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REVIEW

Methyl nutrients, DNA methylation, and cardiovascular disease

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Diet plays an important role in the development and prevention of cardiovascular disease (CVD), but the molecular mechanisms are not fully understood. DNA methylation has been implicated as an underlying molecular mechanism that may account for the effect of dietary factors on the development and prevention of CVD. DNA methylation is an epigenetic process that provides "marks" in the genome by which genes are set to be transcriptionally activated or silenced. Epigenomic marks are heritable but are also responsive to environmental shifts, such as changes in nutritional status, and are especially vulnerable during development. *S*-adenosylmethionine is the methyl group donor for DNA methylation and several nutrients are required for the production of *S*-adenosylmethionine. These methyl nutrients include vitamins (folate, riboflavin, vitamin B12, vitamin B6, choline) and amino acids (methionine, cysteine, serine, glycine). As such, imbalances in the metabolism of these nutrients have the potential to affect DNA methylation. The focus of this review is to provide an overview on the current understanding of the relationship between methyl nutrient status and DNA methylation patterns and the potential role of this interaction in CVD pathology.

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1 Introduction

Diet plays a role in the development of cardiovascular disease (CVD). The underlying molecular mechanisms by which dietary constituents contribute to CVD pathology are only partially understood, but may involve epigenetic processes. Epigenetics can be defined as changes in gene expression that occur without changes in DNA sequence. Epigenetic processes include DNA methylation, chromatin modifications (histone methylation, acetylation), and miRNA. These processes provide "marks" in the genome by which genes are set to be transcriptionally activated or silenced and are heritable, transmitted from parent to daughter cells during mitosis [1]. However, epigenomic marks are also responsive to environmental shifts, like changes in nutritional status [2], and as such, are attractive diet-related targets for the prevention and treatment of CVD. Changes in DNA methylation patterns have been implicated in the pathology of CVD [3–7]. However knowledge regarding the effect of dietary factors on DNA methylation patterns and the role in CVD pathology is in its infancy and further studies are required. Such studies will help improve our understanding of the role of diet in the etiology of CVD and provide new targets for diet-related CVD prevention and treatment.

We will provide an overview of the current state of knowledge regarding dietary factors and DNA methylation in the context of CVD pathology. Dietary factors required for methylation reactions are often referred to as "methyl nutrients" and are required for the generation of *S*-adenosylmethionine (AdoMet), the key methyl donor for DNA, RNA, proteins, and phospholipids [8]. Methyl nutrients include: vitamins, such

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Abbreviations: **AdoHcy**, *S*-Adenosylhomocysteine; **AdoMet**, *S*-adenosylmethionine; **BMI**, body mass index; **CAD**, coronary artery disease; **CBS**, cystathionine-β-synthase; **CVD**, cardiovascular disease; **DNMT**, DNA methyltransferase; **HHcy**, hyperhomocysteinemia; **MTHFR**, methylenetetrahydrofolate reductase; **PBMCs**, peripheral blood mononuclear cells; **PEMT**, phosphatidylethanolamine methyltransferase; **THF**, tetrahydrofolate

