

5th Amino Acid Assessment Workshop

Assessing the Effects of High Methionine Intake on DNA Methylation^{1,2}

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ABSTRACT Methylation of DNA occurs at cytosines within CpG (cytosine-guanine) dinucleotides and is 1 of several epigenetic mechanisms that serve to establish and maintain tissue-specific patterns of gene expression. The methyl groups transferred in mammalian DNA methylation reactions are ultimately derived from methionine. High dietary methionine intake might therefore be expected to increase DNA methylation. Because of the circular nature of the methionine cycle, however, methionine excess may actually impair DNA methylation by inhibiting remethylation of homocysteine. Although little is known regarding the effect of dietary methionine supplementation on mammalian DNA methylation, the available data suggest that methionine supplementation can induce hypermethylation of DNA in specific genomic regions. Because locus-specific DNA hypomethylation is implicated in the etiology of various cancers and developmental syndromes, clinical trials of "promethylation" dietary supplements are already under way. However, aberrant hypermethylation of DNA could be deleterious. It is therefore important to determine whether dietary supplementation with methionine can effectively support therapeutic maintenance of DNA methylation without causing excessive and potentially adverse locus-specific hypermethylation. In the viable yellow *agouti* (A^{vy}) mouse, maternal diet affects the coat color distribution of offspring by perturbing the establishment of methylation at the A^{vy} metastable epiallele. Hence, the A^{vy} mouse can be employed as a sensitive epigenetic biosensor to assess the effects of dietary methionine supplementation on locus-specific DNA methylation. Recent developments in epigenomic approaches that survey locus-specific DNA methylation on a genome-wide scale offer broader opportunities to assess the effects of high methionine intake on mammalian epigenomes. J. Nutr. 136: 1706S–1710S, 2006.

KEY WORDS: • *methionine* • *nutrition* • *DNA methylation* • *viable yellow agouti* • *epigenetic* • *epigenomic*

Nutrition during diverse stages of life can influence epigenetic gene regulation. This review focuses on the role of dietary methionine (MET)⁴ in the epigenetic process of DNA methylation. Consuming excessive quantities of MET may affect DNA methylation and thereby cause dysregulation of gene expression. Conversely, for certain individuals, high intake of dietary MET may be an effective component of therapeutic promethylation dietary regimens to restore appropriate locus-specific DNA methylation. This paper discusses the important role of DNA methylation in mammalian epigenetic gene

regulation and reviews studies examining the effects of high MET intakes on mammalian 1-carbon metabolism and DNA methylation. The potential application of the viable yellow *agouti* (A^{vy}) mouse as a sensitive epigenetic biosensor to detect MET-induced alterations in DNA methylation is discussed. Finally, recent developments in epigenomic approaches to investigate gene-specific DNA methylation on a genome-wide basis provide excellent opportunities to assess more generally the effects of high intakes of MET on mammalian epigenomes.

Epigenetic gene regulation and DNA methylation. Epigenetics refers to the study of mitotically (and potentially meiotically) heritable changes in gene expression that are not caused by changes in DNA sequence. Epigenetic gene regulatory mechanisms are responsible for establishing and maintaining the diverse patterns of gene expression that distinguish different cell and tissue types. The term epigenetics, literally meaning "above genetics," describes mechanisms that are layered on top of the DNA sequence information and perpetuated in daughter cells when DNA is replicated during mitosis. In the last decade it has become clear that just as genetic differences among individuals lead to individual differences in disease susceptibility, so too do interindividual epigenetic differences. Epigenetic processes play an important role in human carcinogenesis and several developmental syndromes (1,2) and are implicated in other complex diseases including type-2 diabetes (3), cardiovascular disease (4), and obesity (5).

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⁴ Abbreviations used: MET, methionine; [MET], concentration of methionine; SAH, S-adenosylhomocysteine; [SAH], concentration of S-adenosylhomocysteine; SAM, S-adenosylmethionine; [SAM], concentration of S-adenosylmethionine.

