to accumulation of mutations. In addition there are epigenetic changes, which affect gene expression without altering DNA sequence, for example via DNA methylation [12].

## **Breast cancer characteristics**

Breast cancer is a heterogeneous disease. The tumors are described according to different characteristics of importance to prognosis and treatment. Tumor type indicates the origin of the tumor. Breast tumors most often develops in the milk ducts, but can also originate from the mammary glands or connective tissue [13, 14]. Tumor stage indicates size, infiltration of the lymph system and prevalence of metastases in other tissues [11]. The Swedish cancer registry defines invasive cancer as all cancer, excluding in situ cancer. In situ cancers may not infiltrate outside the basal membrane of the original site, and may therefore not reach the lymph or metastasize. Since in situ cancer does not necessarily progress into invasive cancer [15], inclusion of in situ cancer may obscure true associations between diet and serious disease. In the MDC-cohort 92 % of all breast cancer cases, diagnosed until the end of 2003, had invasive breast cancer. Tumors are also described according to histology and molecular characteristics (e.g. expression of hormone receptors).

## **Risk factors for breast cancer**

### **Age**

Breast cancer incidence increases with age in premenopausal women, but the increase reaches a plateau around menopause. The incidence increases with age also in postmenopausal women, but the biological mechanisms behind this association is probably different from the ones behind the age related increase in premenopausal women [16].

## **Ionizing radiation**

Studies of survivors from the atomic bombs in Hiroshima and Nagasaki have shown that high-dose ionizing radiation is a risk factor for breast cancer. This has also been shown when ionizing radiation has been used in disease treatment, for example of Hodgkin's disease. The low-dose radiation used in mammography screening is however not known to considerably counteract its benefits [17].

## **Hormones**

High levels of sex hormones increase the proliferative activity in mammary epithelial cells and make them more susceptible to carcinogens. This mechanism explains the associations between reproductive events and breast cancer observed in epidemiological studies [16, 18]. Low age at menarche, high age at menopause, high age at first birth, nulliparity, and short cumulative duration of breastfeeding may contribute to an increased number of menstrual cycles and thereby increased life-time exposure of endogenous sex hormones. Exogenous hormones are also of importance in breast cancer etiology. Menopausal hormone therapy (MHT), previously referred to as HRT (Hormone replacement therapy), is a risk factor, and usage of both estrogen and progestin are associated with increased risk [19]. Recent use of oral contraceptives is also related to an increased risk of breast cancer [16].

## **Obesity**

As circulating ovarian estrogen levels decrease at menopause, the mammary glandular tissue gradually regress [20]. Simultaneously, mammary adipose tissue increase [21]. Consequently, local estrogen synthesis in mammary adipose tissue becomes more important in breast cancer etiology after menopause [22]. This probably explains why obesity is associated with an increased breast cancer risk only in postmenopausal women [23]. BMI, weight, weight gain and height are all risk factors for postmenopausal breast cancer [24].

High insulin concentrations may also be responsible for the increased risk of breast cancer in overweight women, because insulin levels seem to be positively related to mammary epithelial hyperplasia [25].

## **Physical activity**

Epidemiological studies indicate an association between physical activity and breast cancer, especially in postmenopausal women [26]. Physical activity is related to anthropometric data, but it may also reduce breast cancer via more direct effects on hormone levels [16].

## **Smoking**

The epidemiological evidence from studies on smoking and breast cancer is inconsistent [27], but ex-smokers were at higher risk of breast cancer in the Malmö Preventive Project [28].

## **Alcohol**

Alcohol consumption is associated with increased breast cancer risk in most epidemiological studies [29, 30]. Possible mechanisms behind this observation are increased levels of estrogens, direct oxidative damage, DNA damage by the mutagen ethanol metabolite acetaldehyde, but also effects on one carbon metabolism via detrimental influence on folate bioavailability [29].

## **Fat**

Fat intake may also influence sex hormone levels. Studies indicate that total fat intake may be associated with an increased risk of breast cancer, and that intakes of different fatty acids may be of importance [31-34]. The World Cancer Research Fund has concluded that the evidence is limited and only suggestive [35].

## **Components of plants foods**

Other dietary components that may affect breast cancer risk are antioxidants in fruit and vegetables (e.g.  $\beta$ -carotene, vitamin C and vitamin E), but convincing evidence is still missing. Dietary fiber is another component in plant that may have protective effects, via decreased circulating estrogen or weight reduction. Although most prospective studies have not observed overall associations between dietary fiber and breast cancer [36-39], inverse associations between fiber intake and breast cancer incidence has been observed in the MDC study [40, 41]. Plant foods also contain other bioactive components, which are not defined as essential nutrients. Phytoestrogens are substances in plant foods that due to estrogenic effects may reduce breast cancer risk. Phytoestrogens are often mentioned as possible cancer protective agents in “healthy diets” [42].

## Heredity

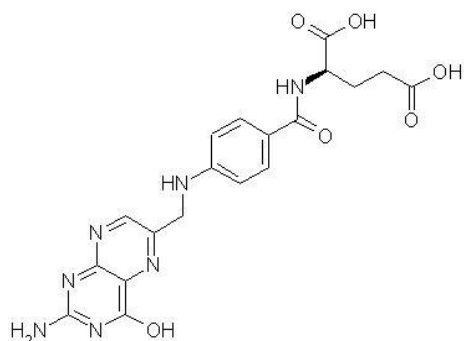
Between 10 and 15 percent of all breast cancer cases have a familial history of breast cancer, and the hereditary cases are more often linked to mutations in the *BRCA1* and *BRCA2* tumor suppressor genes. Both family history and *BRCA1/BRCA2* mutations are, in particular, strong risk factors in younger women [43]. Other identified breast cancer risk genes are *TP53*, *PTEN*, *STK11*, *CDH1* and *CHEK2*. In addition several genes has been identified that may act as low- penetrance genes for breast cancer susceptibility [44]. They influence an individual's risk to a lesser degree per se, but may be of great importance, because of interactions with lifestyle and environmental factors. In addition they have relatively high frequency of genetic variation.

## Folate

The name originates from folium (=leaf), because folate was first isolated from spinach leaves in 1941. However, already in 1931 Lucy Wills found that anemic pregnant Indian women could be treated with yeast extract. The active component was later identified as folate.

## Chemical structure

Folates are compounds consisting of a pteridine ring, p-aminobenzoic acid and glutamate units (**Figure 3**). The synthetic form, folic acid, is a monoglutamate (Pteroylmonoglutamicacid, PGA), while foods mainly contain polyglutamates. Bioactive folates, dihydrofolates (DHF) and tetrahydrofolates (THF), are reduced forms of folic acid with additional glutamate units, dihydropteroylglutamate ( $H_2PteGlu_n$ ) or tetrahydropteroylglutamate ( $H_4PteGlu_n$ ). The structure of folates vary depending on the oxidation state of the pteridine ring, one-carbon substituents at different positions and the number of glutamate residues[45] [46].



**Figure 3.** *Chemical structure of folic acid*

## Folate content in foods

Folate is mainly found in plant foods, but also at lower concentrations in foods of animal origin. Particularly good sources are green leafy vegetables, legumes and liver [47]. Two thirds of the intake in Sweden comes from fruits, vegetables and cereals. Due to the high consumption of dairy products, they are also important folate sources in Swedish diet [48].

## Recommendations of folate intake

Folate intake of 50-100 µg prevents folate deficiency anemia. Because of uncertain values in food tables, it is difficult to assess absolute folate intake in different populations. The mean dietary intake among Swedish women is however estimated to be 217 µg /day [48]. Since knowledge about folate bioavailability in foods is unsatisfying, intake recommendations are also uncertain. In the Nordic Nutrition Recommendations, NNR 2004, the recommended folate intake is 300 µg /day. Since adequate folate supply before conception until 12 weeks after conception, reduces the risk of neural tube defects in infants, the daily recommended intake for women of fertile age is set to 400 µg [49].

## Absorption, distribution and excretion of folate

Folate is primarily absorbed in the small intestine, in the jejunum, by active transport with specific receptors and folate carriers. However, it has recently been shown that folate is also absorbed in the colon [50].



Considering the long passage time through the colon, this may be of importance to consumed folate as well as bacterially produced folate in the colon [51].

After absorption, folate is transported to the liver or to other tissues. Folate is stored in the liver for a few months, and can be recycled in the enterohepatic circulation [52]. Folate is excreted unmetabolized or as catabolites with urine and faeces [53].

## **Folate bioavailability**

The bioavailability of folate is above all considered to depend on the intestinal absorption, but also on tissue uptake, enterohepatic circulation as well as urinary excretion rate. The absorption is mainly influenced by folate stability, deconjugation of glutamates and folate entrapment in food matrixes [54]. Food folates are unstable and very sensitive to heat, light, oxygen and leaching. They are easily destroyed during food preparation, but also in the intestinal tract. Other dietary components (e.g. ascorbic acid) may enhance folate stability [55]. Food folates need to be hydrolyzed to monoglutamates by brush border enzymes, before absorption in the upper part of the small intestine. Other compounds may influence the deconjugation of glutamates [56]. It is however not clear whether the deconjugation has any substantial influence on food folate absorption [54]. The food matrix may also influence the degree of absorption, because the structure and composition could make folate less accessible [56]. The availability of folate is enhanced by disruption of the food matrix [57]. The bioavailability of food folates is generally approximated to 50%, but results from different studies show great variation [56]. In addition recent observations have indicated smaller differences between food folates and folic acid [58]. Dietary folate equivalents (DFE) are calculated based on the assumption that the bioavailability of synthetic folic acid consumed in a meal is 1.7 times higher than that of food folate [59].

## **Biomarkers of folate intake**

The reference value for normal plasma folate is  $>6.8\text{nmol/L}$ . Plasma folate is a biomarker for dietary folate intake and an objective estimate of exposure. It is considered to be a good marker of folate status in large

epidemiological studies, although folate in red blood cells is less influenced by recent folate intake [60]. Folate deficiency has been associated with decreased plasma folate already after 1-3 weeks. In red blood cells a decrease has been observed first after 17 weeks of deficiency [61]. Folate status in red blood cells is also more informative about different folate vitamers [62], because in plasma folate is almost exclusively appearing as 5-methyl THF [63]. Blood levels of homocysteine are inversely related to folate levels.

Most epidemiological studies are only able to collect blood samples at a single occasion. Consequently it is valuable to examine the reliability of using one sample to predict individual mean concentration of a biomarker in a study cohort. The reliability can be estimated by the intraclass correlation coefficient (ICC), defined as the ratio of the between-person and total variability. Use of biomarkers with low ICC often result in attenuation of the relation between exposure and disease [64].

## **Folate and disease**

Since folate is necessary for normal cell division, the vitamin is especially important during growth periods and to rapidly dividing cells such as blood cells and tumor cells. Folate deficiency may lead to megaloblastic anemia, characterized by large erythrocytes. Folate requirement is increased during pregnancy, and low intakes have been associated with several adverse birth outcomes. The association between folic acid supplementation before conception and reduced occurrence of neural tube defects is often considered to be one of the most important findings relating vitamins to disease[65] [66]. The mechanism behind this finding is however unknown, but it probably concerns DNA methylation. Recently it was suggested that changes in methylation of the insulin-like growth factor gene are involved. [67]. Folate may also protect against cardiovascular disease [68, 69]. In addition, it has been related to several other disorders including Alzheimer's disease [70] and bone fractures [71]. Concerning its potential value in cancer development, research has mainly focused on colorectal cancer [72], but studies has also suggested associations with cancers at other sites including cervix, breast, prostate, esophageal and pancreas[73-76].

## **Folate metabolism and cancer**

Folate intake and intake of dietary one-carbon sources (e.g. serine, methionine, choline and betaine) may influence carcinogenesis through their involvement in DNA metabolism [77]. Folate is a coenzyme, that carries one carbon units and is thereby of great importance in DNA metabolism [78] (**Figure 4**).

### **DNA synthesis and repair**

5,10-methylene THF acts as a methyl donor when the pyrimidine thymine is synthesized from uracil. Folate deficiency may lead to changes in DNA, such as misincorporation of uracil, subsequent chromosome breaks and disruption of DNA repair. Unrepaired double-strand breaks enhance cellular transformation, and may contribute to an increased risk of cancer [79].

5,10-methylene THF can also be converted to 10-formyl THF, and thereby act as a methyl donor in purine synthesis [80].

### **DNA-methylation**

5,10-methylene THF is also converted to 5-methyl THF (the main form of folate in blood). 5-methyl THF functions as a methyl donor when homocysteine is converted into methionine. Diminishing levels of methionine lead to decreased formation of S-adenosylmethionine (SAM), the most important methyl donor in biological reactions including DNA methylation [77]. Changes in DNA-methylation patterns are early events in carcinogenesis and may influence the expression of proto-oncogenes and tumor suppressor genes [81, 82].

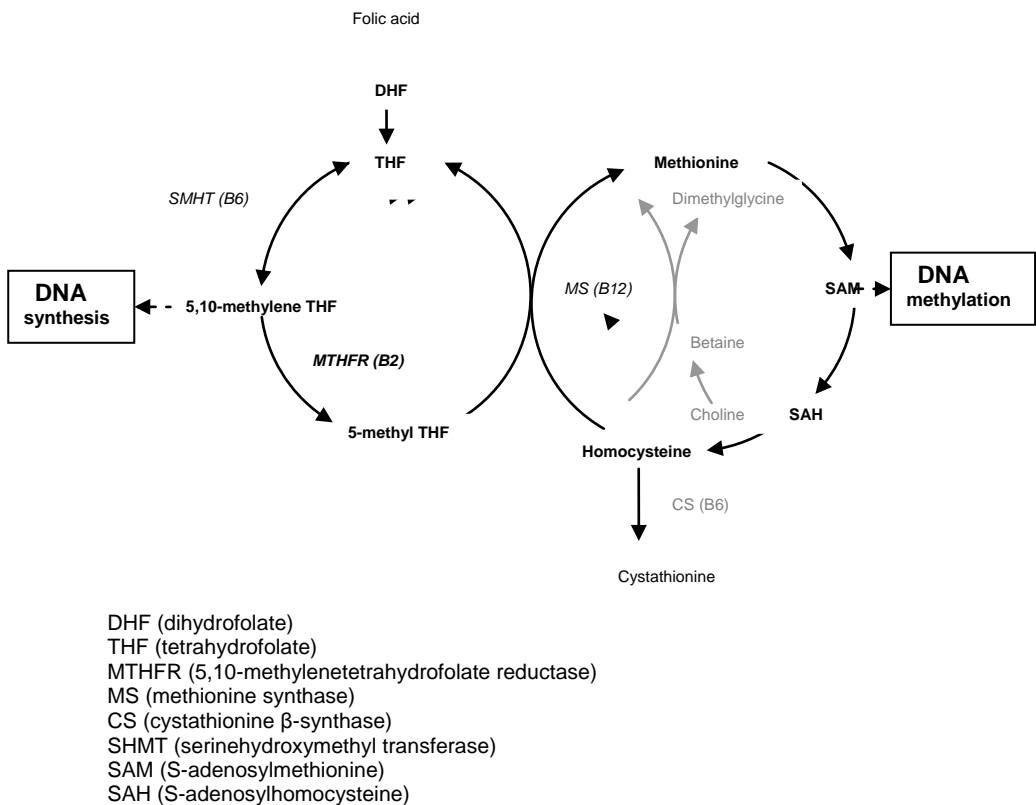
Changes in DNA-methylation patterns involve global genomic hypomethylation, but the changes can also be site specific, such as hypermethylation at cytosine-guanine rich areas (CpG islands) in promoter regions of tumor suppressor genes, causing silencing of these genes [83]. Methyl deficiency may increase the activity of DNA methyltransferase, and the enzyme may facilitate methylation of CpG sites [84-87].

