One-Carbon Metabolism and Breast Cancer

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Dedication

This dissertation is dedicated to my husband and loving daughter, who have patiently supported me with their unconditional love.

Abstract

Breast cancer is the most common cancer among women in the United States. Nutrients in one-carbon metabolism (OCM) have been examined as potential modifiable risk factors because of OCM's important role in DNA methylation and DNA synthesis. However, biologic mechanisms between OCM and carcinogenesis are still not clarified.

The first manuscript tested the hypothesis that OCM nutrient status and genetic variation in methionine adenosyltransferases (*MAT1A*, *MAT2A* and *MAT2B*) are associated with plasma *S*-adenosylmethionine (SAM) levels in a cross-sectional analysis among healthy Singapore Chinese adults. Choline and methionine were strongly and positively associated with plasma SAM levels (p_{trend}<0.0001), and folate and betaine were positively associated with plasma SAM only in men (p_{trend}=0.02). The association between *MAT1A* rs2993763 and plasma SAM was modified by gender and plasma methionine levels.

In the same study population, the second manuscript cross-sectionally tested the hypothesis that plasma SAM levels alone or in combination with genetic variation in DNA methyltransferases (*DNMT1*, *DNMT3A* and *DNMT3B*) are associated with global DNA methylation measured at long interspersed nucleotide element-1 (LINE-1). The LINE-1 methylation index was positively associated with plasma SAM levels ($p\leq0.01$), with a plateau at approximately 78% and 77% methylation in men and women, respectively. Among men, there were statistically significant positive or negative associations between *DNMT1* rs2114724 or *DNMT3A* rs758127 genotype and the LINE-

1 methylation index, respectively (p_{trend}<0.01). The SAM-LINE-1 methylation association was modulated by *DNMT1* rs2114724 genotype in men only.

In a prospective cohort study, the third manuscript attempted to replicate increased breast cancer risk related to higher dietary nitrate intake only among those with low folate intake, which was previously reported by a case-control study. Opposite my hypothesis, among women with total folate intake 400 μg/day or above, breast cancer risk was statistically significantly higher among women in the highest quintile of nitrate intake from public water (HR=1.40, 95%CI=1.05-1.87) and private well users (HR=1.38, 95%CI=1.05-1.82) than those with the lowest nitrate intake from public water.

The projects described in this dissertation contribute evidence describing how OCM may be related to cancer risk, and are a step in pursuit of my long-term goal to enhance our understanding of cancer etiology through OCM.

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I. INTRODUCTION

One-carbon metabolism (OCM) is a series of methylation reactions that consists mainly of folate metabolism and the methylation cycle. OCM has been studied extensively in relation to cancer due to its critical role in transferring methyl moieties to DNA synthesis and DNA methylation. A number of epidemiologic studies have examined folate in relation to various cancers but results have been inconclusive. There may be multiple mechanisms behind the link between OCM and the cancer development process, which have not been clarified yet. In this dissertation, I focused on DNA synthesis and repair and DNA methylation as possible mechanisms behind the association between OCM and cancer and addressed the areas on which our understanding is still evolving.

The aim of this dissertation was to fill the gaps in the current literature to enhance our understanding of the mechanisms of OCM and carcinogenesis. The first and second projects examined 1) which OCM nutrients and genetic variation in OCM are associated with plasma concentrations of *S*-adenosylmethionine (SAM), a universal methyl donor for many biological reactions including DNA methylation, and 2) to what extent plasma SAM concentrations are associated with global DNA methylation in peripheral blood in a subset of participants in the Singapore Chinese Health Study. The third project evaluated to what extent the association between nitrate intake from diet and drinking water and breast cancer risk is modified by total folate intake among postmenopausal women in the Iowa Women's Health Study.

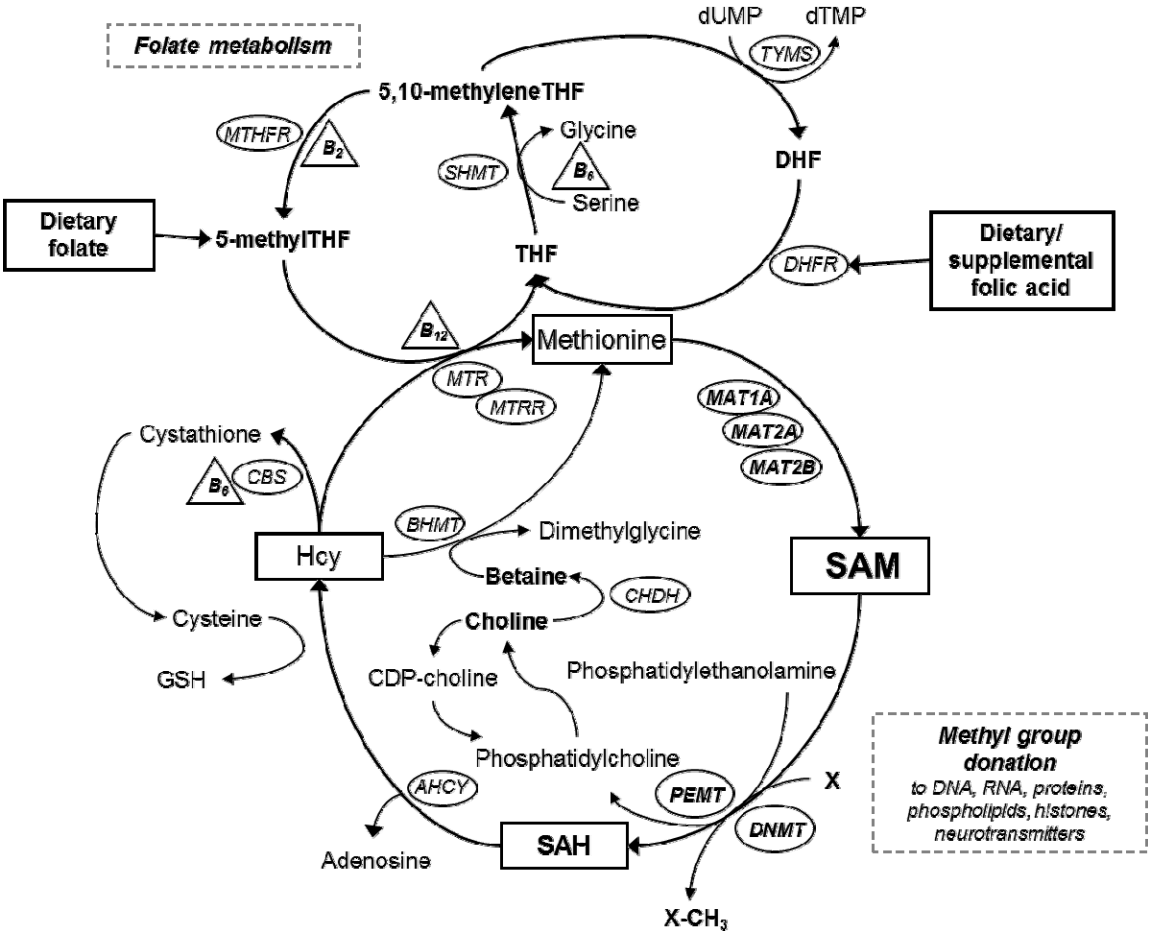
II. BACKGROUND

A. Overview of One-carbon Metabolism (OCM)

OCM is a network of biochemical reactions that involves the transfer of methyl groups essential for DNA synthesis and DNA methylation. OCM consists of two major pathways – folate metabolism and the methylation cycle. Folate metabolism and the methylation cycle are connected via the vitamin B_{12}-dependent methylation of homocysteine (Hcy) to methionine. **Figure II-1** shows an overview of OCM.

Folate plays a central role in OCM as a source of methyl moieties. Folate is the generic term used to describe all forms of the water-soluble vitamin B_9 that naturally occurs in foods such as fruits, vegetables, grain products, legumes and seeds, whereas folic acid refers specifically to the monoglutamyl form of folate found in fortified foods and dietary supplements. Folic acid must be reduced by dihydrofolate reductase (DHFR) via dihydrofolate (DHF) to tetrahydrofolate (THF) in the body to be metabolically active in folate metabolism. Folate is distributed as 5-methyl THF in circulation. 5-methyl THF can donate a methyl group to the methylation of Hcy catalyzed by methionine synthase (MTR), producing THF and methionine. THF is subsequently converted to 5,10-methylene THF, which can donate a methyl group for thymidylate synthesis catalyzed by thymidylate synthase (TYMS), leaving DHF, which must be converted back to THF by DHFR. The remaining 5,10-methylene THF is reduced back to 5-methyl THF by methylenetetrahydrofolate reductase (MTHFR).

Figure II-1. One-carbon metabolism pathway



Substrates are in boxes, enzymes in ovals, and cofactors in triangles. Abbreviations: AHCY = *S*-adenosylhomocysteine hydrolase; BHMT = betaine hydroxymethyltransferase; CBS = cystathione β-synthase; CDP-choline = cytidine diphosphocholine; CHDH = choline dehydrogenase; DHFR = dihydrofolate reductase; DNMT = DNA methyltransferase; GSH = glutathione; Hcy = homocysteine; MAT1A= methionine adenosyltransferase 1A; MAT2A = methionine adenosyltransferase 2A; MAT2B = methionine adenosyltransferase 2B; MTA = methylthioadenosine; MTHFR = methylenetetrahydrofolate reductase; MTR = methionine synthase; MTRR = methionine synthase reductase; PEMT = phosphatidylethanolamine methyltransferase; SAM = *S*-adenosylmethionine; SAH = *S*-adenosylhomocysteine; SHMT = serine hydroxymethyltransferase; THF = tetrahydrofolate; TYMS = thymidylate synthase; dTMP = deoxythymidine monophosphate; dUMP = deoxyuridine monophosphate

Choline and betaine also function as methyl donors in the methylation of Hcy.

Choline and betaine are methyl-rich quaternary amines which are metabolically linked (1,

2). Choline is important for normal membrane function, acetylcholine synthesis and methyl group metabolism, and functions as a precursor of several important compounds including the phospholipid, phosphatidylcholine; the cholinergic neurotransmitter, acetylcholine; and the methyl group donor, betaine. The major metabolism of choline occurs in the cytidine diphosphate (CDP)-choline pathway in which choline is metabolized to phosphatidylcholine (Figure II-1). Otherwise, choline is converted to acetylcholine or oxidized to betaine catalyzed by choline dehydrogenase (CHDH). Betaine plays an important role as a methyl-group donor in the methylation of Hcy to methionine catalyzed by betaine homocysteine methyltransferase (BHMT) in the liver and other tissues. This reaction is an alternate pathway of the folate-dependent methylation of Hcy to methionine.

Choline and betaine can be obtained from diet. Choline exists in foods as free choline or as choline derivatives including phosphatidylcholine, phosphocholine, sphingomyelin, and glycerophosphocholine. Phosphatidylcholine is the primary form of choline in foods and in the body. Choline is rich mostly in animal-origin foods such as meat, liver, milk and eggs (3-5), but also acquired via the *de novo* biosynthesis from phosphatidylethanolamine catalyzed by phosphatidylethanolamine methyltransferase (PEMT). Similarly, the primary source of betaine is diet, but betaine can be formed endogenously from its precursor, choline. Betaine presents most abundantly in plant-origin foods such as cereals, bread and vegetables of the beet family including spinach,

chard, and beetroot (4-6). While choline can supply the requirements for both choline and betaine, betaine cannot serve as a substitute for choline.

Methionine is a sulfur-containing amino acid and a precursor of *S*-adenosylmethionine (SAM) that provides methyl groups for nearly all biochemical reactions, including the methylation of more than 80 molecules such as DNA, RNA, proteins, phospholipids, histones and neurotransmitters (7, 8). Methionine is not synthesized *de novo* in humans; hence it is derived from ingested methionine or methionine-containing proteins, which are rich in animal-origin foods. After releasing a methyl moiety, SAM becomes *S*-adenosylhomocysteine (SAH), and then Hcy. Hcy is remethylated back to methionine receiving methyl moieties from 5-methyl THF, a folate metabolite, or betaine, a choline derivative. Insufficient availability of folate, choline or betaine may cause an imbalance in the pool of methionine and SAM and, as a result, induce aberrant DNA methylation. Thus, blood Hcy level has been considered as an integrated biomarker of the availability of methyl groups in OCM (3, 9, 10).

B. Possible Mechanisms of OCM and Carcinogenesis

A large number of epidemiologic studies have investigated OCM in relation to cancer. However, our understanding of the biologic mechanism behind the association between OCM and carcinogenesis is still evolving. There are multiple possible mechanisms by which altered balance in OCM metabolites may lead to the development of cancer, including: 1) impaired DNA synthesis and DNA repair due to insufficient

supply of methyl moieties; 2) genome-wide DNA hypomethylation because of reduced availability of methyl groups for DNA methylation reactions; 3) alterations in the biosynthesis of the choline metabolite, phosphatidylcholine, leading to an alteration in DNA methylation patterns.

In this section, the two possible mechanisms that are relevant to this dissertation – 1) DNA synthesis and DNA repair and 2) global DNA methylation – are discussed.

B.1. DNA synthesis and DNA repair

Purine nucleotides are required for DNA synthesis. In the *de novo* biosynthesis of purine, 10-formyl-THF provides the methyl group, which is incorporated into the purine ring providing the adenine and guanine nucleotides. Altered balance in OCM metabolites may lead to insufficient supply of methyl groups for the *de novo* biosynthesis of purine and, in turn, impaired DNA synthesis and repair. In OCM, 5,10-methylene THF provides methyl units for the TYMS-mediated reductive methylation of deoxyuridine monophosphate (dUMP) to form deoxythymidine monophosphate (dTMP) (**Figure II-1**). This reaction represents the sole *de novo* source of the thymidine nucleotide (also known as a pyrimidine nucleotide) for DNA replication and repair (11).

Mutations generally arise either during DNA replication at the damaged site or during malfunctioning DNA repair (12). In normal cells, limited availability of folate depletes thymidylate resulting in an increase in uracil content of DNA and promotes the misincorporation of uracil into DNA, and subsequently various types of genetic

instability (including chromosomal instability, point mutations and DNA strand breaks) during DNA repair (13). Thus, folate deficiency may both increase the need for DNA repair and inhibit DNA repair capacity. Animal data showed increased susceptibility to intestinal tumorigenesis resulted from low folate status with increased dUMP/dTMP ratio, double-strand DNA strand breaks and other DNA damage (14).

On the other hand, once a neoplastic process is initiated, folate may accelerate DNA replication in cancer cells. For this reason, TYMS is a critical target of cancer chemotherapy drugs such as 5-fluorouracil (5-FU) in order to inhibit the activity of TYMS to block the DNA replication of cancer cells. The possible deleterious effect of folate in individuals who have already developed some preneoplastic lesions is a new, controversial issue.

B.2. DNA methylation

DNA methylation – epigenetic mechanism

DNA methylation is an important epigenetic mechanism in mammalians that is involved in the regulation of gene expression patterns, and forms the basis of chromatin structure, and thus plays an essential role in maintaining normal cellular function (15-18). Epigenetics refer to heritable changes in gene expression or cellular phenotypes caused by mechanisms other than changes in nucleotide sequence. Unlike genetics, epigenetic mechanisms can be modified by environmental factors and are reversible. DNA methylation involves the addition of a methyl group to the number 5 carbon of the

cytosine pyrimidine ring of DNA (5-methylcytosine, 5-mc). DNA methylation occurs almost exclusively in CpG sites regions where a cytocine and a guanine separated by only one phosphate, in adult somatic tissues. Between 60% and 90% of all CpG sites are methylated in mammals (19). The majority of CpG sites are normally dispersed in repetitive sequences, but some CpG sites are grouped in clustered CpG sites, known as 'CpG islands'. CpG islands are present in the promoter regions of many genes and usually unmethylated, whereas the more scattered CpG sites in repetitive sequences are generally methylated under normal conditions in humans.

Abnormalities in DNA methylation

Alterations in DNA methylation have been recognized as an important component of cancer development (20). In human cancers, abnormal hypermethylation in gene promoter CpG islands and genomic hypomethylation, which is hypomethylation in more dispersed CpG sites, are commonly observed. CpG island hypermethylation may result in transcriptional silencing of oncogene suppressors, while genomic hypomethylation may activate oncogenes. Genomic DNA hypomethylation, also known as global DNA hypomethylation, denotes the genome-wide decrease in overall content of 5-mc, but generally occurs outside promoters and in CpG depleted areas or in repetitive sequences (21). The abnormal hypermethylation of CpG islands in promoter regions has been most frequently implicated in carcinogenesis. Global DNA hypomethylation was largely ignored until recently, however, it is one of the early molecular abnormalities in cancer

development, and is linked to genetic instability (22), increased mutation rates (23), abnormal chromosomal structures (24) and activation of oncogenes (25, 26).

Global DNA hypomethylation particularly reflects changed methylation in repetitive DNA sequences rich in CpG sites, such as long interspersed nucleotide element-1 (LINE-1). LINE-1 is the most common family of retrotransposons in the human genomes and accounts for at least 17% of human DNA (27, 28). LINE-1 has been shown to be responsible for the overall loss of DNA methylation (29, 30). Although a change in cell regulation by DNA hypomethylation is small, considering that LINE-1 segments constitute a significant part of the genome, the magnitude of hypomethylation in LINE-1 could be critical to cell function.

Global DNA hypomethylation and cancer

Global DNA hypomethylation was first demonstrated in human cancer tissues by two laboratories independently in 1983 (20, 26). Subsequently, decreased global DNA methylation levels measured as LINE-1 or genomic 5-mc content were reported in many types of human cancer tissues – colon (25, 29, 31-36), stomach (29, 35, 37), breast (29, 38, 39), lung (29, 40), liver (29, 41), prostate (29, 42, 43), uterine cervix (44), head and neck (29, 45, 46), bladder (29, 47), urothelial (30, 47), and kidney (30). DNA methylation levels seem to be tissue-specific because they varied considerably among different organ tissues (19, 20, 29). Several studies have shown that DNA methylation levels in malignant tissues were not different from those in benign tumor tissues as well

as normal tissues surrounding malignant tissues (31, 34). These results indicate that DNA hypomethylation may be an early event in tumorgenesis and precedes malignancy. Because of its high incidence, global DNA hypomethylation has been suggested to be useful for the diagnostic purpose in certain cancers (41, 47).

Recently, the methylation level of LINE-1 in blood DNA has been used as a surrogate biomarker for global DNA methylation levels because it can be measured using peripheral blood samples, which can be collected less invasively in epidemiologic studies. In a series of case-control studies, decreased LINE-1 methylation in blood DNA compared to cancer-free subjects was observed in patients with cancer of colon and rectum, breast, bladder, liver, stomach, esophagus, prostate, and head and neck (29, 48-54). A recent prospective cohort study reported incidence of all cancers was significantly higher among individuals with low leukocyte LINE-1 methylation (defined as below the median) compared to those with high LINE-1 methylation, and increased in a dose-response manner across quartiles of LINE-1 methylation (55). Meanwhile, LINE-1 methylation was not associated with prevalence of all cancers in this study, indicating LINE-1 hypomethylation may precede cancer occurrence.

OCM and DNA methylation

DNA methylation involves the substrate, SAM and the enzyme, DNA methyltransferase (DNMT). When DNMT transfer a methyl group from SAM to DNA, SAM is converted to SAH, a competitive inhibitor of DNMTs. Therefore, the balance of

metabolites in OCM, such as the SAM/SAH ratio, might be an indicator of DNA methylation capacity (56).

To serve as the universal methyl donor, SAM acquires methyl groups from its precursor, methionine. Methionine can be remethylated back from Hcy requiring either folate or betaine, a choline derivative as a source of a methyl moiety. Therefore, SAM status depends on the availability of methyl groups from these nutrients and methionine. A large number of nutritional studies have shown that dietary methyl insufficiency causes a decrease in hepatic SAM levels and an increase in SAH content (57, 58).

Epidemiologic studies have shown that diet deficient in OCM nutrients, especially folate, was associated with an increased risk of cancer (59-61). Moreover, genetic variation involved in folate metabolism and the methylation cycle may affect the supply of methyl moieties. Indeed, some genetic variants have been associated with cancer risk as is described in section **C.3**. Furthermore, the availability of methyl groups for DNA methylation reactions may be determined by interactions between the status of OCM nutrients and genetic variation involved in OCM.

Folate and DNA methylation

The association between folate deficiency and DNA stability has been well-established *in vitro* (62, 63); however, results of human studies have been inconclusive. Several observational studies have explored associations between folate status and DNA methylation. Fenech *et al.* reported no significant correlations between red blood cell

(RBC) folate levels and lymphocyte DNA methylation in individuals with normal folate status (64). However, Pufulete *et al.* showed that DNA methylation in colon tissues was positively correlated with RBC folate levels in individuals with and without colon adenomas and cancer (33, 48).

Some intervention studies suggested that altered folate status can affect global DNA methylation in humans. Jacob *et al.* fed postmenopausal women a folate-deplete diet (55-111 μg/day of folate) for 9 weeks and reported a significant degree of lymphocyte genomic DNA hypomethylation (65). Rampersaud *et al.* also conducted a feeding study and showed a 10% decrease in global DNA methylation in peripheral blood in response to the 7-week folate-deplete diet (118 μg/day of folate) among elderly women aged 60 to 85 (66). Meanwhile, another feeding study using healthy younger males did not observe a change in DNA methylation in response to severe dietary restriction of folate and methyl groups (25 μg folate/day for 30 days) (67).

Folate supplementation studies in humans have shown restoration of global DNA methylation. An 8-week folate supplementation intervention with 15 mg/day methyltetrahydrofolate in men with pre-existing DNA hypomethylation in peripheral blood reversed hypomethylation to the normal level (68). In another study, a physiological dose of folate supplementation (400 μg/day folic acid) for 10 weeks increased genomic DNA methylation in peripheral blood by 31% (48). On the other hand, Fenech *et al.* reported that folate supplementation at 5 times of daily requirements did not change global DNA methylation in peripheral blood in healthy volunteers (64).

Although the existing data from human studies are not conclusive, these data indicate the potential for folate to modulate DNA methylation.

Gene-folate interaction on global DNA methylation

Global DNA methylation may decrease when the activity level of enzymes in OCM is low, and as a result, the aberrant transfer of methyl groups changes the balance in OCM metabolites. As is described in section **C.3**, some genetic variants have been shown to be associated with enzyme activity levels. Previous studies of genetic variation in OCM focused mainly on the polymorphisms of *MTHFR* because of the critical role of MTHFR in irreversible conversion of 5,10-methylene THF to 5-methyl THF. Several studies reported that DNA methylation in peripheral blood was diminished among individuals carrying the homozygous TT genotype of *MTHFR* 677, compared with those carrying the CC wild type genotype (69-71). In these studies, the correlation between RBC folate and DNA methylation was also observed. When examining concomitant folate status, the effect of the *MTHFR* 677 genetic polymorphism on DNA methylation was observed only in individuals with low folate status. These results indicate that the *MTHFR* C677T genetic polymorphism may influence DNA methylation through an interaction with folate status.

Gene-nutrient interactions affecting DNA methylation may explain different responses to dietary exposure at the molecular level. In other words, genetic variation may modulate the effect of dietary intake of OCM nutrients on DNA methylation and

cancer susceptibility. Thus, evaluating gene-nutrient interactions on DNA methylation is a crucial issue, not only to explain the mechanism of carcinogenesis, but also to identify high-risk individuals for targeted interventions such as lifestyle modification.

Choline, betaine and DNA methylation

Given their roles as methyl donors to the Hcy remethylation, choline and betaine have been investigated as Hcy lowering agents in several feeding studies. A 2-week high dose daily choline supplementation intervention lowered fasting and post-methionine-loading plasma Hcy concentrations by 18% and 29%, respectively, in healthy men with mild hyperhomocysteinemia (72). Similarly, a 6-week betaine supplementation intervention reduced fasting and post-methionine-loading plasma Hcy concentrations by up to 20% and 50% respectively, in healthy men and women (73). Furthermore, plasma Hcy concentrations were reduced only 6 hours after ingestion of high-choline or high-betaine diet (74).

Based on these study results, we may speculate that high intake of choline and betaine increases the synthesis of methionine and, in turn, decreases Hcy status. However, these studies have shown acute, not chronic, beneficial effects of dietary choline and betaine on plasma Hcy concentrations. Several epidemiologic studies have examined chronic effects of dietary choline and betaine on plasma Hcy levels among free-living individuals. In the Framingham Offspring Study, the Nurses' Health Study (NHS) and the Nurses' Health Study II (NHS II), dietary choline and betaine intakes were

inversely associated with plasma Hcy concentrations (3, 75). The inverse association was manifested in individuals with low folate intake. The strongest dose-dependent association was observed in individuals with high alcohol and low folate intake (75). These inverse associations between dietary choline and betaine intake and plasma Hcy concentrations may be due to the long-term effect of higher dietary intake of choline and betaine on decreasing plasma Hcy.

Deficient dietary choline has been associated with altered global and gene-specific DNA methylation in animals (76-79), but human studies have been scarce. One study by da Costa *et al.* observed increased DNA damage and apoptosis in peripheral lymphocytes in humans fed with a choline-deficient diet (80). Future studies are warranted in humans using better markers of DNA methylation capacity such as plasma SAM levels and the LINE-1 methylation index.

DNA methyltransferase (DNMT)

In addition to the availability of SAM as a substrate, interindividual variation in the enzyme activity of DNMTs may impact DNA methylation. In humans, five DNMTs, namely DNMT1, TRDMT1 (tRNA aspartic acid methyltransferase 1), DNMT3A, DNMT3B, and DNMT3L have been identified (81, 82). DNMT1 is the first recognized DNMT, which is a maintenance DNMT expressed in proliferating cells and somatic tissues. DNMT1 has been shown to prefer hemimethylated DNA over unmethylated DNA 15- to 40-fold *in vitro*, indicating its major function in copying the existing

parental-strand DNA methylation pattern onto the daughter strand after DNA replication (83, 84). It alone has little or no function in *de novo* DNA methylation. TRDMT1 was formerly known as DNMT2 because of its strong sequence similarities with 5-methylcytocine methyltransferases, but a recent study showed it does not methylate DNA (85). DNMT3 is a family of DNMTs that could methylate hemimethylated and unmethylated DNA at the same rate and has three members: DNMT3A, DNMT3B, and DNMT3L. DNMT3L has sequential similarities to DNMT3A and DNMT3B, but does not have enzymatic activity because it lacks some critical catalytic motifs. Therefore, DNMT3L appears incapable of methylation. Unlike DNMT1, DNMT3A and DNMT3B methylate DNA without preference for hemimethylated DNA, and thus function mainly in *de novo* methylation (86). DNMT3A and DNMT3B are highly expressed in embryonic cells, early embryos, and germ cells, where *de novo* DNA methylation occurs, but are downregulated in somatic tissues (82). In addition to establishing DNA methylation patterns in *de novo* methylation, DNMT3A and DNMT3B were also shown to play a role in the maintenance of DNA methylation in cooperation with DNMT1 in embryonic cells (87) or cooperation between DNMT3A and DNMT3B (88, 89). DNMT3A and DNMT3B are structurally and functionally similar, yet encoded by separate genes.

Animal studies demonstrated that reduced expression of *DNMT1* resulted in reduced global DNA methylation. Mice carrying a hypomorphic *DNMT1* allele had a reduced enzyme activity by 10%, showed substantial global DNA hypomethylation in all

tissues, and developed aggressive T-cell lymphomas (90). Another study showed that the mice with global DNA hypomethylation induced by a hypomorphic allele of *DNMT1* developed sarcomas at earlier age (91). In humans, mutation of *DNMT1* was observed in two of 29 colorectal cancer tissues, but not found in any stomach cancers and hepatocellular carcinomas (92). Meanwhile, overexpression of *DNMT1*, *DNMT3A* and *DNMT3B* may affect gene-specific DNA methylation and thus impact cancer development. Overexpression of *DNMT1*, *DNMT3A* and *DNMT3B* was reported in multiple types of cancers, but results have been inconclusive (93-104).