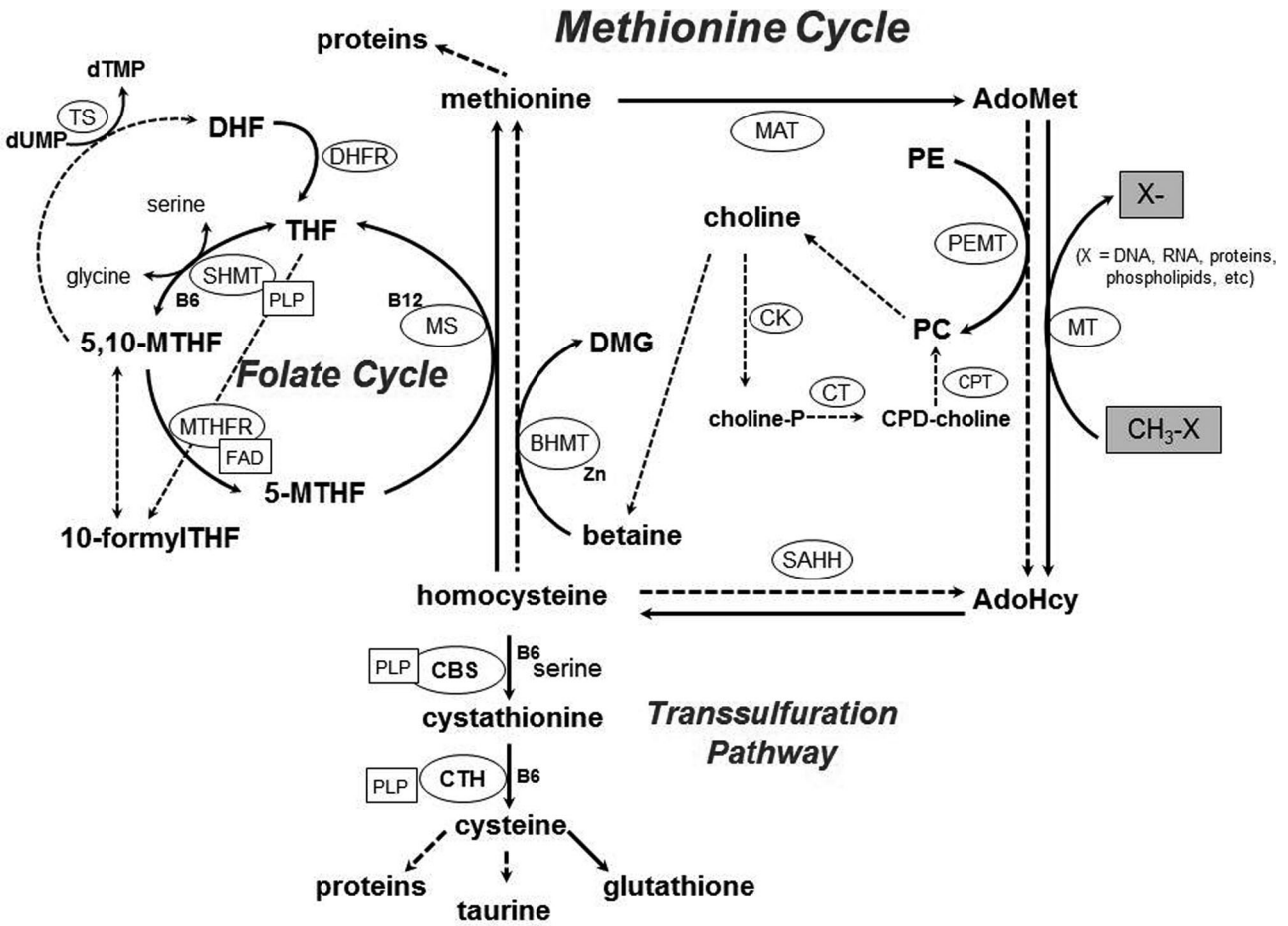

Figure 1. Schematic representation of the metabolic pathways associated with generation of *S*-adenosylmethionine, as characterized in the liver. AdoHcy, adenosylhomocysteine; AdoMet, adenosylmethionine; B12, vitamin B12; BHMT, betaine:homocysteine methyltransferase; CBS, cystathionine β-synthase; CDP-choline, cytidine diphosphocholine; choline-P, phosphocholine; CK, choline kinase; CPT, choline phosphotransferase; CT, CTP:phosphocholine cytidylyltransferase; CTH, cystathionine γ-lyase; DHF, dihydrofolate; FAD, flavin adenine dinucleotide; MAT, methionine adenosyltransferase; MS, methionine synthase; 5-MTHF, 5-methyltetrahydrofolate; 5,10-MTHF, 5,10-methylenetetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; MT, methyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamaine; PEMT, phosphatidylethanolamine methyl transferase; PLP, pyridoxal phosphate; SAHH, AdoHcy hydrolase; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; Zn, zinc.

as folate, riboflavin, vitamin B12, vitamin B6 (pyridoxal phosphate), and choline; and amino acids, such as methionine, cysteine, glycine, and serine. A summary of research assessing the relationships between alterations in methyl nutrient status with changes in DNA methylation, highlighting the consequences of methyl nutrient imbalance and folic acid fortification will be presented. This will be followed by a synopsis of our current understanding of how diet-related changes in DNA methylation patterns affect CVD pathology.

2 Methyl nutrient metabolism

Methyl nutrients are metabolically required for the generation of AdoMet, the primary methyl donor for DNA, RNA, proteins, and lipids [8]. AdoMet generation involves three interrelated biochemical pathways: folate cycle, methionine cycle, and the transsulfuration pathway. Below is a brief description of these pathways and their metabolic relationship is illustrated in Fig. 1.

2.1 Folate cycle

Folate is a water-soluble B-vitamin. The active form of folate is tetrahydrofolate (THF) [9], which functions in the production of purines, pyrimidines, and the remethylation of homocysteine to methionine. THF is used for purine synthesis following conversion to 10-formylTHF and for thymidylate synthesis following conversion to 5,10-methyleneTHF. Conversion to 5,10-methyleneTHF requires glycine and is accomplished by serine hydroxymethyltransferase. Folate, as 5-methylTHF, and vitamin B12 are required for the

methionine synthase catalyzed remethylation of homocysteine to methionine. Conversion of 5,10-methyleneTHF to 5-methylTHF is accomplished by methyleneTHF reductase (MTHFR), which requires riboflavin (as FAD) as a cofactor. Following homocysteine remethylation, 5-methylTHF is cycled back to THF, and this process can be repeated. A deficiency of vitamin B12, in the presence of adequate folate, results in an accumulation of 5-methylTHF because of decreased methionine synthase activity. This is referred to as the "methyl folate trap" hypothesis because the conversion of 5,10-methyleneTHF to 5-methylTHF is unidirectional, trapping 5-methylTHF.