Disturbed DNA methylation may also involve hypomethylation of non-coding DNA sequences. Hypomethylation may activate these regions and

lead to inappropriate recombination, with subsequent spread of the normally non-coding DNA and chromosomal instability [80].

Since SAM is a universal methyl donor, another possible mechanism for the involvement of folate in carcinogenesis, is its effect on non-inherited methylation of for example proteins and lipids [80].

**Figure 4.** *A Simplified scheme of folate metabolism*



## The dual role of folate in carcinogenesis

Depending on site, timing and dose, folate seems to have differing effects [88, 89]. It is possible that folate intake inhibits the initiation of cancer. On the other hand, folate seems to stimulate the progression and growth of already existing pre-stages of cancer [90]. Drugs that interfere with folate

are therefore often used in cancer treatment. The antifolate methotrexate is for example used to treat cancer and other diseases with rapidly dividing cells, because it inhibits the conversion of dihydrofolate into tetrahydrofolate and thereby DNA synthesis.

## **MTHFR polymorphisms, folate metabolism and cancer**

Single nucleotide polymorphisms (SNPs) are variants in a single base pair that occur with a frequency of above 1% in a population [91]. SNPs in genes encoding for enzymes in folate metabolism may alter relations between folate and carcinogenesis. Methylenetetrahydrofolate reductase (MTHFR) is an enzyme that catalyses the irreversible conversion of 5,10-methylene THF to 5-methyl THF. The MTHFR gene is located at chromosome 1, on the short arm at position 36.3 (1p36.3). Particularly two polymorphisms are associated with changes in the activity of MTHFR. MTHFR 677C>T results in substitution of alanine by valine at position 222 in the amino acid sequence. 1298A>C results in substitution of glutamate by alanine at position 429 in the amino acid sequence. The two polymorphisms are in total linkage disequilibrium, i.e., variants never occur on the same chromosome. The variants have been related to reduced enzyme activity, and subsequent decrease in the conversion of 5,10-methylene THF to 5-methyl THF. Thereby methyl groups for DNA methylation are directed towards DNA synthesis/repair [92]. The heterozygote 677 CT-variant has about 65 % of the enzyme activity of the homozygote wild type, and the homozygote 677 TT may be reduced to only about 30% of the activity of the homozygous 677 CC wild-type [93-95]. This variant has been connected to lower levels of plasma folate [96]. The 1298 C allele has also been associated with reduced enzyme MTHFR activity [97]. In addition to genetic influence of the MTHFR activity, concentrations of different metabolites in the folate cycles affect the activity [62, 98, 99]. The frequency of 677 TT homozygotes in Nordic populations is 5-8% [100]. This is somewhat lower than in other European populations [101, 102]. The frequency of the TT-genotype is about 10% among European Whites, but higher in the southern countries [103] [104]. Worldwide the frequency range between 3 and 32 % and seem to depend on ethnicity. The frequency seems to be highest for groups of Hispanic origin, and lowest for those of African origin [104] .

In particular 677C>T has been linked to decreased colon cancer risk [105-107]. Results from studies on the MTHFR 677C>T SNP and breast cancer have been less consistent. In the Long Island Breast Cancer Study the 677TT genotype was associated with increased breast cancer risk independent of menopausal status [92]. In a Japanese study the minor 677T allele was associated with increased breast cancer risk, only among postmenopausal women [108]. In contrast, the 677TT genotype has been associated with an overall decreased risk in Japanese American women [109]. Most other studies have not observed any overall associations [109-115]. Moreover, four meta-analyses conclude that the 677C>T SNP does not seem to influence breast cancer risk among postmenopausal women [116-119], but that the minor allele may have risk elevating effects among premenopausal women [86, 120]. In a recent American study from the Cancer Prevention Nutrition Cohort, tendencies towards an increased postmenopausal breast cancer risk was observed for the 677T allele, and women with variant alleles from both the 677C>T and 1298A>C were at higher risk [121]. On the other hand women with variant alleles from both SNPs had decreased risk in a study from Taiwan [122]. In that study, as well as two other studies, the 1298A>C variant genotypes has been associated with decreased risk [92, 101, 122]. However, most studies have not observed any associations between the 1298A>C SNP and breast cancer [109, 112-116, 123], and a meta-analysis did not support any overall associations [116]. In addition, observed associations between the minor 1298C allele and breast cancer may be explained by linkage to the major 677C allele [92].

Among women at high risk due to MHT, the 677TT genotype has been associated with 40% lower breast cancer risk [109], and among women at high risk due to the BRCA1 gene the 677T allele has been associated with increased breast cancer risk among women below 50y of age [124], whereas the 1298C allele was associated with a decreased risk.

## **Mendelian randomization**

Genetic variation that is linked to levels of a specific exposure may be used as a proxy for the exposure. This approach in epidemiology is known as Mendelian randomization. The advantage of the use of genotypes in stead of exposure measurements is based on Mendel's second law, that inheritance of one trait is independent of other traits, which means that genetic variants generally are independent of environment. Consequently

Mendelian randomization can be applied to minimize the influence of unknown or poorly estimated confounders [125]. In addition it is not affected by measurement errors commonly causing attenuation of diet-disease associations [126].

Since the MTHFR 677T allele is linked to lower levels of plasma folate, which is marker of intake, the 677T allele may be used as a proxy for low folate intake. Concerning associations between folate and cancer the use of Mendelian randomization is however a bit more complicated, because the potential mechanisms behind the association involve both the substrate for the MTHFR catalyzed reaction (5,10-methylene THF, involved in DNA synthesis) and the product (5-methyl THF, involved in DNA methylation). Therefore the interpretation of associations between MTHFR polymorphisms and cancer are not obvious [127]. Associations between the MTHFR SNPs and a cancer would however support the importance of folate in cancer development. Furthermore, detection of gene nutrient interactions contribute evidence of a nutrient specific association, by reducing confounding by correlated food components and other lifestyle factors.

## **Other B-vitamins involved in folate metabolism**

Other B-vitamins are also involved in folate metabolism, and may therefore modify the effects of folate on cancer development. The synthesis of methionine from homocysteine is dependent on folate (5-methyl THF), but also of vitamin B-12, which acts as a cofactor to the enzyme methionine synthase. Since the conversion of 5,10-methylene THF to 5-methyl THF is practically an irreversible reaction, B-12 deficiency may lead to accumulation of 5-methyl THF, and the folate destined for DNA synthesis becomes trapped. This condition is usually referred to as the “folate trap” [128].

Riboflavin is a cofactor for MTHFR.

Vitamin B6 serves as co-factor when tetrahydrofolate is converted to 5, 10-methylene tetrahydrofolate and when homocysteine is converted to cystathionine [80, 106].

## **Alcohol and folate**

Alcohol may not only affect cancer development via effects on DNA by toxic metabolites. Its metabolite acetaldehyde may also interfere with folate metabolism and affect DNA methylation via reduced methionine synthesis [129]. Alcohol may also indirectly have effects on one carbon metabolism, via its detrimental effect on folate bioavailability, and thereby affect DNA synthesis, repair and methylation [130]. It is therefore possible that high folate intake has a more crucial role in cancer suppression among women with high alcohol consumption. These women may need diets high in folate to compensate for the disturbed folate bioavailability and metabolism.

## **Other factors affecting folate requirement**

It is possible that folate inhibits the negative effects of smoking on DNA synthesis. This implies that smokers have higher folate requirements. Smoking may also influence folate metabolism. Lower levels of folate in blood have been observed among smokers, after adjustments for folate intake. This might be explained by effects on enzymes in folate metabolism [131, 132]. In an epidemiological study, the inverse association between dietary folate intake and colorectal cancer was stronger among smokers. A statistically significant interaction between folate intake and smoking was observed [133]. Some drugs may also interfere with folate metabolism (e.g. oral contraceptives) [134, 135]. Catechins in green tea disrupts folate metabolism by inhibiting dihydrofolatereductase, and thereby the conversion of dihydrofolate to tetrahydrofolate [136]. In addition both green and black tea could inhibit the intestinal absorption of folate[137]. It is however unclear whether this substantially influences folate status.

## **Folate intake and breast cancer in epidemiological studies**

Four case control studies suggest that high folate intakes are associated with a decreased risk of postmenopausal breast cancer [138-141], and a Scottish study indicates a non-significant negative association [101]. In two case-control studies, folate intake could not be related to postmenopausal breast cancer [142, 143]. High dietary folate intake was recently associated with a decreased risk of postmenopausal breast cancer in women from a large French cohort [144]. A nested case-control study from Denmark report that total folate intakes below 300 µg/day indicate an increased risk (however not significant) [145]. Results from most other prospective studies have not observed an overall inverse association between folate intake and



postmenopausal breast cancer [142, 146-149]. In fact, four cohort studies have not detected any significant relations at all [142, 146-148]. In addition, a study from a multi center cohort in USA shows that high total intakes are related to a significantly increased risk [149]. Meta-analyses have not found any overall associations between folate intake and breast cancer [119, 150]. However, most cohort studies have observed decreased risks at high folate intakes among women with moderate to high consumption of alcohol [146-148], and in the Danish study an increased breast cancer risk was only associated with alcohol consumption among women with low folate intakes [145]. Results are apparently contradictory, why more studies with high quality dietary information are required.

### **Folate intake - *MTHFR* - breast cancer**

A contributing reason to the differing results, reported by epidemiological studies investigating the association between breast cancer and folate intake, may be that many studies have not taken polymorphisms of *MTHFR* into consideration. Different associations between folate and breast cancer in subgroups with genetic variants of *MTHFR* indicate that an observed effect is connected to folate rather than to other variables correlated to folate intake, such as nutrients found in the same foods as folate. A Chinese case-control study reported an interaction between dietary folate intake and the *MTHFR* 677C>T polymorphism on breast cancer risk ( $P=0.05$ ) [113]. The strongest risk estimate was, similarly to observations from the Long Island Breast Cancer Study Project, the VITamins And Lifestyle study (VITAL) and a Japanese study, found among 677TT women with the lowest dietary folate intake, compared to women with the 677CC wild type and the highest folate intake [92, 108, 151]. Folate intake did not modify the relation between *MTHFR* polymorphisms and breast cancer in the Multiethnic Cohort Study or in a large Polish case control study [109, 116].

### **Folate intake - hormone receptor status- breast cancer**

Prognosis of estrogen receptor  $\alpha$  negative (ER $\alpha$ -) breast tumors is poorer and ER $\alpha$ - tumors seldom respond to hormone therapy [152]. Less is known about ER $\beta$ , but the expression seems to be decreased in tumors and it may act as a tumor suppressor [153]. Folate may affect methylation of CpG islands in ER genes, possibly leading to subsequent gene silencing. It is therefore plausible that adequate folate status is especially important to counteract the development of ER negative tumors [85].

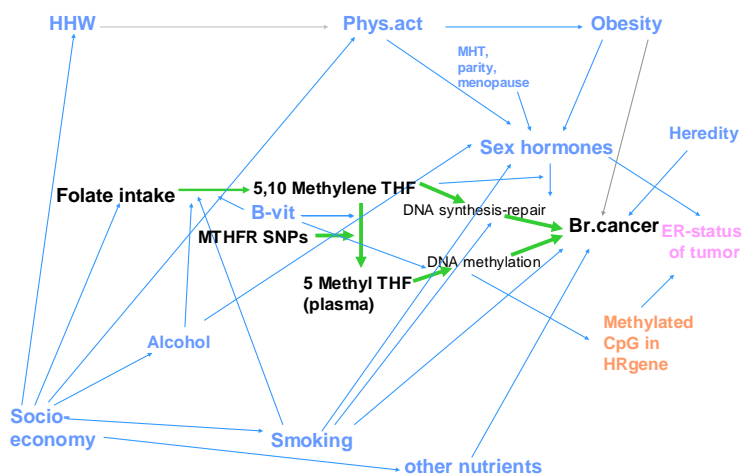
In a case control study of African-American women there was a tendency towards an increased risk of breast tumors with a methylated ER  $\alpha$  gene in women with low folate intake. However, the association was not statistically significant [84]. In both the Nurses Health Study and the VITAL study, women with high folate intake had lower risk of ER $\alpha$ - breast cancer [154, 155]. Similar relations were observed among women with an alcohol intake above the median in the IOWA Women's Health Study [156].

P-folate, folate intake was not associated with overall risk of ER $\alpha$ + breast cancer in three the studies [154-156], but results from the Swedish Mammography Cohort suggest a protective association between folate intake and ER $\alpha$ +PR- tumors [157].

## Folate intake, other B-vitamins and breast cancer

Since folate interacts with other B-vitamins in synthesis and methylation of DNA, possible modifying effects of other B-vitamins on breast cancer-folate associations need to be evaluated. A study in Shanghai showed that the association between high folate intake and decreased risk of breast cancer was stronger among women with high intakes of vitamin B12, vitamin B6 and methionine [141]. The modifying effect of vitamin B12 is supported by other studies [139, 144].