Epigenetic mechanisms include DNA methylation, various modifications to the histone proteins that package and regulate the regional conformation of DNA in accessory chromatin, and feed-forward autoregulatory transcription factors. DNA methylation occurs at cytosines within cytosine-guanine (CpG) dinucleotides. This covalent modification of DNA contributes to transcriptional regulation and chromatin structure by affecting the affinity of methylation-sensitive DNA binding proteins and thereby plays an important role in epigenetic processes including tissue-specific gene expression, genomic imprinting, and X-chromosome inactivation (6). In the early embryo, genome-wide methylation patterns are largely erased and then reset in a lineage-specific fashion (7). The process of cellular differentiation involves the establishment of tissue-specific epigenetic marks that are necessary to maintain appropriate patterns of tissue-specific gene expression through many rounds of DNA replication as cellular proliferation continues during development and, in many tissues, throughout life. Importantly, after their developmental establishment and maturation, tissue-specific patterns of DNA methylation are generally maintained with high fidelity.

An essential amino acid, MET plays a unique role in epigenetic processes by serving as the penultimate methyl donor for mammalian methylation reactions (Fig. 1). In addition to DNA methylation, another important epigenetic mechanism utilizing 1-carbon groups is the methylation of specific residues in histone proteins (8). This review focuses on the potential for high intakes of dietary MET to influence CpG methylation in DNA. It is plausible, however, that dietary MET could also influence epigenetic gene regulation at the level of histone methylation.

In the process of methylating cytosines in CpG dinucleotides, the DNA methyltransferase (DNMT) reaction converts S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH) (Fig. 1). It is important to consider the fundamental mechanisms by which nutritional factors affecting the supply of SAM and/or the removal of SAH might affect DNA methylation. During prenatal and early postnatal development, when tissue-specific DNA methylation patterns are undergoing establishment and developmental maturation, any nutritional factor

that inhibits the efficiency of the DNMT reaction might result in permanent deficits in CpG methylation. Even in adulthood, established patterns of DNA methylation must be mitotically maintained in proliferative tissues, and, therefore, nutritional deficiencies affecting one-carbon metabolism could induce DNA hypomethylation. What is less intuitive is how a nutritionally induced increase in transmethylation efficiency might increase the highly regulated process of DNA methylation. It has been shown, however, that nutritional supplementation during early development can increase methylation at specific genes and cause permanent changes in gene expression (9). This is possible because at certain loci, establishment of epigenotype exhibits a dynamic stochasticity that can be influenced by nutrition. In human adults with a genetic tendency for hyperhomocysteinemia, nutritional supplementation with high doses of folate can increase methylation and normalize gene expression at specific loci (10). Hence, nutritional supplementation in adulthood can in some cases correct for genetically based epigenetic deficiencies.

Effects of methionine supplementation on [SAM] and [SAH]. Because the DNMT reaction is dependent on the supply of SAM and removal of SAH, the [SAM]:[SAH] ratio has been proposed as a 'methylation index' to indicate the likelihood of hyper- or hypo-methylation of DNA. The interpretation of the [SAM]:[SAH] ratio is complicated, however, by both the tissue-specificity of enzymes that regulate mammalian one-carbon metabolism and the low efficiency for transmembrane flux of critical metabolites. For example, in response to diverse dietary intakes of MET, regulation of plasma [MET] occurs by varying the activity of enzymes responsible for homocysteine (HCY) disposal; these metabolic responses appear to occur specifically in the liver (11). Because SAM does not readily cross the plasma membrane, each mammalian cell is responsible for synthesizing its own SAM from circulating MET, HCY, or SAH (12). SAH, in contrast, does leak from the cell once its accumulation exceeds the buffering capacity of specific SAH-binding proteins in the cytoplasm, but only the kidney appears capable of taking up SAH from plasma (12). For all these reasons, the [SAM]:[SAH] ratio must be interpreted on a tissue-specific basis, and the ratio in plasma may not provide a meaningful indication of systemic methylation capacity.

Several studies have examined the effects of high dietary MET intakes in rodent models on hepatic [SAM] and [SAH]. Finkelstein and Martin (11) fed adult rats purified amino-acid-defined diets differing only in MET content. The diets (0.3, 1, 1.5, 2, or 3% MET, wt/wt) were fed for up to 7 d. No change in hepatic [MET] was seen, even at the highest intake level, illustrating effective MET homeostasis in the face of dietary excess. Hepatic [SAM] and [SAH] were maintained near baseline levels until dietary MET was increased to 3%. Hepatic [SAH] showed gradual increases as dietary MET was increased from 0.3% to 1.5 and 2.0%, causing a dramatic overall decrease in the [SAM]:[SAH] ratio with an inflection point around 1.5% dietary MET. In this short-term study, no evidence of MET toxicity was noted until dietary MET reached 3.0%.