2.2 Methionine cycle

Methionine, an essential sulfur-containing amino acid is converted to AdoMet by methionine adenosyltransferase. *S*-adenosyl-homocysteine (AdoHcy) is formed following methyl donation followed by reversible liberation of adenosine and formation of homocysteine, catalyzed by AdoHcy hydrolase. Homocysteine is a nonprotein forming amino acid with two possible fates: remethylation to methionine; or conversion to cysteine and glutathione by the transsulfuration pathway. Remethylation of homocysteine to methionine can be accomplished (as above) by the ubiquitous methionine synthase, which requires vitamin B12 as a cofactor and utilizes 5-methylTHF as the methyl donor. An additional, tissue-specific pathway of homocysteine remethylation to methionine is catalyzed by the zinc-dependent betaine-homo-cysteine methyltransferase that utilizes betaine as the methyl donor [8, 10]. Betaine can be obtained from the diet or endogenously synthesized from choline. Betaine-homocysteine methyltransferase has tissue-specific activity, and is found at high levels in liver and kidney [11]. Choline comes from phosphatidylcholine, which can be obtained from the diet or endogenously synthesized by the cytidine diphosphocholine pathway [12]. An additional pathway of phosphatidylcholine synthesis is present in other tissues, such as liver, and involves the methylation of phosphatidylethanolamine to phosphatidylcholine by phosphatidylethanolamine methyltransferase (PEMT).

2.3 Transsulfuration pathway

Homocysteine is irreversibly converted to cysteine by a two-step process involving the formation of cystathione by cystathionine-β-synthase (CBS) followed by conversion to cysteine by cystathionine-γ-lyase [13, 14]. Both enzymes require vitamin B6 as a cofactor. The transsulfuration pathway is only found in liver, pancreas, kidney, and intestine because of the tissue-specific activity of CBS and cystathionine-γ-lyase. Cysteine is used for protein synthesis or further metabolized to other compounds including taurine and glutathione.

3 DNA methylation

DNA methylation involves the addition of methyl groups to the 5′ position of cytosine within CpG dinucleotides, and it is estimated that 70–80% of CpGs within the genome are methylated [15]. DNA methylation also occurs at cytosine residues not adjacent to guanine residues (termed "non-CG methylation"); however, further studies are required to determine their role in regulating gene expression [16]. Methylation of DNA is accomplished by DNA methyl-transferases (DNMTs). De novo methylation occurs during embryogenesis and is accomplished by DNMT3a and 3b, whereas maintenance methylation occurs during somatic cell division and is accomplished by DNMT1 [17].

Immediately following fertilization, during the initial stages of embryo development, there is a genome-wide demethylation of the parental genomes and reprogramming of the methylation patterns [18, 19]. The mechanisms involved in this "epigenetic reprogramming" are complex and there is asymmetry in the mechanisms and degree to which this happens between maternal and paternal genomes. This phenomenon suggests the presence of heritable epigenetic memory, patterns of which are then transmitted during somatic cell division throughout the lifespan. Furthermore, this epigenetic reprogramming that occurs during early embryonic development maybe especially vulnerable to environmental factors that can affect methylation. There are several examples in the literature whereby changes in nutrition status, either during development or in adulthood, can affect DNA methylation patterns and have profound effects on gene expression and phenotype [2, 20–22]. Furthermore, epigenetic regulation of gene expression and DNA methylation profiles are tissue and cell specific so the vulnerability will be cell and tissue specific and dependent on the developmental stage and sex of the organism.

Clusters of CpGs, referred to as "CpG islands", can be defined as regions of DNA greater than 200 base pairs with a higher than expected C+G dinucleotide content. CpG islands are often associated with gene promoters, are usually unmethylated, and play a role in governing gene expression, especially in tissue-specific and developmental-specific patterns [23]. CpG islands are also found in intragenic and intergenic regions, the functions of which remain to be fully elucidated. Methylation of DNA at intergenic and intragenic regions often occurs in repeat elements such as short interspersed elements, e.g. Alu, and long interspersed elements (LINE), eg. LINE-1, which are usually methylated. Methylation of DNA at sites within a promoter region or at other regulatory sites is often associated with silencing of gene expression [1]. Silencing of gene expression can also occur by alteration of DNA methylation outside a promoter region and not within a CpG island, but in sequences up to 2 kb upstream, often referred to as CpG island shores [24]. In addion, DNA methylation also plays an important role in X chromosome inactivation [25] and in allele-silencing of imprinted genes [26].