Existing data supporting the link between genetic variation in *DNMT*s and DNA methylation or cancer risk are still limited. A recent study examined associations between 22 common single nucleotide polymorphisms (SNPs) in *DNMT1*, *DNMT3A* and *DNMT3B* and breast cancer risk, but none of the SNPs was associated with breast cancer risk (105). A few studies suggested that genetic variants of *DNMT3B* may influence cancer susceptibility, although conflicting results were reported in different tumor types. A C→T SNP at the 149 base pair of the *DNMT3B* promoter (rs2424913) was associated with 30% increase in promoter activity and increased risk of lung cancer (106, 107) and breast cancer (108). Other studies have shown no associations with breast, head and neck, and gastric cancers (109-111). Other SNPs in the *DNMT3B* promoter (rs6058870 in the 283 base pair and rs1569686 in the 579 base pair) were associated with decreased risk of lung cancer and a variant allele of the rs6058870 SNP was associated with up to 50% decrease in promoter activity (107). One study showed a SNP at the 448 base pair

of the *DNMT3A* promoter (rs1550117) was associated with increased risk of gastric cancer but not esophageal cancer (112).

C. OCM and Breast Cancer – Epidemiologic Evidence

C.1. Overview of breast cancer

Excluding skin cancers, breast cancer is the most common cancer, and the second leading cause of cancer deaths among women in the United States. An estimated 230,480 invasive and 57,650 *in situ* incident breast cancer cases, and 39,970 breast cancer deaths are expected in 2011 (113). The survival rate has improved dramatically for the past few decades. The 5-year survival rate for U.S. women has improved from 63% in the early 1960's to 90% today. The 5-year survival rate for women diagnosed with localized breast cancer is 98%, whereas the counterpart for those spread to distant lymph nodes or organs is only 23%. Therefore, identifying risk factors and predictors of breast cancer is particularly important for primary and secondary prevention. Known potentially modifiable risk factors for breast cancer include adult weight gain, body fatness (for postmenopausal breast cancer), use of combined estrogen and progestin menopausal hormone therapy (MHT), physical inactivity, and consumption of one or more alcoholic beverages per day (113). To date, many epidemiologic studies have examined nutrients, metabolites, and/or genetic variation in OCM as potential modifiable risk factors or predictors of breast cancer.

C.2. OCM Nutrients and breast cancer

Folate

Among methyl-containing nutrients, folate is by far the most studied nutrient in relation to cancer including breast cancer. A protective effect of folate at the early stage of carcinogenesis has been shown by a number of animal and epidemiologic studies (60, 114). However, the association between folate intake and risk of breast cancer has been inconclusive among previous cohort and case-control studies. In large prospective cohort studies, folate intake was not significantly associated with breast cancer among premenopausal women in the NHS II (115), and among postmenopausal women in the American Cancer Society (ACS) Cancer Prevention Study II (116), the Iowa Women's Health Study (IWHS) (117), the Malmo Diet and Cancer Study (118), the Diet, Cancer and Health Study, Denmark (119), the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial (120) and the VITamine And Lifestyle (VITAL) cohort study (121). Only one large prospective study, the French European Prospective Investigation into Cancer and Nutrition (EPIC)-E3N study, reported that dietary folate intake was significantly inversely associated with postmenopausal breast cancer (122).

Table II-1 shows results of several meta-analyses that have examined the association between folate intake or status and breast cancer risk. As a whole, case-control studies have found a statistically significant inverse association between folate intake and breast cancer risk, whereas an inverse association was only marginally significant in cohort studies. Case-control studies are more prone to biases compared

Table II-1. Results of meta-analysis of folate intake or status and breast cancer risk

Author	Year	Exposure	Studies	Summary RR/OR (95%CI)
Larsson (61)	2007	Dietary folate intake	8 cohort	0.97 (0.88 – 1.07)
			13 case-control	0.80 (0.72 – 0.89)
			8 cohort	1.01 (0.97 – 1.05)
		Total folate intake	13 case-control	0.93 (0.81 – 1.07)
				By increments of 200μg/day
Lewis (123)	2006	Dietary folate intake	9 cohort	0.99 (0.98 – 1.01)
			13 case-control	0.91 (0.87 – 0.96)
				By increments of 100μg/day
Larsson (61)	2007	Blood folate levels	3 cohort	0.81 (0.59 – 1.10)
			2 case-control	0.41 (0.15 – 1.10)
				High vs. low blood folate levels

with cohort studies. Recall bias is particularly a concern, because folate intake is assessed after cancer diagnosis for cancer cases. Selection bias is another concern because cancer cases may be selected from surviving cancer patients. The need to use next-of-kin interviews for the deceased cases in case-control studies may also raise a problem. All of these biases may result in differential misclassification of folate exposure. The inconsistent study results of the association of dietary folate intake with breast cancer risk between case-control and cohort studies may in part be due to the issues related to a case-control study design.

Folate concentrations in blood have also been assessed in relation to breast cancer; however, results are inconsistent. In a meta-analysis of 3 cohort and 2 case-control studies, the association between blood levels of folate and risk of breast cancer was not statistically significant (61) (Table II-1).

Since the publication of these meta-analyses, two large prospective cohort studies, the PLCO Cancer Screening Trial (120) and the Cancer Prevention Study II Nutrition

Cohort (124), have reported an increased risk of breast cancer related to high total or dietary folate intake. On the other hand, a reduced risk of breast cancer with higher 10-year average total folate intake was reported in the VITAL cohort study (121). But, current folate intake was not associated with breast cancer risk in the same study. Results of these studies are summarized in **Table II-2**. As a whole, the impact of excessively high folate intake on breast cancer risk is still unclear.

Table II-2. Increased risk of breast cancer with high folate intake in previous cohort studies

Author	Year	Cohort	Subjects	Results [a]
Stolzenberg-Solomon (120)	2006	PLCO Cancer Screening Trial	25,400 postmenopausal women, 691 cases	Dietary folate intake: RR=1.19 (1.0 – 1.41) (highest vs. lowest quintile), *p* for trend = 0.04 Total folate intake: RR=1.32 (1.04 – 1.68) (highest vs. lowest quintile), *p* for trend = 0.03 Supplemental folic acid: RR=1.19 (1.01 – 1.41) (≥400μg/d vs. none), *p* for trend = 0.04
Maruti (121)	2009	VITAL	35,023 postmenopausal women, 743 cases	10 y average folate intake: RR=0.78 (0.61 – 0.99) (highest vs. lowest quartile), *p* for trend = 0.05 RR = 0.38 (0.18 – 0.80) (highest vs. lowest quartile), *p* for trend = 0.02 for estrogen-receptor negative cancers Current folate intake: RR=0.90 (0.72 – 1.12) (highest vs. lowest quartile), *p* for trend = 0.52
Stevens (124)	2010	Cancer Prevention Study II Nutrition Cohort	70,656 postmenopausal women, 3,898 cases	Dietary folate intake: RR=1.12 (1.01 – 1.24) (highest vs. lowest quintile), *p* for trend = 0.15 Total folate intake: RR=1.03 (0.93 – 1.15) (highest vs. lowest quintile), *p* for trend = 0.97

DFE = dietary folate equivalent

[a] 95% confidence intervals (CIs) are provided in parentheses

Choline and betaine

While numerous studies have examined the folate-cancer association, choline and betaine have been understudied in relation to cancer. There have been only a few studies on the association between dietary choline and betaine intake and cancer risk mainly because the dietary database of choline and betaine became available only recently. To date, three studies have investigated the association between dietary choline and betaine intake and breast cancer risk (115, 125, 126) (**Table II-3**). The inverse association was reported in a population-based case-control study (the Long Island Breast Cancer Study (LIBCSP)), whereas such an association was not observed in cohort studies (the NHS and the NHS II).

Table II-3. Previous studies on dietary intake of choline and betaine and breast cancer risk

Author	Year	Study design	Subjects	Results[a]
Xu (125)	2008	Population-based case-control LIBCSP	1,508 cases, 1,556 controls	**Choline:** OR=0.76 (0.58 – 1.00) (highest vs. lowest quintile), *p* for trend = 0.29 **Betaine:** OR=0.94 (0.69 – 1.30) (highest vs. lowest quintile), *p* for trend = 0.83
Cho (115)	2007	Cohort NHS II	90,663 premenopausal women, 1,032 cases	**Choline:** RR=0.88 (0.72 – 1.07) (highest vs. lowest quintile), *p* for trend = 0.26 **Betaine:** RR=0.99 (0.79 – 1.22) (highest vs. lowest quintile), *p* for trend = 0.88
Cho (126)	2010	Cohort NHS	74,584 postmenopausal women, 3,990 cases	**Choline:** RR=1.10 (0.99 – 1.22) (highest vs. lowest quintile), *p* for trend = 0.14 **Betaine:** RR=0.98 (0.89 – 1.09) (highest vs. lowest quintile), *p* for trend = 0.96

[a] Multivariate-adjusted OR or RR and 95% confidence intervals (CIs) in parentheses

C.3. Genetic variation in OCM and breast cancer

A growing number of epidemiologic studies on genetic variation in OCM and cancer risk indicate that genetic variation in OCM may affect cancer etiology. Key enzymes in OCM are illustrated in Figure II-1. Genetic variation in these enzymes may alter their enzymatic activity, resulting in the changed balance of OCM metabolites. Although an increasing number of genetic variants of these enzymes have been identified, the number of functional variants which were confirmed to influence transcription, translation, and/or function of resulting protein is limited. To date, most of previous studies have been on functional genetic variants of key enzymes in OCM. **Table II-4** summarizes associations between genetic variation in OCM and breast cancer risk. The most extensively studied genetic variants in relation to breast cancer include *MTHFR*, *MTR*, methionine synthase reductase (*MTRR*) and *TYMS*, all of which are involved in folate metabolism.

Two SNPs of *MTHFR*, 677 C→T (rs1801133) and 1298 A→C (rs1801131) are the most extensively studied genetic variants in OCM in relation to breast cancer risk. Both the *MTHFR* 677 C→T substitution and the 1298 A→C substitution result in a reduced enzymatic activity *in vitro* (127, 128). Individuals with the homozygous variant 677 TT genotype have only about 30% of normal enzymatic activity of MTHFR whereas heterozygotes with the 677 CT genotype have about 65% enzymatic activity as compared with 677 CC individuals (127). Individuals with the 1298 CC genotype have 60 – 70% of the activity level in 1298 AA individuals (23, 127, 129). The *MTHFR* 677C→T

Table II-4. Associations between genetic variation in OCM and breast cancer risk

Genes	Enzyme name	SNP/variant	rs number	Study	OR (95%CI) [a]	G-E Interaction[b]
MTHFR	5,10-methylene THF reductase	677C→T	rs1801133	Zintzaras (130)	1.07 (0.90 – 1.27)[c]	
				Lewis (123)	1.04 (0.94 – 1.16)[c]	
				Lissowska (131)	0.99 (0.86 – 1.15)[c]	
				Qi (132)	1.13 (1.02 – 1.26)[c]	
		1298A→C	rs1801131	Zintzaras (130)	1.00 (0.83 – 1.19)[c]	
				Lissowska (131)	1.00 (0.85 – 1.17)[c]	
				Qi (132)	1.01 (0.92 – 1.11)[c]	
MTR	Methionine synthase	2756A→G	rs1805087	Justenhoven (133)	0.95 (0.53 – 1.71)	
				Shrubsole (134)	0.8 (0.3 – 2.0)	Dietary folate/methionine/ Vit B_6 & B_{12}
				Xu (135)	0.88 (0.56 – 1.41)	
				Lissowska (131)	0.85 (0.62 – 1.16)	Dietary folate/methionine/ Vit B_2, B_6 & B_{12}, alcohol
				Cheng (136)	1.05 (0.77 – 1.43)	
				Suzuki (137)	1.35 (0.68 – 2.68)	Hormone exposure
MTRR	Methionine synthase reductase	66A→G	rs1801394	Shrubsole (134)	1.0 (0.7 – 1.4)	Dietary folate/methionine/ Vit B_6 & B_{12}
				Xu (135)	1.03 (0.80 – 1.32)	
				Lissowska (131)	1.06 (0.87 – 1.21)	
				Kotsopoulos (138)	1.02 (0.78 – 1.34)	
				Suzuki (137)	0.82 (0.52 – 1.27)	
TYMS	Thymidylate synthase	5'-UTR 28 bp tandem repeat	N/A	Zhai (139)	1.13 (0.61 – 2.10)	
				Suzuki (137)	1.49 (0.69 – 3.22)	Dietary folate
				Stevens (140)	Null (OR N/A)	
		3'-UTR 6 bp deletion	N/A	Justenhoven (133)	1.22 (0.81 – 1.85)	
				Zhai (139)	0.58 (0.35 – 0.97)	
DHFR	Dihydrofolate reductase	Intron 1 19 bp deletion	N/A	Xu (135)	1.18 (0.93 – 1.51)	Multivitamin use
				Xu (141)	1.15 (0.90 – 1.47)	
SHMT	Serine	1420 C→T	rs1979277	Xu (135)	0.79 (0.59 – 1.07)	

	hydroxymethyltransferase			Lissowska (131)	1.04 (0.84 – 1.28)	
				Cheng (136)	0.56 (0.39 – 0.80)	Hormone exposure
CBS	Cystathionine-β-synthase	233 C→T	rs234706	Lissowska (131)	1.0 (0.82 – 1.23)	
		360 C→T	rs1801181	Lissowska (131)	0.90 (0.75 – 1.08)	
				Stevens (140)	0.56 (0.35 – 0.91)	
BHMT	Betaine-homocysteine methyltransferase	742 G→A	rs3733890	Xu (135)	0.90 (0.67 – 1.20)	
CHDH	Choline dehydrogenase	432 G→T	rs12676	Xu (125)	1.05 (0.77 – 1.42)	Dietary choline/betaine/methionine
			rs9001	Xu (125)	0.99 (0.52 – 1.87)	Dietary choline/betaine/methionine

[a] Overall OR for homozygous variant vs. wild genotypes
[b] Gene-environmental interaction analysis included in the study
[c] Summary OR by meta-analysis

variant has also been related to circulating Hcy levels because MTHFR is the only producer of 5-methyl THF, which is required for the methylation of Hcy. Reduced MTHFR activity may increase Hcy concentrations via decreasing 5-methyl THF availability for Hcy methylation. A number of studies have reported elevated Hcy levels by up to 70% in 677 TT individuals compared with 677 CC individuals (142, 143).

The association between genotypes of *MTHFR* 677 C→T and 1298 A→C and breast cancer have been reported by a large number of studies. A recently reported meta-analysis indicates that the homozygous variant 677 TT genotype is associated with an increased risk of breast cancer (132). On the other hand, the 1298 A→C genotype was not associated with breast cancer risk in meta-analyses (130-132).

One nonsynonymous variant in exon 26 of *MTR*, the *MTR* 2756 A→G polymorphism (rs1805087) has been described. An association between this genetic variant and lower Hcy levels has been reported by a number of studies (144-146), but others reported no associations (147-150). A nonsynonymous mutation in exon 2 of *MTRR*, the *MTRR* 66 A→G polymorphism (rs1801392) has been described, but the functional impact of the variant has not been well defined (151). An association of the *MTRR* G variant and Hcy levels has been reported by a few studies (152, 153). Nonetheless, previous study results on the association between *MTR* 2756 A→G or *MTRR* 66 A→G polymorphisms and breast cancer risk have been mostly null.

Two genetic variants of *TYMS* – a polymorphic 28-bp tandem repeat in the 5'-UTR and a 6-bp deletion (1494del6) polymorphism in the 3'-UTR – have been described

in relation to cancer risk. A 28-bp tandem repeat functions as a transcriptional enhancer element. The triple repeat (3R) allele was associated with greater *TYMS* gene expression compared with the double repeat (2R) allele (154). A 6-bp deletion polymorphism has been associated with reduced mRNA stability (155). However, evidence of associations between these genetic variants and breast cancer risk is sparse with generally null associations (Table II-4). Genetic variation in other enzymes have been studied rather sporadically, and the observed associations have been mostly null.

As a whole, previously reported associations of genetic variation in OCM and breast cancer are mostly null. There are several possible reasons why a significant association was not observed if these genetic variants were actually associated with breast cancer risk. One possible reason is that the association of a single genetic variant with cancer risk is likely to be too weak to be detected even in a large cohort study. The other reason is possible interactions with other genetic variants and/or OCM nutrients. For example, the positive association between folate intake and breast cancer was observed among women carrying the *MTHFR* 677 CT or TT genotype along with 1298 AA genotype, whereas an inverse association was observed among those carrying both 677 CT and 1298 AC genotypes in the Malmo Diet and Cancer cohort (156). Because multiple nutrients and genes are involved in OCM, gene-nutrient, gene-gene and nutrient-nutrient interactions should be considered in a study of OCM and breast cancer risk. This dissertation built on existing studies examining OCM nutrients or genetic variation in OCM and cancer risk, but extended the analysis to potential gene-nutrient and nutrient-

environment interactions. In addition, few studies have inspected the link between genetic variation in the methylation cycle part of OCM and cancer development. In this dissertation, I examined the associations of genetic polymorphisms in the methylation cycle (and their interactions with the OCM nutrient or metabolite) with plasma SAM levels or global DNA methylation levels.

D. Gaps in the Current Literature Addressed in the Dissertation Projects

OCM has been extensively studied in relation to cancer development, yet there are various knowledge gaps in the currently available literature. The aim of this dissertation was to address these gaps to enhance our understanding of the biologic mechanisms behind the association between OCM and breast cancer. In this section, 2 areas of gaps addressed in this dissertation are discussed.

D.1. Human data on the link between OCM and global DNA methylation

Appropriate indicators for methylation capacity

The etiological background of the association between OCM nutrients and cancer risk is that deficient supply of methyl groups decreases SAM synthesis, leading to decreased global DNA methylation. Diet-related risk of chronic health conditions such as cancer is usually associated with habitual dietary patterns assessed using a food frequency questionnaire (FFQ), because these diseases develop gradually during a long period of time. To validate, values obtained from a FFQ are compared with those

obtained from other measurements such as dietary records or biomarkers. Biomarker measurements, however, may not be highly correlated with dietary intake data obtained from a FFQ or a dietary record because nutrient status in the body depends on many factors, only one of which is dietary intake. This is particularly true for choline and betaine. Choline can be obtained from diet but also from the *de novo* biosynthesis via the PEMT pathway. Betaine can come from diet, but it can also derive from choline. Therefore, dietary intake may not be a good marker of choline and betaine status. This may partly explain the null or inconsistent results of studies on dietary intake of choline and betaine and cancer risk. As an indicator of methylation capacity, assessing the status of all OCM nutrients may have potential, but is not practical. Plasma SAM levels and global DNA methylation might be potential candidates for an indicator of methylation capacity, but data supporting associations among OCM nutrient status, SAM status and global DNA methylation are lacking.

Lack of evidence on relationships between OCM nutrient status and plasma SAM levels

Plasma SAM levels and global DNA methylation might be potential candidates for an indicator of methylation capacity, but data on associations among OCM nutrient status, SAM status and global DNA methylation are lacking. Many *in vitro* studies showed that deficiencies of OCM nutrients, including folate, vitamin B_{12}, methionine and choline, result in depleted concentrations of hepatic SAM (57, 157). But the measurement of SAM in a single tissue does not represent the systematic status. Limited

existing data on the extent to which the status of OCM nutrients are associated with plasma SAM levels in humans are partly because, until recently, there was no reliable method to measure plasma SAM levels. Although a published method for the measurement of plasma SAM is now available (158), no study has yet determined the association between multiple OCM nutrient status and plasma SAM levels in human populations. Investigating the association between plasma levels of OCM nutrients and SAM would add the important evidence for cancer etiology.

Lack of evidence on the association between systematic SAM status and global DNA methylation.

Associations between OCM nutrients or metabolites and global DNA methylation have been studied in a few epidemiologic studies, but results are not conclusive. Deficiency of choline combined with folate, vitamin B_{12} and methionine as well as severe deficiency of folate have been shown to induce genomic DNA hypomethylation in animal studies (78, 159, 160). As for human data, genomic DNA methylation measured as LINE-1 methylation in normal colonic mucosal biopsies were not associated with folate supplementation, circulating level of B vitamins, circulating level of Hcy, alcohol use or selected genotypes as well as demographic characteristics such as age, gender and body mass index (BMI), cross-sectionally in the Aspirin/Folate Polyp Prevention Study (161). Similarly, a 13-month folic acid supplementation (0.8 mg/d) among moderately hyperhomocysteinemic subjects did not change global methylation in leukocyte DNA in a

randomized intervention study (162). Another human intervention study demonstrated that moderate folate deficiency, even for a prolonged period, did not induce global DNA hypomethylation (66). There are few data on associations of choline and betaine with global DNA hypomethylation in humans.

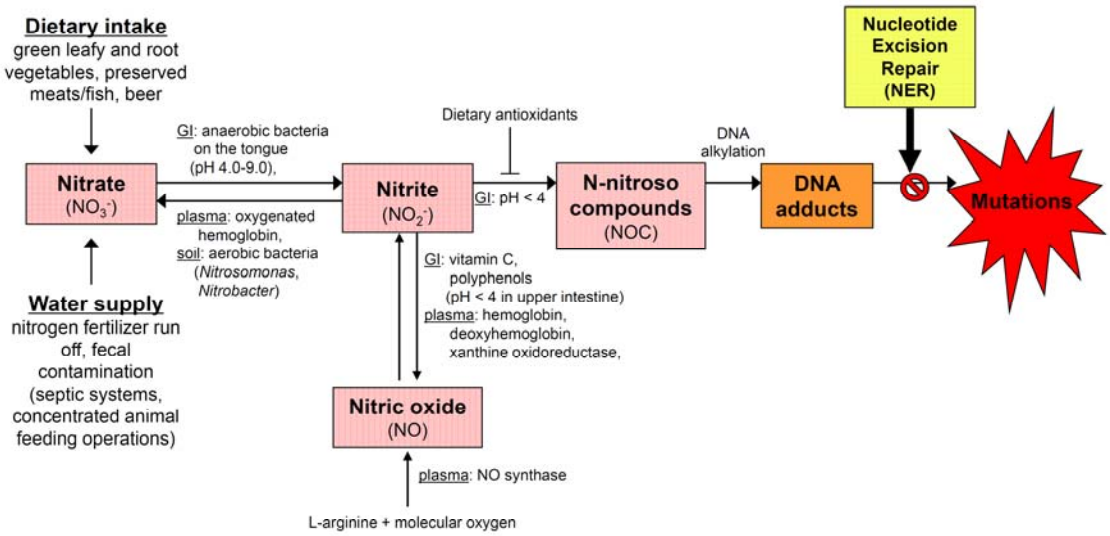
Plasma SAM levels might be a better indicator for methylation capacity than status of each OCM nutrient as mentioned earlier. An animal study showed that higher numbers of tumors in pre-neoplastic intestinal tissues were associated with elevated intracellular SAM levels as well as global DNA hypomethylation in tissue DNA in mice (163). However, very few studies have evaluated the association between circulating SAM levels and global DNA methylation in human.

D.2. Possible interactions between folate and environmental toxic exposures

Beneficial effects of folate on carcinogenesis have been well shown in animal studies, but epidemiologic study results of the association between folate intake and breast cancer have been inconclusive with lack of an association in most cohort studies (61, 123). There are multiple reasons which possibly attribute to the reported null associations in the previous studies, such as potential nondifferential misclassification of folate intake assessed using a FFQ. As one possible reason, I postulate that folate may interact with carcinogenic agents which coexist in dietary folate sources, such as nitrate.

Nitrate is present naturally in soils as a consequence of nitrogen fixation. Nitrates in soils are readily taken up by plant roots or migrate into groundwater. Therefore, our major sources of nitrate are vegetables and drinking water. Nitrate *per se* is not

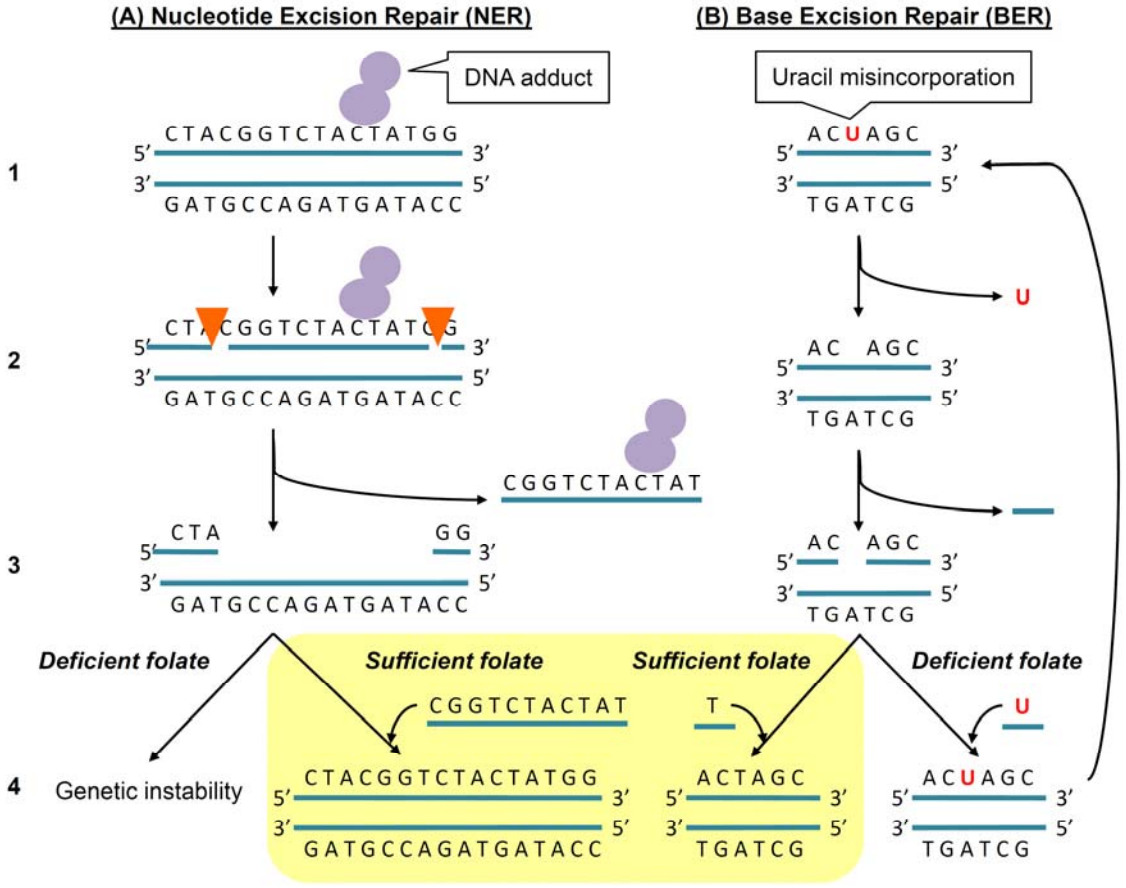
Figure II-2. Nitrate and carcinogenesis



carcinogenic but it can be reduced to nitrite by anaerobic bacteria in the oral cavity. In the stomach, nitrite subsequently nitrosates amines and amides ingested in foods to form *N*-nitroso compounds (NOCs), potent carcinogens in humans (164, 165). NOCs, such as nitrosoamines and nitrosoamides, modify DNA and form DNA adducts, a piece of DNA bounded to a carcinogenic chemical, and may lead to mutations (166) (**Figure II-2**).

DNA adducts can be removed by multiple DNA repair pathways. Nucleotide excision repair (NER) is one of the major repair pathways. NER system can repair many different types of changes in the structure of the DNA double helix by removal and repair of the entire portion of the bulky damaged strand like DNA adducts, while base excision repair (BER) system repairs a single damaged base such as uracil incorporation (**Figure II-3**). Thus, sufficient amounts of folate are essential to provide the necessary methyl group for normal synthesis and replication of nucleotides.

Figure II-3. Two major DNA repair pathways



A = adenine, T = thymine, G = guanine, C = cytosine

(A) Nucleotide excision repair (NER): 1. A bulky lesion of the DNA strand is damaged (such as DNA adduct). 2. The damaged bulky lesion is recognized by a multienzyme complex, and both sides of the lesion are cut. 3. DNA helicase removes the entire portion of the damaged strand. 4. With sufficient folate supply, the gap is filled with appropriate nucleotides by DNA polymerase and DNA ligase. Under the folate deficiency, the gap is not filled and leads to genetic instability such as DNA strand breaks.

