The Role of Dietary Methionine in Cancer Incidence and Treatment

By

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Abstract

Methionine and methionine cycle intermediates are used for various aspects of cell maintenance and growth, including the synthesis of proteins, nucleotides, glutathione, and S-adenosylmethionine, the universal methyl donor for nucleotide and histone methylation. Methionine uptake into cancer cells is heightened, likely owing to its contribution to many functions necessary for rapid cell growth and division. Due to these findings, there is much interest in evaluating the role methionine plays in both cancer development and progression. Here we review the current understanding of methionine restriction in cancer treatment and a concept from our lab, metabolic priming, in which cancer cells are pre-treated to expose metabolic vulnerabilities in response to methionine restriction, creating new drug targets to enhance the utility of currently used and newly developed chemotherapies. We also show preliminary data into the use of methionine deprivation as a way to up-regulate phosphoglycerate dehydrogenase (PHGDH), making it a viable drug target in cell lines normally non-responsive to PHGDH inhibition. High methionine diets have previously been associated with an intracellular increase in S-adenosylhomocysteine (SAH), the byproduct of SAM utilization in methylation reactions, which is capable of inhibiting methyltransferase activity, and this inhibition has been associated with global DNA hypomethylation. Here we investigate the role methionine consumption, in particular high methionine diets, play in post-menopausal breast cancer incidence in the Prostate, Lung, Colorectal and Ovarian (PLCO) cancer screening trial, as well as on cancer cases in the Survey of the Health of Wisconsin (SHOW) study cohort. The SHOW data was also analyzed for global CpG methylation to determine if high methionine diets lower global methylation in humans. Collectively this work indicates lowering dietary methionine may be beneficial for both cancer prevention and treatment.

Table of Contents

Chapter 1: Targeting the Methionine Addiction of Cancer

Abstract

Methionine is the initiator amino acid for all protein synthesis, the methyl source for most nucleotide, chromatin, and protein methylation, and the carbon backbone for various aspects of cellular redox buffering. Methionine is provided via the diet and serum methionine levels change based on dietary methionine content. Within the cell, methionine is recycled from homocysteine via the methionine cycle, which is linked to nutrient status via one-carbon metabolism. Unlike normal cells, many cancer cells, both *in vitro* and *in vivo*, show high methionine cycle activity and are dependent on exogenous methionine for continued growth. However, the molecular mechanisms underlying the methionine dependence of diverse malignancies are poorly understood. Methionine restriction initiates widespread metabolic alterations in cancer cells that enable them to survive despite limited methionine availability, and these adaptive alterations can be specifically targeted to enhance the activity of methionine depletion, a strategy we have termed "metabolic priming". Chemotherapy-resistant cell populations such as cancer stem cells, which drive treatment-resistance, are also sensitive to methionine restriction, suggesting dietary methionine restriction may inhibit metastasis and recurrence. Several clinical trials in cancer are currently underway using methionine restriction in combination with other agents. This review will explore new insights into the mechanisms of methionine dependence in cancer and therapeutic efforts to translate these insights into enhanced clinical activity of methionine restriction in cancer.

Methionine Cycle and One-carbon Metabolism: Linking Carbon Flux to Nutrient Status

Methionine is an essential amino acid for protein synthesis as well as many biochemical reactions required for cell viability and growth (**Figure 1**). S-adenosylmethionine (SAM) is the universal methyl donor for RNA, DNA, and chromatin methylation, and is synthesized from methionine via methionine adenosyltransferase (MAT)[1]. MATI and MATIII are generally expressed in the liver, where high SAM synthesis occurs, and are encoded by *MAT1A,* while MATII is expressed in most other cells types and is encoded by *MAT2A*[2-4]. SAM is converted to S-adenosylhomocysteine (SAH) through various transmethylation reactions. SAH is then hydrolyzed to homocysteine by SAH hydrolase (AHCY or SAHH), which can then be re-methylated to methionine by methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase; MTR or MS) or betaine-homocysteine methyltransferase (BHMT)[1]. Homocysteine can also be diverted into the transsulfuration pathway by cystathionine-β-synthase (CBS) to become cystathionine, which is then converted to cysteine by cystathionase (CTH) for use in glutathione production[1,2]. SAM can also be used for polyamine synthesis, which produces S-methyl-5'-thioadenosine (MTA) as a byproduct that can then be recycled back to methionine in the methionine salvage pathway[1,2]. The immediate precursor to methionine in the salvage pathway, 2-keto-4-methylthiobutyrate (MTOB), can also be converted to methional, which is capable of activating the apoptotic cascade, linking methionine metabolism to cell fate decisions[1]. The methionine cycle is outlined in **Figure 2A**.

The methionine and folate cycles are directly linked to stabilize metabolite levels, particularly during times of low methionine availability to maintain flux through the methionine cycle instead of diverting intermediates towards other pathways[5] (**Figure 2B**). During SAM abundance, indicative of methionine abundance, SAM inhibits the enzymes methylenetetrahydrofolate reductase (MTHFR) and BHMT to limit conversion of homocysteine to methionine, allowing homocysteine to be diverted for transsulfuration[4,5]. SAM also activates CBS, further diverting homocysteine towards transsulfuration[4-6]. During times of low methionine input, SAM levels decline, releasing inhibition on MTHFR and BHMT, and suppressing activation of CBS to maintain flux through the methionine cycle. Low methionine synthesis leads to accumulation of 5-methyltetrahydrofolate (5-mTHF), which inhibits glycine N-methyltransferase

(GNMT) to direct SAM usage towards DNA methyltransferases (DNMTs)[5]. These coordinated interactions allow a cell to buffer declining methionine and SAM levels in times of low methionine input to maintain the synthesis of proteins and polyamines, as well as methylation reactions, all of which are necessary for cell growth and survival.

Cancer Metabolic Vulnerabilities: An Addiction to Methionine

Oncogenic alterations in cancer cells modify their nutrient intake and utilization, leading to altered metabolic states that fuel their enhanced proliferation and growth. One such tumor-specific metabolic alteration is methionine dependence, namely, the inability of transformed cells to grow when methionine is replaced by homocysteine in cell media or the diet[7,8]. This effect appears to be specific to transformed cells, as normal cells maintain growth and viability, even on prolonged methionine deprivation when supplemented with homocysteine[9-12]. When human sarcoma HOS-1A cells were co-cultured with fibroblast FS-3 cells, methionine deprivation selectively suppressed growth of the cancer cells while maintaining normal growth of the fibroblasts[13]. Methionine has also been found to be taken up faster by tumor tissue than surrounding normal tissue[14]. Methionine and other essential amino acids are taken up into cells by L-Type Amino Acid Transporters, LAT1, LAT2, LAT3, and LAT4[15]. LAT1 is commonly overexpressed in various types of cancer[15,16], and this overexpression is commonly associated with shorter survival time[16]. The expression levels of LAT1 is also correlated with intracellular availability of SAM and the level of H3K9me3 within cells[15], suggesting LAT1 expression affects methionine availability or aspects of methionine metabolism. LAT1 down-regulation has also been found to lower the viability of cancer cell lines[16]. Due to the increased uptake of methionine into rapidly growing tumor tissue, the radiotracer L-methyl-^{11}C-methionine (^{11}C-methionine) has also been used as a way to measure treatment responsiveness[17] and to differentiate tumor margins from necrotic areas from previous radiation treatment to more accurately and completely remove cancerous tissue[18]. Viewed from this context, cancer cells are methionine auxotrophs “addicted” to exogenous methionine, a metabolic dependence not seen in genetically-related non-transformed cells[11,19,20].

A study that evaluated the methionine dependence status of 20 different cancer cell lines found 13 cell lines to be fully methionine dependent, while the other 9 cell lines grew to variable extents when methionine was restricted[8]. Another study looking at 23 cancer cell lines found 11 fully methionine dependent, 3 relatively dependent, and 9 relatively independent[9]. It’s worth noting that cell line contamination or un-dialyzed serum, as well as late passage number, may contribute to cancer cell lines appearing methionine independent[21]. To date, most cancer cell lines evaluated, including prostate, breast, bladder, colon, glioma, kidney, melanoma, and leukemia cells, along with fresh patient tumors, have been found to be some level of methionine dependent[1,10,12,22-24]. Methionine restriction has also been found to decrease invasion and wound healing, suggesting it may be useful in targeting metastatic subclones[25,26].

Methionine restriction limits tumor growth in rodent models, including two patient-derived tumor models found to be chemotherapy and radiation resistant[7]. A study of mice with implanted adenocarcinomas found tumor growth significantly reduced to 56% of control tumors when methionine was restricted to 0.1% of the control diet[27]. Another study using Yoshida tumors injected in nude mice found methionine deprivation reduced the growth rate of tumors compared to mice on the control diet, and when prolonged, tumors regressed on average 81% under methionine deprivation and prolonged survival in all mice tested[24]. Methionine deprivation also reduced organ metastasis 32.2% and ablated metastasis in combination with ethionine[28]. Rodent models have also shown dietary methionine restriction or deprivation reduces methionine metabolite levels in serum, liver, and tumor cells[7,29], however, some tissue-specific alterations in methionine cycle metabolites were found. One study using dietary methionine restriction in mice did not find a reduction in liver methionine[30]. Considering the liver is normally the largest consumer of exogenous methionine, it is possible there are liver-specific compensatory mechanisms to maintain methionine levels[4,31]. It is also important to note that in most mammals, including rodents, SAM availability is regulated by threonine metabolism via threonine dehydrogenase (TDH), however in humans TDH is a non-functional pseudogene, suggesting human cells may be more reliant on methionine metabolism than other mammalian cells[2].

Mechanisms of Methionine Dependence in Cancer: Oxidative Stress Derails Methionine Recycling

Rapidly proliferating cancer cells have an increased demand for methionine for protein synthesis, for maintenance and *de novo* methylation of DNA and chromatin, which requires SAM, and for the methionine cycle to generate intermediates and cofactors used in glutathione and nucleotide biosynthesis[32]. Methionine restriction in transformed cells has been found to lower available methionine and SAM[8,33-37]. Methionine still appears to incorporate into newly synthesized proteins at a rate comparable to healthy cells, suggesting endogenous methionine is preferentially utilized or mobilized for protein synthesis in methionine limitation[1,35,38,39]. Many enzymes within the methionine cycle, including MTR and AHCY, as well as enzymes involved in transmethylation, polyamine synthesis, and transsulfuration have been shown to be highly active in many types of tumor cells, suggesting many aspects of methionine metabolism may influence the growth capacity of cancer cells[38,40]. In culture, methionine restriction has also been found to induce apoptosis and increase p53-positive cells; however this induction does not appear to fully depend on p53 status, as p53 knockout only led to a partial rescue of cell death in response to methionine restriction[2,22,41]. Interestingly, wild-type p53 interacts with DNA (cytosine-5)-methyltransferase (DNMT1) to increase the *de novo* DNA methylation and gene silencing capacity of DNMT1, suggesting functional p53 may use DNMT1 and epigenetic plasticity to evade apoptosis in low methionine stress conditions[42].