**Figure 5.** A simplified scheme of the complex relation between folate and breast cancer



## **Folate status and breast cancer**

Similar to results from studies on folate intake, there are also inconsistencies among studies that have investigated the associations between breast cancer and blood levels of folate and homocysteine. An Australian case-control study found that breast cancer risk was lower at higher levels of serum folate [158] and similar, but not statistically significant, observations were made in a case-control study on plasma folate from Taiwan [122]. In addition, four prospective studies have investigated blood folate status in relation to breast cancer. Again, an Australian study observed inverse associations between folate status and breast cancer. This observation was, however, only made for red cell folate and not for serum folate [159]. Three nested case-control studies were conducted in the USA. The Nurses Health study indicated a non significant inverse association between plasma folate and breast cancer [160]. The association was, however statistically significant among women with relatively high alcohol consumption. In the Washington County Study no associations were observed between serum folate and breast cancer [161]. In the Women's Health Study higher plasma folate concentrations were associated with increased risk in premenopausal women, but not in postmenopausal women [162].

The study from Taiwan also reported a positive association between breast cancer risk and plasma levels of homocysteine [163], but these observations are not supported by results from the Nurses Health Study.

A meta-analysis concluded that there was no evidence for an association between folate concentrations in blood and breast cancer [150].

Lack of evidence for the biologically plausible hypothesis of an inverse association between folate status and breast cancer could however be caused by an interaction between the 677C>T SNP and plasma folate. Since lower folate concentrations in plasma have been connected with the variant allele, it may simultaneously (as a consequence of reduced MTHFR activity) indicate higher intracellular concentrations of 5,10-methylene THF. This form can serve as a methyl donor for DNA synthesis and repair and thereby influence breast cancer risk. Consequently, stratified analysis according to MTHFR genotypes is of great relevance. In a case-control study from Taiwan the minor alleles of the 677C>T and 1298A>C SNPs were associated with decreased risk of breast cancer among women below median P-folate concentrations, but no associations were observed among

women above median P-folate concentrations. In the Australian case-control study no interaction was found between serum folate and the 677C>T polymorphism [122, 158].

In a report from the Women's Health study, high plasma folate concentrations were associated with increased risk of ER+ breast tumor development [162].

## **Folate and breast cancer in animal studies**

Results from three animal studies contradict the inverse association observed in several epidemiological studies, because mild folate deficiency suppresses tumor development in a rat model [164-166]. In one study, folate did not affect the mammary tumor incidence, but increased the number of tumors per animal and increased the rate of tumor development [164]. In another study, folate deficiency inhibited the progression of already existing neoplasms in mammary tissue, but did not affect tumor development in the initiation phase [165]. High amounts of folic acid supplementation did not show any effects [165, 166]. The observed effects on established neoplasms are not surprising, because antifolates are used to in cancer chemotherapy to prevent effective DNA synthesis in otherwise rapidly dividing neoplastic cells.

While no effects of folic acid supplementation was observed on mammary tumor development, folate supplementation have protected against colorectal tumor development in a murine model, compared with a mildly folate deficient diet. Contrary, in mice with already existing preneoplastic lesions, the folate deficient diet protected against small intestinal adenomas [90].

Consequently, timing of folate deficient/supplemented diets seems to be crucial in carcinogenesis.

## **Folate and cancer in human trials**

Tendency of an increased risk of death from breast cancer have been observed among women receiving 5 mg folic acid per day during pregnancy [167]. In a clinical trial on folic acid supplementation and colorectal cancer, the incidence of advanced lesions was border-line significantly increased among participants receiving 1 mg of folic acid per

day [168]. In another study folic acid supplementation had no effect on colorectal adenoma recurrence [169]. In a Norwegian trial examining effects of supplementation with folic acid and other B-vitamins on cardiovascular disease, tendencies of an increased total cancer risk have been observed [170]. This might be explained by enhancement of the development of non-diagnosed preneoplastic lesions.

## **Food fortification**

The mean intake in Swedish women seems to lie below the recommended intake, and far below the recommendation of 400 µg /day for women of fertile age. In order to prevent neural tube defects, many countries have ongoing discussions concerning implementation of mandatory fortification of foods. In the USA and Canada mandatory folate fortification of flour and cereals was implemented already in 1998, and since then the incidences of neural tube defects have decreased substantially [171, 172]. It has been estimated that about 5 births of children with neural tube defects could be prevented in Sweden per year by folate fortification. In addition, about 20 abortions could be prevented per year [173]. However several potential adverse effects of folic acid fortification have also been proposed. Intake of folic acid, instead of naturally occurring folates from foods, may lead to high concentrations of unmetabolized folic acid in the blood. This has been observed in the USA after implementation of mandatory fortification [174]. Whether high levels of circulating folic acid are harmful is not known, but folic acid seems to compete with natural folates in the body [175]. Circulating folic acid has also been inversely related to natural killer cell cytotoxicity, and may thereby lead to decreased tumor cell destruction [174]. Moreover, it has been hypothesized that observed increased incidences of colorectal cancer in the USA and Canada after 1996 and 1998 may be related to folic acid fortification [176], but later studies are not conclusive [168, 177]. Another concern has been that high intakes of folic acid may eliminate early symptoms of vitamin B12 deficiency and thereby increase neurological damage [178]. Therefore, also 5-methyl THF have been considered for fortification [179, 180].

# AIMS

The general aim was to study folate, from diet and plasma, in relation to risk of postmenopausal breast cancer among women from the MDC study, and to explore if the associations were confounded by other nutrients or differed according to genetic variation or hormone receptor status of tumors.

## Specific aims

1. **Folate intake and breast cancer**

To examine if folate intakes from foods and supplements were associated with postmenopausal breast cancer (Paper I).

2. ***MTHFR* and breast cancer**

To examine if polymorphisms of the folate metabolizing enzyme MTHFR (677C>T and 1298A>C) were associated with postmenopausal breast cancer. (Paper II).

3. **Folate intake, *MTHFR* and breast cancer**

To examine if associations between folate intake and breast cancer were different in subgroups with variants of the MTHFR 677C>T and MTHFR1298A>C SNPs (Paper II).

4. **Menopausal status, *MTHFR* and breast cancer**

To examine whether the associations between the MTHFR 677C>T and MTHFR1298A>C SNPs and breast cancer were different in perimenopausal and postmenopausal women. (Paper II).

5. ***MTHFR* and plasma folate**

To examine if folate concentrations in plasma were related to variants of the MTHFR 677C>T and MTHFR1298A>C SNPs. (Paper III).

6. **Plasma folate and breast cancer**

To examine if folate concentrations in plasma were associated with postmenopausal breast cancer in a nested case control study (Paper III).

7. **Plasma folate, *MTHFR* and breast cancer**

To examine if the associations between folate concentrations in plasma and breast cancer were different in subgroups with variants of the MTHFR 677C>T and MTHFR1298A>C SNPs (paper III).

8. **Variation of plasma folate**

To examine the variation of folate concentrations in plasma from postmenopausal women, in order to determine the accuracy of using a single sample when ranking subjects according to plasma folate concentrations (Paper III).

9. **Plasma folate, ER receptor status and breast cancer**

To examine the association between folate concentrations in plasma and risk of ER $\alpha$  and ER $\beta$  defined breast cancers. (Paper IV).

10. **Folate, other nutrients and breast cancer**

To examine if the consumption of other nutrients (i.e. B-vitamins involved in folate metabolism, alcohol and nutrients correlated to folate intake) influenced the relation between folate and breast cancer. (Paper I, II, III and IV).

# SUBJECTS AND METHODS

## **Malmö Diet and Cancer**

Malmö Diet and Cancer (MDC) is a population-based prospective cohort study in Malmö, a city in the south of Sweden. The main objective was initially to investigate if diets high in energy and fat, but low in vitamins and fiber lead to cancer of the breast, prostate, colon, rectum, pancreas, ovary or endometrium. The study was planned by the Swedish Cancer Society and the International Agency for Research on Cancer (IARC) and the Faculty of Medicine, Lund University, Sweden [181].

Baseline examinations took part between March 1991 and September 1996. The participants visited the MDC screening centre twice. During the first visit, groups of 6-8 participants were instructed how to register meals in a menu-book and how to fill out the diet questionnaire and the extensive questionnaire covering socio-economic and lifestyle factors. Nurses drew blood samples, registered blood pressure and made anthropometric measurements. All questionnaires were completed at home. During the second visit, approximately 10 days after the first, the socio-economic questionnaire was checked and a dietary interview conducted.

## **Source population**

Malmö is a city in the south of Sweden, which had about 250 000 inhabitants in the 1990's. In 1991, the MDC source population was defined as all persons living in the City of Malmö and born between 1926 and 1945. However in May 1995, the cohort was extended to include all women born 1923-1950, and all men born 1923-1945. With this extension 74138 persons constituted the source population. The MDC study was approved by the Ethical Committee at Lund University (LU 51-90). Details of the recruitment procedures and the cohort are described elsewhere (31). Briefly, participants were invited by personal letters or came spontaneously after invitation by advertisement in local newspapers, in public places or in



primary health care centers. Inadequate Swedish language skills and mental incapacity were the only exclusion criteria (n=1975). However, 3017 persons died or moved before they received their first invitation, 224 persons died before completion of the baseline examination and 17 persons could not be identified. In total 68 905 persons were eligible. In October 1996, when recruitment closed, 28 098 participants (40% of the eligible person, 17 035 women and 11 063 men), had completed the baseline examinations (Dietary assessments, Anthropometric measurements and Questionnaire on socio-economy/lifestyle) [182].

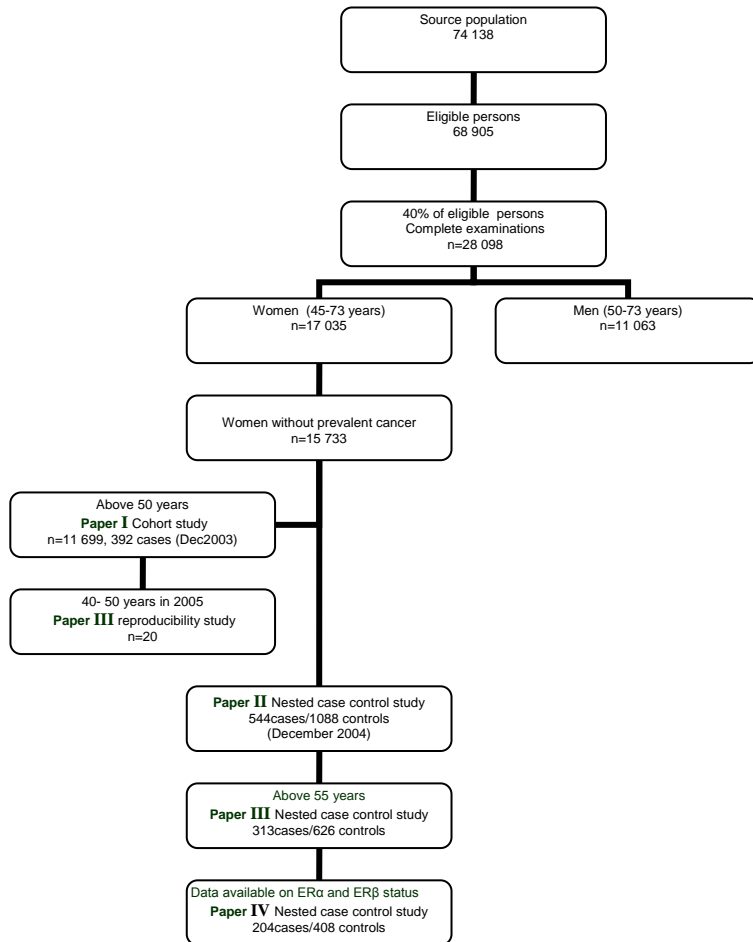
## **EPIC**

The MDC study is together with 26 other prospective studies from 10 countries participating in the European Prospective Investigation into Cancer and Nutrition (EPIC), which altogether includes 500 000 people. EPIC is organized by at IARC in Lyon and Imperial College in London.

## **Cohort study (paper I)**

This study included women who completed the baseline examinations and were 50 years or older. An age criterion for menopause was used due to missing values of self-reported cessation of menses, imprecise cessation of menses in combination with information on menopausal hormone use among premenopausal women and lack of detailed information on all women's medical history of hysterectomy. Fifty years was chosen as the definition for menopause [183], because the median natural age of menopause was 50.0 years in a sub-sample of 2898 women (without surgery and hormone therapy) from the cohort [184]. All women with prevalent cancers at baseline, except those with cervix cancer in situ, were excluded. In total 11 699 women were included. The average follow-up time was 9.5 years. The participants contributed person time until the end of follow up (December 31, 2003), when 392 cases of incident invasive breast cancer had been diagnosed.

**Figure 6.** *Study populations in paper I- IV.*



## Nested case-control study

Women with prevalent cancers at baseline, except those with cervix cancer in situ, were excluded. After this exclusion 15 773 women remained. Cases were all women who had been diagnosed with invasive breast cancer during follow-up (until 31 December 2004). The study included 544 breast cancer cases. Two controls (alive, living in Sweden and without breast cancer at the time of breast cancer diagnosis of the corresponding case) were matched on age at baseline  $\pm$  3 months and date of blood sample  $\pm$  1 month. During follow up 0.5 percent of the MDC study participants had migrated from Sweden.

## **Paper II**

The second paper included all women from the nested case-control study. The women were between 45 and 73 years of age at baseline.

## **Paper III**

In the third paper, the study sample included those who were above 55 years of age at baseline (313 breast cancer cases and 626 controls). Plasma folate concentrations were determined for 312 cases (99.7%) and 623 controls (99.2%).

## **Paper IV**

The last paper included cases from the third paper for whom information about ER $\alpha$  status and ER $\beta$  status of tumors was available, n=204, and their matched controls, n=408.

## **Reproducibility study (paper III)**

Twenty women, between 55 and 65 years of age and without any serious co-morbidity, participated in three non-fasting blood collections during May and June 2005. They were recruited by mail and telephone among 100 women, randomly selected from the MDC cohort.

## **Breast cancer case ascertainment**

The Swedish Cancer Registry and the Southern Swedish Regional Tumor Registry provided data on case definition and ascertainment until end of follow-up. Information on vital status was obtained from the National Tax Board, which provides up-to-date information on vital status for all Swedish residents. Since the registries are well established, the time of follow-up was almost complete. Cases were identified via the Swedish personal identification number.