(B) Base excision repair (BER): 1. A single base is damaged (such as misincorporated uracil instead of thymine under the folate deficient condition). 2. The damaged base is removed by uracil DNA glycosylase; 3. The sugar phosphate with the missing base is cut out by the sequential action of AP endonuclease and phosphodiesterase. 4. With sufficient folate, the gap is filled with the appropriate base by DNA polymerase and DNA ligase. Under the folate deficiency, a base damage occurs again and goes back into the BER system.

Recently, exposure to excess nitrate due to the contamination of soil with nitrogen-containing fertilizers as well as animal or human natural organic wastes has

become an increasing concern. The association between high dietary nitrate intake and cancer risk has been examined in humans but results have been inconclusive (167-170). It has been postulated that antioxidants contained in vegetables, such as vitamin C, E and polyphenols, may negate the deleterious effect of nitrate on cancer because antioxidants inhibit the formation of NOCs in the stomach (171-173). A case-control study of breast cancer in Korea recently reported dietary nitrate intake was not associated with breast cancer risk, but increased nitrate intake relative to folate intake, specifically the nitrate-folate ratio, was associated with elevated risk of breast cancer. In the same study, dietary nitrate intake was positively associated with risk of breast cancer only among women with low folate intake. This finding is biologically plausible considering a critical role of folate in the repair of DNA adducts. The protective effect of folate and the deleterious effect of nitrate on breast cancer risk cancel each other out, and thus become unobservable. Alternatively, folate may accelerate cancer progression in cells that have previously developed DNA mutations as a result of *N*-nitroso DNA adducts. The aforementioned Korean case-control study was the first study reporting an interaction between nitrate and folate on breast cancer risk, but it has several methodological issues including potential biases related to a case-control study design and a relatively small sample size. Therefore, this finding needs to be replicated in a prospective cohort study with a large study population.

E. Summary and Significance

Deficiencies in OCM nutrients and genetic variation in OCM may impact cancer risk through multiple mechanisms such as the aberrant synthesis and repair of DNA and global DNA hypomethylation. Although a number of epidemiologic studies have investigated the link between OCM nutrient deficiencies and/or genetic variation in OCM and risk of breast cancer in humans, evidence supporting the assumed mechanism between OCM and carcinogenesis is still lacking. Furthermore, there are gaps in existing evidence regarding relatively new issues related to folate intake and breast cancer risk including interactions of folate with environmental carcinogenic agents on cancer development.

The aim of this dissertation was to address the current knowledge gaps in etiological mechanisms of OCM and breast cancer in population-based studies. Specifically, in the first and second manuscripts, I evaluated 1) which OCM nutrients and genetic variation in OCM are associated with plasma levels of SAM, a universal methyl donors for many methylation reactions including DNA methylation, and 2) to what extent plasma SAM levels are associated with global DNA methylation in peripheral blood in a subset of healthy individuals within a large prospective cohort study of Singapore Chinese adults. In the third manuscript, I determined to what extent the association between nitrate intake from diet and drinking water and breast cancer risk is modified by folate intake in a large prospective study of postmenopausal women in Iowa. These projects contribute evidence to fill important gaps in the current literature on biologic

mechanisms between OCM and the development of cancer, especially breast cancer.

III. DATA SOURCES

A. The Singapore Chinese Health Study

A.1. Overview of cohort

The Singapore Chinese Health Study (SCHS) is a population-based, prospective cohort investigation to elucidate the role of diet and its interaction with genetic factors in risk of cancer. The study was initiated in 1993 and enrolled 63,257 participants (27,959 males, 35,298 females). The SCHS study cohort represents a population-based sample of ethnic Chinese volunteers who lived in Singapore. Eligibility criteria for cohort enrollment include: 1) ages 45 to 74 years; 2) the Cantonese or Hokkien dialect group; and 3) permanent residents or citizens of Singapore who resided in government-built housing estates (86% of the Singapore population lives in these facilities) (10). Enrollment took place between 1993 and 1998, at which time baseline information was obtained through in-person interviews. Eighty-five percent of eligible subjects participated in the study. The cohort participants have been followed for cancer incidence and vital status through linkage with the Singapore Cancer Registry and the Office of the Singapore Registry of Births and Deaths, respectively.

A.2. Baseline questionnaire data collection

At recruitment, study participants were interviewed in person in their home by a trained interviewer using a structured questionnaire. The questionnaire included demographic information, dietary intake, lifetime use of tobacco, current physical

activity, history of occupational exposures, medical history, a family history of cancer, reproductive history and hormone replacement therapy, and exposure of incense. The structured FFQ was developed based on 165 major categories of Singapore Chinese food and beverage items identified in the pilot study (174). In the FFQ, participants were asked to estimate their usual intake frequencies and portion sizes for each food and beverage item during the past 12 months. The FFQ was validated against a series of 24-hour diet recalls in over 1,000 cohort subjects (174). Correlation coefficients between FFQ- and 24-h recall-based intakes (energy-adjusted) were 0.24 – 0.72 for major nutrients and 0.36 – 0.69 for micro nutrients. During the pilot study, a food composition database of raw and cooked food items, specifically for Singapore Chinese food items was also developed. In 2008, dietary intake values of choline, betaine, methionine and flavonoids were added to the SCHS database by Nutrition Coordinating Center at the University of Minnesota.

A.3. Biospecimen sample collection

To establish a biospecimen subcohort, a 20-mL blood sample was collected from all consenting subjects from a 3% random sample of the cohort enrollee between 1994, a year after the cohort started, and 1999 (175). All of the blood specimen were separated into their various components (plasma, serum, buffy coat, and RBCs) immediately after sample collection. The specimens were dispensed in aliquots and subsequently stored in a liquid nitrogen tank at -180°C until 2001, when they were moved to a -80°C freezer for

long-term storage until used for testing or further processing. If the subject refused to donate blood, she/he was asked for buccal cells, which were collected through the use of a modified "mouthwash" protocol (176) and stored at -30°C.

The study subjects for the first and second projects of this dissertation were selected from 509 Singapore Chinese adults (221 men and 288 women) who donated blood samples as a random 3% sample of the SCHS cohort for the original biospecimen subcohort. **Table III-1** compares selected baseline characteristics between the whole cohort participants of the SCHS and 509 subjects from the biospecimen subcohort. The subcohort participants had somewhat higher education, lower rates of smoking and higher rate of alcohol intake than the overall SCHS participants. Otherwise, they were comparable to the whole SCHS cohort in terms of age, height, weight and body mass index (BMI).

Table III-1. Baseline characteristics of the Singapore Chinese Health Study cohort members and 509 subjects from the biospecimen subcohort

	SCHS		509 subjects	
	Males	Females	Males	Females
Total number of subjects	27,959	35,298	221	288
Mean Age at interview	56.7	56.3	56.4	55.2
Education < 12 years (%)	72.1	79.4	59.3	75.0
Mean height (cm)	165.2	154.8	165.5	155.6
Mean weight (kg)	63.0	55.8	62.4	55.5
Mean BMI (kg/m^2)	23.0	23.2	22.8	22.9
Ever smoker (%)	58.0	8.8	55.2	4.9
Current smoker (%)	36.2	6.3	29.9	4.2
≥ Weekly alcohol consumption (%)	20.5	4.4	30.8	6.6

B. The Iowa Women's Health Study

B.1. Overview of cohort design

The Iowa Women's Health Study (IWHS) is a prospective cohort study of cancer and other chronic diseases among women in Iowa, initiated in 1986. At baseline, a self-administered questionnaire was mailed to 99,826 women aged 55 to 69 who were randomly selected from the Iowa driver's license list. Of these women, 41,836 women (42%) who completed the baseline questionnaire consist of the IWHS cohort. Compared with respondents to the baseline questionnaire, non-respondents were slightly older (3 months) and heavier (0.38 kg/m^2) but otherwise comparable in terms of baseline demographic characteristics and life style factors (177).

Follow-up questionnaires were mailed to the cohort participants in 1987, 1989, 1992, 1997 and 2004 to update vital status, residence, and other exposure status. The corresponding response rates for the each follow-up survey are 91%, 89%, 83%, 79% and 68%, respectively. Data from follow-up surveys showed that the annual emigration rate for the IWHS cohort was less than 1%, which indicates a nearly complete follow-up (177). The vital status of non-responders to the follow-up questionnaires was identified by linkage with the State Health Registry of Iowa, supplemented by National Death Index of the National Center for Health Statistics. Approximately 99% of deaths among the IWHS cohort participants have been identified (178).

B.2. Data collection

At baseline, data on demographic characteristics, lifestyle, dietary intake, medical history and family history of cancers were collected using a self-administered questionnaire. BMI was calculated from self-reported height and current weight.

Dietary intake was assessed using the Harvard FFQ developed by Willett *et al.* at baseline and in 2004. Study participants were asked to report their usual intake frequencies of 127 food items during the past 12 months. There were nine frequency levels of each food item, ranging from "never or less than once per month" to "6 or more per day". To enable participants to obtain a sense of scale, a commonly used portion size for each food item was specified. Dietary nutrient intake was calculated by multiplying the frequency of consumption of the specified unit of each food by the nutrient content of that unit of food. The use of dietary supplements was also asked in FFQs. Micronutrient intake was computed with or without intake from dietary supplements. The Harvard FFQ has been studied for validity and reproducibility in the IWHS (179). The median correlations of energy-adjusted intake were 0.45 for macronutrients, 0.33 for micronutrients without supplements, and 0.64 for micronutrients with supplements.

B.3. Ascertainment of incident cancers

Incident cancer cases were identified through linkage with the State Health Registry of Iowa, a member of the National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) program via annual computer match. The SEER

program provides information on tumor size, extent, grade at diagnosis as well as cancer sites which are defined using International Classification of Diseases for Oncology (ICD-O) codes (180). As of December 31, 2008, a total of 9,821 primary cancers, including 2,992 breast cancers, were identified during the follow-up period.

IV. MANUSCRIPT 1: ONE-CARBON METABOLISM NUTRIENT STATUS AND PLASMA *S*-ADENOSYLMETHIONINE CONCENTRATIONS IN MIDDLE-AGED AND OLDER CHINESE IN SINGAPORE

S-adenosylmethionine (SAM) is a primary methyl donor for the methylation of many molecules including DNA. DNA methylation is believed to play an important role in functions of cells and genes. Dietary, genetic and metabolic factors that influence systematic SAM levels are not fully understood. We conducted cross-sectional analysis to evaluate associations between plasma concentrations of one-carbon metabolism nutrients and metabolites and plasma SAM concentrations using healthy individuals within the Singapore Chinese Health Study. Plasma SAM, betaine, choline, folate, total homocysteine (Hcy), methionine, *S*-adenosylhomocysteine (SAH), vitamin B_6 and vitamin B_{12} concentrations were quantified. Genotypes of methionine adenosyltransferases (*MAT1A*, *MAT2A* and *MAT2B*) were also determined. Linear regression and path analysis were performed to depict the directed dependencies in one-carbon metabolism. Age and body mass index were positively associated while cigarette smoking were inversely associated with plasma SAM concentrations. Plasma choline, methionine and SAH were positively and strongly associated with plasma SAM after adjustment for confounders. Plasma betaine and folate were positively associated with plasma SAM only in men. Men carrying the variant *MAT1A* genotypes had lower plasma SAM concentrations than men carrying the wild type genotype (*p* for gene x gender

interaction = 0.02). This effect modification by gender was restricted to individuals with low plasma methionine. In conclusion, plasma choline, methionine and SAH were strongly and positively associated with plasma SAM concentrations. The *MAT1A* genetic polymorphism may impact plasma SAM concentrations in men with low plasma methionine concentrations.

A. Introduction

S-adnosylmethionine (SAM) is a key metabolite in one-carbon metabolism (OCM) (**Figure IV-1**). OCM consists of two major pathways – folate metabolism and the methylation cycle – which are connected via the methylation of homocysteine (Hcy) to methionine. Hcy is a sulfhydryl-containing amino acid derived from the metabolic demethylation of methionine. It can be remethylated back to methionine, which is subsequently converted to SAM to maintain methyl group supply for methylation reactions.

SAM provides methyl groups for nearly all biochemical reactions, including methylation of more than 80 molecules such as DNA, RNA, proteins, histones and neurotransmitters (7, 8). After transfer of methyl groups to DNA methylation, SAM is converted to *S*-adenosylhomocysteine (SAH), a competitive inhibitor of DNA methylation (181). DNA methylation influences gene expression, DNA integrity and stability, chromosomal modifications and mutations (17). Aberrations in DNA methylation, at both gene-specific and global levels, play a crucial role in carcinogenesis

(20, 21). Global DNA hypomethylation has been associated with many types of cancer including bladder, breast, colon, esophagus, head and neck, liver, lung, prostate and stomach (29, 182), and observed in cancer tissues, benign tumor tissues and normal tissues surrounding cancer tissues, indicating that global DNA hypomethylation may be an early event in carcinogenesis (31, 34).

Methionine is a precursor of SAM and can be derived from diet as well as the remethylation of Hcy in which a methyl group is transferred from either 5-methyl tetrahydrofolate (THF) or betaine, a choline derivative (10, 73). In addition, vitamin B groups (vitamin B_2, B_6 and B_{12}) are cofactors for the remethylation of Hcy and other reactions (10). Therefore, the availability of SAM, as a methyl donor for DNA methylation, depends on the availability of these nutrients involved in OCM. Nutritional studies have shown that low dietary intake of OCM nutrients resulted in decreased levels of SAM and increased SAH contents in the liver (57, 58).

In addition to nutritional intake, interindividual variation in OCM enzyme activity may impact SAM availability. The biosynthesis of SAM from methionine is catalyzed by methionine adenosyltransferases (MATs). Three distinct forms of MATs have been identified: MATI, MAT II and MAT III (183). MAT I and MAT III are predominantly found in the liver and encoded by *MAT1A*. MAT II, encoded by *MAT2A* and *MAT2B*, can be found in the fetal liver and the tissues of many other organs. Differential expression of these genes has been shown to decrease hepatic and intracellular SAM concentrations and enhance global DNA hypomethylation and cancer cell growth *in vitro*

(184-186). In humans, deficiency of MATI and MATIII, caused by mutations in the *MAT1A* gene, was associated with persistent hypermethioninemia (187). A recent study showed that genetic polymorphisms of *MAT1A* were associated with DNA damage, but only among individuals with low plasma vitamin B_6 concentrations (188). However, human data on genetic variation in *MAT*s and plasma SAM concentrations are limited.

To date, human data describing associations between OCM nutrient/metabolite status and circulating SAM concentrations are limited. We therefore examined the associations between plasma concentrations of OCM nutrients and metabolites as well as *MAT* genotypes and plasma SAM concentrations among healthy adults.

B. Subjects and Methods

B.1. Study population

The study subjects were selected from participants of the Singapore Chinese Health Study (SCHS), a population-based prospective cohort study of the role of diet and its interaction with genetic factors in the risk of cancer and other chronic diseases. The detailed study design and subject recruitment of the SCHS have been described elsewhere (10). In brief, 63,257 Chinese men and women aged 45 – 74 years in Singapore were enrolled from April 1993 through December 1998. Eighty-five percent of eligible individuals participated in the study. At recruitment, information on demographic and lifestyle factors, medical history, family history of cancer and dietary intake was collected through in-person interviews. Usual dietary intake during the previous 12

months was assessed using a structured semi-quantitative food frequency questionnaire (FFQ) which was specifically designed to assess the dietary habits of Chinese in Singapore and validated through a series of 24-h dietary recalls (174).

We collected biospecimen from a 3% random sample of the cohort participants between 1994 and 1999. A 20-mL blood sample was collected from the cohort participants. Immediately after blood draw, blood tubes were transported from the homes of the subjects to the laboratory at the National University of Singapore, where plasma was separated from whole blood within approximately 4 hours after blood draw. Eight straws of plasma, each with approximately 0.5 mL, were created and stored in a liquid nitrogen tank at -180ºC until 2001, when they were moved to -80ºC freezers for long-term storage. All samples were kept on dry ice during shipment.

The subjects of the present study were selected from 509 men and women who donated their blood samples in 1994 – 1999. This study was approved by the Institutional Review Boards at the National University of Singapore and the University of Minnesota.

B.2. Laboratory measurements

Plasma folate, vitamin B_6 and B_{12} concentrations were determined at the National University of Singapore in 1996 – 1997. The methods for the assays were described previously (10). All other plasma biomarker determinations were performed by the Institute of Metabolic Disease at Baylor Research Institute in Dallas, Texas. Total

plasma homocysteine (Hcy) concentrations were determined by a rapid, isocratic high performance liquid chromatography (HPLC) coupled to a fluorescence detector (189). Plasma concentrations of betaine, choline, methionine, SAH and SAM were measured by a modified assay of the stable-isotope dilution liquid chromatography-electrospray injection tandem mass spectrometry (LC-ESI-MS/MS) previously described (190).

Calibrators and internal standards ($^2H_3$-SAM, $^2H_3$-choline, $^2H_3$-betaine, $^2H_3$-methionine) were included in each analytical run. One mM stock solutions of each standard were diluted in distilled water to perform a 5-point calibration curve over the following concentration ranges of 12.5 – 400 nmol/L of SAH and SAM and 5 – 80 µmol/L of betaine, choline and methionine, respectively. We utilized microcentrifugal filter units, Microcon YM-10, 10 kDa NMWL (Millipore, USA) for sample cleanup. Samples were prepared by the addition of 100 µL mobile phase A containing 10 – 50 µmol/L labeled-isotope internal standards to 30 µL of standard or plasma in microcentrifugal filter unit. Filter units were mixed by vortex and centrifuged for 20 min at 14800 x g at 4ºC. Sample filtrate was removed and transferred to autosampler vial for analysis. Five µl was injected into the LC-MS system, a Shimadzu Prominence LC System interfaced with a 4000 QTRAP® LC-MS/MS (AB Sciex LLC, Foster City, CA). Chromatographic separation was achieved on a 250 x 2.0 mm EZ-faast analytical column (Phenomenex) maintained at 33°C at a flow of 250 µL/min with a binary gradient with a total run time of 12 minutes. Solvents for HPLC were: (A) 4mM ammonium acetate, 0.1% formic acid, 0.1% heptafluorobutyric acid (pH=2.5); (B) 100% methanol and 0.1%

formic acid. The initial gradient condition was 75% A: 25% B and was increased in a linear fashion to 100%B in 6 minutes and held constant for 1 minute. At 7.1 minutes, the mobile phase was reset to initial conditions for 5 minutes. The flow from the column was delivered to the ESI source from the period of 3 to 8 minutes; otherwise the flow was diverted to waste. The compounds were detected by MRM using positive ESI with a dwell time of 30 milliseconds. The curtain gas was set at 15 L/min, and source gas 1 and 2 were set at 60 L/min. The heater was set to 700ºC with an ionspray voltage of 5000V and collisionally activated dissociation (CAD) gas (nitrogen) was set at "Medium". Analyte specific MRM transitions monitored declustering potentials (DP), entrance potential (EP), collision energy (CE) and collision exit potential (CXP).

Betaine, choline, methionine, SAH and SAM were resolved by a gradient to 100% methanol with retention times of 3.5, 5.4, 5.8, 6.6 and 7.0 minutes, respectively. The observed m/z values of the fragment ions were *m/z* 399→250 for SAM, *m/z* 385→136 for SAH, *m/z* 402→250 for $^{2}H_{3}$-SAM, *m/z* 150→104 for methionine, *m/z* 153→107 for $^{2}H_{3}$-methionine, *m/z* 104→45 for choline, *m/z* 108→49 for $^{2}H_{4}$-choline, *m/z* 118→59 for betaine, and *m/z* 121→61 for $^{2}H_{3}$-betaine. All data of analytes were collected using Analyst software version 1.4.2. (AB Sciex LLC, Foster City, CA).

The relative standard deviations (RSDs) for determinations of plasma betaine, choline, methionine, SAH and SAM concentrations, as determined by analysis of samples ten times across different days, were 6.3%, 6.9%, 6.1%, 10.4% and 9.2%, respectively.

B.3. SNP selection

We selected common single nucleotide polymorphisms (SNPs) of *MAT1A*, *MAT2A* and *MAT2B*. Given the relatively small study sample size, we selected common SNPs with minor allele frequency (MAF) ≥20%. Using data from the International HapMap Project (Tagger Pairwise method, HapMap Data Rel 27 Phase II + III, Feb09, on NCBI B36 assembly, dbSNP b126 for the Han Chinese (CHB) population), a total of 3 tagging SNPs and their proxies (in parentheses) were selected: rs2993763 and (rs4934028) for *MAT1A*, rs2289972 and (rs2028900) for *MAT2A*, and rs4869089 and (rs7733775) for *MAT2B*.

B.4. DNA extraction and genotype determinations

DNA extraction and genotype determinations were performed by the University of Minnesota BioMedical Genomics Center (BMGC). DNA was extracted from buffy coats using a Qiagen Kit (Qiagen Inc., Valencia, CA). Genotype determinations were performed in multiplex using the Sequenom MALDI-TOF mass spectrometry system (Sequenom Inc., San Diego, CA). Positive and negative controls were included in each 96-well plate. A tagging SNP of *MAT2B* (rs4869089) had a low call rate (<95%), and therefore its proxy SNP (rs7733775) was used in the analysis. In this study, we report a total of 3 SNPs – *MAT1A* rs2993763, *MAT2A* rs2289972 and *MAT2B* rs7733775. All 3 SNPs included in the analysis were in Hardy-Weinberg equilibrium ($p \geq 0.05$).

B.5. Statistical analysis

Of the 509 subjects, 14 subjects had missing values in plasma SAM concentrations and therefore were excluded. In addition, 19 subjects who had undetectable (n = 9) or extremely high values (n = 10) in all measured biomarkers, and 15 subjects who had missing (n=14) or extremely high values (n=1) in serum creatinine concentrations were excluded. After these exclusions, 461 subjects were included in the analysis.

The distributions of plasma concentrations of all measured OCM nutrients and metabolites and serum creatinine concentrations were markedly skewed with fewer subjects having high values; therefore, logarithmically transformed values were used when included in the models as covariates to correct the departure from the normal distribution. Plasma concentrations of OCM nutrients and metabolites were compared by gender using multiple linear regression modeling adjusting for age at blood draw and serum creatinine concentrations. Analysis of covariance (ANCOVA) was used to examine the associations between selected demographic, lifestyle and genetic characteristics and plasma SAM concentrations. To test the hypothesis that plasma concentrations of OCM nutrients (betaine, choline, folate, methionine, vitamin B_6 and B_{12}) and OCM metabolites (SAH and Hcy) are associated with plasma SAM concentrations, multiple linear regression modeling was used, adjusting for gender, age at blood draw, body mass index (BMI), smoking status (current, past and never) and serum creatinine concentrations. Geometric means and corresponding 95% confidence intervals

(CIs) of plasma SAM concentrations were presented for quartile groups of plasma OCM nutrient or metabolite concentrations. A trend across quartiles was tested using median values of quartile groups. To examine the associations between *MAT* genotypes and plasma SAM concentrations, multiple linear regression modeling was used with adjustment for age at blood draw, gender, BMI, smoking status, plasma methionine concentrations, and serum creatinine concentrations. To evaluate interactions with gender and/or plasma methionine concentrations, we included interaction terms in the models, and also performed stratified analysis by gender and plasma methionine concentrations (≤ or >the median (=23.4 μmol/L)).

To further depict the directed dependencies among a set of variables involved in the OCM pathway, we analyzed the data using a diagram-based approach of the path analysis (191). We separated the associations between plasma choline or folate and plasma SAM concentrations. In the choline-SAM pathway and the folate-SAM pathway, we further broke down the associations between plasma concentrations of choline or folate and plasma SAM concentrations into associations among intermediate molecules on each causal pathway. The final models for the choline-SAM pathway and the folate-SAM pathway were determined incorporating paths that showed statistically significant associations, controlling for age at blood draw, gender, BMI, smoking status and serum creatinine concentrations.

Linear regression analysis and path analysis were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC) and Mplus version 6.1 (Muthen & Muthen, Los

Angeles, CA), respectively. All reported *p* values were two-sided, and significance was defined as $p < 0.05$. This study had 80% power to detect 7.1 nmol/L (= 0.39 x standard deviation (SD)) difference in plasma SAM concentrations between the lowest and highest quartiles of plasma OCM nutrient or metabolite concentrations and 12.6 nmol/L (= 0.7 x SD) difference in plasma SAM concentrations between homozygous wild type genotype and homozygous variant genotype of *MAT*s assuming 20% MAFs.

C. Results

The study subjects consist of 204 men and 257 women. The mean age of study subjects was 58 y (SD, 7.8 y; range, 46 – 77 y) at blood draw. About 72% of the subjects had BMI below 24 and only 4% had BMI of 28 or above. While 41% of men had secondary school or higher education, 75% of women had no formal education or only primary school education. About 56% of men were previous or current smokers, whereas the majorities (95%) of women were never smokers. Alcohol intake was not common; 70% and 93% of men and women, respectively, reported no alcohol intake.

Table IV-1 shows geometric means of plasma SAM and other OCM nutrient/metabolite concentrations in men and women, separately. The median plasma SAM concentrations for all subjects was 63.3 nmol/L (interquartile range (IQR), 54.9 – 75.9 nmol/L). Serum creatinine concentrations were statistically significantly higher in men (geometric mean, 75.6 μmol/L; 95%CI, 73.6 – 77.7 μmol/L) than women (54.6 μmol/L; 53.3 – 55.9 μmol/L) ($p < 0.0001$). After adjustment for serum creatinine

concentrations and age at blood draw, plasma SAM concentrations were comparable for men and women. Plasma concentrations of betaine, choline, Hcy and methionine were statistically significantly higher in men than women, whereas plasma concentrations of folate, SAH and vitamins B_6 were higher in women than men. Plasma vitamin B_{12} concentrations did not differ by gender. Among OCM nutrients and metabolites, betaine, choline and methionine were positively correlated each other (Spearman partial correlation coefficients (r), 0.27 – 0.31; **Table IV-2**, supplemental table). Similarly, folate and vitamins B_6 and B_{12} were positively correlated each other (r = 0.16 – 0.31). Hcy was inversely correlated with folate, betaine, and vitamins B_6 and B_{12} (r = 0.12 – 0.32). Plasma SAM concentrations were positively correlated with plasma concentrations of choline, folate, methionine, SAH, and vitamins B_6 (r = 0.11 – 0.37).

Table IV-3 describes the associations between selected demographic, lifestyle and genetic characteristics of the study subjects and plasma SAM concentrations in men and women, separately. Higher plasma SAM concentrations were observed in women at older age and in men with high BMI. Current smokers had statistically significantly lower plasma SAM than never smokers in men. The difference in plasma SAM concentrations between current /past and never smokers in women was not statistically significant, most likely due to the very few smokers in women. Alcohol consumption and methylenetetrahydrofolate reductase (*MTHFR*) C677T genotype was not associated with plasma SAM concentrations.

Associations between plasma concentrations of each OCM nutrient or metabolite

and plasma SAM concentrations in men, women and all study subjects from linear regression analyses are shown in **Table IV-4**. Higher plasma concentrations of choline, methionine and SAH were statistically significantly associated with higher plasma SAM concentrations in men and women. Plasma betaine and folate were positively associated with plasma SAM concentrations in men, but not in women. The positive association between plasma vitamin B_6 and SAM was borderline significant in both men and women combined (p for trend = 0.046). Plasma concentrations of Hcy and vitamin B_{12} were not associated with plasma SAM concentrations in either men or women. We further examined the association between overall availability of methyl-containing nutrients in the choline (betaine)-mediated and the folate-mediated Hcy remethylation pathways and plasma SAM concentrations using a sum of scores for quartiles of plasma betaine, choline and methionine concentrations, or a sum of scores for quartiles of plasma folate and methionine concentrations, respectively: zero was assigned to the lowest quartile and 3 to the highest quartile of each biomarker. In the choline-mediated Hcy remethylation pathway, the geometric means of plasma SAM concentrations were 50.4 nmol/L (95%CI, 47.0 – 54.0) for subjects with the lowest summed score (0 to 1) and 70.0 nmol/L (95%CI, 65.5 – 74.7) for subjects with the highest summed score (8 to 9) in all subjects (p for trend <0.0001) (data not shown). Similarly, in the folate-mediated Hcy remethylation pathway, the geometric means of plasma SAM concentrations were 56.2 nmol/L (95%CI, 53.3 – 59.2) for those with the lowest score (0 to 1) and 71.7 nmol/L (95%CI, 67.7 – 75.9) for those with the highest score (5 to 6) in all subjects (p for trend <0.0001). A

similar dose-dependent positive association between the summed score and plasma SAM concentrations was observed in men and women in both pathways (p for trend <0.001).