A common feature of methionine-dependent cancer cells is their increased demand for exogenous methionine for transmethylation reactions[1,8,24,40,43]. When 18 tumor cell lines and 4 fibroblast cell lines were compared, all tumor cell lines showed elevated rates of transmethylation relative to fibroblasts in both normal and methionine-depleted media[44]. Methionine-dependent mammary adenocarcinoma and lymphoma cell lines were found to accumulate SAH five times faster than methionine-independent cell lines in response to AHCY inhibition, suggesting higher rates of transmethylation[38]. The same study isolated a methionine-independent-revertant from the mammary adenocarcinoma cell line and found the major difference between dependent and independent status was decreased transmethylation rates in the independent revertant[38]. Notably, the methionine-independent revertant also lost several hallmarks of malignancy, including colony-formation and increased anchorage-independence[35,45,46]. One study evaluating a panel of 60 cancer cell lines found significantly decreased levels of CBS in methionine-dependent cell lines compared to methionine-independent cell lines, suggesting defects in diverting intermediates into the redox-buffering transsulfuration pathway may impact cancer cell survival in low-methionine conditions[47].

Many studies have also found a lower SAM:SAH ratio in methionine-dependent versus independent cells in response to methionine deprivation, with a methionine-independent-revertant exhibiting a normal SAM:SAH ratio[35,38,39]. Furthermore, SAM supplementation has also been found to rescue the effects of methionine deprivation in a variety of cell types[34,48], including breast cancer stem cells[49]. Interestingly, transmethylation and SAM may play a role in the development of cancer, as global DNA hypomethylation is commonly found in many types of cancer and pre-cancerous cells, and has been hypothesized to result from unbalanced global methylation, leading to the gradual loss of DNA methylation, particularly in genomic areas copied late in replication[35,50,51]. SAM is also used in the synthesis of polyamines, and various cancer cells have been found to have hyperactive polyamine synthesis to support rapid rates of growth and division, however spermidine supplementation during methionine deprivation did not rescue cell growth defects[45].

While the transsulfuration pathway for glutathione synthesis is largely limited to the liver, pancreas, and kidneys in healthy adults, cancer cells appear to have a heightened dependency on transsulfuration of homocysteine to buffer oxidative stress that accumulates as a result of rapid cell growth paired with metabolic dysfunction[52]. In humans, the conversion of homocysteine to cystathionine drops 20% under dietary methyl group restriction[4]. The methionine-dependent phenotype can be partially reversed by over-expression of the cystine transporter xCT, and loss of the xCT transporter leads to a significant decrease in intracellular glutathione[52,53]. Indeed, oncogenic *PI3KCA* confers methionine dependence by reducing xCT expression and cystine import, thereby diverting homocysteine to the transulfuration pathway to buffer oxidative stress rather than recycling methionine[52]. Moreover, knockout of activating transcription factor 4 (ATF4) reduced both glutathione synthesis and xCT activity[54]. Collectively, these findings strongly suggest that at least part of the methionine dependence of cancer cells results from the need to divert homocysteine from the methionine cycle into the transsulfuration pathway to promote glutathione synthesis and buffer oxidative stress, thereby creating a dependence on exogenous methionine.

Methionine deprivation also activates nuclear factor erythroid 2-related factor 2 (NRF2) and enhances complex formation at antioxidant response elements (ARE)[55]. Interestingly, MAT II has been found to associate with MAFK-NRF2 and affect repression of target genes[56]. The oxidative stress induced by methionine restriction may partially result from glutathione depletion. One study found glutathione showed a time-dependent reduction during methionine restriction, followed by an increase in nuclear NRF2[57]. However, data on serum and intracellular glutathione levels in response to methionine deprivation have been variable[58-61], possibly due to ATF4-NRF2 activating multiple genes involved in glutathione biosynthesis[62]. A study of long-term methionine restriction in F344 rats found short-term methionine restriction depletes glutathione, while adaptive mechanisms lead to increased blood glutathione when methionine restriction is prolonged[63]. One consequence of diverting homocysteine away from the methionine cycle is reduced available intermediates for SAM synthesis needed to maintain methylation reactions and polyamine synthesis for cell growth and division[45]. Oxidative stress increases the activity of CBS while MATI and MATIII activity is reduced, creating a further deficit in serum methionine and SAM availability[6,64]. Increased uptake in exogenous methionine commonly seen in cancer may contribute to cancer cell dependence on transsulfuration through high intracellular sulfur load and the generation of cytotoxic sulfur intermediates that can interfere with cellular detoxification proteins and mitochondrial electron transport chain function[6,14]. Interestingly when methionine-dependent and methionine-independent-revertant mammary carcinoma cell lines were compared, methionine-dependent cells showed an increase in total SAH as well as increased conversion of homocysteine back to SAH compared to the revertant, further implicating oxidative stress with methionine dependence, as homocysteine itself can directly contribute to oxidative damage[45]. Homocysteine can also inhibit AHCY to further accumulate SAH[65]. SAH binds and inhibits methyltransferase activity[66], and intracellular SAH concentrations have been found to correlate with DNA hypomethylation[67]. In healthy year-old mice, methionine restriction decreased liver SAH levels 50% compared to control-fed mice; methionine restricted mice also maintained global DNA methylation in the liver comparable to young animals, while control-fed mice gradually lost global DNA methylation marks, similar to what is commonly seen in human aging[68]. Healthy cells in aging animals and humans may benefit from the reduced SAH burden seen during methionine restriction, while cancer cells, that have a generally heightened need for methionine, SAM, and redox buffering, may suffer from methyl shortage leading to cell cycle arrest or cell death. Further research into the role SAH plays in the methionine dependence of cancer cells is needed.

Another factor evaluated for its role in methionine dependence is deletion of the methionine salvage enzyme gene, methylthioadenosine phosphorylase, (*MTAP*), seen commonly in a variety of cancer types, often due to co-deletion with the tumor suppressor gene *CDKN2A* which encodes p16

(INK4A)[1,43]. MTAP uses MTA, a byproduct from the utilization of SAM in polyamine synthesis, to regenerate methionine. While MTAP is frequently mutated or deleted in cancer, cancer cell lines with intact methionine cycle enzymes can still be methionine dependent, suggesting MTAP deletion may facilitate, but is not crucial for, methionine-dependent status[69]. Furthermore, MTAP deletion or re-introduction in various human cancer cell lines did not affect methionine-dependent growth defects[70]. However, *MTAP* deletion does uncover a druggable dependence on the protein arginine methyltransferase PRMT5, which is inhibited by the resultant high levels of MTA[71,72]. Moreover, *MTAP* deletion sensitize tumor cells to depletion of MAT2A, which is needed to produce SAM for PRMT5-mediated methylation[71,72]. PRMT5 has a much higher affinity for MTA than SAM, making its catalytic activity sensitive to the SAM/MTA ratio[72], suggesting methionine restriction may further promote PRMT5 dependence in *MTAP*-deficient cells.

Defects in MTR, impairing methionine generation from homocysteine, do not appear to play a role in methionine dependence[43] as many cancer cell lines evaluated synthesize at least as much methionine as their healthy counterparts and show high MTR activity[11,47]. Furthermore, when looking at cancer cells within a methionine dependent population that have reverted back to a methionine independent status, no change was found in MTR activity[73]. Interestingly, MTR uses SAM as a cofactor, suggesting intracellular SAM depletion may influence the rate of methionine synthesis. The other enzyme responsible for converting homocysteine to methionine, BHMT, may compensate when MTR is lost[73].

These specific enzyme alterations, while they may contribute to methionine dependence, are not necessary for methionine dependent status, as seen in cell lines such as Walker-256 rat carcinoma cells, that have no innate biochemical defects in the methionine or folate cycles, yet still show high methionine dependence and requirement for exogenous methionine input[69]. These cells, when compared to the less methionine dependent TLX5 lymphoma cell line, were unable to maintain intracellular SAM levels following 48-hour methionine deprivation, whereas SAM showed little fluctuation in TLX5 lymphoma cells[69].

One relatively unexplored area that may influence methionine dependent status and flux through the transsulfuration pathway is the regulation the methionine and folate cycles exert over each other, a regulation that can become dysfunctional in cancer cells (**Figure 2B**). With methionine-folate cycle regulation intact, the SAM/SAH ratio varied 2-fold when methionine input was changed 13-fold, however this same change in methionine input caused a 50-fold variation in the SAM/SAH ratio in cells lacking methionine-folate cycle regulation[5]. Furthermore, when methionine-folate cycle regulation is missing, the flux through DNMTs became very sensitive to methionine input, particularly at low methionine input when DNMT flux drops, potentially due to SAH inhibiting DNMTs and histone methyltransferases[5,74,75]. This suggests that cancer cells may be more sensitive to methionine input fluctuations and methionine restriction when methionine-folate cycle regulation is non-functional and may in part explain why SAM levels appear more stable in cell lines that are less methionine dependent, however further work is necessary to investigate this relationship.

Methionine deprivation-specific effects may be ablated in a global amino acid deprivation diet[33,76,77]. Combined methionine-serine-glycine deprivation in MCF7 cells and methionine-serine-glycine-threonine deprivation in PC3 cells abolished the methionine deprivation-specific response and showed only the canonical amino acid deprivation response[34]. A different study with MCF7 and PC3 cells also found combined methionine and arginine deprivation restored the demethylation of histones seen during methionine deprivation alone[5]. This suggests that while the cellular response to methionine restriction may partially overlap with caloric restriction or general protein restriction, the full benefits of methionine restriction may be best seen when other essential and non-essential amino acids are present in sufficient amounts in the diet.