## **Dietary assessment method**

The MDC-study used an interview-based, modified diet history method that combined (i) a 7-day menu-book for registration of meals that varies from day to day (usually lunch and dinner meals), cold beverages including alcohol, drugs, natural remedies, and nutrient supplements, and (ii) a 168-item questionnaire for assessment of consumption frequencies and portion sizes of regularly eaten foods that were not covered by the menu-book (**Figure 7**). Usual meal pattern was also reported in the questionnaire. Finally, (iii) a 45-minute interview completed the dietary assessment.

The participants filled out the questionnaire at home. They were asked to report consumption frequency of all items, as well as portion sizes. A booklet with 48 black and white photographs was used to estimate of portion sizes. Each set of photographs showed four different portion sizes of a dish. During the interview about ten days later, the consistency of the information provided was carefully checked so that the questionnaire and menu-book did not overlap, and so that they were in agreement with the reported meal pattern. A more comprehensive picture book was also used to estimate portion-sizes of foods reported in the menu-book. In addition the participants were asked questions about cooking methods and detailed food choices, such as type of bread and fats. In total 17 trained diet interviewers performed the interviews during the baseline examination period. The interviewers coded the information during the interview, and it was entered using an interactive software (Kostsvar, AIVO, Stockholm).

The mean daily intake of foods was calculated based on frequency and portion size estimates from the questionnaire and menu-book. The food intake was converted to energy and nutrient intakes using the MDC nutrient database where the majority of the nutrient information comes from PC-KOST2-93 from the National Food Administration in Uppsala, Sweden. The MDC-method is described in detail elsewhere [185, 186].

**Figure 7.** Example from the 168-item MDC diet questionnaire.

	Äter sällan eller aldrig	Antal bröd- skivor med pålägg per dag vecka		Antal skivor pålägg per brödskiva
Gräddost; hyvlade skivor typ Åseda, Västervik	✓			
Hårdost; hyvlade skivor		2		2
Mager hårdost; hyvlade skivor	✓			
Påläggskorv typ salami			5	2
Påläggskorv typ medvurst	✓			
Skinka, saltkött, hamburgerkött			2	1
Leverkorv	✓			

Consumption frequency
no. of pieces or portion size

## Relative validity of the MDC-method

The relative validity of the MDC-method was evaluated 1984-85 in a sample of Malmö residents, 105 women and 101 men, 50-69 years old, using 18 days of weighed records, three days every second month during a year, as the reference method [187, 188]. The Pearson correlation coefficients, adjusted for total energy, between the reference method and the MDC-method, were in women 0.75 for folate, 0.69 for dietary fiber, 0.70 for carotene and 0.71 for ascorbic acid.

## Reproducibility of the MDC-method

The reproducibility of the MDC-method was examined by administering the method one year apart on 120 residents in Malmö, 50-69 years old. The Pearson correlation coefficients adjusted for total energy, between the two assessments, were in women 0.71 for folate, 0.70 for dietary fiber, 0.53 for carotene and 0.74 for ascorbic acid [189].

**Table 1.** *Relative validity and reproducibility of the MDC dietary assessment method. Nutrients of relevance in this thesis.*

	Relative validity	Reproducibility
Folate	0.75	0.71
Fiber	0.69	0.70
$\beta$ -carotene	0.70	0.53
Vitamin C	0.71	0.74
Vegetables	0.53	0.76
Fruits	0.77	0.81
Alcohol	0.78	0.82

<sup>1</sup>Energy-adjusted Pearson correlation coefficients between estimated intakes by the MDC method and 18 days of weighed records.

<sup>2</sup>Energy-adjusted Pearson correlation coefficients between estimated intakes by the MDC method at two occasions 12 months apart.

## Handling of blood samples for the biobank

Non-fasting blood samples were drawn at baseline. The samples were separated into fractions within one hour, as previously described [190]. The plasma was stored at -80°C and granulocytes were stored at -80°C. From

August 1995 buffy coats were stored at -140°C, in stead of granulocytes [191].

## **Plasma folate analysis**

The folate concentration in plasma was analyzed by a two step immuno assay with alkaline phosphatase (ALP), enzyme marking and magnetic separation. Blood samples from cases were analyzed together with their matched controls to avoid problems with variability between assays. The laboratory personnel were unaware of case-control status of the specimens.

## **DNA analysis**

DNA was extracted from granulocyte or buffy coat cell suspensions using QiaAmp mini-kits (Qiagen, Hilden, Germany). Genotyping of the MTHFR SNPs 677C>T (rs1801133) and 1298A>C (rs1801131) was performed at the Clinical Chemistry Laboratory at the Malmö University Hospital on a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS) (SEQUENOM MassArray) using iPLEX reagents and protocol (SEQUENOM) and 10ng DNA template. All procedures were performed according to SEQUENOM standard protocols. The MALDI-TOF MS analysis was repeated on 4.2% (N=68) of the samples for rs1801133 and 4.3% (N=70) of the samples for rs1801131. There were no discrepancies between repeated analyses.

A few samples were not successfully genotyped on the MALDI-TOF MS (32 of MTHFR 677C>T and 24 of 1298A>C). The genotyping of these samples was performed on an ABI PRISM 7900HT Sequence detection system (Applied Biosystems, California, USA) using commercial SNP detection assays C\_1202883\_20 for MTHFR 677C>T and C\_850486\_20 for MTHFR1298A>C (Applied Biosystems).

Genotypes for the 677C>T SNP were determined for 540 cases (99%) and 1074 controls (99%), and genotypes for the 1298A>C were determined for 541 cases (99%) and 1072 controls (99%).

## **Estrogen receptor status evaluation**

ER and progesterone receptor (PR) status analysis was performed at the Centre for Molecular Pathology in Malmö.

For the construction of tissue micro arrays (TMAs), two 0.6 mm tissue cores were collected from each tumor block and arranged in a recipient block using a manual tissue arrayer (Beecher Inc., Sun Prairie, WI, USA).

Slides were then automatically stained using ER $\alpha$  (pre-diluted anti-ER 6F11, Ventana, USA), ER $\beta$  (1:25 EMR02, Novocastra, UK) and PR (prediluted anti-PR clone 16, Ventana, USA) antibodies [192]. The ER-antibody has been validated by comparing the immunohistochemical method with Western blot [193]. Receptor status of all breast tumors was evaluated in a standardized way by one person, thereby eliminating inter-observer variation. All arrays were evaluated independently twice, and in case of discrepancy a third examination was performed followed by a final decision, thereby reducing the potential intra-observer bias.

The tumors were classified as positive (+) and negative (-) using the clinically established cut-off value of 10% positive nuclei. ER $\alpha$  and ER $\beta$  was estimated for 204 cases (65 %). For the remaining cases, adequate tumor samples were not available, either because of surgery performed at other hospitals or insufficient amount of tumor material available for histopathological evaluation. These cases were categorized as unknown. PR status was determined for 98% of the ER  $\alpha$  and  $\beta$  defined cases.

## Dietary variables

Analyses included daily dietary and total intakes (including supplements) of *energy* and the following nutrients: *folate* ( $\mu\text{g}$ ), *vitamin B12* ( $\mu\text{g}$ ), *vitamin B6* (mg), *riboflavin* (mg), *dietary fiber* (g),  *$\beta$ -carotene* (mg) and *ascorbic acid* (mg). Energy-adjusted variables were obtained by regressing intakes of all nutrients on total energy intake. Tertiles and quintiles of nutrient residuals were used as exposure categories.

Total folate intakes were calculated by adding nutrient intakes from supplements to the intakes from foods.

In addition, dietary folate equivalents was calculated based on the assumption that the bioavailability of synthetic folic acid consumed in a meal is 1.7 times higher than the bioavailability of food folate [59] (dietary folate equivalents =  $\mu\text{g}$  food folate +  $1.7 * \mu\text{g}$  folic acid from supplements).

*Consumption of folic acid-containing supplements* (yes /no), was based on information on current use of supplements from the menu book.

## **Other variables**

### **Plasma folate**

*Tertiles of P-folate concentration* was used as exposure categories. The categories were defined as tertiles of P-folate concentrations among the controls.

### **MTHFR genotypes**

*MTHFR 677C>T and 1298A>C categories* were defined as genotypes (i.e. 677CC, 677CT, 677TT, 1298AA, 1298AC and 1298CC) and occurrence of the minor allele; yes/no (i.e. 677CC or 677CT+TT, and 1298AA or 1298AC+CC). Combinations of the 677C>T and 1298A>C genotypes were also used to define the MTHFR categories.

### **Methodological variables**

In September 1994, the processing of dietary data was slightly altered [185]. *Method version* (indicating data collection before or after September 1<sup>st</sup> 1994) and *season of data collection* was examined as potential confounders of dietary relations. Since 17 *dietary interviewers* performed the probing, this variable was also examined as a potential confounder.

### **Age and sex variables**

Information on *age* and *sex* was obtained via the Swedish personal identification number. *Age* was divided into 5-year categories.

### **Anthropometric variables**

*Weight* was measured using balance-beam scale with subjects wearing light clothing and no shoes. Standing *height* was measured with a fixed stadiometer calibrated in centimeters. *Body mass index* (BMI; kg/m<sup>2</sup>) was calculated from direct measurement of weight and height and a three category variable was created (BMI≤25, 25-29, ≥30 kg m<sup>-2</sup>). BMI was also dichotomized (≤25, >25).



## Reproduction variables

*Age at menarche* and *age at menopause* was used as continuous variables. *Age at menopause* was also divided into a four category variable (<45, 45-50, 50-55, >55 years). *Lactation* (months) was defined as the total number of months for all children. *Years with menstrual cycles* was defined as the time span between menarche and menopause accounting for interruptions for pregnancies and lactation. *Duration of contraceptive pill use* (years) was divided into four categories with zero-consumption in the lowest category. *Menopausal hormone therapy* (yes/no) was based on the questionnaire item “Which medications do you use on a regular basis?”, in combination with information on drug use from the 7-day menu book [194]. Reported usage in the questionnaire or in the menu book was defined as “yes”. *Age at birth of first child* was divided into four categories with an additional category for women with no children. *Parity* was defined as the number of children with no children in the lowest category, and four or more in the highest.

## Lifestyle variables

The *smoking status* of the participants was defined as smokers (including irregular smokers), ex-smokers and never-smokers. Information on total *alcohol* consumption was converted into a four-category variable. Women reporting zero consumption in the menu book, and indicating no consumption of any type of alcohol during the previous year, was categorized as zero-reporters. The other category ranges was <15 g of alcohol per day (low), 15-30 g alcohol per day (medium) and >30g of alcohol per day (high). Alcohol intake was also dichotomized ( $\leq 4.7$ ,  $> 4.7$ g per day). Since alcohol intake was not correlated with total energy intake, the variable was not energy-adjusted. *Leisure-time physical activity* was assessed using a questionnaire, adapted from the Minnesota Leisure Time Physical Activity Questionnaire [195, 196]. The number of minutes per week of 18 different activities was multiplied with an activity specific intensity coefficient and an overall leisure-time physical activity score was created. The score will be divided into tertiles and categorized as low, medium and high. The score was moderately correlated to accelerometer measurements [197]. *Household activities* were estimated in hours per week and will be divided into four groups with cut points every ten hours (0-9, 10-19, 20-29, 30 or more).

## **Variables related to socioeconomic**

Participants were divided into four categories according to their highest level of *education* ( $\leq 8$  years, 9-10 years, 11-13 years, university degree). Classification of *socioeconomic index* was based on information on job title, tasks and position at work. The procedure was adapted from that of the 1985 Swedish population census [198]. The information was collapsed into five categories: blue collar workers, white collar workers (low, medium and high) and self-employed. Retired and unemployed were classified according to their position before retirement/ unemployment.

## **Statistical methods**

All nutrient variables and plasma folate concentrations were log transformed to normalize the distribution before analysis. A very small amount (0.01) was added before transformation to handle zero intakes.

All statistical tests were two-sided.

The SPSS statistical computer package (version 14.0; SPSS Inc., Chicago, Illinois) and Stata (version 10; StataCorp, College Station, TX) were used for the statistical analyses.

## **Power calculations**

In this population enough “power” (80% and  $\alpha=0.05$ ), to detect a risk gradient from 1 to 1.75 over quintiles of a consumed nutrient, was obtained when more than 283 breast cancer cases had been registered (in 1999), presupposing a true risk gradient from 1 to 3 over quintiles, and a validation coefficient of 0.6.

The test for gene-nutrient interaction with a power of 80% and  $\alpha=0.05$  will be able to detect a risk gradient of 3 between the highest and the lowest quintile, in a study with 200 cases and 400 controls, given that the minor allele frequency is at least 20% [199]

## **Aim 1**

Logistic regression analysis was applied to examine the likelihood of falling in the highest quintile of total folate intake, depending on

socioeconomic, lifestyle and anthropometric characteristics. Adjustments were made for age, method version and season.

We used Cox proportional hazard regression model to estimate hazard ratios (HRs) of invasive breast cancer in quintiles of folate intake. Adjustments were made for age category, method version, season, energy, intake of vitamin B12, intake of vitamin B6, intake of riboflavin, weight, height, leisure time physical activity, household work, smoking, alcohol, socio-economic status, age at menopause and MHT. Intakes of nutrients were adjusted for total energy with the residual method [200]. The covariates were identified from the literature and previous analysis within the MDC cohort [28, 40], indicating potential confounding of the folate-breast cancer association. The analysis was repeated for dietary folate intakes (excluding folic acid from supplements), total folate intakes, dietary folate equivalents and consumption of folic acid supplements.

## **Aim 2**

Genotypes of MTHFR 677C>T and MTHFR 1298A>C were cross-classified.

ORs for breast cancer according to MTHFR genotypes were computed with unconditional logistic regression with adjustments for matching variables. The analysis was repeated with adjustments for weight, height, household work, smoking, alcohol intake, socioeconomic status, age at menopause, parity and MHT.

## **Aim 3**

The examination of ORs for breast cancer according to MTHFR genotypes (Aim 2) was stratified on *age at baseline above or below 55 years*. A test for interaction with regard to breast cancer was performed using the cross product term (age below or above 55 years at baseline × occurrence of the variant MTHFR alleles).

## **Aim 4**

ORs for breast cancer according to joint effects of MTHFR genotypes and folate intake (DFE) were computed with unconditional logistic regression, with wild-type women at low folate intake as the reference. Adjustments were made for the matching variables, method version, season and total energy intake. A second model also included adjustments for established

risk factors and potential confounders (i.e., weight, height, household work, smoking, alcohol intake, socioeconomic status, age at menopause, parity, MHT). The model was stratified on age at baseline above or below 55 years. A test for interaction with regard to breast cancer was performed (tertile of dietary folate equivalents  $\times$  occurrence of the variant MTHFR allele). All analyses were repeated for supplement consumption (yes /no) instead of folate intake.