We performed the path analysis for the choline (betaine)-mediated Hcy remethylation pathway for all subjects and the folate-mediated Hcy remethylation pathway in men and women separately, because the association between plasma folate and SAM was different in men and women in the regression analysis. Including plasma Hcy concentrations in the models did not change the results; therefore Hcy was not included in the final path models. Plasma folate was not statistically significantly associated with plasma SAM through the one-carbon metabolism or other unspecified mechanisms in men and women (data not shown). **Figure IV-2** graphically depicts a final path model for the choline (betaine)-mediated Hcy remethylation pathway. The total association between choline and SAM was statistically significant with a standardized path coefficient of 0.218 (p <0.0001). When associations among intermediate molecules were separated, the associations of choline with betaine, methionine or SAM, the association between betaine and methionine as well as the association between methionine and SAM were all statistically significantly positive. The direct path between betaine and SAM was not statistically significant and thus excluded in the final model. When separated, the association between choline and SAM through the OCM pathway and through unspecified mechanisms other than the OCM pathway were statistically significant; the standardized path coefficients were 0.105 and 0.114, respectively (p <0.01 for both). We did not find any modifying effect of folate in

the choline-SAM path model.

Overall, *MAT1A, MAT2A* and *MAT2B* genotypes were not associated with plasma SAM concentrations in both men and women combined (**Table IV-5**). However, the *MAT1A*-SAM association was modified by gender and plasma methionine concentrations. Plasma SAM concentrations were lower in subjects carrying the variant allele of *MAT1A* rs2993763 only in men (p for trend = 0.047, p for a gene x gender interaction = 0.02). When plasma methionine was below the median (23.4 μmol/L), the *MAT1A* genotype was marginally statistically significantly associated with plasma SAM concentrations in men (p for trend = 0.05); men with the *GA* or *AA* genotype had 13% and 14% decreases in plasma SAM, respectively, compared with men carrying the *GG* wild type genotype (**Table IV-6**). However, the *MAT1A* genotype was not associated with plasma SAM in men when plasma methionine concentrations were above the median. No such modulation of the *MAT1A*-SAM association was observed in women. There was no modifying effect by plasma methionine concentrations or gender on associations between the *MAT2A* or *MAT2B* genotypes and plasma SAM concentrations (data not shown).

D. Discussion

This is the first comprehensive study to examine the relationship between OCM nutrient and metabolite status and circulating SAM levels. Plasma choline, methionine and SAH were strongly positively associated with plasma SAM concentrations among

healthy Singapore Chinese men and women. Plasma folate and betaine concentrations were positively associated with plasma SAM concentrations only in men. Vitamin B_{12} and B_6 had no or minimal positive associations with plasma SAM. In OCM, nutrients and metabolites in the methylation cycle may play a more important role than those in the folate cycle in determining plasma SAM concentrations.

We observed higher concentrations of plasma SAM in women at the age of 60 or higher. The animal study showed a decrease in tissue SAM levels in aging rats compared to adult counterparts because of increased utilization of SAM due to elevated activity of the enzyme, catechol-*O*-methyltransferase (COMT) (192). On the other hand, Van Driel *et al.* reported lack of difference in plasma SAM concentrations by age among women of reproductive ages (20 – 48 y) (193). The mean age of the women in our study was 57.5 y, and about 84% of the women were postmenopausal at the cohort baseline. The differences in age and menopausal status may explain these conflicting results. We also observed a positive association between BMI and plasma SAM concentrations only in men, while Van Driel *et al.* reported a positive association between BMI and plasma SAM concentrations among premenopausal women (193). Future studies are required to unravel the association of age and BMI with plasma SAM concentrations.

SAM is synthesized from its precursor, methionine, via a process catalyzed by the MAT enzymes. In theory, the SAM pool can be affected by the availability of methionine and the activity level of MATs. Methionine is a sulfur-containing essential amino acid furnishing methyl groups and sulfur necessary for normal metabolism.

Methionine can be derived from dietary sources such as fish, meats and nuts, as well as from Hcy remethylation which requires either 5-methyl THF or betaine (choline). Therefore, insufficient availability of these nutrients or dietary methionine may lead to low methionine and SAM status. Although folate-mediated Hcy remethylation is traditionally considered as a major pathway of the remethylation of Hcy, it is estimated that approximately 60% of methyl groups are derived from choline in the liver (194). Therefore, choline and betaine may play a more important role than folate in providing methyl groups for methylation of DNA and other molecules. The choline (betaine)-mediated Hcy remethylation pathway may become even more dominant when the folate-mediated Hcy remethylation pathway is impaired because these two pathways are interrelated. This interrelationship between folate and choline has been demonstrated by the reduction of choline stores following the administration of the folate-deficient diet in animal and human studies (195-198). In the SCHS cohort, the average folate intake at baseline was 156 μg/day, which is significantly lower than the recommended daily intake (RDI) of 400 μg/day from the Institute of Medicine in the United States (199). The low dietary folate intake in our study population could be one of the reasons for weak or no associations between plasma folate and SAM concentrations in the present study. The low folate status might have enhanced the association between plasma choline and SAM in this population.

In adults, MAT I and MAT III, encoded by *MAT1A*, are found in the liver, whereas MATII is encoded by *MAT2A* and *MAT2B* which can be found in many organs

and tissues, including lymphocytes. Experimental studies showed that individuals with persistent hypermethioninemia had deficient MAT activity in the liver but normal MAT activity in red blood cells and lymphoid cells (200, 201), suggesting a critical role of hepatic MAT I and MAT III in methionine metabolism (185). Our findings of the association between the *MAT1* genotype and plasma SAM concentrations, especially in the condition of low methionine status, are consistent with those results. The lack of the association between the *MAT1A* genotype and plasma SAM, when methionine is sufficient, suggests that high plasma methionine concentrations may overcome the reduced MAT enzyme activity level in individuals carrying the *MAT1A* variant genotypes. Therefore, a sufficient supply of methionine might be important for the maintenance of an adequate methyl pool for methylation reactions.

Besides via the methionine-mediated OCM pathway, choline may play a critical role in determining plasma SAM concentrations via a non-methionine mediated OCM pathway. Choline can be acquired from diet, but also from the *de novo* biosynthesis via the methylation of phosphatidylethanolamine to phosphatidylcholine catalyzed by phosphatidylethanolamine methyltransferase (PEMT) (Figure 1). *De novo* choline biosynthesis might explain the lack of correlations between plasma concentrations of choline, betaine and methionine and dietary intake data in the current study (**Table IV-7**, supplemental table). Because PEMT consumes methyl groups from SAM to generate phosphatidylcholine, suboptimal choline intake may enhance the *de novo* biosynthesis of choline via the PEMT pathway that could reduce SAM concentrations. In the present

study, we found a statistically significant positive association between plasma choline and SAM independent of the methionine-mediated OCM pathway. We speculate that this choline-SAM association via the non-methionine-mediated pathway may be in part through the choline biosynthesis pathway and may explain a stronger association of plasma choline than betaine with plasma SAM concentrations in the present study. Further studies are warranted to investigate this choline-SAM association.

After a methyl group is transferred to DNA catalyzed by DNA methyltransferases (DNMTs), SAM is converted to SAH, which is a competitive inhibitor of DNMTs. As expected, plasma SAH concentrations were statistically significantly positively associated with plasma SAM in the present study. SAH is subsequently converted to Hcy. We previously reported inverse associations between plasma Hcy and plasma folate, vitamin B_6 and B_{12} in this Singapore Chinese population (10). In the present study, plasma betaine concentrations were inversely associated with plasma Hcy but plasma choline was not (Supplemental Table 1). Hcy status is traditionally used as an indicator of OCM function in studies of health outcomes such as cardiovascular diseases, but health effects of Hcy on carcinogenesis are still not clear. In the present study, plasma Hcy concentrations were not associated with plasma SAM concentrations. Our result is consistent with the previous study reporting that plasma Hcy concentrations were highly correlated with plasma SAH, but not with plasma SAM among healthy adult females (202). Lack of a correlation between plasma Hcy and SAM in the presnt study may be due to the fact that our study subjects were healthy individuals with normal

plasma Hcy concentrations. We may have observed a higher correlation between Hcy and SAM, if our study had included hyperhomocysteinemic individuals.

The present study is novel in that existing human data describing associations between OCM nutrient status and systematic SAM status are limited. The simultaneous measurement of major compounds in the OCM pathway including SAM, SAH, Hcy, methionine, choline, betaine, folate, vitamins B_6 and B_{12} allowed the comprehensive analysis for their effects on SAM. The inclusion of genetic variation in *MAT* sheds lights on the methionine-SAM association. In addition, the path analysis based on the understanding of the biological function of these OCM metabolites provides further insight on the OCM pathway in humans. Although the present study is a cross-sectional study, which does not allow us to determine cause-effect relationships, it would be extremely difficult to disentangle complicated pathways involving multiple molecules in OCM in an intervention study.

One concern of the present study is the possible degradation of SAM in stored plasma samples over time. The median plasma SAM concentrations in the present study (63.3 nmol/L) was somewhat lower than those found in populations in Europe (70 to 128 nmol/L) (193). However, the wide range of plasma SAM in various studies (50 to 150 nmol/L) might be due to different study populations and different laboratory methods (158, 190, 202-206). It should be noted that a reliable method to measure plasma SAM concentrations became only recently available. Furthermore, previously reported plasma SAM concentrations were mainly in whites; no reference data are available for Asian

populations. In the present study population, we measured plasma Hcy concentrations in the same subjects at two different times in two different laboratories; one at the National University of Singapore in 1996 – 1997 and the other at Baylor Research Institute in 2010. High correlation between the two Hcy measurements ($r = 0.70$) indicates that the degradation of Hcy, and possibly SAM, might have been minimal during prolonged storage. Furthermore, degradation of plasma SAM would not result in the observed associations, given that it occurred in a non-differential manner.

In summary, the present study demonstrates that plasma choline, methionine and SAH were strongly associated with plasma SAM concentrations. Plasma choline may be associated with plasma SAM through OCM (via betaine and methionine), but also through unspecified mechanisms other than OCM. The common genetic variant of *MAT1A* showed interactions with gender and plasma methionine on plasma SAM concentrations. Improving plasma methionine concentrations may increase plasma SAM concentrations in a subset of a population, depending on this *MAT1A* genotype and gender. Future studies are warranted to confirm our findings.

E. Tables

Table IV-1. Geometric means and 95% confidence intervals (CIs)[a] of plasma one-carbon metabolism nutrients and metabolites[b] by gender

	Men (n = 204)	Women (n = 257)	*p*
SAM (nmol/L)	63.7 (61.1 – 66.4)	64.8 (61.6 – 66.2)	0.95
Betaine (μmol/L)	52.1 (49.9 – 54.4)	44.5 (42.9 – 46.2)	<0.0001
Choline (μmol/L)	12.9 (12.4 – 13.4)	11.9 (11.5 – 12.3)	0.006
Folate (nmol/L)	12.9 (12.0 – 13.8)	14.6 (13.8 – 15.5)	0.02
Hcy (μmol/L)	10.2 (9.7 – 10.6)	9.3 (9.0 – 9.7)	0.009
Methionine (μmol/L)	25.0 (23.9 – 26.1)	22.6 (21.7 – 23.4)	0.002
SAH (nmol/L)	26.5 (25.2 – 28.0)	29.2 (27.9 – 30.6)	0.02
Vitamin B_6 (nmol/L)	29.4 (26.5 – 32.6)	36.2 (33.1 – 39.6)	0.009
Vitamin B_{12} (pmol/L)	313.5 (294.8 – 333.5)	336.4 (318.9 – 354.8)	0.13

[a] Adjusted for age at blood draw and serum creatinine levels.
[b] One-carbon metabolism metabolites: SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; Hcy, total homocysteine.

Table IV-2. Spearman partial correlation coefficients (r) among plasma concentrations of OCM nutrients and metabolites, adjusted for age at blood draw, gender and serum creatinine concentrations

	Folate	Choline	Betaine	Methionine	Vitamin B_6	Vitamin B_{12}	SAM	SAH	Hcy
Folate	1								
Choline	0.027	1							
Betaine	0.069	0.271*	1						
Methionine	0.075	0.309*	0.215*	1					
Vitamin B_6	0.313*	-0.049	0.059	0.130*	1				
Vitamin B_{12}	0.161*	0.029	-0.027	0.064	0.204*	1			
SAM	0.126*	0.215*	0.054	0.373*	0.109*	0.054	1		
SAH	0.044	0.298*	0.085	0.095*	-0.102*	0.053	0.233*	1	
Hcy	-0.322*	0.035	-0.119*	-0.045	-0.231*	-0.183*	-0.008	0.217*	1

* Statistically significant (p <0.05)

Table IV-3. Plasma SAM concentrations in men and women by selected demographic, lifestyle and genetic factors

	Men (n = 204)			Women (n = 257)		
	n	Geometric mean (95%CI) (nmol/L)[a]	p_{trend}	n	Geometric mean (95%CI) (nmol/L)[a]	p_{trend}
Age at blood draw						
<50	29	64.9 (59.1 – 71.3)	0.17	43	59.7 (55.3 – 64.5)	0.02
50 – 59	86	67.0 (63.5 – 70.8)		126	58.2 (55.6 – 60.8)	
60 – 69	72	67.2 (63.3 – 71.3)		58	68.0 (63.7 – 72.6)	
≥70	17	74.3 (65.8 – 84.1)		30	63.1 (57.4 – 69.4)	
BMI						
<20	27	63.1 (57.2 – 69.5)	0.02	41	59.0 (54.5 – 63.9)	0.21
20 – <24	119	66.5 (63.5 – 69.6)		147	60.7 (58.3 – 63.3)	
24 – <28	50	71.0 (66.2 – 76.3)		58	63.3 (59.3 – 67.7)	
≥28	8	73.5 (61.5 – 87.8)		11	62.0 (53.2 – 72.3)	
Level of education						
No formal school	26	68.8 (62.0 – 76.4)	0.26	96	60.2 (57.0 – 63.6)	0.59
Primary school	94	68.2 (64.7 – 71.9)		97	61.9 (58.8 – 65.2)	
Secondary school	58	67.0 (62.6 – 71.7)		54	60.4 (56.3 – 64.8)	
Some college or above	26	63.6 (57.4 – 70.5)		10	65.2 (55.5 – 76.6)	
Smoking status						
Never	90	70.9 (67.2 – 74.8)	0.004	243	61.2 (59.2 – 63.2)	0.75
Past	53	67.2 (62.6 – 72.1)		2	56.5 (39.4 – 80.9)	
Current	61	62.6 (58.8 – 66.7)		12	60.0 (51.7 – 69.7)	
Alcohol intake						
None	143	66.7 (63.9 – 69.6)	0.32	239	60.8 (58.9 – 62.9)	0.42
<7 drinks/week	45	68.3 (63.3 – 73.7)		14	65.6 (57.2 – 75.2)	
≥7 drinks/week	16	71.1 (62.6 – 80.7)		4	62.1 (48.2 – 80.0)	

Table IV-3 (cont.)

	Men (n = 204)			Women (n = 257)		
	n	Geometric mean (95%CI) (nmol/L)[a]	p_{trend}	n	Geometric mean (95%CI) (nmol/L)[a]	p_{trend}
MTHFR C677T						
CC	117	67.5 (64.4 – 70.8)	0.82	139	59.9 (57.4 – 62.5)	0.13
CT	69	67.7 (63.6 – 72.0)		89	61.5 (58.3 – 64.9)	
TT	15	65.7 (57.6 – 75.0)		22	65.7 (58.9 – 73.2)	

[a] Adjusted for age at blood draw and serum creatinine levels.

Table IV-4. Geometric means and 95% confidence intervals (CIs)[a] of plasma SAM concentrations (nmol/L) by quartiles of one-carbon metabolism nutrients and metabolites[b]

	Quartiles of plasma concentrations				p_{trend}
	1 (lowest)	2	3	4 (highest)	
Betaine					
Men	63.0 (58.8 – 67.6)	65.9 (61.5 – 70.8)	67.7 (63.0 – 72.6)	70.9 (66.2 – 76.0)	0.02
Women	56.2 (48.9 – 64.6)	59.7 (51.7 – 69.0)	57.5 (50.0 – 66.1)	61.5 (53.2 – 71.0)	0.08
All subjects	60.7 (57.5 – 64.0)	60.2 (57.2 – 63.5)	61.6 (58.3 – 65.0)	65.1 (61.7 – 68.7)	0.03
Choline					
Men	59.5 (55.7 – 63.5)	63.4 (59.1 – 68.0)	74.0 (69.4 – 78.9)	70.7 (66.1 – 75.6)	<0.0001
Women	55.8 (48.5 – 64.2)	56.8 (49.3 – 65.4)	60.1 (52.3 – 69.1)	61.3 (53.0 – 71.0)	0.02
All subjects	57.5 (54.5 – 60.6)	59.3 (56.4 – 62.4)	65.8 (62.3 – 69.4)	65.4 (62.1 – 68.9)	<0.0001
Folate					
Men	64.8 (60.5 – 69.5)	63.8 (59.5 – 68.4)	67.1 (62.5 – 72.0)	72.1 (67.1 – 77.4)	0.02
Women	56.8 (49.2 – 65.6)	57.0 (49.5 – 65.7)	57.7 (49.8 – 66.9)	59.7 (51.9 – 68.8)	0.24
All subjects	60.2 (57.2 – 63.4)	61.1 (57.8 – 64.5)	61.8 (58.6 – 65.1)	64.8 (61.3 – 68.5)	0.03
Hcy					
Men	67.5 (62.8 – 72.4)	67.9 (63.4 – 72.6)	65.5 (61.2 – 70.0)	67.4 (62.7 – 72.5)	0.87
Women	57.8 (49.8 – 67.0)	55.8 (48.3 – 64.5)	59.3 (51.7 – 67.9)	57.2 (49.3 – 66.4)	0.92
All subjects	62.3 (58.9 – 65.9)	60.4 (57.3 – 63.7)	65.0 (61.6 – 68.6)	56.9 (56.9 – 63.4)	0.56
Methionine					
Men	56.9 (53.4 – 60.6)	64.1 (60.1 – 68.3)	71.5 (67.3 – 76.0)	77.1 (72.3 – 82.2)	<0.0001
Women	54.6 (48.0 – 62.2)	57.2 (49.7 – 65.8)	64.0 (55.7 – 73.5)	67.0 (58.2 – 77.2)	<0.0001
All subjects	55.5 (52.9 – 58.2)	59.0 (56.1 – 62.1)	65.9 (62.7 – 69.3)	70.8 (67.3 – 74.6)	<0.0001
SAH					
Men	60.7 (56.4 – 65.2)	63.9 (59.7 – 68.5)	72.9 (68.1 – 78.1)	70.3 (65.3 – 75.6)	0.006
Women	52.7 (45.9 – 60.5)	59.1 (51.3 – 67.9)	61.9 (53.8 – 71.0)	60.7 (52.6 – 69.9)	0.005
All subjects	55.7 (52.8 – 58.7)	61.3 (58.3 – 64.5)	65.6 (62.2 – 69.1)	65.2 (61.7 – 68.8)	<0.0001

Table IV-4 (cont).

	Quartiles of plasma concentrations				p_{trend}
	1 (lowest)	2	3	4 (highest)	
Vitamin B_6					
Men	67.1 (62.5 – 72.1)	65.7 (61.3 – 70.3)	63.5 (59.3 – 68.1)	71.5 (66.5 – 76.9)	0.17
Women	57.3 (49.7 – 66.2)	55.2 (47.8 – 63.7)	59.1 (51.4 – 68.0)	59.6 (51.7 – 68.6)	0.19
All subjects	61.8 (58.8 – 65.1)	59.5 (56.4 – 62.8)	61.7 (58.4 – 65.1)	64.9 (61.4 – 68.6)	0.046
Vitamin B_{12}					
Men	68.3 (63.6 – 73.3)	64.0 (59.8 – 68.5)	65.4 (61.0 – 70.0)	70.3 (65.4 – 75.6)	0.41
Women	57.0 (49.2 – 66.0)	58.2 (50.4 – 67.2)	57.3 (49.8 – 66.0)	58.8 (51.1 – 67.7)	0.56
All subjects	60.9 (57.7 – 64.2)	61.0 (57.9 – 64.3)	62.1 (58.9 – 65.5)	63.5 (60.1 – 67.1)	0.19

[a] Adjusted for age at blood draw, BMI, smoking status, serum creatinine levels and gender (for all subjects).
[b] One-carbon metabolism metabolites: SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; Hcy, total homocysteine.

Table IV-5. Plasma SAM concentrations (nmol/L) by genotypes of *MAT1A*, *MAT2A* and *MAT2B*

	All subjects		Men		Women		
	n[a]	Geometric means (95%CIs)[b]	n[a]	Geometric means (95%CIs)[b]	n[a]	Geometric means (95%CIs)[b]	$p_{interaction}$[c]
MAT1A rs2993763							
GG	134	62.2 (59.4 – 65.1)	57	69.4 (65.5 – 73.4)	77	58.9 (51.7 – 67.2)	0.02
GA	230	63.0 (60.7 – 65.3)	102	67.2 (64.4 – 70.1)	128	61.6 (54.3 – 70.1)	
AA	94	61.8 (58.6 – 65.1)	44	63.5 (59.5 – 67.8)	50	63.5 (59.5 – 67.8)	
p_{trend}		0.88		0.047		0.17	
MAT2A rs2289972							
GG	228	62.7 (60.3 – 65.1)	100	67.4 (64.5 – 70.5)	128	61.3 (56.9 – 66.1)	0.72
GA	174	62.0 (59.5 – 64.6)	83	65.9 (62.8 – 69.1)	91	61.4 (56.6 – 66.7)	
AA	43	61.7 (57.2 – 66.4)	14	67.0 (59.5 – 75.5)	29	51.1 (54.6 – 68.3)	
p_{trend}		0.59		0.63		0.96	
MAT2B rs7733775							
GG	324	62.8 (60.7 – 65.0)	143	68.0 (65.6 – 70.6)	181	60.5 (53.4 – 68.6)	0.22
GA	118	62.6 (59.7 – 65.5)	53	65.0 (61.3 – 68.9)	65	61.8 (54.0 – 70.8)	
AA	10	62.6 (54.1 – 72.4)	5	63.1 (51.9 – 76.6)	5	62.7 (49.2 – 79.8)	
p_{trend}		0.87		0.16		0.51	

[a] Subjects with missing data on a genotype of each SNP were excluded from an analysis.
[b] Adjusted for age at blood draw, BMI, smoking status, serum creatinine levels, plasma methionine levels and gender (for all subjects).
[c] *p* value for an interaction between *DNMT* genotypes and gender.

Table IV-6. Geometric means and 95% confidence intervals (CIs) of plasma SAM concentrations and genotypes of *MAT1A* rs2993763 by plasma methionine concentrations and gender

	Plasma methionine ≤23.4 μmol/L		Plasma methionine >23.4 μmol/L		
	n[a]	Geometric means (nmol/L)[b]	n[a]	Geometric means (nmol/L)[b]	*p*
Men					
GG	23	65.3 (59.5 – 71.6)	34	71.7 (66.4 – 77.5)	0.13
GA	40	57.0 (53.1 – 61.2)	62	74.9 (70.8 – 79.3)	<0.0001
AA	19	56.1 (50.6 – 62.3)	25	68.8 (62.9 – 75.4)	0.004
p_{trend}		0.05		0.66	0.18[c]
Women					
GG	45	54.0 (47.0 – 62.1)	32	62.9 (53.9 – 73.5)	0.009
GA	75	57.0 (49.9 – 65.0)	53	66.2 (57.4 – 76.4)	0.001
AA	28	57.4 (49.1 – 67.0)	22	68.1 (57.9 – 80.1)	0.02
p_{trend}		0.35		0.10	0.86[c]
$p_{interaction}$		0.06[d]		0.14[d]	0.48[e]
All subjects					
GG	68	57.5 (54.0 – 61.1)	66	66.1 (62.0 – 70.5)	0.001
GA	115	57.0 (54.3 – 59.8)	115	69.2 (65.9 – 72.7)	<0.0001
AA	47	56.5 (52.5 – 60.8)	47	67.4 (62.6 – 72.5)	0.0005
p_{trend}		0.85		0.50	0.51[c]

[a] Subjects with missing data on a genotype of each SNP were excluded from an analysis.
[b] Adjusted for age at blood draw, BMI, smoking status, serum creatinine levels and gender (for all subjects).
[c] *p* value for an interaction between the *MAT1A* genotype and plasma methionine.
[d] *p* value for an interaction between the *MAT1A* genotype and gender within plasma methionine groups.
[e] *p* value for an interaction among the *MAT1A* genotype, plasma methionine, and gender in all subjects.