Methionine-Sensing and the Cellular Stress Response: Living with Less (Methionine)

The integrated stress response (ISR) is a highly conserved adaptive response to disparate cellular stressors and is largely mediated by: protein kinase R (PKR) in response to double stranded RNA, protein kinase R-like endoplasmic reticulum kinase (PERK) in response to endoplasmic reticulum (ER) stress, hepatic heme-regulated inhibitor (HRI) in response to heme depletion, and general control nonderepressible 2 (GCN2) in response to amino acid deprivation and ultraviolet (UV) damage[58]. These kinases are all capable of phosphorylating eukaryotic initiation factor 2α (eIF2α), which blocks translation re-initiation by reducing GDP exchange for GTP on eIF2, inhibiting its function. Although global protein translation is suppress, ATF4 mRNA is preferentially translated during times of cell stress, when complete assembly of the translational machinery on ATF4 mRNA is slowed so that the 40S ribosomal subunit bypasses the upstream open reading frame, uORF2, that is translated during non-stress times[78] (**Figure 3**). When the 60S ribosomal subunit attaches after uORF2, the coding sequence of ATF4 is translated, allowing protein levels to rapidly increase in response to eIF2α inactivation, including amino acid limitation[79]. ATF4 dimerizes and binds DNA that contains a cAMP response element (CRE) sequence. A component of the methionine deprivation-specific response induced by ATF4 may depend on its dimerization partner[80-82].

Work from our lab using triple-negative breast cancer cell lines found a rapid induction of ATF4 and Sestrin2 (SESN2), an ATF4 target, in response to methionine deprivation[83]. Methionine deprivation also induced phosphorylation of eIF2α, however this phosphorylation was not dependent on GCN2 or PERK, as knockdown of either or both did not eliminate phosphorylation of eIF2α or ATF4 and SESN2 induction[83]. The roles of PKR and HRI, other known kinases capable of phosphorylating eIF2α, have not been investigated during methionine deprivation, however, there is also some evidence that ATF4 and the ISR can be activated without eIF2α phosphorylation[84] and that part of the methionine deprivation-specific response may be eIF2α phosphorylation-independent[85]. Methionine deprivation in eIF2αknockout mouse embryonic fibroblasts (MEFs) resulted in an increase in ATF4 mRNA (albeit blunted response), and the authors hypothesized ATF4 is induced via lack of initiator $tRNA^{met}$ instead of phosphorylation of eIF2α[85]. Another team reported that hepatic ATF4-target gene induction in response to methionine restriction was not dependent on GCN2 or phosphorylation of eIF2α[86]. They also found glutathione levels were decreased and NRF2 targets were strongly induced, suggesting ATF4-NRF2 may be induced regardless of phosphorylation of eIF2α in response to methionine restriction[86]. GCN2- and eIF2α phosphorylation-independent induction of ATF4 and ATF4-target genes in response to methionine restriction were also observed in mouse liver, along with glutathione depletion and NRF2 induction[87]. NRF2-ATF4 have been found to interact with each other at stress responsive elements[82], possibly in response to phosphoinositide 3-kinase (PI3K)[54] or protein kinase C (PKC)[88] pathway activation; however further work into methionine restriction-specific induction of NRF2-ATF4 is necessary.

The precise intracellular stress response of cancer cells to methionine restriction remains to be elucidated but appears to involve ATF4-NRF2 activation and induction of target genes. While there appears to be agreement that GCN2 and phosphorylated eIF2α play a role in the canonical amino acid deprivation response[89], the methionine deprivation-specific response greatly diverges and appears less reliant on GCN2 and phosphorylation of eIF2α to activate ATF4.

Autophagy functions to recycle macromolecules and organelles within a cell during stress or when nutrients are limited. Methionine restriction has been found to extend the life- and health-span of various organisms, and this effect was attributed to increased autophagic flux[90,91]. Interestingly, methionine restriction led to a decrease in Reactive Oxygen Species (ROS)[90], suggesting the increase in autophagy may promote mitochondrial function to prevent ROS leak. In line with these findings, a recent study found methionine restriction elevated mitophagy specifically, which is the autophagic recycling of mitochondria[92]. Furthermore, cells restricted of methionine no longer showed lifespan extension when key mitophagy genes were deleted or when mitochondrial function was blocked by the mitochondrial toxin, paraquat[92]. Mitochondrial peroxide production, indicative of mitochondrial dysfunction, was reduced by roughly half in cells undergoing methionine restriction compared to controls, and this reduction was not seen in cells with impaired mitophagy[92], suggesting increased mitophagy may increase functional mitochondria. Interestingly, while impaired bulk autophagy blocks tumor progression, it has been found that impaired mitophagy actually promotes tumor progression and increases the Warburg effects[93]. When looking at cancer treatment there has been mixed findings in terms of mitophagy, as mitophagy can promote drug-resistance by removing damaged mitochondria, however, heightened levels of mitophagy can promote tumor cell death via excessive clearance of mitochondria[94], suggesting tumor may respond differently to mitophagy induction based on stage and other genetic factors. While mitophagy induced by methionine restriction has been found to increase longevity of healthy cells, further work is needed to determine the role mitophagy induction plays in cancer cell survival and cancer treatment.

Methionine Restriction and the Cancer Epigenome: Methylation Matters

The bulk of genomic methylation is determined early in development to aid in cell differentiation and repress genes not needed for tissue-specific function. These methylation patterns often remain static throughout life, with changes mainly occurring in stress conditions and injury response[95,96]. Epigenetic plasticity is seen in the normal cellular response to injury, however when prolonged this plasticity can contribute to the hallmarks of cancer[97]. Gene mutations and truncations can also alter gene methylation patterns at a specific loci, leading to heritable epigenetic alterations[95]. Global hypomethylation has been implicated in the acquisition of senescent cells, aging, and various age-related diseases[98]. Cancer cells in general exhibit genome-wide loss of epigenetic stability, often exhibiting global hypo-methylation[99,100] and promoter-specific hyper-methylation[101], which has been implicating in oncogene activation and tumor suppressor silencing respectively[102,103]. These epigenetic changes can also be observed in pre-cancerous cells[97,104], normal tissue adjacent to tumor tissue[105,106], and hereditary human cancers[107]. Loss of heterochromatin, particularly loss of H3K9me2, has also been associated with the epithelial-to-mesenchymal (EMT) transition used by cancer cells for migration and metastasis, further implicating epigenetic dysfunction with malignant progression[108-110]. Multiple studies evaluating the cancer cell epigenome have found a correlation between epigenetic dysfunction and tumor progression, particularly for sustained growth in suboptimal conditions[75,108,111].

Methionine deprivation, through reduced available SAM, has been found to reduce the histone methylation marks H3K4me2, H3K4me3, H3K9me2, and H3K27me3 *in vitro* and in vivo[2,30,34]. Bulk screening for methylation at CpG island promoters or long interspersed nuclear element-1 (LINE-1) repetitive DNA sequences found no significant alterations, suggesting under methionine deprivation cells attempt to recuse growth by salvaging methyl groups preferentially from existing histone marks instead of DNA methylation[34]. Widespread DNA hypomethylation in cancer is associated with gene silencing due to the formation of repressive chromatin structures at these sites, suggesting cancer cells with epigenetic dysfunction may rely more heavily on histone methylation than DNA methylation to maintain viability[50,112]. DNA methylation marks are also generally more stable than histone methylation[113-115], suggesting histone methylation may be more dynamic or replaceable for cell survival than DNA methylation[95,97,116]. Interestingly, MAT II has been found to directly interact with histone methyltransferases and this interaction can alter histone methylation dynamics[117]. Further work evaluating the allele-specific methylation response to methionine restriction over time may better elucidate the role DNA methylation plays in methionine restriction, as bulk methylome sequencing may overlook changes in individual allele methylation patterns.

“Epigenetic persistence” is defined as the ability of a cell to reestablish its epigenetic signature following environmental alterations or metabolic stress[118]. Recent work has found that under methionine restriction both di- and tri-methyl histone marks are preferentially removed and new H3K9 mono-methylation marks are added to maintain heterochromatin stability during times of low SAM availability[118]. When H3K9 mono-methylation is blocked and cells are subjected to low SAM availability, they are no longer capable of epigenetic persistence when returned to full methionine media[118], suggesting H3K9 mono-methylation is necessary for a cell to maintain its heterochromatin state. The role of epigenetic persistence, H3K9 mono-methylation and methionine restriction needs further investigation in cancer cells.

Many studies have found methionine restriction to induce a reversible growth arrest in the late S/G_2 phase of the cell cycle[8,10,13,22,26,65,119,120] ; however, this arrest was not found in lung tumorspheres[33]. It is hypothesized this growth arrest may result from insufficient SAM for DNA and chromatin methylation necessary for cell division with re-creation of the parental cells methylation signature[73]. DNMT1 only complexes with histone deacetylase 2 (HDAC2) and DNMT1-associated proteins in late S phase when most heterochromatin is replicated[42,103,121]. Cells can also arrest in the G_2 phase of the cell cycle while DNA methylation remodeling is occurring[95,122] or when polyamine synthesis is disturbed[119].

It is also important to remember that SAM is used for RNA modifications to generate functional mRNA, control mRNA degradation, and aid in tRNA base-pairing under stress conditions. The most abundant modification to eukaryotic mRNA is methylation of adenosine by guanine 7-methyltransferase (RNMT), which uses SAM to create the 5'-methyl cap on pre-mRNA, where eukaryotic initiation factor 4α (eIF4α) binds and recruits the 40S ribosomal subunit to begin translation and control m^6A-dependent mRNA decay[123-125]. During times of DNA damage, the uridine of tRNAs that encode lysine, glutamine, and glutamic acid can be modified to enhance the wobble of the third position for less exact base pairing in translation, allowing base-pair mutated transcripts to be translated[32,126]. Both uridine modifications, methoxycarbonylmethyluridine and thiolation of uridine, consume SAM, and the abundance of tRNA uridine thiolation correlates with sulfur amino acid availability[91,126]. Lysine, glutamine, and glutamic acid codons are prevalent in ribosome biogenesis genes, suggesting altered tRNA thiolation may impact general translation under reduced SAM conditions[91]. Ribosome thiolation also alters complex I ROS production, suggesting reducing thiolation may alter cellular redox status[127]. This may be another mechanism by which methionine restriction preferentially affects cancer cells that require mRNA methylation to generate mature mRNA transcripts and may depend more heavily on tRNA uridine modifications to translate damaged mRNA transcripts, both of which may contribute to blocking translation in cancer cells in times of methionine limitation.