### **Aim 5**

Mean P-folate concentration according to MTHFR genotypes were calculated with ANOVA and adjusted for age and date of blood sample.

### **Aim 6**

The energy adjusted partial correlation coefficient between intake of dietary folate equivalents and P-folate concentration was computed.

ORs for breast cancer in quintiles of P-folate were computed with unconditional logistic regression, controlling for matching variables. A second model included adjustments for established risk factors and potential confounders (i.e., weight, height, household work, smoking, alcohol intake, socioeconomic status, age at menopause, parity, and MHT).

Mean P-folate concentrations according to genotype combinations of MTHFR SNPs (677C>T and 1298 A>C) and tertiles of folate intakes were calculated with ANOVA and adjusted for age and blood sampling date.

### **Aim 7**

In subgroup analysis according to MTHFR genotype, ORs for breast cancer in tertiles of P-folate were computed with unconditional logistic regression, controlling for matching variables. A second model included adjustments for established risk factors and potential confounders (i.e., weight, height, household work, smoking, alcohol intake, socioeconomic status, age at menopause, parity, and MHT). A test for interaction with regard to breast cancer was performed between tertiles of P-folate (treated as a continuous variable) and MTHFR genotypes (of 677 C>T and 1298 A>C respectively, treated as continuous variables). In order to avoid confounding between the two loci, the analysis was also restricted to women homozygous for the major 1298A allele when the test for interaction between P-folate and the 677 C>T genotype was performed, and to women homozygous for the

major 677C allele when the test for interaction between P-folate and the 1298 A>C genotype was performed.

### **Aim 8**

For the 20 women with repeated blood samples, median folate concentrations, and the number and range of days between blood collections were determined. Pearson correlation coefficients were also calculated. Estimates of between-subject ( $\sigma^2_B$ ), within-subject ( $\sigma^2_W$ ), total variances as well as the intra class correlation coefficient (ICC) (between subject variance divided by total variance), with 95% CIs, were obtained using reliability analysis with one-way ANOVA.

Risk estimate correction for within person P-folate variation was performed using the following formula:  $OR_{\text{observed}} = (OR_{\text{corrected}})^{ICC}$  [201], with the ICC obtained in the reproducibility study

### **Aim 9**

ORs for ER $\alpha$  and ER $\beta$  defined breast cancer in tertiles of P-folate were computed with unconditional multinomial logistic regression, controlling for matching variables. A second model included adjustments for established risk factors and potential confounders (i.e., weight, height, household work, smoking, alcohol intake, socioeconomic status, age at menopause, parity, and MHT). Wald's test was used to examine heterogeneity between the folate -breast cancer associations according to ER  $\beta$  subgroup. A test for interaction with regard to ER $\beta$  breast cancer was performed between tertiles of P-folate (treated as a continuous variable) and carriage of the *MTHFR* 677T allele.

### **Aim 10**

The examination of folate intake in relation to hazard ratios (HRs) of invasive breast cancer (Aim 1) was also repeated with adjustments for either intake of ascorbic acid, carotene or dietary fiber.

Mean P-folate concentrations according to fiber intakes were calculated with ANOVA and adjusted for age, blood sampling date and folate intake (DFE).

When examining folate intake in relation to hazard ratios (HRs) of invasive breast cancer (Aim 1), the full model was replaced by (i) a model without

other B-vitamins or (ii) a model with either intakes of vitamin B12, vitamin B6 or riboflavin.

The analysis of folate intake in relation to breast cancer (Aim 1) was also stratified on alcohol intake ( $\leq 4.7$  and  $> 4.7$  g/day). Sensitivity analysis was made for alcohol intake  $\leq 15$  g/day.

The multivariate model used to examine ORs for breast cancer according to joint effects of MTHFR genotypes and folate intake (Aim 4) was repeated with additional adjustments for vitamin B12 intake, vitamin B6 intake and riboflavin intake.

The multivariate models used to examine breast cancer according to plasma folate in strata of MTHFR genotypes (Aim 7) was repeated with additional adjustments for vitamin B12 intake.

The multivariate models used to examine ER-defined breast cancers according to plasma folate (Aim 9) was repeated with additional adjustments for vitamin B12 intake.

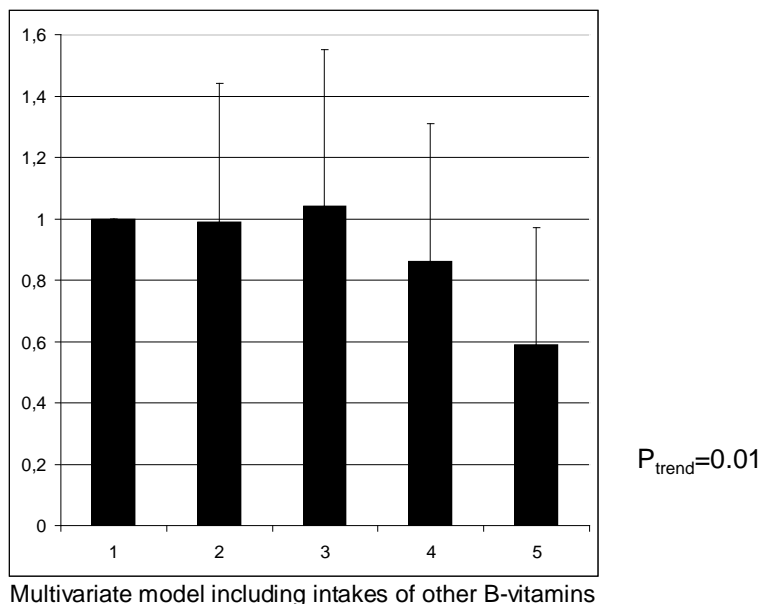
# RESULTS AND DISCUSSION

## Aim1 - Folate intake and breast cancer (paper I)

The estimated total median intake among women above 50 years of age in the MDC cohort was 238 µg per day. This is below the Nordic recommended daily intake of 300 µg per day. Only women in the highest quintile had intakes corresponding to the recommendations. Nineteen percent of the women consumed folic acid-containing supplements. Women with high dietary intakes of folate were more likely to consume supplements. The likelihood of having high folate intakes was associated with several lifestyle factors. Groups of women less likely to have high folate intakes were manual workers, women with low education, overweight women, women who got their first child early in life and women with many children. Groups who were more likely to have high intakes were women with menopausal hormone therapy and high leisure time physical activity, as well as ex-smokers (Information not reported in paper I). These factors have previously been related to breast cancer (see background), and were therefore evaluated as potential confounders in the relation between folate intake and breast cancer.

After adjusting for B-vitamins involved in folate metabolism and other potential confounders, women with high folate intakes had lower risk of postmenopausal breast cancer (highest quintile compared with the lowest). The results were similar for intakes of folate naturally occurring in foods (HR = 0.56; 95% C.I: 0.35, 0.90; P for trend = 0.02) and total folate intake, which included intake of folic acid from supplements. Total folate intakes were expressed both as the sum of dietary and supplemental intake (P for trend = 0.006), and as folate equivalents (P for trend = 0.01) (**Figure 8**). The association was somewhat strengthened in a sensitivity analysis excluding women below 55 years and thereby minimizing inclusion of perimenopausal women (HR = 0.48; 95% CI: 0.28, 0.84; P for trend = 0.004). Consumption of folic acid containing supplements was independently associated with lower risk of postmenopausal breast cancer, but adjustment for this variable did not alter the observed protective association between dietary folate and breast cancer.

**Figure 8.** Hazard ratios of invasive breast cancer across quintiles of total folate intake (expressed as dietary folate equivalents).



Considering breast cancer incidence, results from this study indicate that higher folate intake would benefit this study population. A possible way to increase folate intake would be to recommend consumption of folic acid supplements. In Sweden most supplements with folic acid contain 400 µg, which is in level with the amount related to lower breast cancer incidence in this study. Another way of increasing folate intake would be to follow the example of many other countries and implement mandatory folic acid fortification of foods. With regard to the contradictory results from studies on folate intake and cancer at different sites, including an American study indicating higher breast cancer risk at high folate intakes [149], increased consumption of fruits and vegetables would be a safer way to optimize folate intake. This would probably also be a realistic approach, because the consumption of fruits and vegetables is relatively low in Sweden compared with that of other European countries [202], but tendencies towards an increased consumption has been observed [203, 204].



## Aim 2 - *MTHFR* - and breast cancer (paper II)

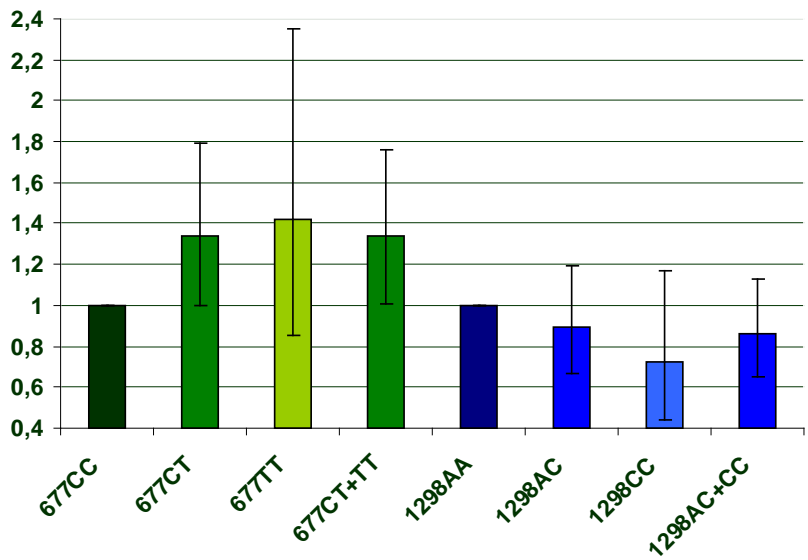
Distributions of genetic variants related to activity of the folate metabolizing enzyme *MTHFR* are presented in **Table 2**. Like in other populations, linkage was observed between the two SNPs (677C>T and 1298A>C) [205-207]. The minor 677T allele, which has been related to the lowest *MTHFR* activity, was linked to the major 1298A allele. No women had genotype combinations with more than two minor alleles.

**Table 2.** *Percentage distribution of persons with genotype combinations of *MTHFR* 677C>T and 1298A>C*

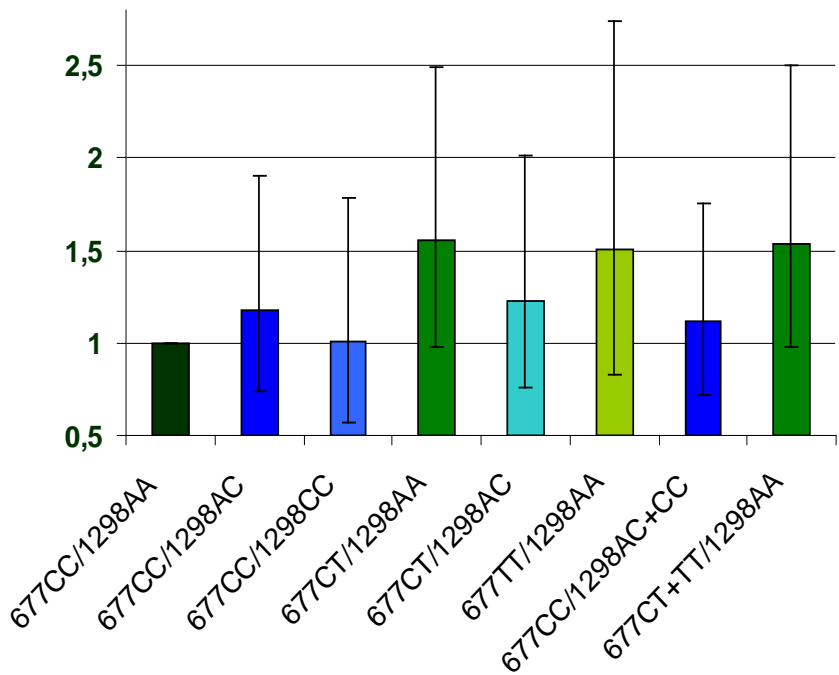
	677C>T			total
	CC	CT	TT	
1298 A>C				
AA	15%	21%	9%	45%
AC	24%	21%	0	45%
CC	10%	0	0	10%
Total	49%	42%	9%	

In postmenopausal women (above 55y of age at baseline), carriage of the 677T allele was associated with increased risk of breast cancer (OR: 1.34; 95%CI: 1.01-1.76) (**Figure 9**). In analysis of genotype combinations of the two SNPs, the 677T allele was associated with a non-significant increased risk (P= 0.06; 677CT+TT-1298AA women compared with 677CC-1298AA women) (**Figure 10**). No significant association was observed between the 1298A>C polymorphism and breast cancer.

**Figure 9.** ORs of breast cancer in women above 55 y according to MTHFR 677C>T and 1298A>C genotypes



**Figure 10.** ORs of breast cancer in women above 55y according to combinations of the MTHFR 677C>T and 1298A>C genotypes



Since the balance between DNA synthesis and DNA methylation is pushed towards DNA synthesis in women with the 677T allele (due to decreased MTHFR activity), the high risk among these women may indicate that DNA methylation is especially crucial in the development of postmenopausal breast cancer.

### **Aim 3 - Menopausal status, *MTHFR* and breast cancer (paper II)**

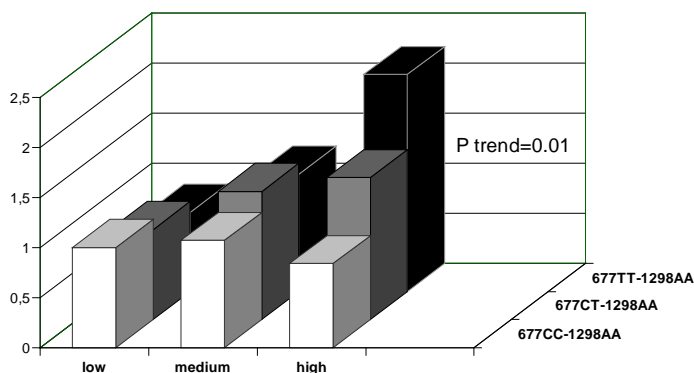
Contrary to the findings among women above 55 years of age, no association between the MTHFR 677C>T allele and breast cancer was seen in women between 45 and 55 years of age at baseline. In addition statistical interaction with regard to breast cancer risk was observed between the MTHFR 677C>T polymorphism and age (below or above 55 years at baseline) ( $P=0.03$ ). Furthermore, borderline statistical interaction was seen between the MTHFR 1298A>C genotype and age ( $P=0.07$ ); in the younger age group homozygosis for the minor C allele was associated with increased risk. Women with the 677CC-1298CC genotype combination was at higher risk compared with 677CC-1298AA women ( $P=0.04$ ).