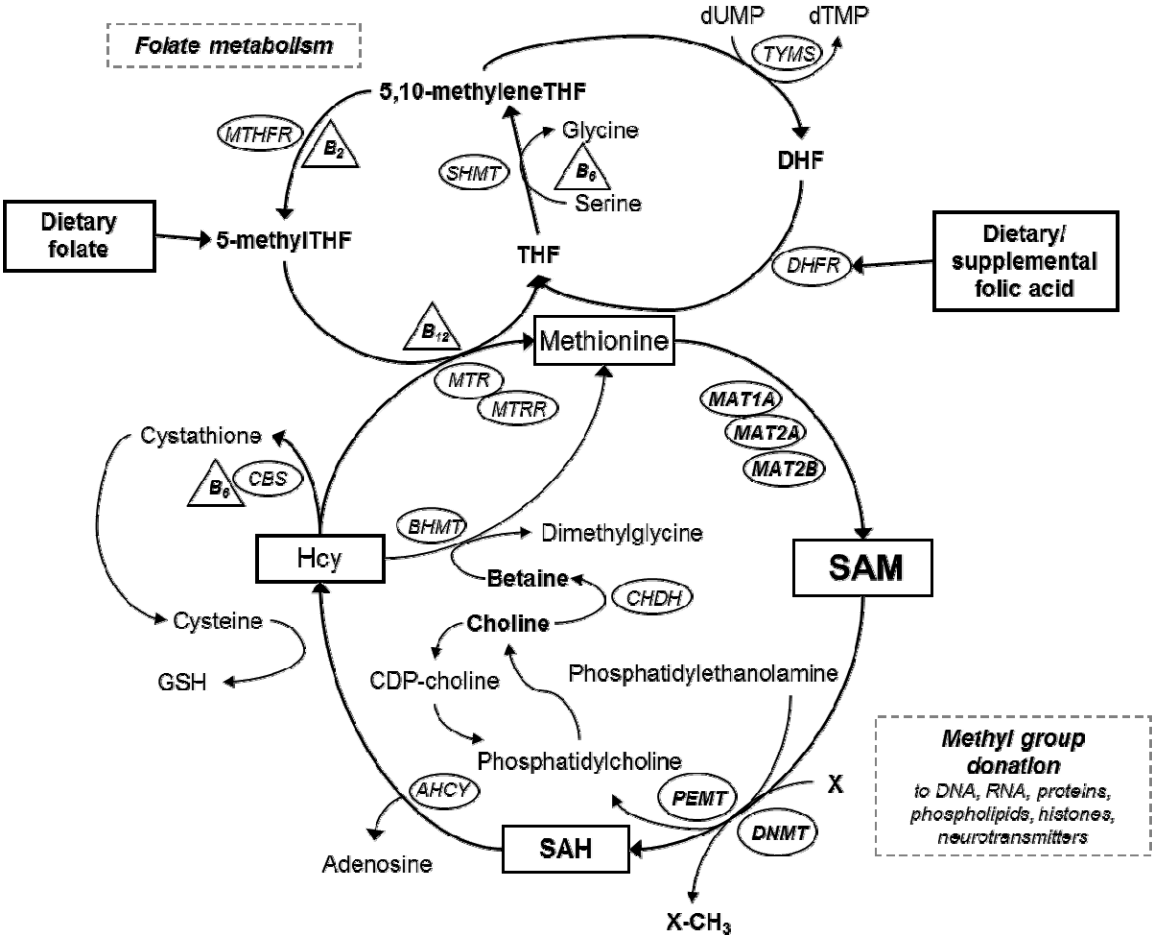
Table IV-7. Spearman partial correlation coefficients (r) between plasma concentrations and dietary intake of one-carbon metabolism nutrients, adjusted for age at blood draw, gender and serum creatinine concentrations

	Plasma concentrations					
Dietary intake	Folate	Choline	Betaine	Methionine	Vitamin B_6	Vitamin B_{12}
Folate	0.149*					
Choline		-0.035				
Betaine			0.066			
Methionine				0.047		
Vitamin B_6					0.158*	
Vitamin B_{12}						0.188*

* Statistically significant (p <0.05)

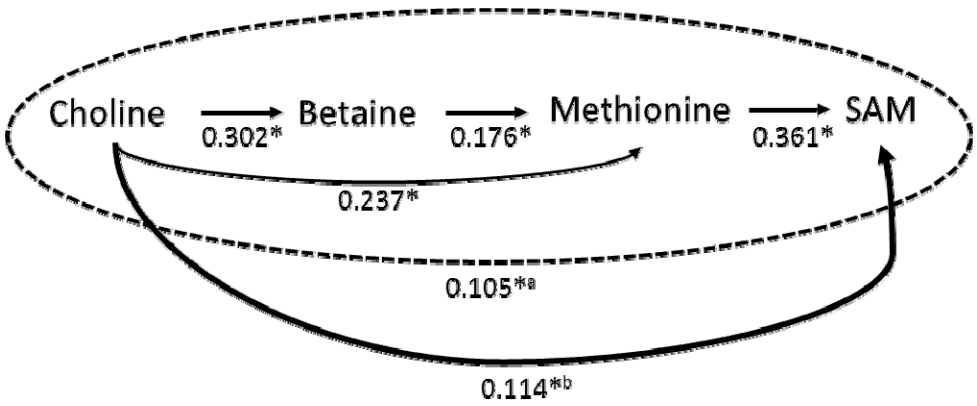
F. Figures

Figure IV-1. Overview of one-carbon metabolism and cholin metabolism



Substrates are in boxes, enzymes in ovals, and cofactors in triangles. Abbreviations: AHCY = *S*-adenosylhomocysteine hydrolase; BHMT = betaine hydroxymethyltransferase; CBS = cystathione β-synthase; CDP-choline = cytidine diphosphocholine; CHDH = choline dehydrogenase; DHFR = dihydrofolate reductase; DNMT = DNA methyltransferase; GSH = glutathione; Hcy = homocysteine; MAT1A= methionine adenosyltransferase 1A; MAT2A = methionine adenosyltransferase 2A; MAT2B = methionine adenosyltransferase 2B; MTA = methylthioadenosine; MTHFR = methylenetetrahydrofolate reductase; MTR = methionine synthase; MTRR = methionine synthase reductase; PEMT = phosphatidylethanolamine methyltransferase; SAM = *S*-adenosylmethionine; SAH = *S*-adenosylhomocysteine; SHMT = serine hydroxymethyltransferase; THF = tetrahydrofolate; TYMS = thymidylate synthase; dTMP = deoxythymidine monophosphate; dUMP = deoxyuridine monophosphate

Figure IV-2. The final path analysis model for the choline-SAM pathway (figures were standardized regression coefficients for each path, adjusted for age at blood draw, gender, BMI, smoking status and serum creatinine concentrations)



* p <0.01

[a] Standardized regression coefficient for the impact of choline on SAM through the one-carbon metabolism pathway.
[b] Standardized regression coefficient for the impact of choline on SAM through unspecified pathways other than the one-carbon metabolism pathway.

Total impact of choline on SAM (= a + b): standardized regression coefficient = 0.218 (p <0.0001).

V. MANUSCRIPT 2: PLASMA *S*-ADENOSYLMETHIONINE LEVELS, GENETIC POLYMORPHISMS OF *DNMT1, DNMT3A* AND *DNMT3B* AND GLOBAL DNA METHYLATION AMONG CHINESE IN SINGAPORE

Global DNA hypomethylation is believed to be one of the early molecular abnormalities in carcinogenesis, and there is a great interest in identifying traits and exposures that contribute to global DNA hypomethylation. Here we have tested the hypothesis that plasma *S*-adenosylmethionine (SAM) levels alone or in combination with genetic variation in DNA methyltransferases (*DNMT1*, *DNMT3A* and *DNMT3B*) are associated with the methylation levels at the long-interspersed element 1 (LINE-1) of DNA. Plasma SAM levels and the LINE-1 methylation index were measured using stored blood samples collected from 440 healthy Singapore Chinese adults during 1994-1999. Thirteen genetic variants of *DNMT1*, *DNMT3A* and *DNMT3B* were genotyped. The LINE-1 methylation index was significantly higher in men than in women (p=0.002). The LINE-1 methylation index was positively associated with plasma SAM levels ($p \leq 0.01$), with a plateau at approximately 78% (55 nmol/L plasma SAM) and 77% methylation (50 nmol/L plasma SAM) in men and women, respectively. In men only, the variant genotypes of *DNMT1* rs21124724 were associated with statistically significantly higher LINE-1 methylation index (p_{trend}=0.001), while the variant genotypes of *DNMT3A* rs7581217 were associated with significantly lower LINE-1 methylation index in men (p_{trend}=0.008). The *DNMT1* rs2114724 genotype modified the association between

plasma SAM and the LINE-1 mehtylation index at low levels of plasma SAM in men only. Our findings support a role of SAM on global DNA methylation. The *DNMT1* genetic polymorphism may exert a modifying effect on the association between SAM and DNA methylation, especially when plasma SAM level is low.

A. Introduction

Inter-individual variation in DNA methylation extent has been associated with increased risk of many chronic diseases including cancer (55, 207, 208). Global DNA hypomethylation, the genome-wide loss of methylcytosine, has been observed in cancer, benign tumors and normal tissues surrounding cancer tissues, indicating that global DNA hypomethylation may be one of the early molecular abnormalities described in carcinogenesis (20, 31, 34). Global DNA hypomethylation particularly reflects changed methylation in repetitive DNA sequences rich in CpG dinucleotide such as long interspersed nucleotide element-1 (LINE-1). DNA methylation extent, as captured by methylation extent at LINE-1, varies by gender, age and environmental and lifestyle factors (53-55, 209-211).

Methylation of DNA requires the methyl donor, *S*-adenosylmethionine (SAM), a key metabolite in one-carbon metabolism (OCM). Transferred from SAM, a methyl moiety is added to the number 5 carbon of the cytosine pyrimidine ring of DNA. A number of epidemiologic studies have evaluated the association between OCM nutrients, such as folate, and the risk of cancer (60, 61). Molecular mechanisms for the OCM-

cancer association are not completely understood, but the altered balance in OCM metabolites may cause insufficient supply of methyl moieties for DNA methylation, resulting in global DNA hypomethylation.

Besides the supply of methyl moieties, inter-individual variation in the activity level of enzymes that regulate methylation reactions may impact global DNA methylation. DNA methylation reactions are catalyzed by DNA methyltransferases (DNMTs). Three active DNMTs, namely DNMT1, DNMT3A and DNMT3B, have been identified in mammals (81, 82). DNMT1 is considered to be a key enzyme for the maintenance of DNA methylation, whereas DNMT3A and DNMT3B function primarily as *de novo* methylatransferases which are responsible for the establishment of DNA methylation patterns in early embryonic development (83, 84, 86). Although DNMT3A and DNMT3B are downregulated in somatic tissues, DNMT3A and DNMT3B may also play a role in maintaining DNA methylation levels (87-89). Therefore, genetic variation in *DNMT1, DNMT3A* and *DNMT3B* may alter the enzyme activity and thus influence global DNA methylation. The genetic variation in *DNMT*s might modify the association between SAM status and global DNA methylation as well.

To date, human data on the link between SAM status or genetic variation in *DNMT*s and global DNA methylation are limited. In this study, we have tested the hypothesis that plasma SAM levels alone or in combination with *DNMT1*, *DNMT3A* and *DNMT3B* genetic variation are associated with DNA methylation levels at LINE-1.

B. Subjects and Methods

B.1. Study Subjects

The Singapore Chinese Health Study (SCHS) is a population-based prospective cohort investigation of diet and the risk of cancer and other chronic diseases. The detailed study design of the SCHS has been described previously (10). In brief, Chinese men and women aged 45 – 74 years who were permanent residents in Singapore were invited to participate in the study from April 1993 through December 1998. A total of 63,257 participants (85% of the eligible individuals) were enrolled. Baseline information including demographic and lifestyle factors, medical history, family history of cancer and usual dietary intake was collected through in-person interviews at recruitment. Usual dietary intake during the previous 12 months was assessed using a structured semi-quantitative food frequency questionnaire (FFQ) which was specifically designed to assess the dietary habits of Chinese in Singapore and validated through a series of 24-h dietary recalls (174).

Biospecimen were collected from a 3% random sample of the cohort between 1994 and 1999. A 20-mL blood sample was collected in tubes from 509 subjects at their home and immediately placed on ice during transport to the laboratory at the National University of Singapore. At the laboratory, blood samples were processed and separated into plasma, serum, buffy coat and red blood cells and stored in a liquid nitrogen tank at -180ºC until 2001, when these blood samples were moved to -80ºC freezers for long term

storage. This study was approved by the Institutional Review Boards at the University of Minnesota and the National University of Singapore.

B.2. SNP selection and genotype determinations

We selected common single nucleotide polymorphisms (SNPs) of *DNMT1*, *DNMT3A* and *DNMT3B* with minor allele frequency (MAF) ≥20%, given the relatively small sample size of the present study. A total of 13 tagging SNPs for the Han Chinese (CHB) population were selected using the International HapMap Project database (Tagger Pairwise method, HapMap Data Rel 27 Phase II + III, Feb09, on NCBI B36 assembly, dbSNP b126): rs2228611, rs2288350 and rs7253062 for *DNMT1;* rs6722613, rs7575625, rs7581217, rs7587636, rs12987326, rs12999687, rs13036246 and rs34048824 for *DNMT3A;* and rs2424908 and rs6141813 for *DNMT3B*. Additional 6 SNPs were chosen based on the reported association with cancer (112, 212). They were rs2114724, rs2241531, rs1863771, rs1699593, and rs75616428 for *DNMT1* and rs1550117 for *DNMT3A*. One SNP of *DNMT1* from the literature (rs1863771) failed in the sequenom design. After genotype determinations, two SNPs of *DNMT1* from the literature (rs1699593 and rs75616428) were excluded due to no genetic variation among the study subjects. Two tagging SNPs of *DNMT3A* (rs12987326 and rs12999687) were excluded from the analysis because they were not in Hardy-Weinberg equilibrium (p <0.05). In addition, one tagging SNP of *DNMT1* (rs2114724) was excluded in the analysis because of its linkage (Spearman correlation coefficient (r), 0.996) with another *DNMT1* SNP

(rs2114724), which was selected via literature search. As a result, we report a total of 13 SNPs (4 SNPs of*DNMT1,* 7 SNPs of *DNMT3A,* and 2 SNPs of *DNMT3B*).

DNA was extracted from stored buffy coats using a Qiagen QIAmp 96 DNA Blood Kit (Qiagen Inc., Valencia, CA), and genotype determinations were performed in multiplex using the Sequenom MALDI-TOF mass spectrometry system (Sequenom Inc., San Diego, CA) by the University of Minnesota BioMedical Genomics Center (BMGC). Each 96-well plate contained positive and negative controls for the quality control purpose.

B.3. Laboratory measurements

Plasma SAM levels were measured by a modified assay of the stable-isotope dilution liquid chromatography-electrospray injection tandem mass spectrometry (LC-ESI-MS/MS) previously described (190) (please see **Manuscript 1**, **C.2.**).

LINE-1 DNA methylation was quantified using the quantitative bisulfite PCR pyrosequencing method developed by Yang *et al.* (213). Genomic DNA from peripheral lymphocytes was sodium bisulfite treated using the EZ-96 DNA Methylation Kit, converting non-methylated cytosine residues into uracil, according to the manufacturer's protocol (Zymo Research, Orange, CA). Bisulfite converted DNA was PCR amplified using Hotstar Taq Polymerase (Qiagen Inc., Valencia, CA). For pyrosequencing, the PCR product was purified using a biotin-labeled primer and Streptavadin Sepharose beads (GE Healthcare, Waukesha, WI). The bead immobilized PCR product was

purified, washed, denatured using a NaOH solution, and washed again using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Inc., Westborough, MA). PCR amplifications were done in triplicate and the extent of methyl cytosine relative to the total cytosine and thymine (%) at each of 4 CpG sites was measured. The average of methylation across the four CpG sites was computed for each replicate, and the average of three replicate measurements of LINE-1 DNA methylation was used as the LINE-1 methylation index (%) for each sample in the analysis.

B.4. Statistical analysis

Twenty-nine subjects with missing values of the LINE-1 methylation index were excluded from the 509 subjects. Of the remaining 480 subjects, 16 subjects who had missing values (n = 14) or extremely high values (n = 2) of plasma SAM levels and 24 subjects whose serum creatinine values were missing (n = 23) or extremely high (n = 1) were also excluded. As a result, a total of 440 subjects were included in the analysis of plasma SAM levels and the LINE-1 methylation index. In addition, 8 subjects with missing values for 2 or more genotypes were omitted in the analysis of *DNMT1*, *DNMT3A* and *DNMT3B* genotypes or haplotypes and the LINE-1 methylation index.

All analyses were conducted in men and women separately, given the consistently reported difference in the LINE-1 methylation index between sexes (214-216). We examined the association between demographic and lifestyle factors and the LINE-1 methylation index and found that age and smoking status were statistically significantly

associated with the LINE-1 methylation index. These factors were included as covariates in the analysis for the association between plasma SAM levels and the LINE-1 methylation index. Serum creatinine levels were associated with both plasma SAM levels and the LINE-1 methylation index and therefore were also included as a covariate in the model. Spline curves were created to visualize the association between plasma SAM and the LINE-1 methylation index and to determine a cut-off value of low LINE-1 methylation index (at 78% in men and at 77% in women). The multivariate linear regression method was used to compare the LINE-1 methylation index by plasma SAM levels using the cut points identified in the spline curves. Similarly, the LINE-1 methylation index was compared across 3 genotypes. Haplotypes with a 5% or more frequency were constructed for each of *DNMT1*, *DNMT3A* and *DNMT3B*. Logistic regression models were used to calculated odds ratio of being at low LINE-1 methylation index for each haplotype comparing with the most frequently observed haplotype. A series of spline curves for relationship between plasma SAM and the LINE-1 methylation index by *DNMT* genotypes were created to evaluate a potential modifying effect of *DNMT* genotypes on the association between plasma SAM levels and the LINE-1 methylation index.

Haplotype analysis was performed using R version 2.13.2, haplostat package. All other analyses were conducted using SAS version 9.2 (SAS Institute Inc., Cary, NC). All reported *p* values are 2-sided, and those that were <0.05 were considered to be statistically significant.

C. Results

The average age of the 440 study subjects was 58.1 y (standard deviation (SD), 7.8 y; range, 46 – 77 y). Approximately 72% of the study subjects had body mass index (BMI) below 24 while only 4% had BMI of 28 or above. Men had slightly higher education levels than women; 40% of men had secondary school or some college education, while the counterpart in women was 25%. About 56% of men were past or current smokers, but ever (current or past) smoking was reported by only 7% of women. The majorities of men (70%) and women (93%) reported no alcohol intake. The geometric means of plasma SAM levels after adjustment for age at blood draw and serum creatinine levels were 66.9 nmol/L in men and 61.2 nmol/L in women (p <0.001 for the difference between men and women).

Table V-1 describes selected demographic and lifestyle factors and the LINE-1 methylation index in the 440 study subjects. The LINE-1 methylation index was normally distributed ranging from 68.7% to 83.4% with the average of 77.7% in all subjects. The mean LINE-1 methylation index was statistically significantly higher in men (78.1%) than in women (77.3%) (p = 0.001). The LINE-1 methylation index was positively associated with age in men, but not in women. High BMI was associated with slightly lower LINE-1 methylation index in women only. There was no statistically significant association of level of education, smoking status or alcohol consumption with the LINE-1 methylation index either in men or women.

Figure V-1 shows spline curves of the LINE-1 methylation index by plasma SAM levels. The LINE-1 methylation index appears positively associated with plasma SAM levels, with a plateau at approximately 78% methylation (55 nmol/L plasma SAM) in men and 77% methylation (50 nmol/L plasma SAM) in women. Based on the observed associations in Figure 1, men were grouped into 2 groups according to their SAM levels (<55 nmol/L and ≥55 nmol/L), and women were categorized into 3 groups (<50 nmol/L, 50 – 90 nmol/L and ≥90 nmol/L plasma SAM). **Table V-2** shows the LINE-1 methylation index by plasma SAM levels in men and women separately. Compared with men with SAM below 55 nmol/L, men with plasma SAM 55 nmol/L or above had statistically significantly higher LINE-1 methylation index after adjustment for age and serum creatinine levels ($p = 0.01$). In women, there was a statistically significant positive trend in the LINE-1 methylation index across 3 categories of plasma SAM levels (p for trend = 0.005).

Table V-3 shows the association between 13 SNPs of *DNMT*s and the LINE-1 methylation index after adjustment for age. There was no statistically significant association between any of the 13 SNPs and the LINE-1 methylation index in women. In men, variant genotypes of *DNMT1* rs2114724 and *DNMT3A* rs7581217 were statistically significantly, and positively and negatively respectively, associated with the LINE-1 methylation index (both p for trend <0.01). No statistically significant association was observed for other SNPs with the LINE-1 methylation index in men. Based on the selected SNPs for the 3 *DNMT* genes studied, multiple haplotype groups were

constructed. The LINE-1 methylation index was comparable across different haplotypes of the given *DNMT*s in both men and women (data not shown).

The association between plasma SAM levels and the LINE1 methylation index stratified by *DNMT1* rs2114724 genotype was presented for men and women separately in **Figure V-2**. Below the threshold of 78% LINE-1 metylation index (55 nmol/L plasma SAM), the LINE-1 methylation index appeared to be positively associated with plasma SAM in men carrying the CC genotype of *DNMT1* rs2114724, while there was no association between plasma SAM and the LINE-1 methylation index in men possessing the CT or TT genotype. Among men carrying variant genotypes, the LINE-1 methylation index was constantly at or above 78% regardless of plasma SAM levels. Such an effect modification by *DNMT1* rs2114724 genotype was not observed among women. Other SNPs of *DNMT1*, *DNMT3A* and *DNMT3B* did not modify the SAM-LINE-1 methylation association (data not shown).

D. Discussion

Inter-individual variation in DNA methylation is a risk trait for several diseases, including cancer. We hypothesized that this variation is associated with the availability of SAM, and genetic polymorphisms of *DNMT*. Our results showed that the LINE-1 methylation index was positively associated with plasma SAM levels and plateaued at approximately 78% for men (55 nmol/L SAM) and 77% for women (50 nmo/L SAM), respectively. The variant genotypes of *DNMT1* rs2114724 and *DNMT3A* rs758127 were

statistically significantly, and positively and negatively respectively, associated with the LINE-1 methylation index in men. Furthermore, the modifying effect of *DNMT1* rs2114724 genotype on the association between plasma SAM and the LINE-1 methylation index existed only at low levels of plasma SAM (<55 nmol/L) in men.

A large number of epidemiologic studies examined nutrients involved in OCM in relation to cancer risk, but very few studies have evaluated the association between circulating SAM levels and global DNA methylation. Results from our study showed a positive association between plasma SAM levels and the LINE-1 methylation index, at levels of methylation below ~78%. There are several possibilities for the observed methylation plateau. It is possible that DNA methylation above a certain level is detrimental to the cell. Alternatively, it is possible there is an active inhibition of DNA methylation at high SAM levels. In other work, we have observed a significant positive association between plasma levels of SAM and *S*-adenosylhomocysteine (SAH) (Inoue-Choi *et al.* under review) (217). Elevated levels of SAH have been shown to inhibit DNA methylation *in vitro* and *in vivo* (202, 218, 219), and might explain the plateaued LINE-1 methylation index at higher plasma SAM levels observed in our study. A combination of increased intracellular SAH and decreased SAM, measured as the ratio of SAM: SAH, may be a better predictor of global DNA methylation compared with plasma SAM alone, however, neither plasma SAH levels nor the SAM:SAH ratio were associated with the LINE-1 methylation index in our study. We previously found that higher plasma choline and methionine levels were strongly associated with higher plasma

SAM levels among healthy Singapore Chinese men and women (217). Combined with these previous findings, our data provide support to the hypothesis that OCM nutrient status may impact the availability of circulating SAM and thus global DNA methylation.

DNMT1 prefers hemimethylated DNA over unmethylated DNA 15- to 40-fold *in vitro*, indicating its major function in maintaining DNA methylation patterns after DNA replication (83, 84). Although DNMT3A and DNMT3B were also shown to play a role in the maintenance of DNA methylation in cooperation with DNMT1 or with each other (87-89), these enzymes do not have preference for hemimethylated DNA, and thus function mainly in the *de novo* methylation and are downregulated in somatic tissues (82, 86). Hence, genetic variation in *DNMT1* may have a greater impact on global DNA methylation in adults compared with *DNMT3A* and *DNMT3B*. In the present study, we found genetic polymorphism of the *DNMT1* gene was positively associated with the LINE-1 methylation index independently and in combination with plasma SAM levels in men. We also found the negative association between a genetic polymorphism of the *DNMT3A* gene and the LINE-1 methylation index in men, but this association was no longer statistically significant after adjusting for multiple testing (n = 13).

Existing data on the link between genetic variation in *DNMT*s and global DNA methylation or cancer risk are limited. A recent study examined the association between 22 common genetic variants of *DNMT1*, *DNMT3A* and *DNMT3B* and breast cancer risk, but none of the SNPs was associated with breast cancer risk (105). A few studies

suggested that genetic variation in *DNMT3B* may influence cancer susceptibility, although conflicting results are reported in different tumor types (105-111).

As previously reported by others, our findings showed that the LINE-1 methylation index was lower in women compared with men (210, 214, 215). The mechanism of this sex difference in the LINE-1 methylation index is still not clear. However, El-Maarri *et al*., showed that natural hormone cycles did not appear to explain the sex difference in the LINE-1 methylation index (216). Recently, Singer *et al.* demonstrated that major LINE-1 promoter regions, L1Hs, which contain a high density of CpG sites, were significantly hypomethylated on inactivated X chromosomes compared with activated X chromosomes (214). They also reported that LINE-1 methylation extent was lower in women compared with men, but LINE-1 CpG sites at autosomes were not differentially methylated by gender, indicating the sex difference in LINE-1 methylation were specific to the X chromosome. LINE-1 elements are believed to be involved in X chromosome inactivation by spreading or maintaining inactivation signals (220). The X chromosome is enriched nearly 2-fold for LINE-1 elements compared with autosomes, and the highest density of LINE-1 were observed for the subset of LINE-1 which is the center of X inactivation (221). Hence, the genomic loci that escape X inactivation are reduced in LINE-1 compared with X chromosome segments containing loci subject to X inactivation. These findings provide support for the link between LINE-1 and X chromosome inactivation and strongly support analyses stratified by gender. The higher density of inactivated genomic loci in LINE-1 in women may also explain an interaction

between the *DNMT1* genotype and plasma SAM levels on the LINE-1 methylation index observed only in men in the present study.

As with most studies, the present study has several limitations. One concern is the possible degradation of SAM in stored plasma samples during prolonged storage. The median plasma SAM level in the present study (63.3 nmol/L; interquartile range, 54.5 – 76.0 nmol/L) was somewhat lower compared with the reference values observed in populations in Europe (70 – 128 nmol/L) (193). However, the wide range of plasma SAM values (50 – 150 nmol/L) in various studies might be due to different study populations and laboratory methods (158, 190, 202-206). In our study population, we measured plasma homocysteine (Hcy) levels in the same subjects at two different times with more than 10 years apart (in 1996 – 1997 and in 2010). High correlation between the two Hcy measurements (r, 0.70) indicates that the degradation of Hcy, and possibly SAM, might have been minimal. Furthermore, the degradation of plasma SAM would not result in the observed associations, given that it occurred in a non-differential manner. LINE-1 is dispersed across the genome, however, because there is a mix of both active and inactive LINE-1 elements present in the genome, it cannot be viewed as either a passive dosimeter of methylation processes, nor a reflection of methylation processes at active chromatin. Therefore, we are limited in our interpretation of the LINE-1 methylation index in examining exposures and disease risk. Another limitation of the present study is potentially limited variability in the LINE-1 methylation index because all of the study subjects were cancer-free, healthy individuals. Meanwhile, our aim here

was to determine the association between SAM status or genetic variation in *DNMT*s and global DNA methylation in a free-living human population. It is also important to report the distribution of the LINE-1 methylation index and its correlates in a general population. A relatively small sample size is also a limitation. We could not have enough power to test an interaction between plasma SAM levels and *DNMT* genetic polymorphisms on the LINE-1 methylation index in the regression models. Nonetheless, our data provide important information on frequencies and identifications of genetic variation in *DNMT*s associated with the LINE-1 methylation index in peripheral blood in a Chinese population in South Asia.

In conclusion, our findings provide supporting data for the association between circulation SAM levels and global DNA methylation. The link between plasma SAM levels and global DNA methylation may be modified by *DNMT1* genetic polymorphisms. To our knowledge, this is the first study to show that systematic SAM status, alone or in combination with the genetic variant of *DNMT1*, was associated with global DNA methylation in healthy humans. Our findings should be replicated in future studies.

E. Tables

Table V-1. Demographic and lifestyle factors and the LINE-1 methylation index (%) in the Singapore Chinese Health Study

	Men			Women		
	n	Mean (95%CI)[a]	p_{trend}	n	Mean (95%CI)[a]	p_{trend}
LINE-1 methylation index (%)	192	78.1 (77.8 – 78.4)[b]	-	248	77.3 (77.1 – 77.6)[b]	-
Age						
<50	25	77.8 (77.0 – 78.7)	0.04	41	77.0 (76.3 – 77.8)	0.82
50 – 59	79	77.8 (77.3 – 78.3)		123	77.6 (77.1 – 78.0)	
60 – 69	71	78.2 (77.7 – 78.7)		55	77.2 (76.5 – 77.8)	
≥70	17	79.3 (78.2 – 80.3)		29	77.1 (76.3 – 78.0)	
BMI						
<20	23	78.3 (77.4 – 79.2)	0.31	38	77.3 (76.5 – 78.1)	0.08
20 – <24	112	77.8 (77.4 – 78.3)		143	77.6 (77.2 – 78.0)	
24 – <28	49	78.4 (77.7 – 79.0)		56	76.9 (76.3 – 77.5)	
≥28	8	78.9 (77.4 – 80.4)		11	76.1 (74.7 – 77.6)	
Level of education						
No formal school	27	77.8 (76.9 – 78.6)	0.28	93	77.0 (76.5 – 77.6)	0.19
Primary school	89	77.9 (77.5 – 78.4)		94	77.5 (77.0 – 78.0)	
Secondary school	53	78.5 (77.9 – 79.1)		52	77.6 (76.9 – 78.3)	
Some college or above	23	78.2 (77.2 – 79.1)		9	77.6 (76.0 – 79.2)	
Smoking status						
Never	84	78.2 (77.7 – 78.7)	0.60	234	77.4 (77.1 – 77.7)	0.21
Past	49	78.0 (77.4 – 78.7)		2	78.5 (75.1 – 81.9)	
Current	59	78.0 (77.4 – 78.6)		12	76.4 (74.9 – 77.8)	
Alcohol intake						
None	135	78.1 (77.7 – 78.5)	0.98	231	77.4 (77.1 – 77.7)	0.12
<7 drinks/week	42	78.1 (77.4 – 78.8)		13	77.1 (75.7 – 78.4)	
≥7 drinks/week	15	78.0 (76.9 – 79.2)		4	75.3 (72.9 – 77.7)	

[a] Adjusted for age at blood draw.
[b] *p* for difference between men and women was 0.001.