Cancer Stem Cells: A Dependency on SAM Biosynthesis

Pluripotent embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) both appear more methionine dependent than differentiated cells, and self-renewal capacity correlates with SAM concentration[2]. Methionine deprivation in ESCs and iPSCs primes cells for differentiation, and once differentiated, total cellular methionine input decreases[2]. This suggests that part of a cancer cells dependence on methionine may be due to the acquired stem-like state frequently observed in cancer cells. It also suggests healthy stem cell maintenance may be affected when methionine restriction is prolonged, however healthy stem cells with intact methionine cycle regulation may more readily compensate for methionine cycle metabolites than cancer cells with dysregulated methionine cycle activity[5].

Cancer stem cells or tumor-initiating cells commonly exhibit resistance to a variety of cytoxic agents and undergo latent growth periods, making them treatment-resistant and critical to eliminate to reduce risk of recurrence[128,129]. Redox state is thought to aid in cancer stem cell therapeutic resistance, with intermediary levels of ROS being used to transition between highly proliferative and latent growth phases[129], suggesting further induction of metabolic stress may increase ROS to a toxic level. Tumorspheres, cancer cells grown on ultra-low-attachment plates, are a common way to enhance propagation of tumor-initiating and cancer stem cells[128,129]. Work from our lab using triple negative breast cancer (TNBC) tumorspheres showed methionine deprivation robustly decreased the number of tumorspheres as well as $CD44^{hi}/CD24^{low}$ cells[49]. Both effects showed at least a partial rescue with SAM supplementation, underscoring that SAM, rather than methionine per se, is the critical metabolite for cancer stem cell survival[49]. These effects of methionine restriction were associated with H3K4me3 demethylation and suppression of Sox9 expression, a regulator of plasticity in cancer stem cells. It remains to be determined whether these epigenetic events contribute functionally to the observed effects of methionine depletion on cancer stem cells.

Lung cancer tumorspheres are also enriched for SAM and SAH as well as methionine cycle activity and intermediates relative to isogenic, adherent cells, suggesting cancer stem cells may have a heightened requirement for methionine and SAM when compared to their more differentiated counterparts[33]. Following 48 hours of methionine depletion, lung cancer tumorspheres showed reduced methionine cycle activity and a 30-fold reduction in SAM accompanied by decreased histone methylation, suggesting exogenous methionine depletion greatly affects cancer stem cell methionine metabolism[33]. Furthermore, when tumorspheres were injected into NOD Scid Gamma (NSG) mice following methionine deprivation they greatly lost their tumor-forming abilities, decreasing the tumor burden 94% compared to mice injected with tumorspheres grown in control media, and this effect was specific to methionine deprivation[33]. While work into the effects of methionine restriction on cancer stem cells is a relatively recent venture, it appears promising as a way to target cancer stem cells and reduce their self-renewal and tumor-forming capacity, at least partially through reduction in SAM availability.

Methionine Restriction: Priming Cancer Cells to Die

Despite preclinical activity against diverse malignancies, methionine restriction has been largely disappointing in clinical trials to date (see section below). We postulated we could enhance the activity of methionine restriction by targeting molecular vulnerabilities uncovered by low methionine stress, a strategy we have termed “metabolic priming”. To this end, we performed a proteomics analysis of triple-negative breast cancer cells cultured in methionine-free media[130]. One of the proteins induced by methionine restriction is the proapoptotic TRAIL receptor-2 (TRAIL-R2/DR5). Methionine depletion, either through methionine-free media or the addition of methioninase, sensitized cells to the agonistic TRAIL-R2 monoclonal antibody lexatumumab by increasing cell surface expression of TRAIL-R2[130]. At least part of this increase in TRAIL receptor expression is attributed to a loss of MAGE family member D2 (MAGED2) protein seen during methionine deprivation[130]. Interestingly, non-transformed breast epithelial cells were largely resistant to the combination of methionine restriction and lexatumumab likely due to the lack of cell surface induction of TRAIL-R2. Mice fed a methionine-free diet and treated with lexatumumab for 3 weeks had smaller primary tumors and reduced lung metastases compared to mice treated with diet or lexatumumab alone in a triple-negative mCherry-MDA-MB-468 tumor model[130]. Similar results were recently reported for oral methioninase and the agonistic TRAIL-R2 mAb tigatuzumab in a mouse model of pancreatic cancer[131].

Another protein identified in our proteomic screen is MAT2A[130], the widely expressed enzyme that converts methionine into SAM. Methionine deprivation increases MAT2A mRNA stability[125] as well as protein and mRNA levels in response to declining SAM availability[49,132]. Our lab used the MAT2A inhibitor, cycloleucine, in combination with methionine deprivation and found synergistic inhibition of cancer stem cell-enriched mammosphere formation and demethylation of H3K4me3[49]. Moreover, the combination methionine deprivation with cycloleucine was more effective than either treatment alone at suppressing lung metastases in NSG mice with orthotopic GILM2 breast cancer tumors[49].

Both lexatumumab and cycloleucine had previously been abandoned as anti-cancer treatments due to lack of efficacy[133-137] and unacceptable toxicity[138,139] respectively in clinical trials. When paired with methionine deprivation, both drugs showed enhanced cytotoxicity at much lower doses, suggesting methionine deprivation may be a useful strategy to enhance efficacy and lower toxicity of various anti-cancer drugs[49,130]. Overall, these studies provide proof-of-concept preclinical evidence that methionine restriction can be used to metabolically prime tumor cells to respond to rationally selected agents that target vulnerabilities exposed by methionine stress.

Methionine Restriction in Combination with Cytotoxic Agents

Altered cell metabolism is a hallmark of cancer that contributes to tumor initiation/progression and chemotherapy-resistance[140]. Methionine restriction alters multiple aspects of cancer cell metabolism, leading to its use in combination with various commonly used chemotherapy drugs[20] (**Table 1**).

DNA and chromatin methylation have been implicating in resistance to DNA-damaging agents by limiting access to DNA[14]. Methionine restriction reduces various histone methylation marks, possibly altering DNA access to enhance the activity of DNA-damaging drugs such as doxorubicin and cisplatin. TC71-MA multi-drug-resistant colon tumors grown in mice showed prolonged survival and tumor growth inhibition when subjected to methionine deprivation, cisplatin and the methionine analog ethionine, with the greatest effect seen for combination treatment[141]. Mice bearing SCLC6 small cell lung cancer tumors normally refractory to doxorubicin showed 51% growth inhibition and 1.7-fold longer survival when treated with methionine deprivation, doxorubicin and ethionine in combination[141]. A mouse model of gemcitabine-resistant pancreatic cancer showed both tumor growth inhibition and regression using combination gemcitabine and methionine deprivation[142].

Methionine restriction also induces a reversible growth arrest in late S/G_2 phase of the cell cycle[35,73] and/or G_1 arrest[24,143]. Since many chemotherapies act during DNA replication or on metaphase microtubules, using methionine restriction to synchronize cells around the S and M phase of the cell cycle may enhance cancer cell sensitivity to cell cycle phase-specific chemotherapies such as 5-fluorouracil and vincristine[1,13]. A study using combined methionine restriction and 5-fluorouracil found synergistic inhibition of the growth of two different chemotherapy and radiation resistant patient-derived tumor models[7]; however, this effect may at least in part be attributed to the reduced protein level and activity of thymidylate synthase (TS) observed during methionine restriction[144]. SAM supplementation has been found to increase resistance to 5-fluorouracil through upregulation of DNMTs[145], suggesting methionine restriction may synergize with 5-fluorouracil administration by maintaining low intracellular SAM levels. Other chemotherapies that target the folate cycle, such as methotrexate and pemetrexed, inhibit dihydrofolate reductase, leading to accumulation and diversion of 5,10-methylene tetrahydrofolate into thymidylate synthesis, reducing available intermediates for one-carbon biosynthesis and to re-methylate homocysteine into methionine[146], suggesting the effects of methionine restriction may be more pronounced when used with antifolate drugs.

Co-culture of HOS-1A sarcoma and FS-3 fibroblast human cell lines in combination methionine deprivation, doxorubicin and vincristine successfully eliminated cancer cells while maintaining growth and proliferation of fibroblasts[13]. This combination also eliminated MCF7 breast cancer, A2182-5D lung cancer and PC3 prostate cancer cells co-cultured with FS-3 fibroblasts[13]. Interestingly, co-culture of FS-3 fibroblasts with HOS-1A-R3 cells, which have reverted to a methionine independent status, treated with combination methionine deprivation, doxorubicin and vincristine virtually eliminated all tumor cells[13]. Methionine restriction in combination with doxorubicin and vincristine prolonged survival of Yoshida sarcoma-bearing rats despite only partially depleting circulating methionine (18.4 μM in methionine-depleted TPN-treated rats versus 87.7 μM in control rats)[147].

Methionine, through transsulfuration to cysteine, contributes to the production of glutathione and elevated glutathione has also been implicated in the development of drug resistance[22]. Due to the finding that methionine restriction lowers intracellular glutathione levels in cancer and tumor cells, methionine restriction may further prove to be a useful strategy to enhance the sensitivity of cancer cells to various chemotherapeutic agents[22,141]. Many commonly used classes of chemotherapy, such as anthracyclines (ex. doxorubicin and epirubicin), alkylating agents and platinum compounds (ex. cisplatin, carboplatin, and oxaloplatin) generate oxidative stress through production of ROS and superoxide radicals[148]. Moderate levels of oxidative stress induced by these drugs can decrease drug effectiveness through various protective adaptive mechanisms[148]. Use of these drugs in combination with methionine restriction may enhance oxidative stress, pushing cancer cells towards apoptosis. One study using drug resistant xenograft tumors in nude mice found reduced intracellular tumor glutathione levels along with reduced tumor growth and prolonged survival following 3-week methionine deprivation[141].

Dietary methionine restriction appears effective in enhancing the anti-tumor activity of different types of chemotherapeutic agents. Considering the anti-cancer in vitro and in vivo effects of methionine restriction alone and in combination with various chemotherapies, several clinical trials have been conducted or are ongoing.