Silencing of gene expression by DNA methylation can occur by several means. For example, methylation can impede binding of regulatory factors that are required for transcriptional activation. Along this line, it is important to note that methylation silencing does not always involve CpG islands and that methylation at just one CpG site contained within an important regulatory factor binding site is associated with silencing gene expression [27]. Methylated DNA can also serve as a target for the methyl-binding proteins, MeCP1 and MeCP2, which can also interfere with transcriptional activation [28]. Furthermore, methylated DNA can influence chromatin structure such that it is in a repressive state and not transcriptionally active.

4 Methyl nutrient imbalance and DNA methylation

Most studies investigating methyl nutrient imbalance and DNA methylation have focused on folate status and hyperhomocysteinemia (HHcy). For example, Jacob et al. conducted a metabolic balance study in which postmenopausal women ($n = 8$) were placed on low-folate diets (56 μg/day) for 41 days. From baseline to 41 days, plasma folate decreased (19.5 ± 4.2 to 9.3 ± 1.8 nmol/L), homocysteine increased (9.8 ± 0.4 to 12.5 ± 1 μmol/L), and lymphocyte global DNA methylation decreased [29]. Similarly, lower global DNA methylation in leukocytes was reported in elderly women fed a low-folate diet for 7 wk (118 μg/day) and was accompanied by low serum folate concentrations (12.1 ± 7.2 nmol/L) and elevated serum total homocysteine concentrations (11.0 ± 3.0 μmol/L) [30].

Further evidence that methyl nutrient imbalance can affect DNA methylation comes from studies in subjects homozygous for the common (10–20% in Caucasian populations) *MTHFR* 677C>T variant (rs1801133). This variant encodes for a thermolabile form of the enzyme and results in an impaired ability to synthesize 5-methylTHF and is often accompanied by elevated plasma total homocysteine [31, 32] and global DNA hypomethylation [33–35]. Subjects homozygous for the *MTHFR* 677C>T variant (TT genotype) with plasma folate concentrations less than < 12 nmol/L and elevated plasma total homocysteine concentrations (19.64–22.98 μmol/L) were reported to have lower global DNA methylation in leukocytes compared to subjects with the CC genotype [35]. This suggests that the effect of the variant on global methylation may be dependent on the folate status of the individual; a classic example of a gene–nutrient interaction.

Several studies have shown that with HHcy, intracellular concentrations of AdoHcy increase, resulting in a lower AdoMet/AdoHcy ratio [21, 36–40]. In vitro studies demonstrated that elevated AdoHcy concentrations and a reduced AdoMet/AdoHcy ratio inhibits DNA methyltransferase reactions in liver [41] and in cultured human fibroblasts [42], suggesting that a lower AdoMet/AdoHcy ratio is an indicator of reduced DNA methylation capacity. However, the relationship between increased intracellular AdoHcy concentrations and a lower AdoMet/AdoHcy ratio with DNA methylation is unclear. Conflicting reports in the literature suggest that the effect of AdoMet/AdoHcy ratio on DNA methylation is tissue specific. For example, studies in subjects with HHcy reported elevated plasma and lymphocyte AdoHcy concentrations were associated with reduced global DNA methylation in leukocytes and lymphocytes, respectively [36, 37], whereas other studies have reported no relationship [43, 44]. One study has reported gene-specific associations of HHcy on DNA methylation in human subjects [45]. Subjects with renal failure on hemodialysis and HHcy ($n = 32$), had global DNA hypomethylation compared to control subjects without HHcy ($n = 11$), and a subset ($n = 3$) with the most severe HHcy (≥ 62.5 μmol/L) had biallelic expression of the imprinted *H19 gene* because of changes in DNA methylation in leukocytes. Interestingly, lowering plasma total homocysteine concentrations with 5-methylTHF supplementation returned *H19* to monoallelic expression in this study.