Table V-2. Plasma SAM levels and the LINE-1 methylation index in the Singapore Chinese Health Study

Men				Women			
SAM (nmol/L)	n	LINE-1 methylation[a]	*p*	SAM (nmol/L)	n	LINE-1 methylation[a]	p_{trend}
< 55	38	77.3 (76.6 – 78.0)	0.01	< 50	48	76.6 (75.9 – 77.2)	0.005
≥ 55	154	78.3 (77.9 – 78.6)		50 – < 90	180	77.5 (77.1 – 77.8)[b]	
				≥ 90	20	78.3 (77.2 – 79.4)[c]	

[a] Mean (95%CI) adjusted for age at blood draw and serum creatinine levels

[b] $p = 0.02$ compared to plasma SAM levels <50 nmol/L

[c] $p = 0.01$ compared to plasma SAM levels <50 nmol/L

Table V-3. Genotype of *DNMT1*, *DNMT3A* and *DNMT3B* and the LINE-1 methylation index (%) in the Singapore Chinese Health Study

	Men (n = 186)				Women (n = 246)			
	n[a]	Mean (95%CI)[b]	*p*	p_{trend}	n[a]	Mean (95%CI)[b]	*p*	p_{trend}
DNMT1								
rs2114724								
CC	90	77.7 (77.2 – 78.1)		0.001	126	77.2 (76.8 – 77.6)		0.38
CT	71	78.3 (77.8 – 78.8)	0.06		98	77.6 (77.1 – 78.1)	0.25	
TT	21	79.3 (78.4 – 80.3)	0.002		20	77.4 (76.3 – 78.5)	0.75	
rs2241531								
GG	44	77.9 (77.2 – 78.5)		0.11	61	77.1 (76.5 – 77.7)		0.71
CG	94	78.1 (77.6 – 78.5)	0.60		121	77.5 (77.1 – 78.0)	0.29	
CC	46	78.6 (78.0 – 79.2)	0.11		63	77.3 (76.7 – 77.9)	0.70	
rs2288350								
CC	55	78.5 (77.9 – 79.1)		0.05	74	77.4 (76.8 – 78.0)		0.57
CT	93	78.1 (77.6 – 78.6)	0.31		115	77.5 (77.0 – 77.9)	0.85	
TT	38	77.6 (76.9 – 78.3)	0.05		55	77.1 (76.5 – 77.8)	0.53	
rs7253062								
GG	109	78.3 (77.9 – 78.7)		0.12	149	77.3 (76.9 – 77.7)		0.92
GA	72	77.8 (77.3 – 78.3)	0.13		83	77.5 (76.9 – 78.0)	0.71	
AA	4	77.6 (75.4 – 79.8)	0.54		11	77.1 (75.7 – 78.6)	0.78	
DNMT3A								
rs1550117								
CC	113	78.3 (77.8 – 78.7)		0.10	146	77.5 (77.1 – 77.9)		0.30
CT	58	78.0 (77.4 – 78.5)	0.39		92	77.1 (76.6 – 77.6)	0.24	
TT	15	77.3 (76.2 – 78.4)	0.12		8	77.3 (75.6 – 79.0)	0.83	

Table 3 (cont.)

	Men (n = 186)				Women (n = 246)			
	n[a]	Mean (95%CI)[b]	*p*	p_{trend}	n[a]	Mean (95%CI)[b]	*p*	p_{trend}
rs6722613								
GG	93	78.0 (77.6 – 78.5)		0.87	101	77.4 (76.9 – 77.9)		0.69
GA	69	78.2 (77.7 – 78.8)	0.58		112	77.4 (76.9 – 77.8)	0.93	
AA	24	78.0 (77.1 – 78.9)	0.93		33	77.2 (76.3 – 78.0)	0.65	
rs7575625								
AA	115	78.2 (77.8 – 78.6)		0.29	148	77.3 (76.9 – 77.7)		0.79
AG	59	78.2 (77.7 – 78.8)	0.86		83	77.3 (76.8 – 77.8)	0.98	
GG	11	77.0 (75.7 – 78.2)	0.08		15	77.6 (76.4 – 78.9)	0.66	
rs7581217								
CC	59	78.5 (78.0 – 79.1)		0.008	83	77.5 (77.0 – 78.0)		0.92
CT	94	78.1 (77.7 – 78.6)	0.27		114	77.1 (76.7 – 77.6)	0.26	
TT	33	77.2 (76.4 – 77.8)	0.006		49	77.6 (76.9 – 78.3)	0.90	
rs7587636								
GG	95	78.1 (77.6 – 78.5)		0.89	116	77.2 (76.8 – 77.7)		0.95
GA	68	78.2 (77.6 – 78.7)	0.86		103	77.6 (77.1 – 78.0)	0.32	
AA	22	78.1 (77.2 – 79.1)	0.94		27	77.0 (76.0 – 77.9)	0.57	
rs13036246								
CC	102	78.0 (77.5 – 78.4)		0.26	129	77.5 (77.1 – 77.9)		0.14
CT	68	78.2 (77.7 – 78.7)	0.47		106	77.3 (76.8 – 77.7)	0.48	
TT	15	78.6 (77.5 – 79.7)	0.31		10	76.1 (74.6 – 77.6)	0.08	
rs34048824								
TT	116	78.1 (77.7 – 78.5)		0.61	150	77.3 (76.9 – 77.7)		0.70
TC	58	78.3 (77.8 – 78.9)	0.59		77	77.3 (76.8 – 77.9)	0.99	
CC	10	77.1 (75.8 – 78.5)	0.18		18	77.7 (76.5 – 78.8)	0.57	

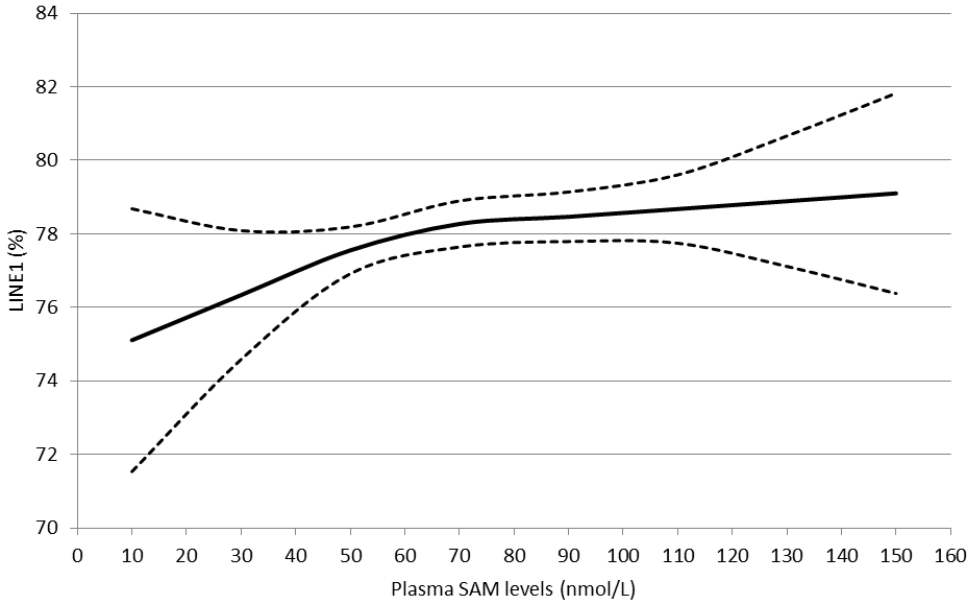
Table 3 (cont.)

	Men (n = 186)				Women (n = 246)			
	n[a]	Mean (95%CI)[b]	*p*	p_{trend}	n[a]	Mean (95%CI)[b]	*p*	p_{trend}
DNMT3B								
rs2424908								
CC	61	77.9 (77.3 – 78.4)		0.83	88	77.4 (76.9 – 77.9)		0.88
CT	79	78.4 (77.9 – 78.9)	0.18		121	77.3 (76.9 – 77.8)	0.88	
TT	44	77.9 (77.2 – 78.6)	0.96		37	77.3 (76.5 – 78.1)	0.91	
rs6141813								
AA	82	78.0 (77.5 – 78.5)		0.76	103	77.2 (76.8 – 77.7)		0.74
AG	73	78.3 (77.8 – 78.8)	0.35		115	77.4 (77.0 – 77.9)	0.56	
GG	30	78.0 (77.2 – 78.8)	0.99		27	77.3 (78.3 – 78.2)	0.94	

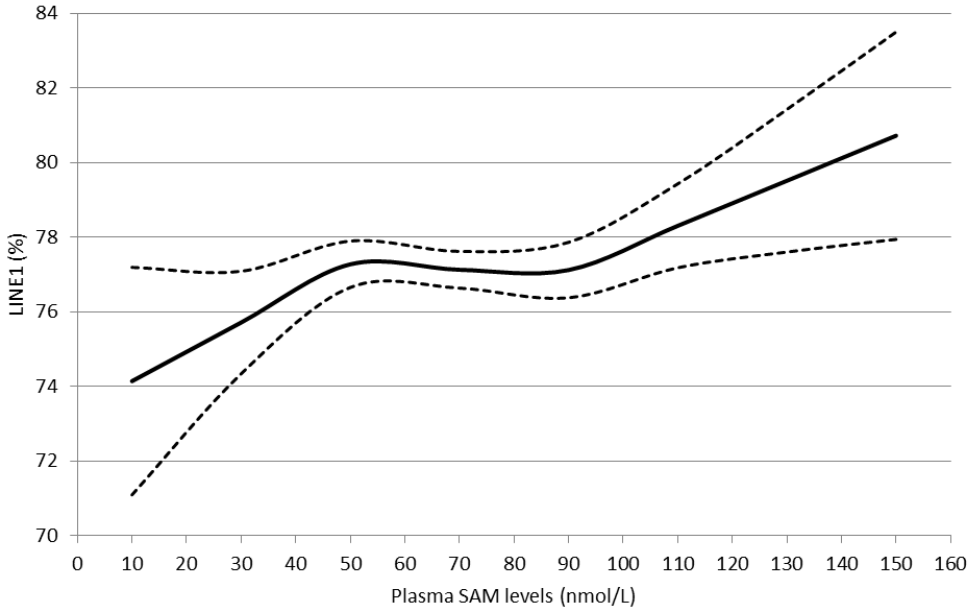
[a] Subjects with missing data on a genotype of each SNP were excluded from an analysis.
[b] Adjusted for age at blood draw.

Figures

Figure V-1. Plasma SAM levels and the LINE-1 methylation index in A) men and B) women in the Singapore Chinese Health Study



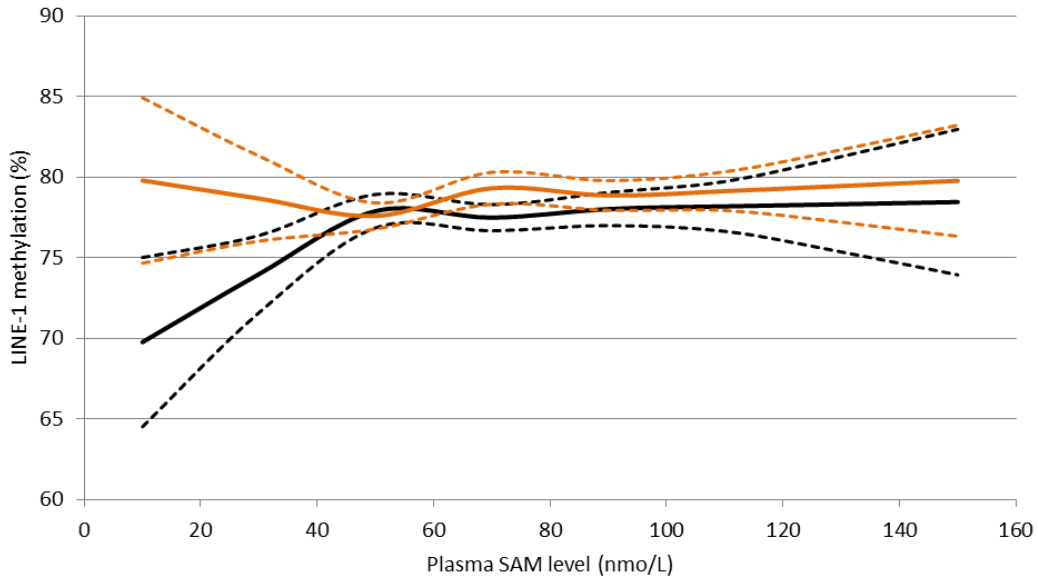
B.



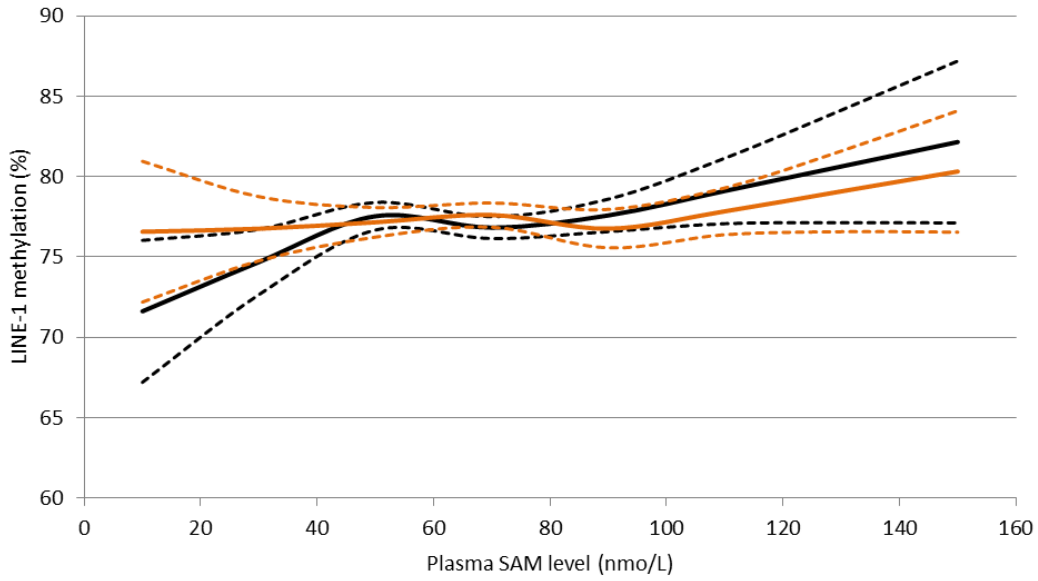
* Adjusted for age at blood draw and serum creatinine levels

Figure V-2. Plasma SAM levels and the LINE-1 methylation index by *DNMT1* rs2114724 genotype in A) men and B) women in the Singapore Chinese Health Study

A.



B.



* Adjusted for age at blood draw and serum creatinine levels.
** Black line: the wildtype genotype (CC) (N=90 for men and N=126 for women); orange line: variant genotypes (CT and TT) (N=93 for men and N=118 for women); dotted lines indicate 95% confidence intervals.

VI. MANUSCRIPT 3: INTERACTION OF NITRATE AND FOLATE ON THE RISK OF BREAST CANCER AMONG POSTMENOPAUSAL WOMEN

Ingested nitrate can be endogenously reduced to nitrite, which may form *N*-nitroso compounds, known potent carcinogens. However, some studies have reported no or inverse associations between dietary nitrate intake and cancer risk. These associations may be confounded by a protective effect of folate, which plays a vital role in DNA repair. We evaluated the interaction of dietary and water nitrate intake with total folate intake on breast cancer risk in the Iowa Women's Health Study. Dietary intake was assessed at study baseline using a food frequency questionnaire. Nitrate intake from public water was assessed using a historical database on Iowa municipal water supplies. After baseline exclusions, 34,388 postmenopausal women and 2,875 incident breast cancers were included. Overall, neither dietary nor water nitrate was associated with breast cancer risk. Among those with folate intake ≥400 μg/day, breast cancer risk was significantly increased in public water users in the highest nitrate quintile (HR=1.40, 95%CI=1.05-1.87) and private well users (HR=1.38, 95%CI=1.05-1.82) compared to public water users in the lowest nitrate quintile; in contrast, there was no association among those with lower folate intake. Our findings do not support a previous report of increased risk of breast cancer among individuals with high dietary nitrate but low folate intake.

A. Introduction

Breast cancer is the second leading cause of cancer death among women in the United States (222). Identifying risk factors and predictors of breast cancer is particularly important for primary and secondary prevention. Many epidemiologic studies have examined various dietary factors as potential modifiable risk factors or predictors of breast cancer. There are convincing or suggestive evidence for alcoholic drinks and total fat (postmenopausal breast cancer); however, the effects of other dietary factors are still not conclusive (223).

High intake of nitrate has been a growing concern in relation to cancer as ingested nitrate can be reduced in the oral cavity to nitrite, which subsequently reacts with amines and amides in the stomach forming *N*-nitroso compounds (NOCs), most of which are known potent carcinogens (164, 165). Exposure to industrial NOCs was associated with DNA adduct formation and benign and malignant tumor incidence in mammary glands in animal studies, suggesting potential relevance for risk of breast cancer (224-226). Nitrate is a natural component of the diet, and nitrate and nitrite are added as preservatives to meats. Nitrate is also a common contaminant of drinking water as a result of excessive application of nitrogen fertilizers to crops, animal waste, pollution from inadequately treated municipal wastewater effluent and air pollution. Nitrogen from all these sources can leach into groundwater or run off to surface waters, which are sources of drinking water. Agricultural and urban nitrogen use has rapidly increased since 1950 and is currently estimated to exceed nitrogen fixed by natural sources by 30% (227). The

maximum contaminant level (MCL) of 10mg/L (or 10 ppm) nitrate-nitrogen for public water supplies was determined based on the prevention of methemoglobinemia, an acute health effect (228). Research performed over the decade has demonstrated therapeutic indications from vasoprotective benefits of the short-term treatment with low-dose nitric oxide, a recycled product of nitrate *in vivo* (229). However, long-term effects of chronic intake of moderately high levels of nitrate (5-9 ppm nitrate nitrogen) from drinking water, in addition to dietary nitrate intake, on risk of chronic diseases such as cancer are still not clear.

A number of epidemiologic studies have reported no associations or inverse associations between dietary nitrate intake and cancer risk with most focusing on gastric cancer (167, 168, 170, 173, 230) and postulated that these results may be due to antioxidants, which co-exist with nitrate in foods (167, 170, 171, 173, 231, 232). The major dietary sources of nitrate are green leafy and root vegetables, which may contribute to up to 93% of total nitrate intake (232-234). Vegetables are also a major source of antioxidants such as vitamin C, which has been shown to inhibit the endogenous formation of NOCs in the stomach (171, 235, 236). Folate is also abundant in dietary sources of nitrate. NOCs are known to yield multiple DNA adducts and induce mutations in activated oncogenes (237-239). DNA adducts must be repaired to avoid proceeding to mutations. Folate plays an important role in DNA repair by providing one-carbon moieties for purine and pyrimidine nucleotide synthesis; therefore, deficient folate status may result in aberrant DNA synthesis and repair functions (13, 14, 240). Thus, no or

inverse associations between nitrate intake and cancer risk reported by previous epidemiologic studies may be confounded by the protective effect of antioxidants or folate through the enhanced DNA repair system, as well as the inhibition of NOC formation by antioxidants.

Recently, a case-control study of breast cancer reported an interaction between dietary nitrate and folate intakes (241). Dietary nitrate intake was not associated with breast cancer risk, but increased intake of nitrate relative to folate, as a nitrate-folate ratio, was associated with elevated risk of breast cancer. Dietary nitrate intake was positively associated with risk of breast cancer only among women with low folate intake. These findings suggested that this dietary pattern may be a potential risk factor for breast cancer. However, this study had several limitations, including a relatively small sample size (362 cases and 362 matched controls), potential biases related to the case-control study design, and lack of information on nitrate intake from drinking water. In the present study, we evaluated a modifying effect of folate on the association between nitrate intake and risk of breast cancer in the Iowa Women's Health Study.

B. Subjects and Methods

B.1. The Iowa Women's Health Study (IWHS)

The IWHS is a prospective cohort study among women in Iowa, initiated in 1986; the research focus was on cancer incidence. Details of the study design of the IWHS have been described elsewhere (242). Briefly, a self-administered questionnaire was

mailed to 99,826 women aged 55 to 69 who were randomly selected from the Iowa driver's license records. Of these women, 41,836 women (42%) who completed the baseline questionnaire comprise the IWHS cohort. The questionnaire contained questions on demographics, anthropometry, medical history, reproductive history, hormone use, family history of cancer, residence location, physical activity, smoking, alcohol consumption and usual dietary intake. Compared with respondents to the baseline questionnaire, non-respondents were slightly older and had a higher body mass index (BMI), but were otherwise comparable in terms of baseline demographic characteristics and life style factors (177). The IWHS was approved by the Institutional Review Board of the University of Minnesota.

B.2. Dietary intake data

Dietary intake was assessed at study baseline using the Harvard food frequency questionnaire (FFQ) developed by Willett *et al.* Cohort participants were asked to report their usual intake frequencies of 127 food items during the past 12 months. There were 9 frequency levels of each food item, ranging from "never or less than once per month" to "6 or more per day". To enable participants to obtain a sense of scale, a commonly used portion size for each food item was specified. Dietary nutrient intake was calculated by multiplying the frequency of consumption of the specified unit of each food by the nutrient content of that unit of food. The use of dietary supplements was also asked. Total intake of folate, vitamin C and E were computed by combining intakes from foods

and dietary supplements. This FFQ has been shown to be valid and reproducible in the study population (179).

To compute dietary intake of nitrate and nitrite, the nitrate and nitrite contents of FFQ foods were determined by reviewing the literature focusing on published reports for the United States and Canada. We computed means of the published values weighted by the number of samples analyzed as previously described (243, 244). The nitrate and nitrite contents of foods comprising a FFQ line item (for example, weighted means for canned, raw, and cooked tomatoes) were combined by weighting the food-specific nitrate and nitrite values by sex-specific intake amounts from the 1994-1996 Continuing Survey of Food Intake by Individuals (CSFII) (245). Dietary intakes of nitrate and nitrite were calculated by multiplying the nitrate or nitrite content of each line item by consumption frequency, and summing values across all line items. We also computed dietary intakes of nitrate and nitrite from plant and animal sources separately.

B.3. Nitrate intake from drinking water

The estimation of nitrate ingestion from public drinking water supplies was previously described in detail (246). Information on usual source of drinking water was collected in the second follow-up questionnaire in 1989. The cohort participants were asked the main source of drinking water at home (municipal water system, private wall, purchased bottled water from a store or dealer, and other) and how long they drank the type of water they indicated (<1 year, 1-5 years, 6-10 years, 11-20 years, and >20 years).

A total of 36,127 women (89% of eligible participants) responded to the questionnaire. The primary source of drinking water among these women was public water (76%) followed by private well (18%) and bottled water (6%). Of the women using municipal water, 82% and 69% used the municipal water supply for >10 years and >20 years, respectively. Of the women using a municipal water supply for >10 years, 79% resided in a total of 484 communities.

To estimate nitrate intake from drinking water, we used a historical database of Iowa municipal water supplies, which contains nitrate measurements from water samples collected during 3 time periods (1955-1964, 1976-1982, and 1983-1988). All water samples were analyzed at the University of Iowa Hygienic Laboratory. Water samples during 1955-1964 were analyzed using the phenoldisulfonic spectrophotometry method (247). The 1976-1988 water samples were analyzed using the cadmium reduction method (248). The mean values from the 3 time periods were averaged to calculate a mean nitrate concentration in each community's water supply for the entire 33-year period (1955-1988). There were no nitrate concentration data available for private wells.

B.4. Study population

Of the 41,836 cohort participants, we excluded women who reported at baseline: 1) history of cancer except non-melanoma skin cancer (N = 3,830); 2) premenopausal status (N = 547); or 3) previous mastectomy or partial breast resection (N = 354). For accuracy of dietary intake data, we also excluded 2,717 women for one or both of the

following reasons; 1) left more than 30 items blank on the FFQ; or 2) reported implausible energy intake estimates (<600 or >5,000 kcal/day). As a result, a total of 34,388 postmenopausal women were included in the analysis of dietary nitrate intake.

For the analysis of nitrate intake from drinking water, we further excluded those who did not respond to or died before the 1989 follow-up survey and those who had used their public or private well water supply for 10 years or less to evaluate long-term exposure to nitrate from drinking water. We also excluded women living in 47 communities that were served by multiple water sources and 41 communities for which no nitrate measurement data were available (246). After these exclusions, a total of 20,147 women (15,151 women using public water and 4,996 using private well water) were included in the drinking water analysis.

Through 2008, a total of 2,875 incident breast cancer cases were identified by annual computer match with the State Health Registry of Iowa's cancer database, a member of the National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) program. The vital status of the cohort participants was determined via linkage with the State Health Registry of Iowa, supplemented with the National Death Index of the National Center for Health Statistics. Person-years of follow-up were assigned for each participant from the date of return of the baseline questionnaire to: 1) the date of first breast cancer diagnosis, 2) date of emigration from Iowa, 3) date of death, or 4) December 31, 2008, whichever came first.

B.5. Data Analysis

We used Cox proportional hazard regression modeling (SAS, PROC PHREG) to compute hazard ratios (HRs) and their 95% confidence intervals (CIs) for breast cancer. Associations between baseline demographic, lifestyle and dietary factors and breast cancer risk were evaluated to determine potential confounders for an association between nitrate intake and breast cancer risk. We assessed breast cancer risk in relation to nitrate intake from diet and drinking water separately, adjusting for confounders and biologically relevant risk factors for breast cancer including age (continuous), BMI (kg/m^2), waist-hip-ratio (WHR) (continuous), education (<high school, high school, >high school), smoking (never, previous, current), alcohol intake, family history of breast cancer, age at menopause (continuous), age at first live birth (continuous), estrogen use, total energy intake (continuous), total intake of folate (except for analyses of the nitrate-folate ratio and nitrate-folate interaction), vitamin C and E and flavonoids (continuous), cruciferous vegetable and red meat intakes (servings/week, continuous). The distributions of dietary intake (folate, vitamin C and E, flavonoids, cruciferous vegetables and red meat) were markedly skewed towards high values; therefore, logarithmically transformed values were used as covariates in the analysis. HRs and 95% CIs were calculated for quintiles of nitrate intake using the lowest quintile as a reference group. We also tested for trends across quintile categories by using the median intake level in each quintile as a continuous variable in the models. We conducted similar analyses of dietary nitrite intake.

Nitrate intake from drinking water for public water supply users was computed by multiplying the average nitrate level in the public water supply by an estimated daily water consumption of 2 liters (L) per day; data on individual daily water consumption were not obtained. In addition, HRs and 95% CIs were computed for private well users compared to those in the lowest quintile of nitrate intake from public water. For public water supply users, total nitrate intake was also calculated by summing dietary and water nitrate intakes. Total and dietary nitrate intakes were highly correlated (Spearman correlation coefficient (r) = 0.93) because the primary nitrate source was diet for most women (percent of total nitrate intake from diet; median = 91.2%, interquartile range (IQR) = 78.9 – 97.0%). Therefore, we do not present results for total nitrate intake.