In a study relating MET toxicity to hepatic accumulation of SAM, Regina et al. (13) measured [SAM] and [SAH] in various tissues after feeding weanling rats casein-based diets containing either 0.6 or 1.5% MET for 14 d. Contrary to the study of Finkelstein and Martin (11), intake of the 1.5% MET diet caused a 20-fold increase in hepatic [MET]; the same increment was observed in plasma. The 1.5% MET diet also elicited signs of toxicity because it decreased growth rate in the young animals. In the liver, the 1.5% MET diet increased both [SAM] (7-fold) and [SAH] (4-fold), causing an 80% increase in the hepatic [SAM]:[SAH] ratio. [SAH] was doubled in

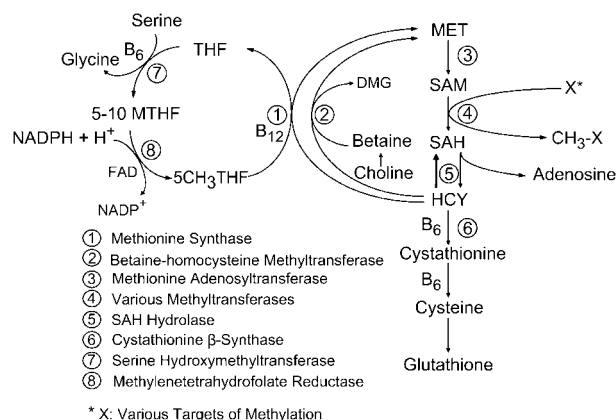


FIGURE 1 The relation between the methionine cycle and methylation of various substrates (including DNA). B vitamins, including folate [shown as tetrahydrofolate (THF), 5–10 methylenetetrahydrofolate (5–10 MTHF), and 5-methyltetrahydrofolate (5-CH₃THF)], B-6, and B-12 are required as cofactors. The liver responds to excess MET at the level of HCY, by increasing transsulfuration (reaction 6) and decreasing remethylation (reactions 1 and 2). Published with permission from Waterland and Jirtle (9).

kidney and skeletal muscle. [SAM] in kidney, small intestine, and skeletal muscle did not respond to dietary MET excess, however, despite increases in [MET] similar to that seen in liver. The liver-specific increase in [SAM] is consistent with the observation that, because of its unique SAM-synthase isozyme, only the liver can increase SAM synthesis in response to excessive MET (12). Interestingly, Regina et al. (13) concluded that MET hepatotoxicity may actually result from uncontrolled (and potentially nonenzymatic) methylation reactions caused by hepatic accumulation of SAM.

In their study examining the effect of excess dietary MET on hepatic glycine N-methyltransferase activity, Rowling et al. (14) provided data on hepatic [SAM] and [SAH] in young rats fed casein-based diets of varying MET contents (0.3, 1.3, and 2.3%) for 10 d. Compared to pair-fed control rats fed the 0.3% MET diet, hepatic [SAM] increased linearly in response to MET excess, increasing 10- and 20-fold in the 1.3% and 2.3% MET groups, respectively. Hepatic [SAH] showed relatively moderate increases of 30% in the 1.3% MET and 4-fold in the 2.3% MET groups. Consequently, the [SAM]:[SAH] ratio in liver was elevated ~8-fold at both levels of MET excess.

All 3 of these studies found that MET supplementation causes moderate and progressive increases in hepatic [SAH]. Regina et al. (13) and Rowling et al. (14) both showed that [SAM] and [SAH] in extrahepatic tissues are much more refractory to dietary MET excess than are those in liver. What is most striking about these 3 studies, however, is the quantitative and qualitative inconsistencies of their findings regarding the effects of moderate MET excess on hepatic [SAM] and the [SAM]:[SAH] ratio. Comparing the results at the ~1.5% MET supplementation level, Finkelstein and Martin (11) found no change in hepatic [SAM], whereas Regina et al. (13) and Rowling et al. (14) found 7- and 10-fold increases, respectively (Table 1). The discordance is even more striking in terms of the [SAM]:[SAH] “methylation index” (Table 1). These inconsistencies might be explained by the age differences of the animals used (only Finkelstein and Martin used adult rats) or by the specific dietary composition (amino-acid–defined vs. casein-based). In any event, this comparison illustrates that potential effects of dietary MET supplementation on transmethylation efficiency are complex and, in addition to being tissue specific, may also depend on multiple factors including age and overall dietary composition. The contradictory effects of MET supplementation on the [SAM]:[SAH] ratio suggest that high dietary intake of MET may induce DNA hypermethylation in some circumstances and hypomethylation in others.