Similar discrepancies have been reported in mouse models of HHcy where there are tissue-specific relationships between circulating total homocysteine concentrations, intracellular AdoMet and AdoHcy concentrations, and global DNA methylation [39, 46]. For example, $Cbs^{-/-}$ mice with severe HHcy were reported to have elevated concentrations of AdoHcy in liver, kidney, and brain with reduced levels of global DNA methylation in liver and kidney, but not in brain [46]. In addition, F1 *Castaneous/EiJ* × $Cbs^{+/-}$ mice with diet-induced HHcy had higher AdoHcy, and lower AdoMet/AdoHcy ratios in liver and this was accompanied by lower maternal *H19* allele methylation, and lower *Igf2* mRNA levels, and loss of *Igf2* maternal imprinting in liver [22]. In contrast, no differences were detected for AdoMet and AdoHcy in brain; however, *Cast* × $Cbs^{+/-}$ mice with diet-induced HHcy had higher maternal *H19* methylation and lower *H19* mRNA concentrations in brain. Furthermore, $Mthfr^{+/-}$ mice with diet-induced HHcy were reported to have lower AdoMet concentrations in liver and higher AdoHcy concentrations in brain but no differences in global DNA methylation in either tissue [39]. Together, these studies indicate that the effect of HHcy on AdoMet and AdoHcy concentrations is tissue specific and can result in gene-specific hypermethylation, global DNA hypomethylation, or no effect on DNA methylation, all of which may contribute to a gene expression profile that promotes CVD risk (Fig. 2).

The tissue specificity of the relationship between circulating total homocysteine concentrations and intracellular AdoHcy concentrations with DNA methylation is an important factor when interpreting results in human studies because DNA methylation is often assessed in peripheral blood mononuclear cells (PBMCs) (leukocytes), which are a heterogeneous cell population. More elaborate studies in specific blood cell types, e.g. monocytes or lymphocytes, are required to clarify the effects of circulating total homocysteine concentrations and intracellular changes in AdoHcy concentrations on global and gene-specific DNA methylation.

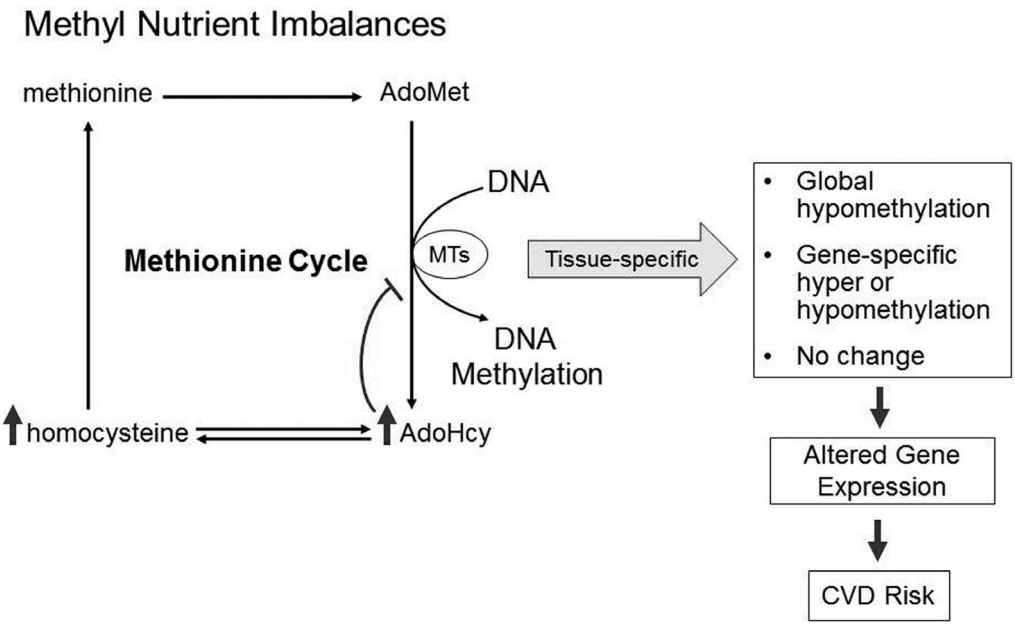

Figure 2. Schematic representation illustrating the potential mechanism whereby methyl nutrient imbalances can alter gene expression through changes in DNA methylation and contribute to cardiovascular disease. Hydrolysis of *S*-adenosylhomocysteine (AdoHcy) to homocysteine is a reversible reaction, with synthesis of AdoHcy in favored. Inadequate removal of homocysteine results in an increase in homocysteine and AdoHcy concentrations. AdoHcy has been reported to inhibit methyltransferases and as such may inhibit DNA methylation reactions. Effects of methyl nutrient imbalance on AdoMet and AdoHcy concentrations and DNA methylation patterns are tissue specific and may result in global DNA hypomethylation, gene-specific hypermethylation, or no change. Changes to DNA methylation capacity may alter gene expression and produce a gene expression profile that promotes CVD pathology.