We performed three different types of analyses to evaluate an interaction of nitrate and folate. First, we repeated the analysis performed by Yang *et al* (241), the Korean case-control study that evaluated the association between a nitrate-folate ratio and breast cancer risk. Second, we evaluated an interaction between nitrate and folate for dietary and water nitrate separately, by including an interaction term for the nitrate intake quintiles and low (<400 μg/day = U.S. Recommended Daily Allowance (RDA)) and adequate or higher (≥400 μg/day) total folate intake, in models adjusted for potential confounders. Finally, we stratified the analysis of dietary and water nitrate intake by low and adequate or higher total folate intake to evaluate differential effects of folate on the nitrate-breast cancer association by intake levels.

All statistical procedures were performed using SAS version 9.2 (SAS Institute, Inc., Cary, NC). All reported *p* values were two-sided, and significance was defined as $p < 0.05$. This study had 80% power to detect HR of 1.20 in the highest quartile category of dietary nitrate intake compared with a reference group of women with total folate intake lower than the median (350.7 μg/d).

C. Results

The average age of the study population at baseline was 61.6 (standard deviation (SD), 4.2; range, 52-71). The study population was predominantly Caucasian (99.2%). Approximately 85% of the participants had completed high school or a higher level of education. Age, BMI, WHR, education, family history of breast cancer, age at menopause, age at first live birth and estrogen use were statistically significantly positively associated with breast cancer risk, while physical activity was inversely associated with breast cancer risk. Cigarette smoking, alcohol intake, age at menarche, and oral contraceptive use were not associated with breast cancer risk. The use of dietary supplements containing folic acid (folic acid or multivitamin) was reported by 8.3% and 69.7% of the women with low and adequate total folate intake, respectively. There was a trend toward decreased risk of breast cancer with moderate total folate intake (400 – 600 μg/d) compared with low folate intake, but risk of breast cancer among those with high folate intake was not different from low folate intake. Total energy, antioxidants

(vitamin C and E and flavonoids), cruciferous vegetable and red meat intakes were not associated with breast cancer risk (data not shown).

The average dietary intakes of nitrate and nitrite were 123.5 mg/day and 1.2 mg/day, respectively. Nitrate intake from plant sources accounted for 97% of dietary nitrate intake. Approximately 63% of dietary nitrite intake was from plant sources. The major contributors to dietary nitrate intake included lettuce (23.2%), celery (16.6%), beets (5.4%), spinach (3.2%) and broccoli (2.9%), while the major contributors of dietary nitrite intake were red meat (beef, pork, and lamb; 11.2%), milk (11.0%), cereals (9.3%), apples (7.1%) and processed meat (salami, sausage, bologna, bacon, hot dog, etc.; 4.4%). Because major contributors of dietary nitrate intake were vegetables, which are also major sources of folate and antioxidants, we evaluated correlations of these nutrient intakes and dietary nitrate intake. Spearman correlation coefficients (r) for dietary nitrate intake with dietary folate, vitamin C, vitamin E and flavonoid intakes were 0.40, 0.36, 0.29 and 0.46, respectively (**Table VI-1**, supplemental table).

Table VI-2 shows demographic, lifestyle and dietary factors by dietary nitrate intake quintiles. Age, BMI, age at menopause and age at first live birth were not substantially different across quintiles of dietary nitrate intake. The prevalence of the completion of college or some college education, alcohol intake and moderate or high physical activity were 62%, 33% and 62% higher, respectively, while never cigarette smoking was 42% lower in the highest quintile of dietary nitrate intake than the lowest quintile. The prevalence of estrogen use was slightly higher in the highest vs. lowest

quintile of dietary nitrate intake; whereas, there were no differences by family history of breast cancer, parity and oral contraceptive use. As expected, total energy intake, total folate, vitamin C, E, flavonoid and cruciferous vegetable intake were positively associated with dietary nitrate intake (p for tend <0.0001). Total folate and vitamin C and E intakes in the highest quintile of dietary nitrate were double and flavonoid intake was almost triple of that in the lowest quintile. Higher red meat intake was also associated with higher nitrate intake (p for tend <0.0001). The distributions of these baseline factors among public water users showed little associations with public water nitrate levels (246).

Overall, neither dietary nitrate nor nitrite intake was associated with risk of breast cancer after adjustment for potential cofounders (**Table VI-3**). There was a statistically significant inverse trend in breast cancer risk across quintiles of the nitrate to total folate ratio, but none of the risk estimates were statistically significant. The ratio of dietary nitrite intake to total folate intake was not associated with breast cancer risk (data not shown). Similarly, breast cancer risk was not associated with nitrate intake from drinking water or with private well use (**Table VI-4**).

There were no statistically significant interactions of nitrate intake from diet (in quintiles) and total folate intake (<400 μg/day or ≥400 μg/day) on risk of breast cancer (data not shown). A marginally statistically significant interaction between water nitrate intake and total folate intake (<400 μg/day or ≥400 μg/day) was observed in the highest (p for interaction = 0.055) and fourth (p for interaction = 0.053) quintiles of nitrate intake

from public water. Among women with adequate or higher total folate intake (≥400 μg/day), breast cancer risk was statistically significantly increased in women using public water with the highest quintile of nitrate and in those using private wells compared to those using public water with the lowest quintile of water nitrate intake; whereas, such an association was not observed among women with low total folate intake (<400 μg/day) (**Table VI-5**). There was no effect modification by total folate intake on the association between dietary nitrate intake and breast cancer risk.

D. Discussion

In this large prospective cohort study, we did not observe associations between nitrate intake from diet or drinking water and breast cancer risk. Among women with adequate or higher total folate intake (≥400 μg/day), breast cancer risk was statistically significantly increased among those using public water with the highest quintile of nitrate and among private well users compared to those using public water with the lowest nitrate level. Effect modification by total folate intake was not observed in the association between dietary nitrate intake and risk of breast cancer.

High nitrate intake has been a concern in relation to risk of methemoglobinemia (or blue-baby syndrome), a potentially fatal illness in infants, and therefore nitrate levels in public water supplies have been regulated. However, health effects of chronic intake of moderately high nitrate intake have not been considered when regulatory levels have been determined. Approximately 8% of ingested nitrate from foods and water is

endogenously reduced to nitrite, which can then react with amines and amides to form NOCs that may alkylate DNA forming DNA adducts (249). If DNA adducts are not removed by the DNA repair system, they may lead to chromosomal instability, which elevates genome-wide mutation rates (166).

The level of DNA adducts is influenced by the consumption of fruits and vegetables. In a large population-based study, DNA adduct level was negatively correlated with fruit and vegetable intakes (250). One potential mechanism that explains the preventive effect of fruits and vegetables against DNA adduct formation is that high folate levels in fruits and vegetables interfere with DNA adduct formation via enhanced function of the DNA repair system. The DNA repair system can identify DNA adducts and replace the entire portion of the damaged strand of the double helix with normal nucleotides (21).

In the previous case-control study in Korea, high intake of dietary nitrate relative to total folate was associated with increased risk of breast cancer (241). They also reported increased breast cancer risk only among individuals with low folate intake. In the present study, we did not observe such an interaction of folate and nitrate intakes from foods or from drinking water. One possible reason for the differences between our results may be the lower dietary nitrate intake in our study population compared with the Korean study population. The average dietary nitrate in our study population was 123.5 mg/day, while the average in Korea was approximately 420 mg/day (241), which is approximately twice the Acceptable Daily Intake (ADI, 3.7 mg/kg of body weight;

equivalent to 222 mg/day for a 60-kg person) established by the Joint Food and Agriculture Organization / World Health Organization (WHO) Expert Committee on Food Additives (251). The high dietary nitrate intake in Korea stems from frequent consumption of high nitrate vegetables including kimchi, traditional Korean fermented vegetables. The major vegetables in kimchi include cabbage and radishes that contain high levels of nitrate as well as NOC precursors and preformed NOCs (241, 252). According to the European Food Safety Authority, the WHO recommendation for fruit and vegetable consumption (400 gm/day) (253) would not exceed the ADI of nitrate, but high consumers of high-nitrate vegetables such as green leafy vegetables could exceed the ADI (254). In our study population, the average vegetable consumption was 26 servings/week (approximately 75% of the United States Department of Agriculture recommendation)(255), and the average consumptions of major contributors of dietary nitrate intake were lettuce (192 gm/week), celery (78 gm/week), and beets (34 gm/week).

Another hypothesized mechanism to explain the protective effect of fruit and vegetable intake against DNA adducts is the inhibition of oxidative damage by antioxidants contained in fruits and vegetables. Vitamin C and other antioxidants such as vitamin E and polyphenols inhibit the endogenous formation of NOCs and DNA adducts (171, 256). When taken between 2 h before and 1 h after administration of nitrate and amino acids, vitamin C inhibited the formation of NOCs (257). There are other constituents of fruits and vegetables that potentially explain the protective effect of fruits and vegetables against cancer. Isothiocyanates, potent anti-carcinogens rich in

cruciferous vegetables such as broccoli and cabbage, have shown to induce metabolic enzymes involved in the metabolism of dietary carcinogens and were associated with decreased risk of cancer (258, 259). In the present study, we adjusted for cruciferous vegetable intake as well as antioxidant intakes (vitamin C and E and flavonoids). When we did not adjust for intakes of folate, vitamin C and E, flavonoids and cruciferous vegetables, a statistically significant overall inverse association was observed between dietary nitrate intake and breast cancer risk, especially among women with low total folate intake (p for trend <0.05; data not shown). This inverse association might be due to confounding by antioxidants and other constituents of fruits and vegetables. In fact, such an inverse association with breast cancer risk was not observed in the water nitrate analysis. In addition, modest correlations of dietary antioxidant intakes with dietary nitrate intake (r = 0.29 – 0.46) and higher correlations with dietary folate intake (r = 0.33 – 0.72) might have caused residual confounding, which may partly explain lack of an interaction between dietary nitrate and folate intakes in our study.

The formation of NOCs and DNA adducts may be inhibited by antioxidants, and the repair of DNA adducts may be enhanced by an increased supply of folate. Nitrate intake from drinking water would, in theory, be more "carcinogenic" because water can be consumed without antioxidants and folate, but dietary sources of nitrate usually contain these nutrients abundantly. Some epidemiologic studies have shown increased risk of cancer related to higher nitrate levels in public water supplies (246, 260, 261), while others reported no associations (168, 169, 244). Nitrate levels in public water

supplies are strictly monitored and regulated, and thus nitrate intake from drinking water usually represents only a small portion of total nitrate intake from both diet and water among public water supply users.

In our study population, dietary nitrate and total nitrate intakes were highly correlated ($r = 0.93$), and an average of only 14% of total nitrate intake was accounted for by nitrate intake from drinking water. On the other hand, nitrate levels in private well water are not monitored or regulated, and some wells are high in nitrate concentrations because private wells are often located in rural agricultural areas. Recent data indicate approximately 22% domestic wells in U.S. agricultural area exceeded the MCL (262), and the Iowa Statewide Rural Well Water Survey from 1988 – 1989 showed that 18% of the well samples had higher nitrate levels than the MCL (263). Therefore, individuals using private well water could be considered as a group to have higher nitrate exposure in drinking water (acknowledging a wide range within the group).

In the current study, we found an increased risk of breast cancer in women with the highest nitrate intake from public water and in private well users compared to those using public water with the lowest nitrate level only among those with adequate or higher total folate intake (≥400 μg/day). The use of dietary supplements containing folic acid (folic acid or multivitamins) were higher (69.7%) in those with adequate or higher total folate intake compared to those with lower folate intake (8.3%). Among women with adequate or higher folate intake, the median of total folate intake was 689 μg/d (IQR = 596-805 μg/d) and 467 μg/d (IQR = 428-533 μg/d) in folic acid-containing supplement

users and nonusers, respectively. Folate has been considered to have a double-edged effect on cancer development. Higher folate intake may be protective in the preneoplastic stage, whereas high folate intake could accelerate cancer development once neoplastic lesions appear (264). The increased risk of breast cancer in users of public water with the highest nitrate level and in private well users among those with adequate or higher total folate intake may be related to a cancer promotion effect from excess folate intake and increased formation of NOCs. It should be also noted that elevated nitrate levels in water supplies can be an indicator of other potential carcinogenic contaminants in water such as pesticides. Increased risk of breast cancer related to higher water nitrate might be not only related to nitrate but to other water contaminants (265, 266). We did not have actual nitrate measurements in private well water. Future studies are needed to evaluate a nitrate-folate interaction as well as the potential effect of nitrate intake on breast cancer risk in private well users.

A major strength of this study is a large prospective cohort study design. Dietary intake was assessed prior to breast cancer diagnoses and more than 2,800 accumulated breast cancer cases were available for analysis. In addition to dietary nitrate intake, we analyzed nitrate in drinking water for public water supply users using a historical analytical database on municipal water supplies. The present study also has limitations. The study population was predominantly elderly Caucasians, and thus the findings may not be generalizable to other populations. Dietary nitrate intake in this study population was low compared to the Korean study that evaluated this hypothesis, which might be, in

part, why we observed different results. In addition to possible nondifferential misclassification, dietary intake assessed by a FFQ may not capture the information most relevant to breast cancer risk such as food preparation and storage methods as well as timing of food and water consumption. Dietary intake assessed at study baseline may have changed over the follow-up period, which may have caused nondifferential misclassification in dietary intake. We compared dietary intake at the baseline (1986) assessment and the 2004 assessment, and found the two measurements to be fairly well correlated suggesting little change in dietary intake patterns. Correlation coefficients for intakes of total calories, macronutrients, and folate with or without dietary supplements were 0.44, 0.39 – 0.42, 0.29 and 0.34, respectively. Nitrate intake from public drinking water was computed based on the annual average nitrate levels in public water supplies in each community and an estimated daily consumption of 2 L drinking water because information on the daily consumption of drinking water was not available. Therefore, water nitrate data used in this study did not reflect annual variations in nitrate levels in public water supplies or patterns of individual water consumption such as the amount and timing of water consumption. Lack of nitrate data in private wells is another limitation.

In summary, our findings do not support the previously reported increased risk of breast cancer risk among individuals with high dietary nitrate but low folate intakes. Among women with total folate intake of 400 μg/day or higher, risk of breast cancer was increased in users of public water with the highest quintile of nitrate and in private well users compared to women with the lowest quintile of nitrate from public water supplies.

Future studies are warranted and should consider multiple potential confounders including intakes of antioxidants and other nutrients contained in nitrate-rich foods.

E. Tables

Table VI-1. Spearman correlation coefficients (r) among selected dietary nutrient intake

	Nitrate	Nitrate-a[a]	Nitrate-p[b]	Nitrite	Nitrite-a[c]	Nitrite-p[d]	Folate	Vitamin C	Vitamin E	Flavonoids
Nitrate	1	0.308	0.999	0.499	0.246	0.543	0.403	0.359	0.288	0.457
Nitrate-a[a]		1	0.282	0.710	0.918	0.387	0.309	0.160	0.208	0.210
Nitrate-p[b]			1	0.482	0.222	0.536	0.397	0.357	0.284	0.456
Nitrite				1	0.731	0.883	0.397	0.250	0.305	0.401
Nitrite-a[c]					1	0.368	0.265	0.100	0.161	0.159
Nitrite-p[d]						1	0.379	0.286	0.320	0.465
Folate							1	0.615	0.715	0.334
Vitamin C								1	0.597	0.305
Vitamin E									1	0.230
Flavonoids										1

[a] Nitrate intake from animal sources
[b] Nitrate intake from plant sources
[c] Nitrite intake from animal sources
[d] Nitrite intake from plant sources

Table VI-2. Baseline demographic, lifestyle and dietary factors and dietary nitrate intake (quintiles)

	Total	Dietary nitrate intake quintiles					*p*
		Q1	Q2	Q3	Q4	Q5	
Age, mean (SD)	61.6 (4.2)	61.4 (4.2)	61.5 (4.2)	61.5 (4.2)	61.6 (4.2)	61.8 (4.2)	<0.0001
BMI, mean (SD)	27.0 (5.1)	26.8 (5.0)	26.9 (53.0)	27.1 (5.1)	27.0 (5.0)	27.1 (5.2)	<0.0001[a]
WHR, mean (SD)	0.84 (0.08)	0.84 (0.09)	0.83 (0.08)	0.84 (0.08)	0.84 (0.08)	0.83 (0.08)	<0.0001[a]
>High school education[c], %	39.8	29.7	36.6	39.8	44.6	48.2	<0.0001
Cigarette smoking, %							<0.0001
Never	65.6	62.6	65.3	66.3	67.6	66.4	
Former	19.4	17.6	18.4	19.3	19.4	22.2	
Current	15.0	19.8	16.3	14.4	13.0	11.4	
Alcohol intake, %	45.1	37.4	42.6	46.4	49.0	49.9	<0.0001
Physical activity, %							<0.0001
Low	47.5	60.6	52.9	46.8	41.1	36.0	
Moderate	27.5	22.9	26.5	28.3	30.3	29.6	
High	25.0	16.5	20.6	24.9	28.6	34.4	
Family history of breast cancer, %	23.0	22.5	22.3	23.9	23.1	23.3	0.14
Age at menarche, mean (SD)	12.8 (1.5)	12.9 (1.5)	12.9 (1.4)	12.8 (1.4)	12.8 (1.4)	12.8 (1.4)	<0.0001[a]
Age at menopause, mean (SD)	47.7 (6.4)	47.3 (6.5)	47.7 (6.3)	47.7 (6.3)	47.9 (6.4)	47.9 (6.3)	<0.0001[a]
Parity, %	91.0	90.4	91.3	91.1	91.0	91.2	0.41
Age at first live birth, mean (SD)	20.7 (7.6)	20.2 (7.7)	20.7 (7.5)	20.8 (7.6)	20.9 (7.6)	21.0 (7.6)	<0.0001[a]
Estrogen use, %	38.6	36.8	37.9	38.5	40.1	39.9	0.0002
Oral contraceptive use, %	19.3	19.0	19.3	19.1	19.3	19.7	0.81
Total energy (kcal), median	1,721	1,379	1,597	1,744	1,873	2,068	
Total folate (µg/d), median	350.7	242.4	296.8	335.2	378.1	468.9	<0.0001[b]
Vitamin C (mg/d), median	190.9	127.8	159.0	184.1	208.5	264.6	<0.0001[b]
Vitamin E (mg/d), median	9.7	6.5	8.1	9.2	10.6	13.1	<0.0001[b]
Flavonoids (mg/d), median	10.3	5.8	8.4	10.1	12.0	15.4	<0.0001[b]
Cruciferae (servings/week),median	2.5	1.5	2.0	2.5	3.5	5.0	<0.0001[b]

Red meat (servings/week), median	5.0	4.0	5.0	5.0	5.5	6.0	<0.0001[b]

BMI = body mass index; WHR = waist-hip-ratio

[a] *p* value for trend in lsmeans from an age-adjusted linear regression model

[b] *p* value for trend from an age- and energy-adjusted linear regression model using a log-transformed values.

[c] The completion of college or some college education.

Table VI-3. Dietary intake of nitrate and nitrite (quintiles) and breast cancer risk

	Q1 (reference)	Q2	Q3	Q4	Q5	p_{trend}
Nitrate						
Median (mg/d)	49.3	78.7	106.1	140.2	209.9	
Range (mg/d)	3.9 – 65.2	65.2 – 91.8	91.8 – 121.8	121.8 – 165.6	165.6 – 2,346.4	
Cases	604	541	575	601	554	
Person-years	123,277	125,273	124,907	125,466	124,333	
HR (95%CI)[a]	1.0	0.86 (0.76 – 0.98)	0.90 (0.79 – 1.02)	0.96 (0.84 – 1.10)	0.86 (0.74 – 1.01)	0.31
Nitrite						
Median (mg/d)	0.6	0.9	1.1	1.4	1.8	
Range (mg/d)	0.1 – 0.8	0.8 – 1.0	1.0 – 1.2	1.2 – 1.5	1.5 – 7.1	
Cases	532	604	573	589	577	
Person-years	123,423	124,696	124,948	124,903	125,284	
HR (95%CI)[a]	1.0	1.12 (0.98 – 1.28)	1.06 (0.92 – 1.22)	1.10 (0.94 – 1.28)	1.05 (0.86 – 1.29)	0.28
Nitrate-folate ratio						
Median	0.12	0.22	0.31	0.40	0.57	
Range	0.01 – 0.17	0.17 – 0.26	0.26 – 0.35	0.35 – 0.47	0.47 – 2.66	
Cases	569	575	608	560	563	
Person-years	122,949	124,816	124,405	126,670	124,415	
HR (95%CI)[a]	1.0	0.96 (0.84 – 1.10)	0.98 (0.84 – 1.14)	0.87 (0.75 – 1.02)	0.87 (0.74 – 1.03)	0.04

[a] Adjusted for age (continuous), total energy intake (continuous), BMI (continuous), WHR (continuous), education (<high school, high school, >high school), smoking (never, previous, current), physical activity level (low, moderate, high), alcohol intake (yes, no), family history of breast cancer (yes/no), age at menopause (continuous), age at first live birth (<20, 20 - <30, ≥30, nulliparous), estrogen use (never, ever), total intake of folate (except for nitrate-folate ratio), vitamin C and E and flavonoids, intakes of cruciferae and red meat.

Table VI-4. Nitrate intake from drinking water (quintiles) and breast cancer risk[a]

	Median (mg/2L)	Range (mg/2L)	Cases	Person-years	HR (95% CI)[b]
Water nitrate intake					
Q1	1.6	0 – 2.8	253	57,345	1.0 (reference)
Q2	4.1	3.0 – 7.9	255	56,101	1.07 (0.89 – 1.28)
Q3	9.4	8.0 – 14.1	244	57,163	0.96 (0.80 – 1.16)
Q4	21.2	14.3 – 33.3	250	54,775	1.05 (0.88 – 1.27)
Q5	57.8	33.5 – 145.3	286	57,902	1.14 (0.95 – 1.36)
p_{trend}					0.11
Private well users	N/A	N/A	463	96,326	1.14 (0.97 – 1.34)

[a] Analysis includes 20,147 women (15,151 public water supply users and 4,996 private well users) who responded to the 1989 follow-up survey, had used public water private well water supply for more than 10 years, and lived in communities which were served by one water source and had nitrate measurement data.

[b] Adjusted for age (continuous), total energy intake (continuous), BMI (continuous), WHR (continuous), education (<high school, high school, >high school), smoking (never, previous, current), physical activity level (low, moderate, high), family history of breast cancer (yes/no), estrogen use (never, ever), and total intakes of folate, vitamin C and E and flavonoids, intakes of cruciferae and red meat.

Table VI-5. Dietary and water nitrate intake (quintiles) and breast cancer risk by total folate intake

	Total folate intake			
	<400 μ/d		≥400 μg/d	
	Cases	HR (95%CI)[a]	Cases	HR (95%CI)[a]
Dietary nitrate (mg/d)				
Q1 (≤65.2)	456	1.0 (reference)	148	1.0 (reference)
Q2 (65.2 – 91.8)	374	0.81 (0.69 – 0.94)	167	1.00 (0.79 – 1.28)
Q3 (91.8 – 121.8)	398	0.93 (0.79 – 1.09)	177	0.82 (0.64 – 1.05)
Q4 (121.8 – 165.6)	311	0.83 (0.70 – 1.00)	290	1.18 (0.93 – 1.49)
Q5 (≥165.6)	192	0.83 (0.67 – 1.02)	362	0.94 (0.73 – 1.22)
p_{trend}		0.19		0.87
Water nitrate (mg/2L)[b]				
Q1 (≤2.8)	169	1.0 (reference)	84	1.0 (reference)
Q2 (3.0 – 7.9)	151	0.97 (0.77 – 1.22)	104	1.25 (0.92 – 1.68)
Q3 (8.0 – 14.1)	140	0.88 (0.70 – 1.12)	104	1.12 (0.83 – 1.52)
Q4 (14.3 – 33.3)	150	0.92 (0.73 – 1.16)	100	1.31 (0.97 – 1.78)
Q5 (≥33.5)	168	1.00 (0.79 – 1.25)	118	1.40 (1.05 – 1.87)
p_{trend}		0.71		0.04
Private well users	283	1.01 (0.83 – 1.24)	180	1.38 (1.05 – 1.82)

[a] Adjusted for age (continuous), total energy intake (continuous), BMI (continuous), WHR (continuous), education (<high school, high school, >high school), smoking (never, previous, current), physical activity level (low, moderate, high), alcohol intake (yes, no), family history of breast cancer (yes/no), age at menopause (continuous), age at first live birth (<20, 20 - <30, ≥30, nulliparous), and estrogen use (never, ever), and total intake of vitamin C and E and flavonoids, intakes of cruciferae and red meat.

[b] Analysis includes 20,147 women (15,151 public water supply users and 4,996 private well users) who responded to the 1989 follow-up survey, had used public water private well water supply for more than 10 years, and lived in communities which were served by one water source and had nitrate measurement data.

VII. SUMMARY

A. Summary of Results

Human data are lacking to support the hypothesis that deficient nutrient intake or/and genetic variation in OCM may alter the balance in OCM metabolites, and thus influence cancer risk via aberrant DNA methylation and DNA synthesis. Also, potential interactions between OCM nutrients and environmental factors such as carcinogenic agents on cancer risk have been understudied. This dissertation addressed these gaps in the current literature.

In the first manuscript, I hypothesized that OCM nutrient status and genetic variation in *MAT1A, MAT2A and MAT2B*, genes encoding enzymes catalyzing the synthesis of SAM from its precursor, methionine, are associated with plasma SAM levels among healthy Singapore Chinese adults in the SCHS. I found that choline and methionine were strongly and positively associated with plasma SAM levels (both *p* for trend <0.0001 in all subjects), and plasma folate and betaine levels were associated with plasma SAM only in men (both *p* for trend = 0.02). I did not find associations between the genotypes of *MAT1A* rs2993763, *MAT2A* rs2289972 and *MAT2B* rs7733775 and plasma SAM levels, but the association between *MAT1A* rs2993763 genotypes and plasma SAM was modified by gender and plasma methionine levels.

In the same healthy Singapore Chinese population, the second manuscript tested the hypothesis that plasma SAM levels alone or in combination with genetic variation in *DNMT1*, *DNMT3A* and *DNMT3B*, genes encoding enzymes catalyzing DNA methylation

reactions, are associated with global DNA methylation (measured as the LINE-1 methylation index). I found that the LINE-1 methylation index was positively associated with plasma SAM levels (p <0.01), with a plateau at approximately 78% methylation (55 nmol/L plasma SAM) in men and 77% methylation (50 nmol/L plasma SAM) in women. Men carrying at least one copy of the variant allele of *DNMT1* rs2114724 and *DNMT3A* rs758127 had significantly higher and lower LINE-1 methylation index, respectively, compared with men carrying the wild type genotype (both p for trend <0.01). I also found a modulating effect of *DNMT1* rs2114724 genotype on the association between plasma SAM and the LINE-1 methylation index only among men.

Combined findings from the first and second manuscripts together provide evidence supporting the association between OCM nutrients/metabolites and global DNA methylation through the availability of SAM, the methyl donor for DNA methylation. To my knowledge, no previous studies comprehensively examined the link between OCM nutrients and global DNA methylation via circulating SAM levels. These findings contribute evidence to fill an important knowledge gap on the biologic mechanisms behind the OCM-cancer association. In addition, my finding of interactions between the OCM nutrient/metabolite and genetic variation will serve as the basis for identifying sub-populations for whom improving OCM nutrient/metabolite status may be particularly meaningful for the maintenance of an adequate methyl pool for DNA methylation.

The third manuscript addressed potential interactions between OCM nutrients and environmental factors on cancer risk. In the IWHS, a large prospective cohort study, I

tested a hypothesis that higher intake of nitrate from diet or drinking water is associated with breast cancer risk only among those with low total folate intake, which was previously shown in a case-control study in Korea. Overall, I did not observe associations between nitrate intake from diet or drinking water and breast cancer risk. Effect modification by total folate intake was not observed in the association between dietary nitrate intake and breast cancer risk. Among women with adequate or higher total folate intake (≥400 μg/day), however, breast cancer risk was statistically significantly increased among those in the highest quintile of nitrate intake from public water (HR = 1.40, 95% CI = 1.05 – 1.87, *p* for trend = 0.04) and among private well users (HR = 1.38, 95% CI = 1.05 – 1.82), compared to those in the lowest quintile of nitrate intake from public water. These findings do not support the previously reported increased risk of breast cancer risk among individuals with high dietary nitrate but low folate intakes.