Women between 45 and 55 years of age are probably a heterogeneous group with regard to menopausal status, including both pre-, peri- and postmenopausal women. Consequently no conclusions could be drawn concerning this age group, but the results indicate that age modifies associations between the MTHFR 677C>T/ 1298A>C polymorphisms and breast cancer.

### **Aim 4 - Folate intake, *MTHFR* and breast cancer (paper II)**

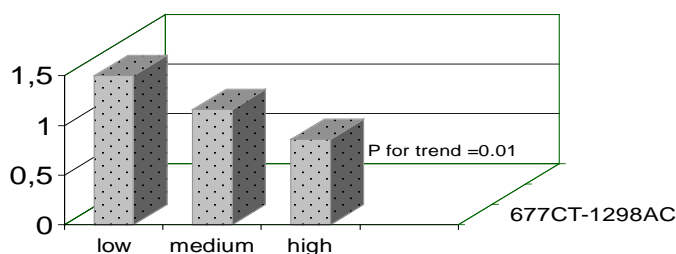
It would be logical to expect folate intake to be particularly important in women with increased risk due to the MTHFR 677T allele, because of their decreased methyl supply for DNA methylation. However, we found high folate intake to be associated with increased risk of breast cancer among women with the 677T allele ( $P$  for trend=0.01 among 677CT+TT-1298AA women), but no association was observed in women without this minor allele ( $P=0.95$  among 677CC-1298AA) (**Figure 11**). Borderline interaction was observed between the 677T allele and folate intake when the analysis was restricted to 1298AA women to avoid confounding by the 1298A>C SNP ( $P=0.07$ , data not included in paper II).

**Figure 11.** OR of breast cancer according to joint effects of folate intake (DFE) and genotypes of MTHFR polymorphisms among cases and controls. In order to elucidate the effect of the 677C>T SNP, the illustration is restricted to 1298AA women.



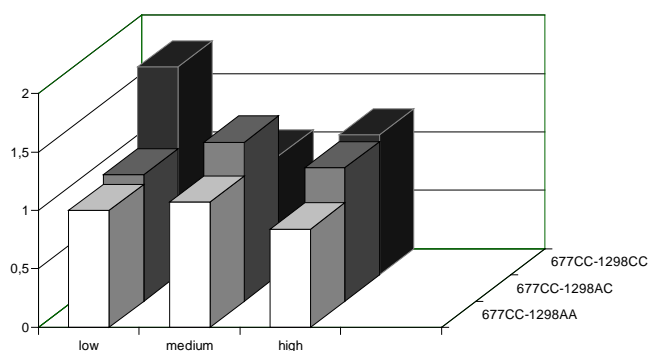
However, among compound heterozygous women (677CT-1298AC), high folate intake was associated with decreased risk ( $P=0.01$ ) (**Figure 12**).

**Figure 12.** OR of breast cancer according folate intake among compound heterozygous women



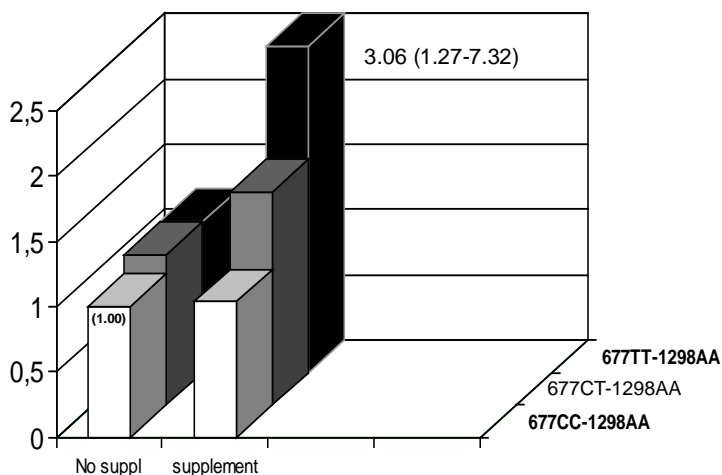
An observed interaction between the minor 1298C allele and folate intake was probably due to linkage between the two MTHFR SNPs, because no such tendencies were observed in analysis of genotype combinations (**Figure 13**).

**Figure 13.** *OR of breast cancer according to joint effects of folate intake (DFE) and genotypes of MTHFR polymorphisms. In order to elucidate the effect of the 1298A>C SNP, the illustration is restricted to 677CC women.*



Women homozygous for the 677T allele who consumed folic acid containing supplements had a significantly increased risk of breast cancer compared with wild type women who did not consume supplements (OR, adjusted for dietary intake: 3.06; 95% CI, 1.27-7.32) (**Figure 14**) (data not shown in paper II). No interaction was observed between the MTHFR genotypes and supplement consumption (P=0.28).

**Figure 14.** ORs of postmenopausal breast cancer according to joint effects from consumption of folic acid containing supplements and genotypes of the MTHFR polymorphisms. In order to elucidate the effect of the 677C>T SNP, the illustration is restricted to 1298AA women.



The results suggest that folate intake is of importance in breast cancer development, but that its role is complex. The observed associations indicate that we are capturing folate intake rather than intakes of correlated nutrients, because we have no reason to expect that the activity of a folate metabolizing enzyme would influence associations with other nutrients. The protective association seen among compound heterozygous women (677CT-1298AC) suggests that adequate folate intake may compensate women at increased risk related to carriage of alleles denoting suboptimal DNA methylation. In women with disturbed folate metabolism due to homozygosis for the 677T allele (677TT), it may not be possible to counteract the imbalanced distribution of folate between DNA synthesis and methylation. In combination with a more pronounced reduction of the MTHFR activity in 677TT women, high folate intake may above all result in high levels of other forms of folate than the one needed for DNA methylation. The consequences of such imbalance are not known, but

folate levels may affect the activity of enzymes in folate metabolism [175], and thereby influence both DNA synthesis and repair, as well as DNA methylation.

The especially high risk estimates among 677TT women consuming folic acid supplements may reflect the comparably high doses, but it may also indicate that consumption of synthetic folic acid has specific health implications. To become bioactive, folic acid must be reduced to tetrahydrofolate, and some studies have indicated that high levels of unmetabolized folic acid may have negative consequences on health [175].

## Aim 5 - MTHFR and plasma folate (paper III)

The MTHFR 677TT genotype was associated with lower P-folate concentration compared with the 677CC genotype, and a significant trend for decreased P-folate over 677C>T genotypes was observed (P=0.001. When the analysis was limited to 1298 AA women (**Table 3**), the same associations between the 677T allele and P-folate were observed. In analysis limited to 677CC women (**Table 3**), the variant C allele at locus 1298A>C also tended to be associated with lower P- folate concentrations (P for trend=0.07).

**Table 3.** Plasma folate concentrations according to genotype combinations of the MTHFR 677C>T and 1298A>C SNPs among control women above 55 years of age from the Malmö Diet and Cancer cohort.

Genotypes	1298 AA		1298 AC		1298 CC		P for trend
	n	Plasma folate (nmol/l) <sup>1</sup>	n	Plasma folate (nmol/l) <sup>1</sup>	n	Plasma folate (nmol/l) <sup>1</sup>	
677 CC	96	12.0 <sup>a</sup>	154	11.8 <sup>a</sup>	71	10.4 <sup>a</sup>	0.07
677 CT	125	9.8 <sup>a,b</sup>	121	10.0 <sup>a,b</sup>			
677 TT	48	8.1 <sup>b</sup>					
P for trend		<0.01					

<sup>1</sup>Geometric means. Homogenous subsets are indicated by letters. Analysis of variance.

Multiple comparison was done with Tukey's test,  $\alpha=0.05$ . Adjustments for age and date of blood sample.

Folate in plasma almost entirely occur as 5-methyl THF, and the minor alleles of the MTHFR 677C>T and 1298A>C SNPs has been linked to reduced MTHFR activity and consequently reduced conversion of 5,10-methylene THF to 5-methyl THF [93, 96]. Consequently, our findings relating the minor alleles to low plasma folate levels were expected and are therefore an indication of high validity data, regarding plasma folate concentrations as well as genotypes.

## Aim 6 - Plasma folate and breast cancer (paper III)

No significant overall association was observed between plasma folate concentrations and postmenopausal breast cancer. **Table 4** shows associations between quintiles of P-folate concentrations and postmenopausal breast cancer (data not reported in paper III).

**Table 4.** *ORs of breast cancer across quintiles of plasma folate concentrations among cases and control women above 55 years of age from the Malmö Diet and Cancer cohort.*

Women >55y	Cases/ controls	Tertiles of plasma folate concentration					P-trend
		1	2	3	4	5	
Basic model	312/	1.0	1.22	0.80	1.39	1.21	0.29
	623		0.80-0.88	0.51-1.25	0.91-2.13	0.79-1.86	
Multivariate model	312/	1.0	1.21	0.79	1.29	1.11	0.58
	621		0.77-1.90	0.50-1.27	0.82-2.02	0.71-1.75	

The energy-adjusted correlation coefficient between folate intake (DFE) and plasma folate was 0.52 among the controls.

If P-folate is seen as a marker of folate intake, the results were not in agreement with the results from paper 1. However, Since folate in plasma almost exclusively appear as 5-methyl THF, P- folate is above all a marker of folate available for DNA methylation, and the levels depend on both



intake and MTHFR activity (**Table 5**)(Not included in paper III). The differing results may highlight the importance of stratification on *MTHFR* genotypes.

**Table 5.** Mean plasma folate concentration<sup>1</sup> according to tertile of folate intake and *MTHFR* groups

Genotype combination	Cases/controls	Tertiles of folate intake			P trend
677CC, 1298 AA	68/165	9.1 <sup>bc</sup>	10.4 <sup>bcd</sup>	15.3 <sup>f</sup>	0.04
677CC, 1298AC	130/257	9.1 <sup>bc</sup>	10.8 <sup>bcd</sup>	15.3 <sup>f</sup>	<0.001
677CC, 1298CC	57/105	8.2 <sup>ab</sup>	9.5 <sup>bcd</sup>	13.1 <sup>def</sup>	0.28
677CT, 1298AA	122/227	8.0 <sup>ab</sup>	9.3 <sup>bc</sup>	13.9 <sup>ef</sup>	<0.001
677TT, 1298AA	50/91	6.3 <sup>a</sup>	9.2 <sup>bc</sup>	12.0 <sup>cdef</sup>	— <sup>2</sup>
677CT, 1298AC	112/223	8.1 <sup>ab</sup>	9.0 <sup>bc</sup>	13.6 <sup>ef</sup>	0.004

<sup>1</sup>Geometric means. Homogenous subsets are indicated by letters. Analysis of variance.

Multiple comparison was done with Tukey's test,  $\alpha=0.05$ . Adjustments for age and date of blood sample.

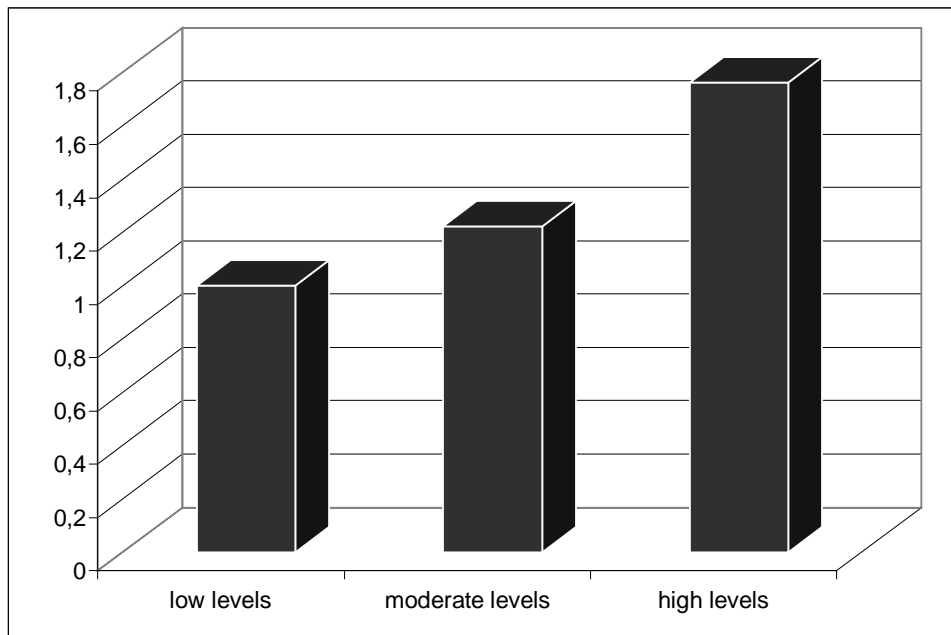
<sup>2</sup>The trend analysis was not performed because of too few subjects in one of the strata.

## Aim 7 - Plasma folate, *MTHFR* and breast cancer (paper III)

Among women with the 677T allele, the results for P-folate were in line with those for folate intake. Women with high P-folate concentrations, despite the P-folate reducing T-allele, had increased breast cancer risk (OR: 1.76; 95% CI, 1.07-2.88;  $P=0.03$  among 677CT/TT women) (**Figure 15**) (Risk estimates for analysis stratified according to *MTHFR* genotypes are not shown in paper III). No significant associations were observed in women without the 677T allele. A statistical interaction was observed between the *MTHFR* 677C>T SNP and plasma folate concentration ( $P=0.002$ ), and the interaction remained significant when the analysis was restricted to 1298AA women ( $P=0.006$ ). No significant interaction was observed between the 1298A>C SNP and plasma folate concentration. In

contrast to the findings for folate intake, high plasma folate concentrations were not associated with decreased breast cancer risk among compound heterozygous women (677CT-1298AC).