Methionine Restriction in Humans and Clinical Trials

Human fasting plasma methionine concentration ranges from 3-30 micromolar and correlates with SAM concentration[30]. This variation in plasma methionine is partially explained by diet (~30%), as well as clinical variables such as age, gender and body composition (~30%), and genetic variables[30]. A study with six healthy, middle-aged individuals on a low methionine diet (approximately 2.92 mg/kg/day) resulted in lower serum methionine and methionine cycle metabolites, as well as diminished reduced glutathione and N-acetyl cysteine (NAC)[7]. Dietary methionine restriction (2 mg per kg per day) in 12 patients with metastatic solid tumors lead to a 58% reduction in plasma methionine by 2 weeks, with only mild losses in body weight that were regained upon return to a full methionine diet[120]. Dietary methionine restriction was accomplished by providing 75% of total protein through a methionine-free medicinal beverage (Hominex-2, Abbott Nutrition) with the remaining 25% of protein through low-methionine foods fruits, vegetables and grains[7,149].

Preclinical data looking at drug-refractory solid tumors in both xenograft mouse models on a methionine-free diet[141] and carcinoma-bearing rats on a methionine-free TPN[150] found methionine deprivation re-sensitized tumors to alkylating chemotherapies, leading to clinical investigation into methionine deprivation in combination with the alkylating agent, cystemustine. In phase I and phase II clinical trials of glioma and melanoma patients on 24-hour methionine deprivation in combination with cystemustine, adherence to the diet was acceptable, there was no significant loss of body weight, and blood and inflammation markers remained stable, while plasma methionine dropped by about half in both studies[151,152]. In the phase I study, 8 of 10 patients showed disease stabilization after the second cycle of treatment, 2 of 7 showed stabilization after the fourth cycle of treatment, and the median overall survival was 6.5 months[151]. In the phase II trial, of the 22 patients treated, 3 showed disease stabilization and the median survival time was 4.6 months[152]. Based on preclinical data showing O(6)-methylguanine-DNA methyltransferase (MGMT) was decreased in both adherent human cancer cell lines[153] and a xenograft mouse model of glioblastoma[154] following methionine deprivation, a study with combined methionine deprivation and cystemustine was conducted in 6 glioma and melanoma patients and found a decrease in MGMT (from 553 fmol/mg before treatment to 413 fmol/mg after), the expression of which is commonly associated with drug resistance[155].The decreases in MGMT as well as plasma methionine were not significantly different when methionine deprivation extended beyond 24-hours, suggesting transient methionine deprivation may be enough to re-sensitize cancer cells to chemotherapy[155]. The purpose of this study was only to evaluate MGMT activity and plasma methionine levels, so response was no evaluated[155].

Preclinical evaluation of 5-fluorouracil in combination with dietary methionine deprivation[156] or recombinant methioninase[41,157] to deplete circulating methionine found increased anti-cancer activity in various human cancer cell lines and mouse models of both human gastric cancer and lung carcinoma. Methionine deprivation and cisplatin, as well as other alkylating agents, have been used in various types of solid tumors in mice[141,158,159] and in lung carcinoma-bearing rats[150], showing enhanced efficacy of alkylating agents, even in drug-refractory tumors. Due to the success of methionine deprivation in combination with both 5-fluorouracil and alkylating agents, the FOLFOX combination of 5-fluorouracil, leucovarin, and oxaloplatin, was tested in combination with 48-hour dietary methionine restriction in 11 metastatic colorectal cancer patients, and found lower plasma methionine while maintaining good compliance and tolerance to the diet[160]. Of the 11 patients, 4 were evaluated for a response, 3 of which showed a partial response and the other one showed stabilization[160].

A methionine- and cystine-free total parenteral nutrition (TPN) infusion, where most dietary nutrients are given directly via intravenous solution, was given to 7 advanced gastric cancer patients[161]. Thymidylate synthase, the primary molecular target of 5-fluorouracil, showed lower activity in the methionine-free TPN group (1.12 pmol/g) compared to control (2.35 pmol/g)[161]. Methionine-free TPN also enhanced necrotic area within tumors, shown by higher histological grades, without any major complications[161].

Overall, clinical data has shown dietary methionine restriction lowers circulating methionine and is well tolerated even in combination with various chemotherapies. Considering many of the studies are pilot studies of small patient size, larger trials are necessary to evaluate anti-cancer outcomes in response to methionine restriction. There are currently multiple clinical trials ongoing that will help to better elucidate the full potential of methionine restriction in cancer treatment (**Table 2**)[162-168].

A currently recruiting trial (NCT03733119) was designed to examine whether methionine restriction metabolically primes patients to respond to a novel TRAIL agonist (Onc201) based on preclinical findings from our lab[130]. Another trial (NCT03186937) conducted by our lab using dietary methionine restriction in TNBC was designed to evaluate the impact of methionine restriction on TRAIL receptor surface expression and its impact on cancer stem cells based on preclinical findings that methionine restriction targets many properties of cancer stem cells[49,130]. Methionine restriction is also being tested in combination with radiation therapy in lung, prostate and breast cancer patients (NCT03574194) to determine if dietary methionine restriction can be used to radio-sensitize tumors.

There are several recently completed clinical trials involving dietary methionine restriction. One study (NCT00508456) in patients with glioblastoma multiforme was conducted to test the safety and efficacy of combination methionine restriction with temozolomide, an alkylating agent commonly used to treat glioblastoma. To better understand the impacts of methionine metabolism on life- and health-span, two studies were conducted to evaluate energy and glucose metabolism in overweight or diabetic adults using dietary methionine restriction (NCT00640757) or dietary methionine and cysteine restriction (NCT03629392). One study also evaluated metabolic changes during combination methionine-cysteine restriction in healthy adults (NCT02192437).

Alternatives to Dietary Methionine Restriction

L-Methionine-γ-lyase or methioninase, a bacterially derived enzyme that degrades sulfur-containing amino acids including methionine, has found success in depleting both media and serum methionine and may be a more convenient approach to clinical methionine restriction[158,169]. Co-culture of human embryonic lung fibroblasts and acute lymphoblastic leukemia cells treated with methioninase eliminated malignant cells to roughly 5% that of control[21]. In vivo serum methionine was depleted following a single injection of 300 units methioninase and this decrease was maintained by 8 hours[41]. Methioninase also depleted serum homocysteine, showing it effectively reduces methionine cycle intermediates[170]. Methioninase in non-tumor-bearing healthy mice did not significantly alter body weight following 21 days of treatment and showed minimal antigenicity in immune-competent mice following repeat exposures[41]. In humans, methioninase depleted serum methionine within 30 minutes and was maintained for up to 4 hours after infusion had stopped without outward signs of toxicity[171].

Oral recombinant Methioninase was tested in mice, reducing recurrent tumor volume from 936.7 mm^3 in control animals to 450.9 mm^3 in treated animals, without weight loss in either group[172]. In an orthotopic model of TNBC oral recombinant Methioninase reduced recurrent tumor weight and the number of metastatic lung nodules (5.3 in control versus 1.3 in treated mice)[173].

To enhance immune tolerance and prolong catalytic function, Methioninase has also been encapsulated in human erythrocytes[174]. Human erythrocyte encapsulated Methioninase maintained lower serum methionine with 34% depletion compared to control still present 9 days after administration[175]. Human erythrocyte encapsulated Methioninase inhibited the growth of human glioblastoma tumor xenografts by 85% compared to control at day 45[175].

Methioninase (oral and systemic) has also been tested in combination with various chemotherapies. Methioninase and 5-fluorouracil inhibited tumor growth and prolonged survival in mice bearing lung carcinoma xenografts compared to treatment with each agent individually (17% survival with methioninase alone, 30% survival with 5-fluorouracil alone, and 78% survival with the methioninase and 5-fluorouracil combination)[41]. Methioninase acted synergistically with cisplatin in SW620 and Colo205 colon cancer cell lines[158]. Moreover, the combination methioninase and cisplatin in nude mice bearing injected human-derived colon tumors significantly reduced tumor growth, especially when cisplatin was given on the first day of methioninase treatment[158]. Overall, methioninase may be a safe and effective way to reduce circulating and intracellular methionine without dietary alterations.

Conclusion

Cancer cells have a high demand for methionine for protein synthesis and SAM generation, which is used for RNA, DNA, and histone methylation, as well as glutathione and nucleotide synthesis. When this demand for methionine cycle metabolites is paired with dysfunctional methionine cycle regulation in cancer cells, it becomes more difficult for transformed cells to buffer declining methionine availability induced by methionine restriction. This leads to a reduced capacity to synthesize glutathione through transsulfuration, leading to an increase in oxidative stress that contributes to the methionine dependence of cancer cells. Interestingly, methionine restriction in rats reduced ROS leak and hydrogen peroxide production from heart and liver cells, and both showed decreased markers of oxidative damage[61,176-180], suggesting healthy cells benefit from methionine restriction. This effect is possibly due to methionine being a target for oxidation by ROS[176]. Methionine supplementation in healthy rats increased mitochondrial ROS production and oxidative stress markers[181], suggesting lowering total available methionine may itself reduce oxidative stress. Healthy cells also generally have a lower demand for methionine, SAM, and glutathione, particularly if the cells are terminally differentiated and non-replicating, and likely have intact methionine cycle regulation capable of buffering falling levels of methionine, allowing them to better adapt under times of methionine shortage.

Epigenetic dysregulation in cancer cells appears to result in a heightened dependency on exogenous methionine in order to maintain sufficiently high levels of SAM to support rapid rates of cell growth and division. When cancer cells are deprived of exogenous methionine they arrest in the S/G_2 phase of the cell cycle, possibly due to methyl group shortage and loss of histone methylation in an attempt to compensate and overcome this cell cycle block. While methionine restriction has been shown to reduced histone methylation, its role in altering DNA methylation is somewhat debated.

Methionine restriction has been used both *in vivo* and in a clinical setting in combination with various chemotherapies and appears to be a safe and tolerable way to enhance the cytotoxicity and specificity of targeted agents and chemotherapies, even in chemotherapy-resistant tumors. While many trials evaluating the safety of dietary methionine restriction or methioninase have been conducted, there is a great need for larger studies evaluating response and for rational selection of cancer drugs in combination with methionine restriction. Our group views methionine restriction as a unique nutritional strategy to metabolically prime tumor cells to respond to drugs that target adaptive responses to low methionine stress ("metabolic priming"). Further work is also needed to definitively determine the molecular mechanisms responsible for the methionine dependence of cancer cells, specific biochemical alterations that occur during methionine restriction, and the role that both transmethylation and transsulfuration play in cancer cell growth inhibition and apoptosis during methionine restriction.