MET excess and DNA methylation. Very few studies have examined the effects of high MET intake on DNA methylation. Several years ago, Tremolizzo et al. reviewed studies from the 1960s using high doses of MET in attempts at pharmacotherapy of schizophrenic patients and found that MET supplementation actually exacerbated the symptoms of schizophrenia (15).

Based on this and other observations, they proposed that schizophrenia has an epigenetic basis and that, among genetically susceptible individuals, MET supplementation precipitates schizophrenic behavior by altering DNA methylation at specific gene regions. In their epigenetic mouse model (15) they examined the effect of subcutaneously injecting 60-d-old male mice with MET (2 g/kg body weight per day, for 15 d) on [SAM] and [SAH] and the methylation status and expression of the *reelin* gene in frontal cortex. Interestingly, after the 15-d MET treatment, brain [SAH] was doubled relative to vehicle-injected controls, whereas [SAM] was not affected. This MET-induced reduction in the brain [SAM]:[SAH] ratio would predict DNA hypomethylation, but specific CpG sites in the *reelin* promoter were actually hypermethylated in cortex of MET-treated mice. The significant increase in CpG methylation appeared to down-regulate *reelin* expression. In a follow-up study from the same group, Dong et al. (16) showed that the 15-d MET treatment increases binding of methyl CpG binding protein 2 (MeCP2) to the *reelin* promoter (consistent with CpG hypermethylation). This effect did not occur in control genes including *Gad65* and *β-globin*.

In another model examining MET-induced alterations in DNA methylation, Devlin et al. (17) compared liver and brain [SAM], [SAH], and genome-wide DNA methylation in wild-type and heterozygous methylenetetrahydrofolate reductase (*Mthfr*) knockout mice. Because *Mthfr*^{+/-} mice have impaired remethylation of HCY (see Fig. 1), they were expected to have an enhanced susceptibility to diet-induced changes in DNA methylation. MET supplementation was achieved by providing half the animals with 0.5% MET in their drinking water, starting at weaning, for 7–15 wk. Although MET supplementation significantly decreased the [SAM]:[SAH] ratio in liver and brain, no significant dietary effects on genome-wide DNA methylation were found. This result does not exclude potential effects on CpG methylation occurring only at specific loci.

The viable yellow agouti mouse as an epigenetic biosensor.

Because of the complexity of the effects of high intakes of MET on the [SAM]:[SAH] methylation index and the dearth of data directly linking dietary MET to DNA methylation, it is desirable to identify appropriate animal model systems in which to test the hypothesis that excess MET intake affects DNA methylation. An ideal animal model would provide an easily detectable yet highly sensitive indicator of nutritional perturbations of epigenotype. The viable yellow agouti (A^{vy}) mouse is 1 such system.

The *agouti* gene encodes a paracrine signaling molecule that stimulates the production of yellow pigment. *Agouti* is normally expressed only in the cells surrounding the hair follicle and only during a specific stage of hair growth; the resulting yellow bands on otherwise black hairs produce the brown “agouti” color of wild-type mice. In viable yellow agouti (A^{vy}) mice, the *agouti* gene is genetically and epigenetically dysregulated by an intracisternal A particle (IAP) retrotransposon insertion (18).

TABLE 1
Responses of hepatic [SAM] and [SAH] to MET supplementation in rats

Study	Protocol	[SAM]	[SAH]	SAM:SAH
Finkelstein and Martin (11)	Adult male Sprague-Dawley rats, purified amino-acid defined diets: control 0.3% MET; supplemented 1.5% MET, 7 d supplementation	No change	↑ 3×	↓ 65%
Regina et al. (13)	Weanling male Wistar rats, casein-based semipurified diets: control 0.61% MET; supplemented 1.5% MET, 14 d supplementation	↑ 7×	↑ 4×	↑ 80%
Rowling et al. (14)	Young male Sprague-Dawley rats, casein-based diets: control 0.3% MET; supplemented 1.3% MET, 10 d supplementation	↑ 10×	↑ 30%	↑ 8×

The IAP contains a cryptic promoter that drives ectopic *agouti* expression, causing yellow coat color and other pleiotropic effects. To study the A^{vy} allele, it is convenient to maintain it in heterozygosity with the “non-*agouti*” (*a*) allele, which does not encode a functional *agouti* protein. Although the wild-type *agouti* gene displays a very predictable and individually invariant pattern of DNA methylation, A^{vy} DNA methylation is highly variable among A^{vy}/a mice (9). Because A^{vy} methylation is inversely correlated with ectopic *agouti* expression, coat color of A^{vy}/a mice provides a convenient readout of A^{vy} methylation (Fig. 2).