5 Methyl nutrient imbalance and cardiovascular disease (CVD)

5.1 Hyperhomocysteinemia (HHcy)

Much attention in the past is focused on homocysteine and its possible role in CVD pathology. In observational studies, HHcy is a risk factor for CVD [47]. It has been speculated that changes in DNA methylation may contribute to the vascular complications associated with HHcy. However, recent B-vitamin intervention trials designed to lower circulating total homocysteine concentrations failed to reduce secondary CVD events [48, 49]. The negative findings of these trials may be a consequence of the fact that the circulating total homocysteine concentrations at baseline in these subjects were not elevated enough to affect CVD pathology and therefore lowering the concentrations further would have no subsequent effect on CVD events. Despite these findings, several studies in animal models and humans have reported an association of HHcy with vascular endothelial dysfunction (defined in these studies as impaired vascular endothelium-dependent vasodilatation), an early indicator of vascular disease, which often precedes primary cardiovascular events [50]. For example, subjects ($n = 24$) with acute HHcy (23.1 ± 5.4 μmol/L) following an oral methionine load were reported to have vascular endothelial dysfunction [51]. Similarly, studies in mouse models of HHcy, produced by target disruption of genes-encoding enzymes required for methyl nutrient metabolism such as *Cbs*, *Mthfr*, or *Mtr* (encodes methionine synthase) and diet-induced HHcy have also reported endothelial dysfunction [38, 39, 52–55]. More recently, folate has also been implicated in endothelial dysfunction because of its effect on nitric oxide metabolism. A study in subjects ($n = 218$) with coronary artery disease (CAD) reported that those homozygous for the *MTHFR* 677C>T variant ($n = 24$) had elevated vascular 5-methylTHF concentrations but no differences in vascular total homocysteine concentrations, and this was associated with endothelial dysfunction and vascular oxidative stress [56].

5.2 Methyl nutrient imbalance and lipid metabolism

Lipid metabolism is linked to methyl nutrient metabolism through the methionine cycle and the synthesis of phosphatidylcholine (PC). Hepatic PC synthesis can be synthesized two ways. The first is the PEMT pathway which is responsible for synthesizing ~30% and the second is the CDP-choline pathways which are responsible for the remaining ~70% [57, 58]. Liver PC is required for the synthesis and secretion of lipoproteins by the liver [59, 60], and therefore, plays an indirect role in the delivery of lipids, such as long chain polyunsaturated fatty acids, to the vascular endothelium and other extrahepatic tissues.

HHcy is reported to be associated with disturbances in lipid metabolism including decreased plasma HDL cholesterol and apolipoprotein A-I concentrations and hepatic lipid accumulation [61–63]. Furthermore, studies by our group have reported that $Cbs^{\pm}$ mice with mild and moderate HHcy have disturbances in hepatic fatty acid and phospholipid metabolism and involve methylation-silencing of the gene that encodes delta-6 desaturase (*Fads2*) [21], and enzyme required for the metabolism of the n-6 and n-3 essential fatty acids, linoleic acid and linolenic acid, respectively. Others have also reported regulation of cholesterol metabolism by DNA methylation in other models. One study reported methylation silencing of liver X-receptor alpha (*Lxra*) expression, a nuclear receptor regulating cholesterol and fatty acid metabolism, in liver from fetal mice exposed to maternal protein restriction compared to fetuses from control-fed dams [64].

5.3 Cysteine and CVD risk factors

A relationship between total cysteine and CVD risk was first described by two studies that identified an association between circulating total cysteine concentrations and body mass index (BMI) [65,66]. A BMI $\geq$ 30 kg/m^2 is used to define obesity in adults and obesity is a well-known risk factor for CVD. Taking this further, the Hordaland Homocysteine Study (n = 5179) reported an independent positive association between plasma total cysteine concentrations and BMI and fat mass at baseline and at the 6-year follow-up [67]. Increased weight gain has also been reported in animals models supplemented with high dietary cysteine or cysteine-rich protein diets [68–70]. Taken together these studies suggest that circulating total cysteine concentrations may be a marker of adiposity, which is an important risk factor for CVD.

The direct relationship between cysteine and CVD disease has not been well studied. Case control studies have reported higher levels of plasma total cysteine concentrations in subjects with cerebral infarction [71], peripheral arterial disease [72], and myocardial infarction [73] compared to healthy controls. A cross-sectional study in humans with hyperlipidemia reported higher circulating cysteine concentrations among those with CVD [74]. Furthermore, the European Concerted Action Project, a case-control study in subjects with CVD (n = 750) and control subjects (n = 800), reported a U-shaped association between plasma total cysteine concentrations and CVD [65], with the greatest risk observed in subjects with plasma total cysteine concentrations < 225 μmol/L and > 300 μmol/L. Similarly, a smaller case-control study assessed the relationship between plasma total cysteine concentrations and atherosclerosis [66]. Cases were defined as subjects with >90% occlusion in one vessel and >40% occlusion in the second vessel (n = 131) and two control groups were assessed: coronary controls, which were subjects with < 50% occlusion in one vessel (n = 88); and population-based healthy controls (n = 101). The study reported that cases had significantly higher plasma total cysteine concentrations than controls and that subjects in the highest tertile of plasma total cysteine concentrations had an odds ratio of 2.5 for coronary atherosclerosis compared to subjects in the lowest tertile of plasma total cysteine concentrations; however, the relationship disappeared after adjustment for other CVD risk factors including BMI. At present, the significance of the relationship between plasma total cysteine and CVD pathology is unclear, and may exist secondary to the association between adiposity and plasma total cysteine concentrations.