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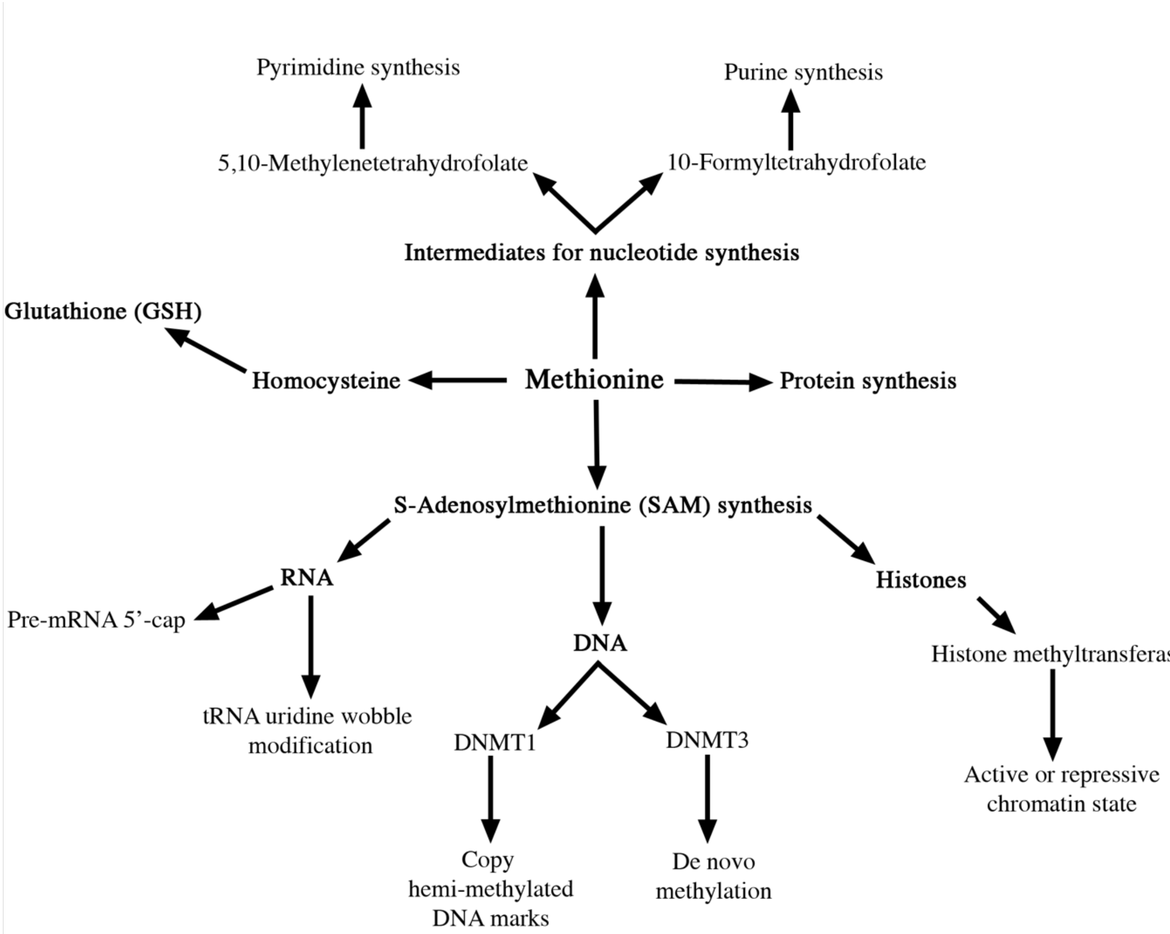
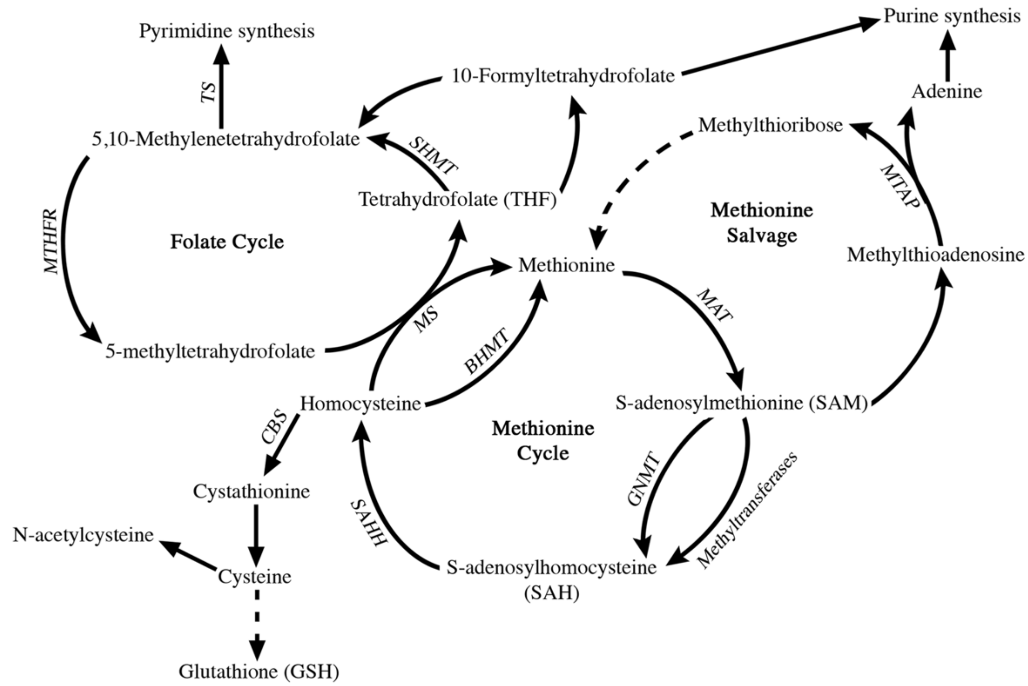


Figure 1. Roles of methionine in cell maintenance, growth, and division.
Methionine is an essential amino acid, meaning it must be provided through diet. The main uses for methionine are for protein synthesis, to generate intermediates and cofactors for nucleotide and glutathione synthesis, and to generate SAM, the universal methyl donor for methylation of RNA, DNA, and histones.

A. Methionine and Folate Cycles



B. Regulation during low methionine input

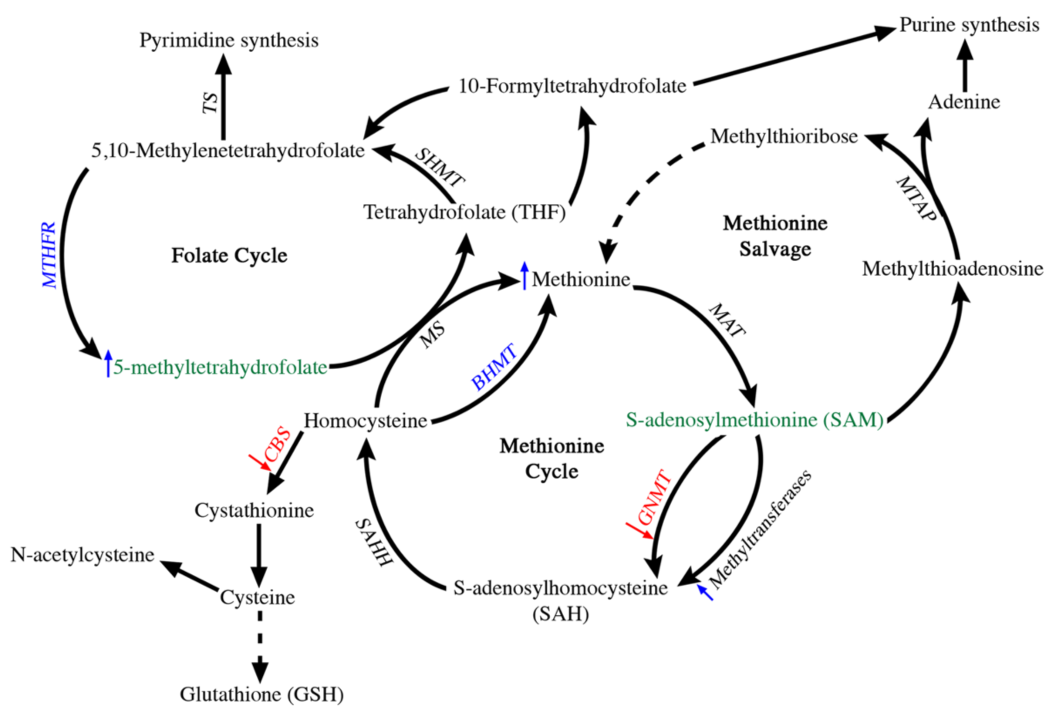


Figure 2. Methionine and Folate Cycles.

A. The methionine and folate cycles interact to generate intermediates and cofactors for various anabolic processes. Available methionine largely depends on dietary input. **B.** The methionine and folate cycles interact to divert intermediates based on methionine availability. SAM normally inhibits MTHFR and BHMT and promotes CBS to divert intermediates towards nucleotide and glutathione synthesis when methionine and SAM are plentiful. During times of low SAM, this inhibition in released, increasing the synthesis of methionine from homocysteine as well as allowing 5-methyltetrahydrofolate to begin to accumulate. 5-methyltetrahydrofolate blocks GNMT to allow methyltransferases to utilize available SAM to maintain RNA, DNA, and histone methylation. Together, this regulation maintains intermediates around the folate and methionine cycle in times of low methionine input.