**Figure 15.** *OR of breast cancer according to plasma folate concentration in strata of MTHFR 677CT/TT women.*



Similar to the findings for folate intake these results may reflect the complex role of folate in breast cancer development due to its involvement in both DNA synthesis and methylation. Again we would have expected high plasma levels to counteract the high risk among 677TT-carriers, by increasing DNA methylation. However, in combination with MTHFR genotype information, P-folate levels may also give an indication of total folate status. Women with the 677TT genotype will probably need very high folate intakes to achieve high plasma folate concentrations. Although not significantly different, the highest mean intake among women with high plasma folate concentrations was actually observed for 677TT women. Consequently it is possible that 677TT women with high plasma folate

concentrations have especially high levels of intracellular 5,10-methylene tetrahydrofolate, or circulating unmetabolized folic acid due to consumption of folate supplements. Since 5,10-methylene tetrahydrofolate is needed in DNA synthesis, and since effective DNA-synthesis is especially important in rapidly dividing cells such as neoplasms [208], it is possible that accumulation of 5,10 methylene THF promotes the progression of neoplastic cells that would otherwise not necessarily progress into cancer cells [88]. Little is known about the importance of circulating unmetabolized folic acid, but this inactive form may interfere with bioactive forms of folate by binding to folate carriers [175]. In addition circulating folic acid has been related to impaired natural killer cell cytotoxicity [174].

## Aim 8 - Variation of plasma folate (paper III)

The results in **Tables 6 and 7** from the reproducibility study indicate that the single non fasting blood sample drawn at baseline can be used to rank women according to plasma folate concentrations. The correlation between the first and third sample, drawn with a median interval of 34 days, was 0.63 (Pearson correlation coefficient) (**Figure 16**).

**Table 6.** *Number of days and correlations between repeated non fasting plasma folate samples from 20 women from the MDC cohort.*

Blood samples	Number of days between samples		Correlation *
	Median	Range	
First and second	14	9-33	0.78
Second and third	18	5-27	0.92
First and third	34	19-46	0.63

\* Pearson correlation coefficients for e-log-transformed plasma folate concentrations.

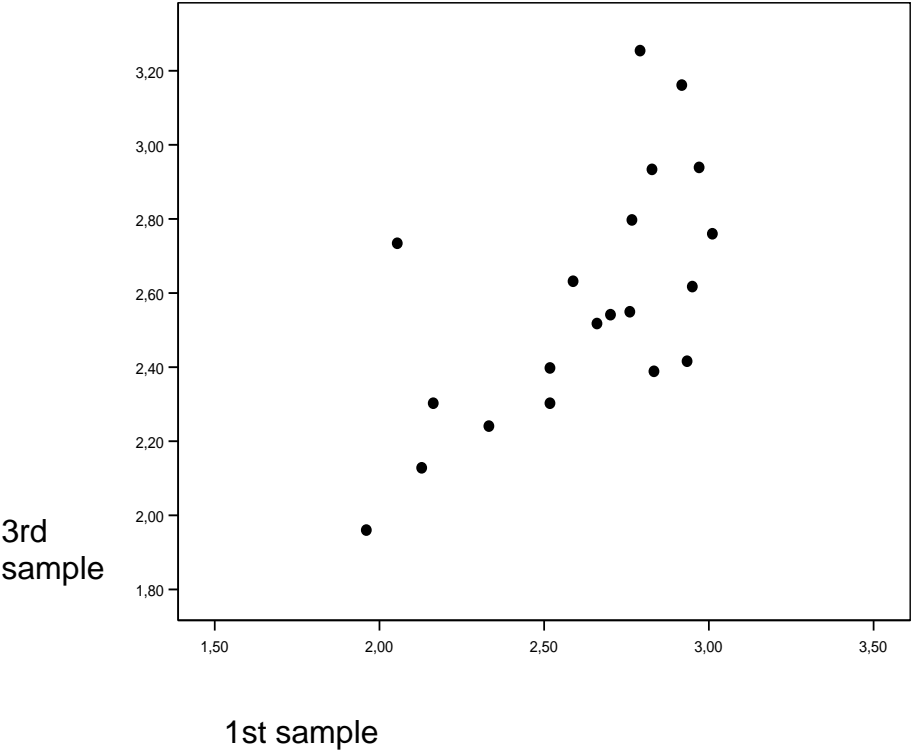
The within person variation was much smaller than the between person variation, and resulted in an intra class correlation coefficient of 0.77. We will consequently not expect our observed associations between plasma folate levels and breast cancer to be severely attenuated, because it has been

suggested that special attention should be given to values below 0.6 [64]. In spite of this, corrections by the ICC of 0.77 resulted in a risk estimate of 4.17 instead of 3.00 for 677CT/TT-1298AA women at high folate intakes compared with compound wild type women at low folate intakes (data not shown in paper III).

**Table 7.** *Non fasting plasma folate concentration, coefficients of variation and intraclass correlation coefficient for 20 women from the MDC cohort.*

Median (nmol/l)	Within person CV	Between person CV	ICC (95%CI)
13	15%	38%	0.77 (0.59-0.89)

**Figure 16.** *In transformed plasma folate concentrations in the first and third blood samples from 20 women from the MDC cohort.*



## Aim 9 - Plasma folate, ER receptor status and breast cancer (paper IV)

Among breast cancer cases above 55 years of age, ER $\alpha$  was highly expressed in most tumors, but high expression of the  $\beta$ -receptor was only seen in about half of the tumors (**Table 8**).

**Table 8.** *Distribution of ER $\alpha$  and ER $\beta$  status of the breast tumors*

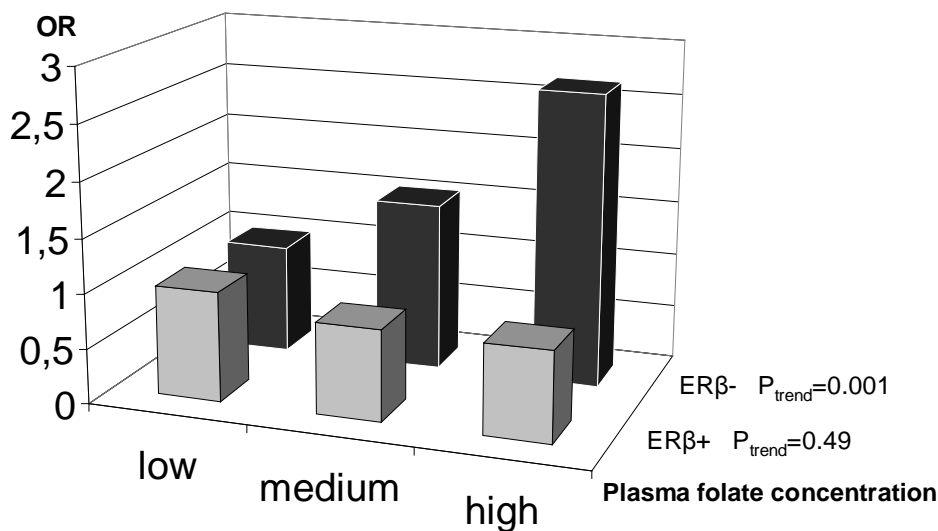
	ER $\beta$ +	ER $\beta$ -	Total
ER $\alpha$ +	91 (45%)	87 (43%)	178 (88%)
ER $\alpha$ -	13 (6%)	13 (6%)	26 (12%)
Total	104 (51%)	100 (49%)	204

High P-folate concentrations were associated with increased risk of ER $\beta$ -breast cancer (P for trend=0.001), but not associated with risk of ER $\beta$ + breast cancer (P for trend= 0.49). A significant difference between the risk for ER $\beta$ + and ER $\beta$ - cancer was seen (P for heterogeneity = 0.003) (**Figure 17**). The positive association between P-folate and ER $\beta$ - breast cancer was also seen when the analysis was restricted to women with ER $\alpha$ + tumors (P for trend=0.007), and a similar tendency was seen in the small group of women with ER $\alpha$ - tumors (P for trend 0.07). We did not observe any significant associations between plasma folate concentrations and breast cancers defined by ER $\alpha$  status. The positive association between P-folate concentration and ER $\beta$ - breast cancer was seen in women both with (P for trend = 0.01) and without the MTHFR 677T allele (P for trend = 0.005).

By influencing DNA methylation, folate levels may affect silencing of tumor suppressor genes [84, 175]. Since ER $\beta$  may act as a tumor suppressor [153], one possible interpretation of our observations might be that the high plasma folate levels have resulted in breast cells with low concentrations of

the possibly protective  $\beta$ -receptor, and that such cells have been transformed into tumor cells.

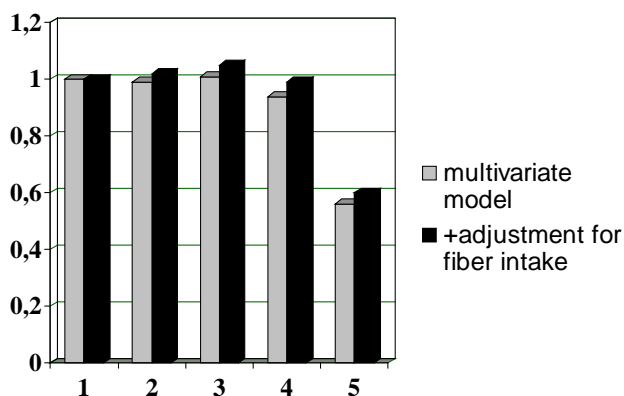
**Figure 17.** *ER $\beta$  defined breast cancer according to plasma folate concentrations.*



## Aim 10 - Folate, other nutrients and breast cancer (paper I, II, III and IV)

Since fiber intake has previously been associated with breast cancer in the MDC cohort and since fiber intake is correlated to folate intake, adjustments for fiber intakes were made. The overall reduced risk seen at high folate intakes remained significant (**Figure 18**). In addition the results were not substantially changed when fiber was replaced by  $\beta$ -carotene or ascorbic acid in the multivariate model.

**Figure 18.** Hazard ratios of invasive breast cancer in quintiles of total folate intakes, with and without adjustments for fiber intake.



Fiber intake may also influence the bacterial growth in the colon [209, 210]. Fiber may thereby promote the bacterial folate production leading to increased colonic folate absorption. In a previous study high fiber intake was associated with increased plasma folate concentrations independently of folate intake [51]. In an additional analysis we also observed higher plasma folate concentrations at high fiber intakes (**Table 9**) (among cases and controls (45-73y) with information on plasma folate concentrations).

**Table 9.** *Plasma folate concentration according to quintile of fiber intake*

Quintile of fiber intake	n	Plasma folate (nmol/l) <sup>†</sup>	P for trend
1	323	8.8a	
2	327	10.3 b	
3	325	10.6 b	
4	321	10.7 b	
5	325	12.4 c	0.04

<sup>†</sup>Geometric means. Homogenous subsets are indicated by letters. ANOVA Adjusted for folate intake (DFE), age and blood sampling date

Other B-vitamins involved in folate metabolism, and vitamin B12 in particular, seemed to influence associations between folate intake and postmenopausal breast cancer. A tendency of decreased risk at high intakes of folate from foods was seen in the multivariate model without inclusion of intakes of other B-vitamins (HR 5<sup>th</sup> quintile: 0.70; 95% CI, 0.48-1.02; P for trend=0.11) (data not shown in paper I). However, the association was only statistically significant after adjustment for vitamin B12 (HR 5<sup>th</sup> quintile: 0.63; 95% CI, 0.43-0.93; P for trend=0.04) (data not shown in paper I). As reported in paper I, the strongest association was seen after simultaneous adjustment for vitamin B12, vitamin B6 and riboflavin (P=0.02). Similar observations were made for total folate intakes: only tendencies of protective associations were seen before addition of vitamin B12 intakes to the multivariate models (P for trend = 0.13 for total folate, P for trend = 0.20 for dietary folate equivalents) (data not shown in paper I).

In subgroup analysis according to MTHFR genotypes (paper II), the increased risk at high folate intake among women with the 677T allele remained significant after adjustment for vitamin B12 intake, but not after additional adjustment for vitamin B6 and riboflavin. However, simultaneous inclusion of all these B-vitamins may not be sensible in subgroup analyses, because dietary intakes are always connected with a



certain degree of misreporting and may lead to unstable results. This is especially problematic in small groups. Adjustments for the other B-vitamins did not alter the protective association seen between folate intake and breast cancer in compound heterozygous women (677CT-1298AC).

Adjustments for intakes of vitamin B12 did neither substantially influence the association between high plasma folate and breast cancer among women with the 677T allele, nor the association between high plasma folate and ER $\beta$  negative breast cancer (paper III and IV).

Our observed protective association remained after exclusion of women with high alcohol consumption (above 15g /day). Besides, similar non significant decreasing risks with higher folate intakes were seen in women below (P for trend = 0.11) and above the median intake of alcohol (P for trend = 0.10). No significant interaction was seen between alcohol consumption and plasma folate (P = 0.13), but we saw a tendency of increasing risk with higher plasma folate concentrations in women with alcohol intakes above the median intake (P for trend = 0.11).

# METHODOLOGICAL CONSIDERATIONS

## **Misreporting and dietary measurement error**

Error in diet measurements is a major problem in nutritional epidemiology. Random errors may occur due to inaccurately measured intakes. In addition, large day to day variation of dietary intakes makes it difficult to estimate mean daily intake, and could be regarded as random measurement error if the intention is to assess long-term intake [211].

Systematic errors may for example occur when a dietary questionnaire fail to cover frequently consumed foods, which leads to underestimations of dietary intakes. In addition people may consistently underreport or overreport intakes of certain foods.

Non-differential misclassification occurs when the errors are randomly distributed among subjects. Non-differential misclassification of the main exposure leads to attenuation of diet-disease associations. Statistical methods can be applied in order to correct for random measurement errors, and thereby reduce attenuation of diet-disease associations [211]. However, if other covariates (in addition to the main exposure) are measured with error associations may also be strengthened.

Systematic errors that affect all persons equally do not affect diet-disease associations [211].

In prospective cohort studies measurement errors are usually random with respect to disease. However, the measurement errors may be correlated to true intakes or influence certain groups of individuals more than others, leading to differential misclassification. Contrary to non-differential misclassification, differential misclassification may either attenuate or strengthen diet-disease associations. Although attenuations due to non-differential measurement errors in general are regarded to have greater impact on associations, both types of misclassifications usually exist simultaneously. People with high intakes tend for example to underreport intakes, whereas those with low intakes tend to overreport [212]. It has also

been shown that overweight persons tend to underreport energy intake [213].

Although the MDC dietary assessment method has shown high relative validity and reproducibility, all dietary methods are prone to errors. In addition there is no golden standard, meaning that methods of high relative validity may have the same errors as the reference method [214].