Wolff et al. (19) showed that providing excess folic acid, vitamin B-12, choline, and betaine to female mice before and during pregnancy shifts the coat color of their A^{vy}/a offspring toward the brown (pseudoagouti) phenotype. Waterland and Jirtle later demonstrated that this nutritionally induced change in phenotype occurs by increasing methylation at the A^{vy} locus (9). Hence, the establishment of CpG methylation at A^{vy} is sensitive to a dietary supplementation regimen designed to increase the efficiency of HCY remethylation. The developmental sensitivity of A^{vy} to nutritional factors is probably not unique but is likely to be a general characteristic of “metastable epialleles” (20). Because changes in A^{vy}/a offspring coat color can be induced by nutrition in utero and are indicative of A^{vy} epigenotype, the A^{vy}/a mouse can be used as an epigenetic biosensor to assess the effects of high MET intake on DNA methylation. In short, the convenient coat color readout of the A^{vy}/a mouse (Fig. 2) enables investigators to screen for nutritionally induced changes in DNA methylation without employing molecular assays.

Rather than being constitutively labile to environmental influences, the methylation status of A^{vy} is sensitive only during the critical ontogenic period coinciding with stochastic establishment of A^{vy} methylation, which appears to occur during preimplantation embryonic development. An important question is whether the A^{vy} mouse is an appropriate model in which to assess potential effects of MET supplementation on DNA methylation in adulthood. The preimplantation A^{vy}/a embryo is essentially acting as a sensor of the oviduct fluid environment. Hence, to the extent that epigenetically labile loci within specific tissues in adult mammals are sensitive to peripheral metabolite concentration, they may respond similarly. The greatest advantage of the A^{vy} mouse as an epigenetic biosensor, however, is its sensitivity. Dramatic changes in A^{vy} methylation in offspring can be induced by relatively moderate supplementation of the maternal diet with dietary methyl donors and cofactors (9). Hence, the system should be appropriate for determining the minimum level of MET supplementation capable of inducing gene-specific changes in DNA methylation.

Epigenomic approaches. Environmental perturbations of DNA methylation do not appear to affect the entire genome to the same extent. Rather, genomic and/or epigenetic characteristics of specific loci render them especially susceptible to environmental perturbation (20). The challenge of nutritional epigenetics is to identify these epigenetically labile gene regions in humans. Adding further to the complexity, epigenetic gene regulation is, by nature, tissue specific and developmentally regulated. Hence, it is likely that specific environmental stimuli, such as high intake of dietary MET, will affect DNA methylation only in specific gene regions, in specific tissues, and during specific life stages. Metastable epialleles associated with transposable elements and genomically imprinted genes have been identified as two potential subsets of candidate genes with enhanced epigenetic lability to nutrition (20). Recent developments in epigenomic approaches to assess DNA methylation on a genome-wide scale, such as restriction landmark genome