Abbreviations: TS – tetrahydrofolate synthase; MTHFR – methylenetetrahydrofolate reductase; SHMT – serine hydroxymethyltransferase; MS – methionine synthase; MAT – methionine adenosyltransferase; GNMT – glycine N-methyltransferase; SAHH – adenosylhomocysteinase; BHMT – betaine-homocysteine S-methyltransferase; CBS – cystathionine-β-synthase; MTAP – S-methyl-5’-thioadenosine phosphorylase

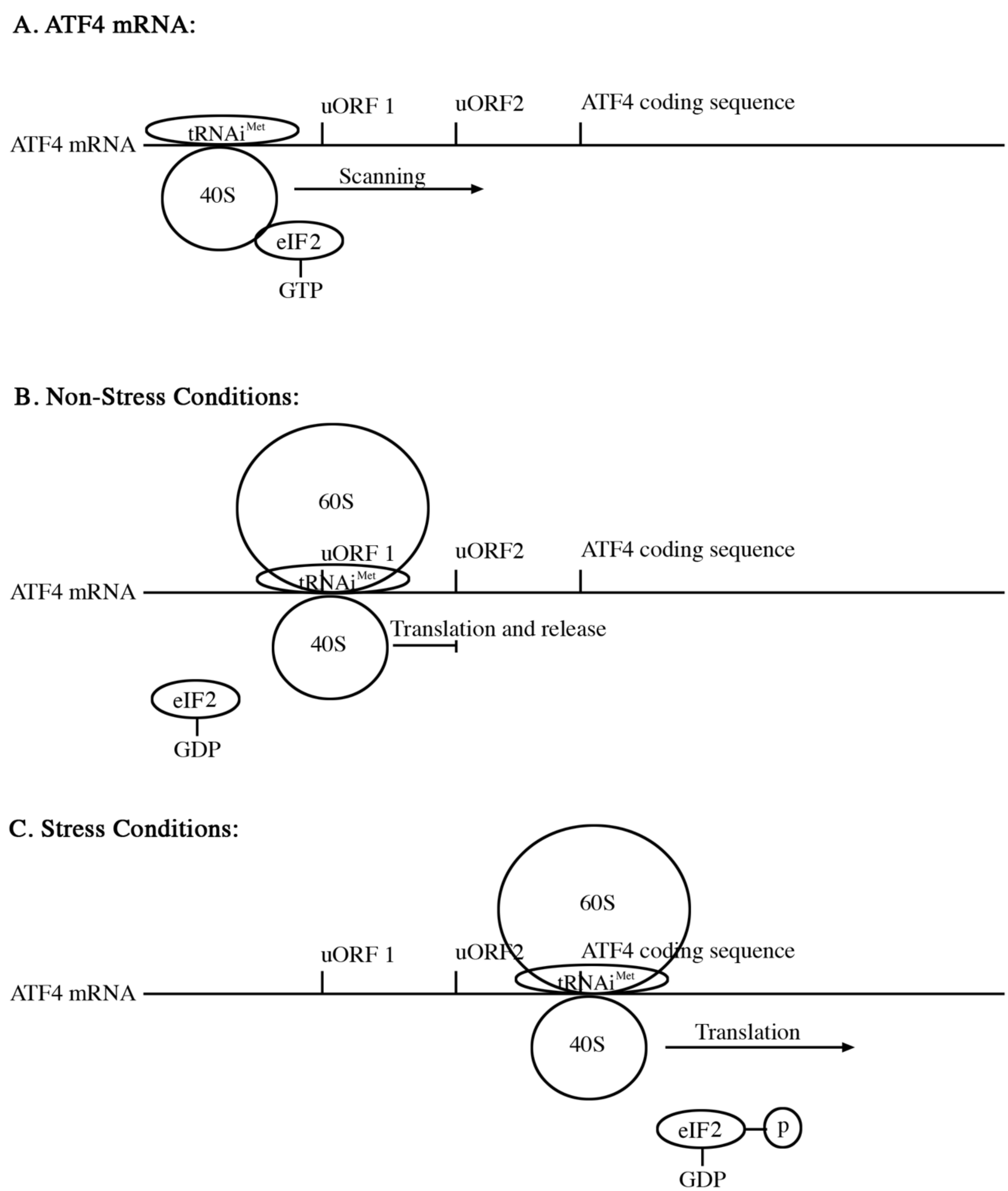


Figure 3 - ATF4 mRNA translation in stress and non-stress conditions.

A. ATF4 mRNA contains two upstream open reading frames (uORF1 and uORF2). The 40S ribosomal subunit, in complex with initiator tRNA-methionine, eukaryotic initiation factors (eIF1, eIF3, eIF4), and eIF2-GTP, binds the 5'-cap on ATF4 mRNA and begins scanning for a translation initiation sequence (AUG). When a complete 40S complex encounters an AUG sequence, the GTP on eIF2 is hydrolyzed and the phosphate is released. This allows the eIFs to dissociate for re-use and the 40S subunit to bind the 60S subunit to commit to translation. **B.** In non-stress conditions, GDP is readily exchanged for GTP on eIF2, allowing translation machinery to assemble quickly. This results in translational machinery assembling at and translating the uORFs on ATF4 mRNA. The machinery hits a stop codon and releases before the ATF4 protein coding sequence can be translated. **C.** In stress conditions, eIF2α phosphorylation blocks GDP exchange for GTP, decreasing the pool of free eIF2-GTP to initiate translation. This increases the likelihood the 40S ribosome-initiator tRNA complex will bypass the uORFs before binding eIF2-GTP to initiate translation, allowing the ATF4 protein coding sequence to be preferentially translated.

Table 1. Methionine restriction or deprivation in combination with various chemotherapies *In Vitro* and *In Vivo*.

Methionine restriction to metabolically prime cancer cells		
Chemotherapy	**Drug Target**	**Reference(s)**
Lexatumumab	Monoclonal agonist antibody for the death receptor TRAIL receptor 2	Strekalova et al 2015[130]
Cycloleucine	Inhibits methionine adenosyltransferase (MAT)	Strekalova et al 2019[49]
Methionine restriction and standard of care chemotherapy		
Chemotherapy	**Drug Target**	**Reference(s)**
5-Fluorouracil	Thymidylate synthase (TS) inhibitor	Gao at al 2019[149]
		Yoshioka et al 1998[41]
Cisplatin	Binds DNA and interferes with repair	Poirson-Bichat et al 2000[141]
		Tan et al 1999[158]
Doxorubicin	Binds and inhibits DNA Topoisomerase II	Poirson-Bichat et al 2000[141]
		Stern et al 1986[13]
		Nagahama et al 1998[147]
Vincristine	Interacts with tubulin to prevent mitotic spindle formation	Stern et al 1986[13]
		Nagahama et al 1998[147]

Table 2. Ongoing clinical trials involving methionine restriction.

Identifier	**Recruiting Location**	**Study Title**	**Condition(s)**	**Study Goals**
NCT03186937	University of Wisconsin Madison Carbone Cancer Center	A window of opportunity study of methionine deprivation in triple negative breast cancer	Triple negative breast cancer (TNBC)	• Effect of methionine restriction on TRAIL R2 cell surface expression • Effect on cancer stem cells and metabolic health
NCT03574194	West Virginia University Cancer Institute	Methionine- restricted diet to potentiate the effects of radiation therapy	Lung cancer Prostate cancer Breast cancer	• Safety and adherence of methionine-restricted diet to radio-sensitize tumors

NCT03733119	University of Wisconsin Madison Carbone Cancer Center	ONC201 with and without methionine-restricted diet in patients with metastatic triple negative breast cancer	Metastatic triple negative breast cancer (TNBC)	• Phase II trial combination methionine restriction and ONC201 (Akt/ERK inhibitor) for response rate
NCT00508456	U.T.M.D. Anderson Cancer Center Houston Texas	Dietary Methionine Restriction Plus Temozolomide for Recurrent GBM	Glioblastoma Multiforme	• Safety and tolerability of methionine restriction in combination with temozolomide • Correlate response and survival with serum methionine and peripheral blood lymphocyte methylation
NCT00640757	Pennington Biomedical Research Center	Methionine-Restriction Diet (MRD) in Obese Adults with Metabolic Syndrome	Metabolic syndrome	• Impact of methionine restricted diet on weight loss and glucose metabolism/tolerance
NCT02192437	Penn State University State College Milton S. Hershey Medical Center	Dietary Methionine and Cysteine Restriction in Healthy Adults	Healthy Individuals	• Evaluate short- and long-term metabolic changes associated with health-span in response to dietary methionine or methionine/cysteine restriction
NCT03629392	University of Oslo, Norway	Methionine and Cysteine Restriction in Humans	Overweight Individuals	• Effect of methionine and cysteine restriction on energy and macronutrient metabolism in overweight humans

Chapter 2: Evaluating the impact of dietary methionine consumption on breast cancer incidence and mortality in post-menopausal women from the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial

Joni C. Sedillo, Kristen M. Malecki, Vincent L. Cryns

Abstract

Background: Breast cancer is the leading cancer in women worldwide and the second most prevalent cancer overall; despite significant progress, much remains unknown about factors contributing to the development and progression of breast cancer. Diet has been identified as a key determinant of breast cancer risk. Methionine is an essential amino acid used for protein synthesis and is the precursor to S-adenosylmethionine (SAM). Intracellular SAM availability has been found to directly correlate with dietary methionine consumption, suggesting dietary methionine content may play a role in SAM availability. Epigenetic dysregulation and alterations have been implicated in both cancer progression and development, with global DNA hypomethylation commonly seen in many types of cancer, including hereditary cancers and pre-cancerous lesions. Previous work has found high methionine diets may actually promote DNA hypomethylation through accumulation of S-adenosylhomocysteine (SAH), which can interfere with DNA methyltransferase activity. Based on these findings, we hypothesize high methionine consumption will increase the incidence and mortality of breast cancer and all cancer types pooled in post-menopausal women in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial population. *Methods:* This prospective study included 52,243 post-menopausal women from the PLCO Cancer Screening Trial. Dietary data were obtained from a food frequency Dietary History Questionnaire (DHQ) to determine average daily intake of methionine. Cox proportional hazard modeling was conducted for breast cancer and all cancer incidence and survival with age as the underlying time variable to estimate hazard ratios (HR) and 95% confidence intervals (95% CI). Similar analyses of all cancer types were used to determine if dietary methionine more broadly impacts cancer

development and progression. *Results:* High dietary methionine was associated with increased risk of breast cancer incidence and mortality in post-menopausal women. High methionine also increased total cancer incidence, however showed little association with total cancer mortality. *Conclusion:* This study found higher levels of dietary methionine may increase the risk of breast cancer and general cancer development. There does not appear to be an association between dietary methionine and all cancer mortality, however this study only considered cancer cases diagnosed after DHQ completion, so dietary changes following cancer diagnosis were not accounted for.

Introduction

Breast cancer is the leading cancer in women worldwide and the second most prevalent cancer overall[1], however much is unknown about factors contributing to the development and progression of breast cancer. Methionine is an essential amino acids and is used for protein synthesis, is the precursor to S-adenosylmethionine (SAM), the universal methyl donor for nucleotide and protein methylation, and contributes to both the transsulfuration pathway that synthesizes glutathione (GSH) and the salvage pathway that synthesizes polyamines through methionine cycle intermediates[2]. Considering the number of pathways influenced by methionine metabolism that contribute to cell growth and division, along with the increased dependency of cancer cells on methionine[2-5], there may be therapeutic utility in determining the role methionine plays in cancer development.