6 Atherosclerosis and DNA methylation

Atherosclerosis is characterized by proliferation of vascular smooth muscle cells, lipid accumulation, connective tissue development, inflammatory cell infiltration, and calcification [75]. Changes to global and gene-specific DNA methylation patterns are implicated in the pathology of atherosclerosis, and may occur without changes in methyl nutrient metabolism. Acquiring tissue samples (e.g. coronary vessels) to assess changes in DNA methylation in human studies is not always feasible. Often only blood samples are collected. As such, it is advisable to target a specific blood cell types as opposed to quantifying DNA methylation changes in PBMCs, a heterogeneous cell population. For example, monocytes/macrophages and lymphocytes are the most predominant leukocytes within atherosclerotic arteries; both have been reported to play a key role in the pathology of atherosclerosis [76]. As such, assessing changes in DNA methylation patterns in specific blood cell types, such as monocytes, may inform as to the pathology of atherosclerosis. These changes in DNA methylation patterns may produce a gene expression profile that promotes the development or progression of atherosclerosis (Fig. 3).

Many studies in animals and humans have linked atherosclerosis to DNA methylation. For example, one study reported genomic DNA hypomethylation in advanced atherosclerotic lesions from human subjects (n = 55), atherosclerotic lesions from mice deficient in apolipoprotein E ($ApoE^{-/-}$), and in aortic neointima from balloon denuded New Zealand White rabbits [4]. Lund et al. assessed DNA methylation in the early stages of atherosclerosis [5] and reported that $ApoE^{-/-}$ mice with vascular lesions have global DNA hypomethylation in aorta and PBMCs and were also able to detect changes in DNA methylation patterns in both tissues, prior to the appearance of the vascular lesions [5]. Taken together, these findings in mice suggest a role for DNA methylation in the development of atherosclerosis.

In addition, human studies such as the Singapore Chinese Health Study (n = 286) have reported that male subjects with a history of CVD or those with CVD risk factors have higher leukocyte global DNA methylation status [77]. Furthermore, findings of the VA Normative Aging Study reported a negative association between leukocyte LINE-1 methylation status and circulating vascular cell adhesion molecule-1 (VCAM-1)

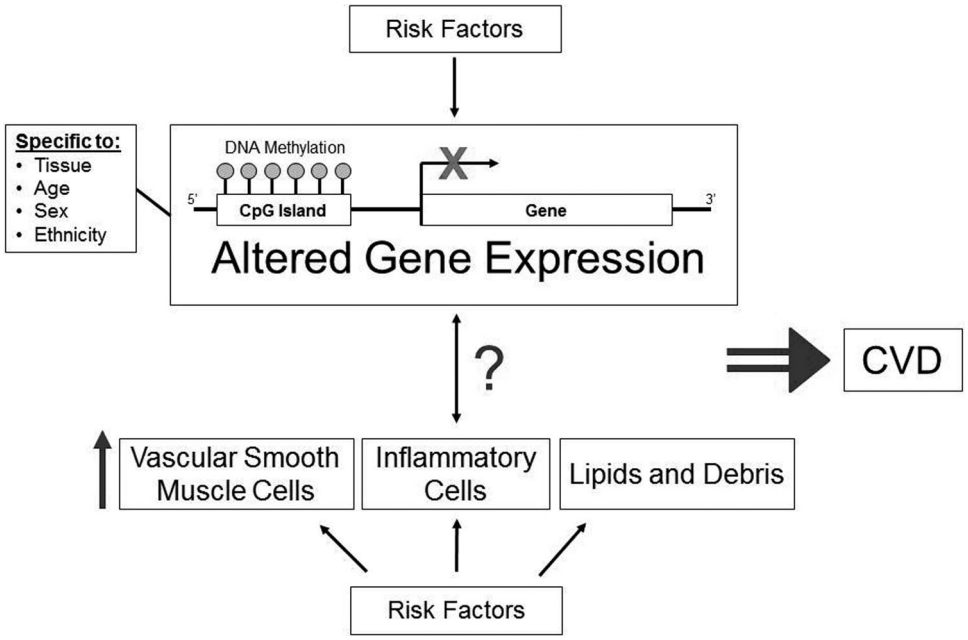

Figure 3. Schematic model illustrating how altered DNA methylation may contribute to cardiovascular disease. Altered DNA methylation patterns and gene expression are specific to tissue, age, sex, and ethnicity.

concentrations in male subjects of European descent that had no CVD ($n = 480$) but not in men with established CVD ($n = 262$) [78]. Similarly, findings from the Samoan Family Study of Overweight and Diabetes ($n = 355$) reported that leukocyte LINE-1 methylation status was negatively associated with plasma LDL and positively associated with plasma HDL [79]. These findings further support the concept that global changes in DNA methylation may contribute to the early stages of CVD pathology.