## **Energy adjustment**

The proportion of a nutrient in relation to the energy content of diet may be of greater importance to health than the absolute amount of a nutrient. This is because the need in absolute terms usually depends on body size and physical activity, which are the most important determinant of energy intake. It is therefore in most cases correct to adjust for total energy intake when examining nutrient intake in relation to risk of disease. Otherwise, extraneous variation due to body size and physical activity may attenuate the association. Since intakes of practically all nutrients are positively correlated with total energy intake, an even more important reason for energy adjustment is that energy intake may be associated with disease, and thereby confound associations between specific nutrients and disease [200]. In addition, energy-adjustments are recommended to reduce the impact of measurement errors, because a substantial part of the variation in estimated energy intakes is caused by measurement error and measurement errors of nutrient intakes and energy intake are strongly correlated [215].

Among women above 50y of age from in the MDC cohort the Pearson correlation coefficient between dietary folate intake and total energy intake was 0.59. Several methods for energy adjustment have been described [216]. In this thesis the nutrient residual energy-adjusted method was used [200]. This approach examines dietary composition, minimizes measurement error and avoids confounding by energy intake.

## **Confounding**

Dietary habits usually reflect lifestyle patterns and are consequently correlated to other lifestyle factors. As previously discussed folate intake in the MDC study was for example associated with socio-economic status,

smoking status, physical activity, weight and parity. Consequently, access to information on different lifestyle factors is of great importance, because accounting for these factors may have great impact on the results. Since the baseline examinations included anthropometric measurements and an extensive questionnaire about lifestyle factors, we were able to adjust for several potential confounders.

### **Residual confounding**

Despite adjustments for established risk factors for breast cancer and other potential confounders, residual confounding may still be a problem. Residual confounding may occur due to poorly measured confounders. Unmeasured variables may also have confounded associations between folate and breast cancer, because unknown risk factors could covary with diet. In addition, categorization may in some cases make variables too crude. Residual confounding may bias associations in either direction [212].

### **Estrogen receptor status**

Associations with ER defined breast cancer may be influenced by misclassification of ER status. However, for the evaluation of ER status, two tumor tissue samples were used from each patient. Besides, the receptor status was evaluated in a standardized way by one person, thereby eliminating inter-observer variation. In addition, all tissue microarrays were evaluated independently twice, and in case of discrepancy a third examination was performed followed by a final decision, thereby reducing potential intra-observer bias.

There is some controversy regarding ER $\beta$ , because the ability to detect the ER $\beta$  protein varies between different ER $\beta$  antibodies [217]. The ER $\beta$  antibody used in this study has been validated by comparing the immunohistochemical method with Western blot [193], and can be regarded as valid and specific for ER $\beta$  [192, 218]

Tumor status of both ER $\alpha$  and ER $\beta$  could only be determined for two thirds of the cases, and the majority of the undefined cases were due to missing data on ER $\beta$ . This may arouse questions concerning selection of tumors related to other characteristics, such as histology, size or stage of the tumors. An observation that potentially reduces the risk of differences in tumor characteristics is that the cases with information on ER $\beta$  status did

not differ from those without ER $\beta$  status regarding major breast cancer risk factors (age, weight, height and MHT). Anyhow differences in tumor characteristics would not explain the different associations seen between folate and cancers with high or low expression of ER $\beta$ .

## **Misclassification of breast cancer status**

Misclassification of breast cancer status is potentially very low, because the Swedish cancer registry is almost 100 % complete. However, undiagnosed cancers at baseline may have affected dietary habits and blood levels in some women. To handle this problem, cancers diagnosed during the first year of follow-up were excluded in sensitivity analysis.

## **Generalizability**

Regarding the etiological significance of observed diet-disease associations, the internal validity of dietary intake and disease status may be of greater importance than the external validity. However, from a public health point of view the importance of observed associations may depend on whether the exposure levels are in range with those of other Malmö citizens, Swedish women in general or other populations.

The socio-demographic structure, weight distribution and smoking habits were similar in the MDC cohort and a health survey cohort in Malmö with higher participation rate (75%). However, the MDC participants reported better health and higher degree of social participation than the participants in the other cohort [182].

## **Power**

Although calculations indicate a satisfying power at the present number of breast cancer cases (see Subjects and Methods), power might be a problem due to linkage between the MTHFR 677C>T and 1298A>C SNPs. Linkage between the SNPs made analysis of genotype combinations valuable. On the other hand, this reduces the number of cases in strata of MTHFR genotypes. It is therefore possible that the small number of cases in some subgroups have prevented as from detecting associations seen in other subgroups.

## **Plasma folate**

Folate in plasma almost exclusively appears as 5-methyl THF [63], and may therefore above all reflect the amount of methyl groups available for DNA methylation. Erythrocytes are more informative about different folate vitamers [62]. They may thereby also give indications of methyl groups available for DNA synthesis, and potentially also its importance in breast cancer development. In addition, erythrocyte folate is less influenced by recent intake. However, P-folate is considered to be a good biomarker of folate status in large epidemiological studies

## **Folate data in food tables**

Folate data in most food tables are based on microbiological assessment methods. However, microbiological methods are sometimes associated with poor precision [219]. One reason might be rigid cell structures of certain foods, that make folate less accessible [220]. However for some foods the conventional microbiological assay seems to overestimate the folate content compared to a LC-MS (liquid chromatography-mass spectrometry) method [221]. In addition to total folate concentration, chromatographic methods give information on concentrations of different folates. This might be important, because the bioavailability may vary between the vitamers.

The Swedish Food Administration uses a microbiological method for analysis of folate concentrations in foods, in which the growth of *Lactobacillus casei* is measured. Enzymatic deconjugation of polyglutamates is performed before analysis [222]. However, data from other information sources, concerning nutrient content in foods, are also used in the Swedish food database. This may also induce error, because the nutrient value for certain foods might not be comparable to that of other foods.

The instability of folates may also be of concern. Although food items with different cooking methods may be included in food databases, all differences in storage and preparation (e.g. chopped foods or purees) could not be accounted for.

Since there are shortcomings in analysis methods as well as important variation of folate content within food items, it is advantageous to complement estimated folate intakes with biomarkers of folate intake in epidemiological studies.

## **Other nutrients in one-carbon metabolism**

Folate's ability to transport one-carbon groups between essential biological reactions may be limited by the content of different important one-carbon containing compounds in diet. Methionine is an essential amino acid, but also a one-carbon nutrient. It is abundant in foods of animal origin as well as in cereals. Choline is another one-carbon compound found in foods of animal origin. Although choline can be synthesized in the body, it is classified as an essential nutrient in the USA, and low intakes might be more frequent than previously stated [223, 224]. Choline can be converted to betaine, but diet also contribute betaine (e.g. seafood, cereals and spinach)[225]. We did not have information about intake of one-carbon containing compounds. However, inadequate intakes are generally considered to be rare, because of the wide distribution in different foods.

Low vitamin B12 status may results in disturbances in the folate cycles and subsequent decreased transfer of methyl groups for both DNA synthesis and methylation. Although information on intakes of vitamin B12 was available, information on blood concentrations of this vitamin would have been a valuable complement, because vitamin B12 deficiency might occur despite adequate intakes. Unfortunately blood concentrations of vitamin B12 were not analyzed.

## **Alcohol**

We were not able to explore if the association between high folate intake and breast cancer incidence was stronger among women with high consumption of alcohol, because the number of women with high alcohol intakes were too few. We could however see similar protective associations in women below and above the median intake. Nevertheless, self-reported alcohol consumption should be carefully interpreted. Although the relative validity and the reproducibility of alcohol intake were high in the MDC study, the intake was higher with the reference method [188, 189]. This indicates that women with high true daily alcohol intakes might be found among those reporting lower intakes.

# MAIN FINDINGS, INTERPRETATION AND IMPLICATIONS

Our observations bring further understanding of the complex role of folate in cancer development. With regard to postmenopausal breast cancer, folate intakes at the recommended level may contribute to reduced risk. On the other hand, this beneficial role of folate may not be applicable to women who are genetically predisposed to disturbed folate metabolism. Besides, high blood levels of folate may be linked to an increased risk of developing ER $\beta$  negative breast tumors. The results also indicate that possible negative effects of high folate intakes are more pronounced among women consuming folate supplements.

Considering the multiple role of folate in biological methylation reactions, it is not surprising that the results from experimental and epidemiological studies on folate and cancer development, including ours, are conflicting. It is well known that, depending on timing, dose, site of the tumor, folate's chemical structure and tumor characteristics, the vitamin seems to have differing effects [88, 89].

## **Timing and the dual role of folate in carcinogenesis**

It is possible that a high folate intake inhibits the initiation of cancer, by transferring one carbon groups for biologically essential methylation reactions in normal cells. This may explain our findings of an overall protective association between folate and breast cancer. On the other hand, folate also seems to stimulate the progression and growth of already existing pre-stages of cancer [90], by transferring one-carbon groups for DNA synthesis. It is possible that it is folate's role as a one-carbon provider for DNA synthesis in precancerous cells, that explains the high risks seen among MDC women with the MTHFR 677TT genotype and high folate intake. The other mechanism through which folate may promote carcinogenesis is by providing one carbon groups for methylation and subsequent silencing of tumor suppressor genes. This mechanism may explain our observed risk increase of ER $\beta$  negative tumors at high plasma folate concentrations, because the  $\beta$ -receptor may act as a tumor-suppressor.



## **Dose - possible non-linear association**

If a true protective association exists between folate intake and breast cancer, it may be the relatively low folate intake in the MDC cohort that allowed us to detect the association. In populations including few individuals with intakes below the recommended intakes, protective associations may not be detected [226]. On the contrary, we could probably not detect possible adverse effects of very high folate intakes. A cohort study in France also observed an overall decreased risk at high dietary folate intakes [144], but other prospective studies have not seen any overall protective associations [150]. The median dietary intake in the French cohort was higher than in the MDC cohort, but they observed a decreased risk already in the second quintile. They did not examine total folate intakes. However, probably fewer women achieved intakes comparable to those seen in American cohorts, because France has no mandatory folate fortification of foods and only 10 % consumed B-vitamin supplements. In contrast, higher levels of total intakes have been associated with increased risk in an American study [149]. Together these three studies may reflect a non-linear association between folate intake and breast cancer.

## **Folate's role depend on tumor site**

Associations between genetic variants of MTHFR and cancer seem to depend on cancer site, because the importance of DNA synthesis and DNA methylation is linked to characteristics of specific cells. The 677T allele seems to protect against colon cancer [227], probably due to the rapid cell division of colonic epithelial cells. In contrast, and as indicated in this study, the T allele may promote development of breast cancer, as well as cancer at several other sites [76]. The impact of the T allele probably depends on diverse optimal balances between folate distribution for DNA synthesis and methylation. It is however reasonable to assume that the most favorable distribution in mammary tissue is altered during periods with increased mammary cell division, such as puberty or pregnancy. In addition, and independent of tumor site, joint effects of folate intakes and genetic variation may be crucial [76, 105, 113, 228].

## **Folate from foods or synthetic folic acid?**

The high risk seen among 677TT women consuming folate supplements, may be attributed to the high dose in combination with a distribution of

folate that favors DNA synthesis, as previously discussed. Alternatively, it may reflect the difference between naturally occurring folate versus folic acid. It has been suggested that unmetabolized inactive folic acid may interfere with the active form of folate [175], and that high blood levels of folic acid may promote tumor development [174].

Moreover, regardless of chemical structure, there is always a difference between nutrients naturally occurring in foods and nutrients in supplements or fortifiers, because the natural environment in foods as well as the balance between different dietary components is impossible to copy [229]. Knowledge concerning optimal contents of specific nutrients in relation to that of other nutrients is limited, but high concentrations of one nutrient may counteract the bioavailability and function of other nutrients. In addition co-existing nutrients also show synergistic effects. Concerning folate, at least alcohol, fiber and other B-vitamins seem to influence folate status and metabolism. Moreover, food texture may be critical for the bioavailability of folate.

Finally plant foods contain both several well examined nutrients, as well as hardly known bioactive components which may have positive health effects. Consequently, recommendations of high intakes of folate containing foods, such as green leafy vegetables and legumes, would not only be the safest way to increase folate intakes, at a population level. This approach would probably also have additional beneficial health implications. The benefits may be particularly apparent in Sweden, because of the rather low intake of fruits and vegetables compared to that in other European countries [202]. However, since the preventive effect of folic acid fortification on neural tube defects is well established, targeted initiatives, including recommendations of folic acid supplementation, are most likely necessary towards women of fertile age as well as other groups with increased folate requirements.

The findings of this thesis indicates that high folate intake may have both beneficial and detrimental effects on breast cancer development, depending on genetic predisposition and tumor characteristics, as well as dosage or chemical structure. These observations may be valuable in the ongoing discussions, in many countries, regarding mandatory folate fortification of foods.

# CONCLUSIONS

We made the following observations among women from the MDC cohort

1. Women achieving the Nordic recommended intake of folate had lower incidence of postmenopausal breast cancer.
2. Women with the 677T allele of the folate metabolizing enzyme MTHFR, had higher risk of postmenopausal breast cancer risk.
3. High folate intake was associated with increased risk of postmenopausal breast cancer among 677T carriers. This association was especially pronounced among women consuming folic acid containing supplements. High folate intake was associated with decreased risk of breast cancer among women compound heterozygous for the variant alleles of the MTHFR 677C>T and 1298A>C SNPs.
4. The increased breast cancer risk in MTHFR 677T carriers was only seen in women above 55 years of age, and a statistical interaction was observed between the T allele and age.
5. Women with the MTHFR 677T allele had lower plasma folate concentrations.
6. No overall association was observed between plasma folate concentration and breast cancer.
7. In line with the observations for folate intake, high folate concentrations in plasma were associated with increased risk of breast cancer among 677T carriers, and statistical interaction was seen between the 677T allele and plasma folate.
8. The reproducibility study indicates that a single non fasting blood sample drawn at baseline can be used to rank women according to plasma folate concentrations, because the within person variability was much smaller than the between person variability.

9. High folate intake was associated with increased risk of developing breast tumors with low concentrations of ER $\beta$ .
10. The inverse association between folate intake and postmenopausal breast cancer was only statistically significant after adjustments for vitamin B12. Adjustments for fiber,  $\beta$ -carotene or ascorbic acid did not substantially influence the association.

Taken together these observations indicate a multifaceted role of folate in breast cancer development. The findings may influence current discussions regarding mandatory folate fortification of foods.

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