B. Main Strengths and Limitations

As with most studies, the three projects in this dissertation have strengths and limitations. Using the same study population, the first and second manuscripts share common limitations. One concern is the possible degradation of plasma SAM, which was the outcome and exposure of interest in the first and second manuscripts, respectively, during a long-term storage of plasma samples. The relatively low mean plasma SAM levels observed in the study subjects (63.3 nmol/L) compared with reference values observed in populations in Europe (70 – 128 nmol/L) (193) may appear

to reinforce this concern, but the facts that 1) the wide range of plasma SAM in various studies (50 to 150 nmol/L) might be due to different study populations and different laboratory methods (158, 190, 202-206), 2) no reference data on plasma SAM levels are available for Asian populations, and 3) two measurements of Hcy using aliquots from the same study participants performed in different laboratories more than 10 years apart showed high correlation ($r = 0.7$) in our study population, indicate that possible degradation was minimal. Furthermore, the degradation of plasma SAM would not result in the observed associations, given that it occurred in a non-differential manner. Another limitation is its cross-sectional study design, which does not allow us to determine cause-effect relationships. However, it would be extremely difficult to disentangle a complicated OCM pathway involving multiple nutrients and metabolites in an intervention study. For example, the number of intervention arms would significantly increase to assess impacts of each OCM nutrient separately controlling for other nutrient status or/and genetic variation, and thus a large number of study subjects would be required to have statistical power. Thirdly, all of the study subjects were cancer-free healthy individuals, and thus variability in plasma levels of OCM metabolites and the LINE-1 methylation index was potentially limited. However, my aim here was to evaluate associations among OCM nutrients/metabolites and genetic variation and global DNA methylation in a free-living human population. Reporting the distributions of plasma SAM levels and the LINE-1 methylation index, and their correlates, in a general population is also important. Next, a relatively small sample size limited statistic power

to analyze interactions between OCM nutrient/metabolite status and multiple genetic variants. Lastly, the LINE-1 methylation index used as a marker of global DNA methylation in the second manuscript might not be a perfect reflection of global DNA methylation because there is a mix of active and inactive LINE-1 elements in the genome, and inactive elements have been associated with hypomethylation.

Nonetheless, these two studies are novel, because, for the first time, almost all major compounds in OCM and several genetic variants were simultaneously measured to analyze their associations with plasma SAM or the LINE-1 methylation index comprehensively. The diagram-based approach using path analysis in the first manuscript provided further insight on the directed dependencies among multiple molecules in OCM, and is an additional strength. Furthermore, data from these two projects provide important information on frequencies of genetic variants of *MAT*s and *DNMT*s, and identification of their association with plasma SAM or the LINE-1 methylation index in the understudied Chinese population in South Asia.

A major strength of the third manuscript is a large prospective cohort study design, where folate and nitrate intake was assessed prior to breast cancer diagnosis, and over 2,800 incident breast cancer cases accumulated during the 22-year follow-up. In addition to dietary nitrate intake, data on nitrate intake from public water were obtained using a historical analytical database on municipal water supplies. A major limitation is lack of information on nitrate levels in private well water, though nitrate levels in private well water sometimes may be higher compared to public water, where nitrate levels are

strictly regulated and monitored. Other limitations include limited generalizability of study results because of the Caucasian dominant population, relatively low dietary nitrate intake, and lack of data on volume of daily water consumption.

C. Future Directions

Findings from the three projects in this dissertation have no clinical relevance but provide us further insights and support to the mechanisms between OCM and carcinogenesis. One of the possible reasons for conflicting results from previous studies on OCM and cancer risk is the complex carcinogenic mechanism via OCM. OCM is a multi-step process involving a number of intermediate compounds and reactions, and possibly interactions with other nutrients and environmental and genetic factors. When examining the association between dietary OCM nutrient intake and cancer risk, such as dietary choline intake and cancer incidence, in a population-based study, the effect of dietary intake on cancer risk may not be detected, because many intermediate factors may modify the effect of the OCM nutrient, if there actually is any effect. For example, choline status is not determined solely by ingested choline from diet due to the *de novo* biosynthesis of choline. Choline metabolism involves multiple reactions, from choline to methionine, and then to SAM, which donates methyl groups to DNA methylation. All these metabolic reactions require catalyzing enzymes whose activity levels may be affected by genetic and/or environmental factors.

To understand mechanisms between OCM and carcinogenesis, investigating associations among intermediate molecules on the OCM pathway is essential for future research. The intriguing findings from the first and second manuscripts, the associations between plasma choline or methionine and plasma SAM levels and the association between plasma SAM levels and the LINE-1 methylation index, are an important step in pursuit of understanding the impact of OCM nutrients on carcinogenesis via DNA methylation. Particularly interestingly, choline had a stronger association with plasma SAM than did folate in our Singapore Chinese population. Whether this finding holds true in a population with high folate intake from folic acid food fortification and/or dietary supplement use needs to be tested in future studies. In addition, it will be of benefit to examine the *PEMT*-catalyzed choline *de novo* biosynthesis, because it might affect the availability of SAM, and thus DNA methylation. Premenopausal women are known to be relatively resistant to choline deficiency, because *PEMT* gene expression is induced by estrogen (267). Therefore, it is important to investigate to what extent *de novo* biosynthesized choline affects choline status and whether demographic/genetic/environmental factors such as gender, genetic variation in *PEMT*, BMI and estrogen levels impact on the *de novo* choline biosynthesis.

In the third manuscript, I found the increased risk of breast cancer related to the combination of high water nitrate intake and high total folate intake. The association between extremely high folate intake, especially from fortified food and dietary supplements, and cancer risk is still not understood, but the increased risk of breast

cancer observed among women with high nitrate intake from public water and private well users among those with adequate or higher total folate intake in this project may be related to a cancer promotion effect from excess folate intake and increased formation of NOCs. Data on nitrate levels in private well water were not available in the present project, but considering that private wells are often located in rural agricultural areas where water contamination with nitrate from nitrogen fertilizers and animal wastes are more likely, nitrate levels in some private wells are likely to be high. Future studies should be conducted on nitrate intake from private well water and its possible interaction with total folate intake on breast cancer risk, especially in a population with high folate intake from fortified food and dietary supplements. In the IWHS, the future research plan is to assess nitrate levels in private well water by geocoding from street address and zip codes using Geographic Information Systems, and to evaluate 1) whether women drink private well water have higher water nitrate intake than public water users and 2) nitrate intake from drinking water (public and private well) is associated with breast cancer risk.

OCM is a complicated pathway, and there are a number of gaps in our understanding of the mechanisms behind its link with cancer risk. Findings from this dissertation research contributed evidence to fill these gaps. This dissertation is a step in pursuit of my long-term goal to enhance our understanding of cancer etiology via OCM.

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IX. APPENDICES

A. Selected SNPs of *DNMT1*, *DNMT3A* and *DNMT3B* in the Singapore Chinese Health Study (Manuscript 2)

Originally selected SNPs: n = 33

***DNMT1* (n=17)**

Tagging SNPs	rs2228611	Linkage w/ rs2114724 (r=0.996); excluded
	rs2288350	
	rs7253062	
Proxies	rs10418707	r^2=1.0 w/ rs2288350
	rs4804490	r^2=1.0 w/ rs2288350
	rs4804125	r^2=1.0 w/ rs2288350
	rs8101626	r^2=1.0 w/ rs7253062
	rs2288349	r^2=1.0 w/ rs7253062
	rs6511677	r^2=1.0 w/ rs7253062
	rs2290684	r^2=1.0 w/ rs2228611
	rs759920	r^2=1.0 w/ rs2228611
	rs8101866	r^2=1.0 w/ rs2228611
Literature	rs2114724	Chu *et al.* 2009 – HBV clearance
	rs2241531	Chu *et al.* 2009 – HBV clearance
	rs1863771	Failed in genotyping set-up
	rs1699593	No variation in the SCHS
	rs75616428	No variation in the SCHS

***DNMT3A* (n=13)**

Tagging SNPs	rs6722613	
	rs7575625	
	rs7581217	
	rs7587636	
	rs12987326	Dropped (HW=0.0218); no proxy
	rs12999687	Dropped (HW=0.0422); no proxy
	rs13036246	
	rs34048824	
Proxies	rs749131	r^2=1.0 w/ rs7587636
	rs13401241	r^2=1.0 w/ rs7587636
	rs6713377	r^2=0.971 w/ rs7575625
	rs6711622	r^2=1.0 w/ rs34048824
Literature	rs1550117	Fan *et al.* 2010 – gastric cancer risk

***DNMT3B* (n=3)**

Tagging SNPs	rs2424908	

	rs6141813	
Proxy	rs6119954	r^2=0.906 w/ rs6141813

SNPs included in the analysis and the manuscript: n = 13

DNMT1 **(n=4)**		
Tagging SNPs	rs2288350	HW=0.6986
	rs7253062	HW=0.1270
Literature	rs2114724	HW=0.2532
	rs2241531	HW=0.8860
DNMT3A **(n=7)**		
Tagging SNPs	rs6722613	HW=0.1327
	rs7575625	HW=0.1059
	rs7581217	HW=0.5469
	rs7587636	HW=0.0550
	rs13036246	HW=0.2974
	rs34048824	HW=0.0652
Literature	rs1550117	HW=0.6369
DNMT3B **(n=2)**		
Tagging SNPs	rs2424908	HW=0.3067
	rs6141813	HW=0.3263

* HW = *p* value from Hardy-Weinberg equilibrium chi-square test

Orders of SNPs in the chromosome for haplotype analysis

Gene	Chr	Order	SNP	Position
DNMT1	19	1	rs7253062	15632
		2	rs2288350	26824
		3	rs2241531	39722
		4	rs2228611	43679
		5	rs2114724	45508
DNMT3A	2	1	rs1550117	4553
		2	rs12987326	19465
		3	rs7575625	21558
		4	rs6722613	31103
		5	rs34048824	34917
		6	rs13036246	37491
		7	rs7581217	45516
		8	rs7587636	51598
		9	rs12999687	58022
DNMT3B	20	1	rs2424908	15193
		2	rs6141813	21053

B. Haplotypes of *DNMT1*, *DNMT3A* and *DNMT3B* and the LINE-1 methylation index (%) in the Singapore Chinese Health Study (Manuscript 2)

DNMT1

	SNP				Men			Women		
	rs7253061	rs2288350	rs2241531	rs2114724	%	OR (95%CI)[a]	*p*	%	OR (95%CI)[a]	*p*
ht1	G	T	G	C	0.454	1.00 (reference)	-	0.457	1.00 (reference)	-
ht2	G	C	C	T	0.288	0.64 (0.40 – 1.05)	0.08	0.276	0.94 (0.61 – 1.45)	0.78
ht3	A	C	C	C	0.214	1.40 (0.78 – 2.52)	0.27	0.215	1.00 (0.64 – 1.58)	0.99
Others	-	-	-	-	0.043	0.54 (0.18 – 1.59)	0.54	0.052	1.13 (0.50 – 2.56)	0.76

DNMT3A

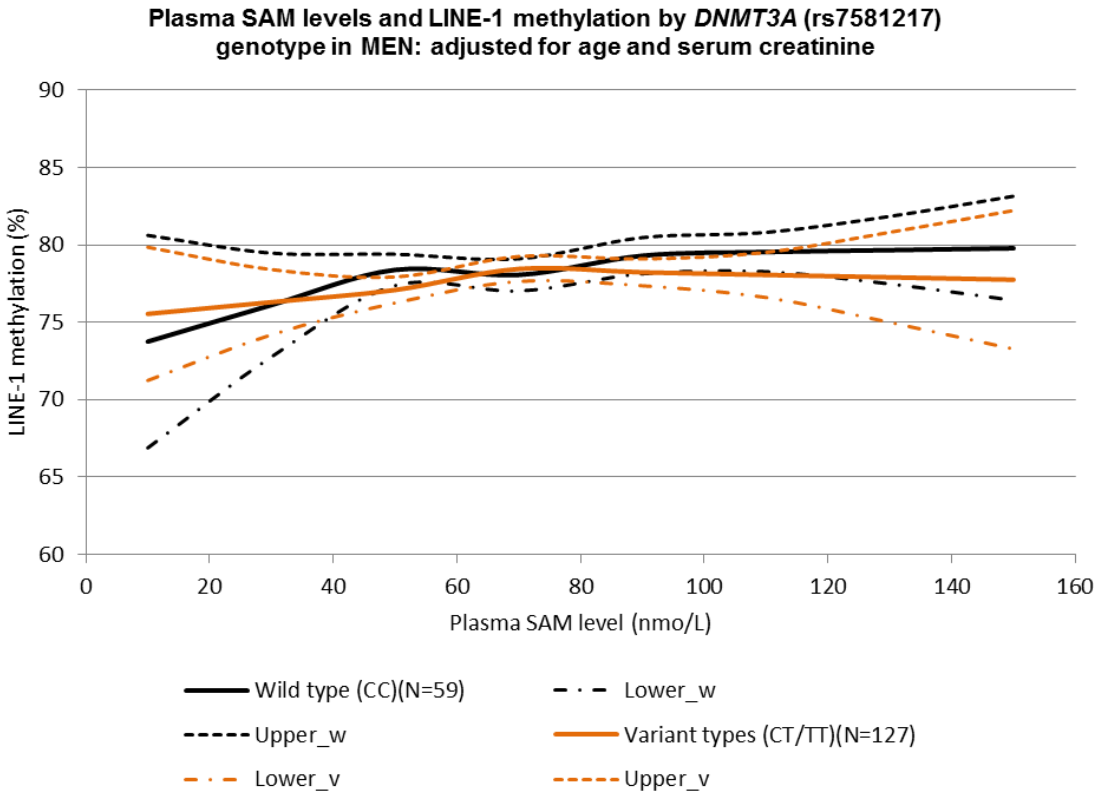
	SNP							Men			Women[b]		
	rs1550117	rs7575625	rs6722613	rs34048824	rs13036246	rs7581217	rs7587636	%	OR (95%CI)[a]	*p*	%	OR (95%CI)[a]	*p*
ht1	C	A	G	T	C	C	G	0.374	1.00 (reference)	-	0.359	1.00 (reference)	-
ht2	T	A	G	T	C	T	G	0.181	0.64 (0.89 – 3.03)	0.12	0.168	1.07 (0.60 – 1.90)	0.82
ht3	C	G	A	C	T	T	A	0.107	1.75 (0.83 – 3.70)	0.14	0.087	1.79 (0.88 – 3.64)	0.13
ht4	C	A	A	T	T	C	A	0.102	0.92 (0.44 – 1.90)	0.82	0.094	0.78 (0.38 – 1.60)	0.47
ht5	C	G	A	C	C	T	A	0.065	1.37 (0.60 – 3.81)	0.38	0.098	0.71 (0.37 – 1.36)	0.28
ht6	T	A	G	T	C	C	G	0.053	0.86 (0.31 – 2.34)	0.76	0.041	1.95 (0.78 – 3.24)	0.34
Others	-	-	-	-	-	-	-	0.118	1.43 (0.68 – 3.00)	0.34	0.154	1.11 (0.65 – 1.89)	0.71

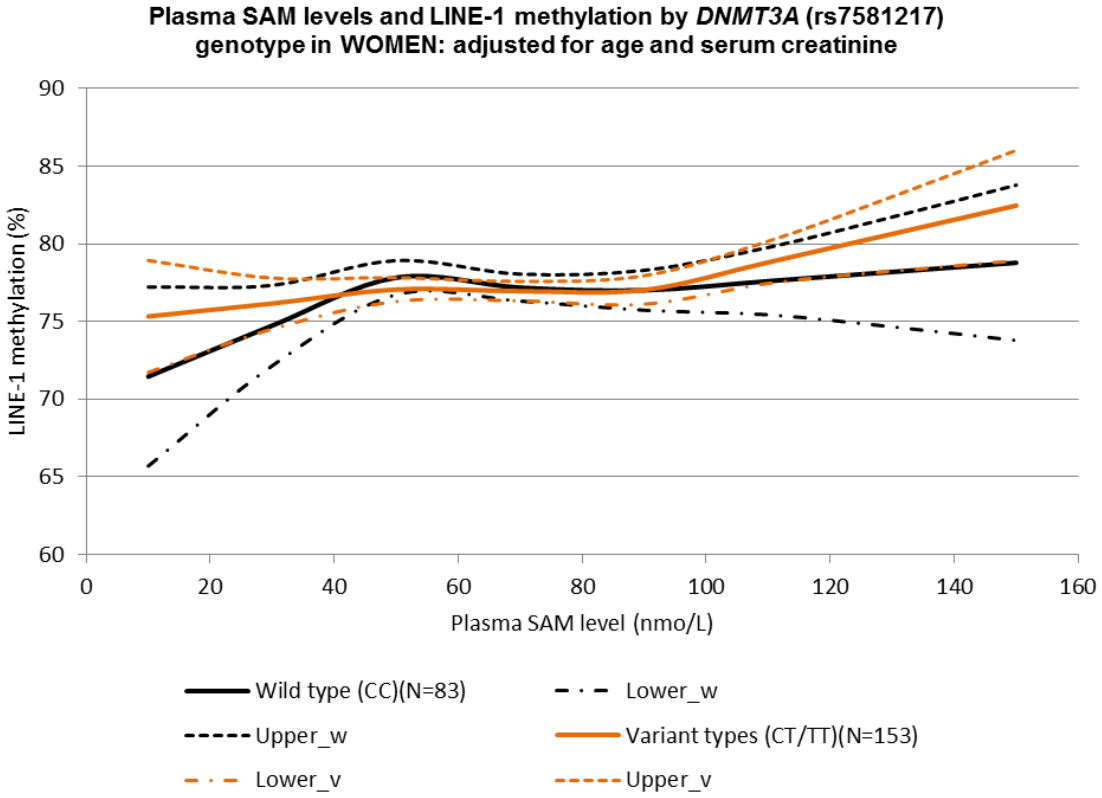
DNMT3B

	SNP		Men			Women[b]		
	rs2424908	rs614813	%	OR (95%CI)[a]	*p*	%	OR (95%CI)[a]	*p*
ht1	C	A	0.548	1.00 (reference)	-	0.604	1.00 (reference)	-
ht2	T	G	0.359	0.95 (0.63 – 1.44)	0.82	0.343	1.08 (0.73 – 1.59)	0.69
ht3	T	A	0.093	1.04 (0.49 – 2.23)	0.92	0.053	1.27 (0.57 – 2.82)	0.55

[a] OR of having the low LINE-1 methylation value (<78% in men and <77% in women), adjusted for age at blood draw.
[b] The minimal frequency of haplotypes were set at 0.04 because the frequency of haplotype 6 among women was <0.05.

C. Spline curves for plasma SAM levels and the LINE-1 methylation index by *DNMT3* rs7581217 (Manuscript 2)




Plasma SAM levels and LINE-1 methylation by *DNMT3A* (rs7581217) genotype in WOMEN: adjusted for age and serum creatinine
LINE-1 methylation (%)
90
85
80
75
70
65
60
0
20
40
60
80
100
120
140
160
Plasma SAM level (nmo/L)
Wild type (CC)(N=83)
Lower_w
Upper_w
Variant types (CT/TT)(N=153)
Lower_v
Upper_v

D. Additional data and analysis results for nitrate intake and breast cancer risk in the Iowa Women's Health Study (Manuscript 3)

Dietary nitrate analysis

P values for nitrate-folate interactions (continuous variables, multivariate model)

Nitrate	0.48
Nitrate from plant sources	0.31
Total nitrate & nitrite	0.31

Stratified by supplemental folic acid intake (multivitamin or folic acid)

	Supplemental folic acid intake		Total
	Yes (%)	No (%)	
Total	11,468 (33.4)	22,920 (66.6)	34,388
Total folate intake			
Low (< median)	1,272 (7.4)	15,921 (92.6)	17,193
High (≥ median)	10,196 (59.3)	6,999 (40.7)	17,195
Q1	495 (5.8)	8,101 (94.2)	8,596
Q2	777 (9.0)	7,820 (91.0)	8,597
Q3	2,372 (27.6)	6,226 (72.4)	8,598
Q4	7,824 (91.0)	773 (9.0)	8,597
< 400 µg/d	1,679 (8.2)	18,664 (91.8)	20,343
400 - < 600 µg/d	2,534 (41.2)	3,623 (58.8)	6,157
≥ 600 µg/d	7,255 (92.0)	633 (8.0)	7,888

P values for nitrate-folate interactions (two-level variables for total folate intake, multivariate model) by folic acid intake from dietary supplements

Dietary intake	Supplemental folic acid intake	
	Yes	No
Nitrate	0.11	0.65

Water nitrate analysis

Data on main source of water

	N	%
Municipal water	14,712	42.8
Rural water system	439	1.3
Subtotal	***15,151***	*44.1*
Private well	4,996	14.5
Subtotal	***20,147***	*58.6*
Others[a]	14,241	41.4

[a] Including 1) women living in 47 communities that were served by municipal water sources and 41 communities for which no nitrate measurement data were available and 2) and women who had used their public or private well supply for 10 years or less (Ward *et al*. *Epidemiology* 2010; 21(3): 389-95).

Duration of use of drinking water (N=20,147)

	N	%
11 – 20 y	14,712	42.8
> 20 y	439	1.3

Statistics of nitrate in drinking water (N=15,151)

	Mean	SD	Median	Range
Mean nitrate-N (mg/L)	2.10	2.35	1.06	0 – 16.41
Nitrate-N intake from water[a] (mg/L)	4.21	4.70	2.12	0 – 32.82
Nitrate intake from water[a] (mg/L)	18.64	20.83	9.39	0 – 145.33

[a] Estimated daily consumption of 2L water

Correlation coefficients between total nitrate intake and dietary nitrate intake

Spearman	0.934
Pearson	0.971

Dietary nitrate intake / total nitrate intake

Minimum	0.097
Maximum	1.0
Mean	0.859

Baseline characteristics and breast cancer risk

	Cases, (%) (n=2,875)	Person-years	HR[a] (95% CI)	p[b]
Age				
< 60	1,011 (35.2)	239,572	1.0	0.0095
60 – < 65	1,108 (38.5)	221,665	1.2 (1.1 – 1.3)	
≥ 65	756 (26.3)	162,018	1.1 (1.0 – 1.2)	
< 60	1,011 (35.2)	239,572	1.0	0.0002
≥ 60	1,864 (64.8)	383,683	1.2 (1.1 – 1.2)	
BMI				
< 25	990 (34.4)	247,856	1.0	< 0.0001
25 – < 30	1,139 (39.6)	233,038	1.2 (1.1 – 1.3)	
≥ 30	746 (26.0)	142,360	1.3 (1.2 – 1.4)	
WHR				
Q1 (0.34 – 0.77)	680 (23.7)	160,951	1.0	0.14
Q2 (0.77 – 0.83)	701 (24.5)	157,761	1.0 (0.9 – 1.2)	
Q3 (0.84 – 0.89)	714 (76.9)	155,027	1.1 (1.0 – 1.2)	
Q4 (0.90 – 2.41)	769 (26.9)	147,304	1.2 (1.1 – 1.4)	
Education				
< High school	442 (15.4)	111,836	1.0	< 0.0001
High school	1,193 (41.5)	265,004	1.1 (1.0 – 1.3)	
> High school	1,240 (43.1)	246,415	1.3 (1.2 – 1.4)	
Cigarette smoking				
Never	1,893 (66.5)	417,943	1.0	0.11
Former	574 (20.2)	115,348	1.1 (1.0 – 1.2)	
Current	379 (13.3)	81,614	1.1 (0.9 – 1.2)	
Never	1,893 (66.5)	417,943	1.0	0.07
Ever	982 (34.2)	205,312	1.1 (1.0 – 1.2)	
Alcohol intake				
No	1,542 (53.6)	342,324	1.0	0.11
Yes	1,333 (46.4)	280,931	1.1 (1.0 – 1.1)	
Regular physical activity				
No	1,695 (59.5)	358,675	1.0	0.13
Yes	1,153 (40.5)	256,770	0.9 (0.9 – 1.0)	
Physical activity				
Low	1,360 (47.6)	286,822	1.0	0.038
Moderate	827 (29.0)	170,670	1.0 (0.9 – 1.1)	
High	670 (23.4)	156,336	0.9 (0.8 – 1.0)	

	Cases, (%) (n=2,875)	Person-years	HR[a] (95% CI)	p^b
Family history of breast cancer				
No	2,026 (70.5)	480,625	1.0	< 0.0001
Yes	849 (29.5)	142,630	1.4 (1.3 – 1.5)	
Age at menarche				
< 13	1,251 (43.9)	262,476	1.0	0.12
≥ 13	1,602 (56.1)	354,670	0.9 (0.9 – 1.0)	
Estrogen use				
Never	1,705 (59.4)	382,625	1.0	0.023
Ever	1,164 (40.6)	238,917	1.1 (1.0 – 1.2)	
Oral contraceptive use				
Never	2,311 (80.5)	499,644	1.0	0.65
Ever	561 (19.5)	122,924	1.0 (0.9 – 1.1)	
Residence location				
Farm	604 (21.2)	128,348	1.0	0.44
Rural (not farm)	220 (7.7)	44,931	1.0 (0.9 – 1.2)	
Town (< 1,000)	269 (9.4)	67,532	0.8 (0.7 – 1.0)	
Town (1,000 – 2,499)	281 (9.8)	65,763	0.9 (0.8 – 1.0)	
Town (2,500 – 9,999)	477 (16.6)	106,116	1.0 (0.8 – 1.1)	
Town (≥ 10,000)	1,009 (35.3)	206,685	1.0 (0.9 – 1.1)	
Total energy intake (kcal/d)				
601 – 1,379	691 (24.0)	155,095	1.0	0.35
1,379 – 1,721	725 (25.2)	155,734	1.0 (0.9 – 1.2)	
1,721 – 2,128	723 (25.2)	155,666	1.0 (0.9 – 1.2)	
2,128 – 4,998	736 (25.6)	156,760	1.1 (1.0 – 1.2)	
Folate intake (μg/d)				
43.4 – 250.9	730 (25.4)	155,768	1.0	0.57
250.9 – 350.7	715 (24.9)	156,747	1.0 (0.9 – 1.1)	
350.7 – 572.1	714 (24.8)	156,281	0.9 (0.8 – 1.1)	
572.1 – 2,555.2	716 (24.9)	154,459	1.0 (0.9 – 1.1)	
< 400	1,731 (60.2)	369,731	1.0	0.08
400 – < 600	490 (17.0)	111,931	0.9 (0.8 – 1.0)	
≥ 600	654 (22.8)	141,593	1.0 (0.9 – 1.1)	
Vitamin C intake (mg/d)				
2.2 – 126.3	723 (25.2)	156,116	1.0	0.31
126.4 – 190.9	750 (26.1)	157,091	1.0 (0.9 – 1.1)	
191.0 – 324.6	710 (24.7)	156,859	1.0 (0.9 – 1.1)	
324.7 – 4,683.6	692 (24.1)	153,189	1.0 (0.9 – 1.1)	

	Cases, (%) (n=2,875)	Person-years	HR[a] (95% CI)	p[b]
Vitamin E intake (mg/d)				
1.2 – 6.4	722 (25.1)	152,431	1.0	0.63
6.5 – 9.6	747 (26.0)	158,537	1.0 (0.9 – 1.1)	
9.7 – 30.3	694 (24.1)	158,163	0.9 (0.8 – 1.0)	
30.4 – 1,212.0	712 (24.8)	154,124	0.9 (0.8 – 1.1)	
Total flavonoid intake (mg/d)				
0 – 6.5	695 (24.2)	155,212	1.0	0.84
6.6 – 10.3	762 (26.5)	157,642	1.1 (1.0 – 1.2)	
10.4 – 16.5	706 (24.6)	155,810	1.0 (0.9 – 1.1)	
16.6 – 228.0	712 (24.8)	154,591	1.0 (0.9 – 1.1)	

[a] Age-adjusted HR and 95% CI; additional adjustment for total energy intake (kcal) for nutrient intakes

[b] Wald χ^2 test p values for trend