Only a few studies have addressed gene-specific changes in atherosclerosis. Tissue collected from patients undergoing coronary artery bypass grafting reported hypermethylation of *ESR1* (encodes estrogen receptor alpha) in aorta, internal mammary artery, and saphenous vein, with greater levels observed in coronary artery atherosclerotic plaques [6]. Findings from the Verona Heart Project reported an inverse relationship between elevated plasma activated coagulation factor VIIa concentrations (mean 37.86 mUI/mL) and hypomethylation of the *F7* (encodes coagulation factor VII) promoter in PBMCs from men and women with CAD ($n = 165$) compared to CAD-free subjects ($n = 88$) [80]. A study in rabbits with atherosclerosis showed hypomethylation of *Sod3* (encodes extracellular superoxide dismutase) and global hypomethylation in atherosclerotic tissue from aorta [3]. As such, additional studies are required to more definitely assess a role for gene-specific changes in DNA methylation in the pathology of atherosclerosis.

7 Folic acid fortification

Mandatory fortification of grain products with folic acid was introduced in North America for the prevention of neural tube defects. Recent estimates have reported a 46% reduction in neural tube defects in Canada since the inception of the mandatory fortification program [81]. Furthermore, the folate status of Canadians has risen, with recent studies reporting that less than 1% of the population is folate deficient and 40% have high red blood cell folate levels (>1360 nmol/L) [82]. Similar findings have been observed in the United States [83, 84]. However, little is known regarding the effects of high folic acid intakes, the folate form used in the fortification of grains and in supplements, on methyl metabolism and DNA methylation. To date, one study has reported no effect of folic acid supplementation (800 μg/day) on leukocyte global DNA methylation [85]. In contrast, another study in women of child-bearing age from China reported an effect of folic acid supplementation (ranging from 100 to 4000 μg/day) on whole blood global DNA methylation in coagulated blood samples but not in uncoagulated blood samples [86]. Similarly, a study in subjects ($n = 7$) homozygous for the *MTHFR* 677C>T variant with high plasma total homocysteine concentrations (mean 51.1 μmol/L) reported a significant lowering of plasma total homocysteine following 8 wk of 5 mg/d folic acid supplementation but no effect on leukocyte global DNA methylation [87]. Additional studies are required to determine the effects of high folic acid intakes on gene-specific DNA methylation patterns and DNA methylation patterns in specific blood cell types, i.e. monocytes, rather than heterogeneous whole blood leukocytes.

The metabolism of folate and cobalamin (also called B12) are tightly linked together. In the case of cobalamin deficiency, with adequate folate status, folate becomes trapped as 5-MTHF, and is unable to participate in further metabolic reactions [88]. Furthermore, high folic acid intakes can mask the hematological signs of cobalamin deficiency. In a study of

older adults ($n = 1459$), data collected from the US National Health and Nutrition Examination Survey (NHANES) conveyed lower serum cobalamin (<148 pmol/L) and elevated folate (>59 nmol/L) concentrations associated with anemia and greater cognitive impairment [89]. In addition, The Canadian Health Measures Survey reported 4.6% of participants ($n = 5600$) are cobalamin deficient (<148 pmol/L), but less than 1% had RBC folate deficiency (>320 nmol/L) and 63.5% had high (>1090 nmol/L) for RBC folate status [90]. As such, the differential effects of folate/cobalamin imbalance on DNA methylation patterns and relevance to CVD warrants further investigation.

8 Conclusions

Currently, our knowledge of how diet plays a role in changing DNA methylation patterns and how this may translate to disease pathology is in the early stages. Data obtained from both human and animal studies suggest that changes in DNA methylation patterns are responsive to changes in nutritional status and are highly tissue and cell specific. Studies in mice have provided us with more detailed findings in tissues that are not easily obtainable in human studies. For this reason, it is advisable to target the primary blood cell type involved in the disease, for example targeting monocytes when investigating DNA methylation in CVD. Detection of at-risk individuals will serve as a means by which we can prevent future cardiovascular events (i.e. myocardial infarction and stroke) and can provide insight into the potential for the reversibility of altered DNA methylation patterns leading to treatment of CVD. The field of epigenetics is growing rapidly; however, further investigations pertaining to epigenetic changes in DNA methylation patterns need to be elucidated before treatment and/or biomarkers for CVD are implemented in the clinical setting.

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