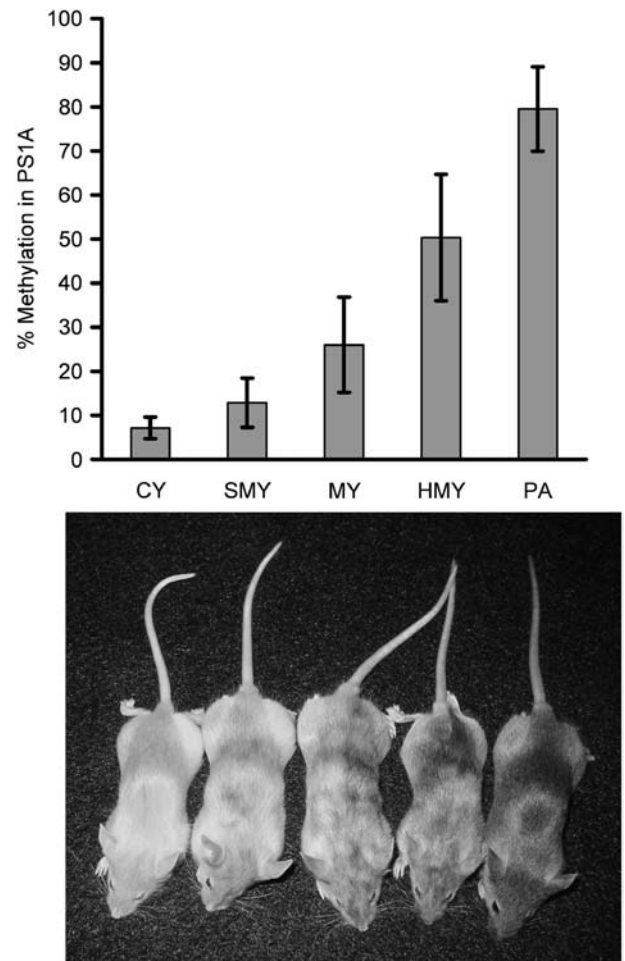


FIGURE 2 A^{vy}/a mice vary in coat color phenotype from clear yellow (CY), to slightly mottled yellow (SMY), mottled yellow (MY), heavily mottled yellow (HMY), and pseudoagouti (PA). The top panel shows percentage methylation (mean $\pm$ SEM) of 7 CpG sites in the A^{vy} region in A^{vy}/a mice according to the coat color classifications displayed in the bottom panel. The photograph was reproduced from Waterland and Jirtle (9) with permission. Methylation of the A^{vy} locus is responsive to nutrition and tightly correlated with coat color phenotype, rendering the A^{vy}/a mouse useful as an epigenetic biosensor.

scanning (21) and various microarray-based techniques (22), will provide an effective complement to the candidate gene approach.

In 1 such approach that shows particular promise, bacterial artificial chromosome (BAC) microarrays are employed in a methylation-sensitive manner to interrogate specific CpG sites throughout the genome (23). In this assay, genomic DNA is first digested with 2 restriction enzymes, 1 that is methylation sensitive (such as *NotI*) and 1 that is methylation insensitive. The unmethylated (*NotI* cleaved) fragments are isolated, labeled with a fluorescent dye, and hybridized to microarrays printed with BAC clones. To compare different groups (for example, genomic DNA from animals fed a MET-supplemented vs. a control diet), DNA samples from the 2 groups are labeled with different fluorophores and cohybridized. A positive signal at any spot on the array indicates a BAC clone containing a *NotI* site that is methylated more frequently in 1 group relative to the other. The *NotI* recognition sequence (5'-GCGGCCGC-3') is so rare that each BAC usually contains no more than a few such sites from which the signal may be originating. Hence, identification of the specific genomic region and verification of differential methylation—literally plucking an epigenetic needle from a genomic haystack—is fairly straightforward.

In a recent study by Ching et al. (23), the BAC microarray approach was used to identify gene-specific methylation differences between human brain and peripheral blood lymphocytes. Tissue-specific methylation in the identified genes was verified by bisulfite sequencing, and gene-specific hypomethylation in brain was found to correlate with increased expression relative to blood. Ching et al. then focused on 1 of the human genes identified, *SHANK3*, and found similar tissue-specific methylation and gene expression in the mouse, providing a compelling example of comparative epigenomics (23). Notably, in human studies, the BAC microarray approach identified genes whose degree of methylation (verified and quantitated by bisulfite sequencing) differed by as little as 40%. Hence, this approach clearly has sufficient sensitivity to identify subtle but potentially important gene-specific CpG methylation changes and could be used to investigate such changes induced by MET excess or other nutritional exposures.

Conclusion. High dietary MET intake alters mammalian 1-carbon metabolism. MET supplementation has the potential, therefore, to induce physiologically relevant changes in CpG methylation and expression of epigenetically labile genes. It remains to be determined whether high MET intakes have a greater tendency to induce DNA hyper- or hypomethylation. MET-induced changes in DNA methylation may not be solely detrimental. Rather, in certain cases supplementation with MET together with other metabolites and/or cofactors may provide an efficacious dietary therapy for epigenetic diseases. Before such approaches can be realized, nutritional epigenomic research in appropriate animal model systems will be an important step toward identifying the genes, tissues, and developmental stages in which high MET intake affects DNA methylation and gene expression in humans.

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