Dietary methionine restriction is currently being used in the treatment of breast cancer[6]; however, less is known about methionine uptake and metabolism in the development of breast cancer. Previous work has found high methionine diets cause an accumulation of both homocysteine and S-adenosylhomocysteine (SAH), particularly in extrahepatic tissues[7-10]. SAH can inhibit both DNA[11,12] and histone[13] methyltransferases and has been found to correlate with global DNA hypomethylation in mice[14]. Global DNA hypomethylation is significantly associated with tumor progression and initiation of aneuploidy[15]. Methionine supplementation for 15 days in humans with schizophrenia doubled the concentration of SAH in the brain while SAM was unaffected, and this increase in SAH was predictive of DNA hypomethylation as well as site-specific promoter hypermethylation[9]. Interestingly, methionine restriction in aging mice was found to increase global DNA methylation and abolish the general loss of methylation seen with aging, and this effect was attributed to the 50% reduction in SAH observed in these animals relative to control[16]. Other study into the effects of feeding a high methionine diet, found an increase in peroxide levels and measures of oxidative stress in various animal models[17], suggesting there may be more than one mechanism by which methionine excess could impact cancer development.

Due to its high demand in protein synthesis, and the generation of nucleotide and reduced glutathione, methionine is considered an essential amino acid even though it can be synthesized from homocysteine[18]. The recommended daily intake of methionine in humans is 12.6 mg/kg of body weight[19]. The main sources of methionine in the diet are from meat, fish and dairy, with intermediate levels in nuts, grains and legumes, and low levels in fruits and vegetables; a more complete review of the methionine content of specific foods can be found elsewhere[2,20]. In general, both Mediterranean and vegan diets contain low levels of methionine[21,22], making them a convenient approach to reducing dietary methionine burden. In rodent models, lower dietary methionine has been found to extend longevity and improve age-associated loss of methylation marks[16] as well as improve parameters of age-related oxidative stress[23]. It's important to note that diets deplete of methionine are lethal if used for extended periods of time, making it necessary to consume adequate levels of methionine to maintain liver function and methylation reactions. High methionine diets contributed to the onset of fatty liver, hypoglycemia, and aminoacidemia[18], as well as increasing both oxidative stress and peroxide levels[17]. In humans, high methionine diets have also been found to exacerbate schizophrenia symptoms at least partially through epigenetic mechanisms[9] and impair multiple vascular functions, potentially contributing to cardiovascular disease[18].

While methionine restriction has been considered in many experimental models, data on human consumption of methionine and cancer risk are mixed. Many previous studies looking at methionine consumption and breast cancer incidence have found no association[24-35]. A meta-analysis of 13 papers found a significant association between methionine consumption and breast cancer risk in post-menopausal women, with high methionine having significantly lower risk than the lowest methionine group (0.89 95% CI:0.82-0.97)[36]. A prospective study also found high methionine consumption was significantly associated with a lower risk of breast cancer (0.88 95% CI:0.79-0.98)[37]. A case-control study looking at Methylenetetrahydrofolate reductase (MTHFR) polymorphisms found an increased risk of breast cancer with low methionine intake with the TT genotype, but not other variants of MTHFR (2.10 95% CI:1.07-4.14)[38]. And one study found breast cancer risk was significantly higher when dietary methionine intake was high (1.31 95% CI:1.02-1.68)[39]. The impact of methionine consumption on cancer development requires further research to better understand the roles of both low and high methionine diets on the development of breast cancer. The objective of this study was to evaluate the relationship between dietary methionine consumption and breast cancer risk and mortality using the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial prospective cohort.

Materials and Methods

Study Design

A detailed description of the PLCO study describing eligibility, consent, design, screening, and follow-up can be found elsewhere[40]. The trials primary objective was to determine the effects of screening for prostate, lung, colorectal, and ovarian cancer on cancer mortality. The trial was conducted at 10 different screening centers across the United States starting in 1993 with follow-up through 2009 and mortality follow-up through 2015. Recruited patients were 60-74 years old from the start of the trial until 1996 when they expanded the eligibility to include 55-59 years old. The study enrolled 154,897 total participants (76,682 men and 78,215 women). Informed consent was obtained at each study center at trial entry for every participant. This study evaluated post-menopausal women with a valid baseline questionnaire (BQ) and diet history questionnaire (DHQ), no history of cancer prior to administration of the BQ and DHQ, and excluded individuals with extreme caloric intakes, leaving 52,243 women eligible for analysis. Within this population the median follow-up time was 11.78 years. Information about trial follow-up can be found elsewhere[41].

Study Population and Dietary Assessment

The baseline questionnaire (BQ) was completed by 97% of participants in the PLCO screening trial, with 82% completed within one month of trial enrollment. A diet history questionnaire (DHQ) was given starting in 1998, 5 years into the start of the trial, to both the intervention and control arms of the trial. Participants enrolled in or after 1998 completed the DHQ at trial enrollment. Previously enrolled participants completing the DHQ at the anniversary of their trial enrollment. The average time from trial entry to competition of the DHQ was 2.95 years. The DHQ was completed by 77% of all trial participants. Participants were only included in our analysis if they completed a valid DHQ (113,446 valid of 154,897 total). A valid DHQ required a DHQ completion date available and prior to the death of the participant, less than 8 missing food frequency responses, and excluded those with extreme calorie consumption (top and bottom 1% for each gender). Participants diagnosed with any type of cancer prior to administration of the DHQ were also excluded as cancer diagnosis could influence dietary habits. Because breast cancer is far more prevalent in women, only female participants were used in this study, leaving 52,243 participants eligible for our analysis. Based on age and other questions asked, no woman in the study was considered pre-menopausal, with most verified as post-menopausal.

The DHQ was used to report the intake frequency of 124 foods[42]. Individual nutrient intake was calculated from DHQ responses using the DietCalc software, which used food frequency responses, portion size and gender in conjecture with nutrient amounts from the USDA Survey Nutrient Database and the Nutrition Data Systems for Research from the University of Minnesota to create a total daily value. Methionine intake was adjusted for total caloric consumption using the residuals method[43].

Cancer Ascertainment

Breast Cancer cases were determined from annual questionnaires for self-reporting cancer diagnosis, as well as reports from family and review of death certificates. Any available medical records were used to follow-up on cancer diagnosis. Death certificates were obtained by annual searched of the National Death Index and verified by a Death Review Committee.

All cancer incidence and mortality included data from all cancer types evaluated in the PLCO Cancer Screening Trial based on exit status at trial exit for first diagnosed cancer. Cancer types included in the PLCO Cancer Screening Trial were prostate, lung, colorectal, ovarian, head and neck, pancreatic, upper GI, liver, biliary, melanoma, breast, endometrial, bladder, renal, glioma, thyroid, and hematopoietic. The PLCO Cancer Screening Trial included in total 30,542 confirmed cases of cancer and 14,206 cancer-related deaths.

Statistical Analysis

Baseline characteristics are presented comparing the 2434 women who developed breast cancer with the remaining 49,809 women who did not develop breast cancer. Continuous variables are presented as mean and standard deviation if normally distributed and median and interquartile range if nonnormally distributed with p-values calculated by ANOVA and Kruskal-Wallis ANOVA respectively. Categorical variables are presented as percentages and p-values were calculated by chi square test.

Cox proportional hazard modeling was conducted for breast cancer and all cancer incidence and survival with age as the underlying time variable to estimate hazard ratios (HR) and 95% confidence intervals (95% CI). Covariates included in this analysis were based on significant associations with breast cancer in a univariate analysis and based on literature implicating various factors with breast cancer risk and methionine consumption. Covariates included were age at menopause, total years of cigarette smoking[44,45], race[46-48], body mass index (BMI)[49-51], whether or not they consume alcohol[52], regular consumption of aspirin and ibuprofen[53,54], family history of breast cancer, the number of pregnancies, daily caffeine consumption[55], and frequency of strenuous physical activity. Hazard modeling was also conducted stratified by quartile for energy adjusted methionine consumption with the first quartile as a reference.

Statistical analyses were conducted in R (version 3.6.2 GUI 1.70 El Capitan build)[56,57]. The baseline characteristic table was created using the R program tableone[58] other packages used within R were survival[59,60] and tidyverse[61].

Results

Baseline characteristics of the study population comparing breast cancer cases to no breast cancer in post-menopausal women are presented in **Table 1**. Participants with incident breast cancer over the 13 years of follow-up were younger at trial exit and had a higher mortality rate compared to those without breast cancer. Breast cancer patients also showed a greater prevalence of family history of breast cancer among female relatives, a higher age at menopause, a lower number of pregnancies, were more likely to drink alcohol and consume more alcohol per day and spent less time per week doing strenuous physical activity. All other factors did not differ substantially by breast cancer incidence.

Breast cancer incidence was significantly associated with energy-adjusted methionine consumption in both the unadjusted univariate model (1.65 95% CI:1.40-1.94) and multivariate model (1.53 95% CI:1.27-1.85) (**Table 2**). This heightened risk of breast cancer increased with methionine consumption as seen when methionine consumption was stratified by quartile (**Table 3**). High methionine consumption was significantly associated with higher breast cancer incidence compared to low methionine consumption in the unadjusted univariate model (1.39 95% CI:1.24-1.55) and in the multivariate model (1.34 95% CI:1.17-1.53). All quartiles showed significantly increased risk of breast cancer compared to the lowest methionine group in both the univariate and multivariate models, with breast cancer risk increasing by methionine consumption level.

Breast cancer mortality was significantly associated with methionine consumption in the univariate analysis (1.80 95% CI:1.06-3.06) and borderline significant in the multivariate analysis (1.97 95% CI:0.97-3.98) (**Table 2**). When stratified by quartile, both the 3rd and 4th quartile were borderline significantly associated with breast cancer mortality in both the univariate (3rd: 1.40 95% CI:0.97-2.02; 4th: 1.38 95% CI:0.94-2.01) and multivariate analysis (3rd: 1.61 95% CI:0.99-2.64; 4th: 1.61 95% CI:0.97-2.68) (**Table 3**).

The incidence of all types of cancer was also significantly associated with methionine consumption in both univariate (1.46 95% CI:1.31-1.64) and multivariate analysis (1.46 95% CI:1.27-1.68) (**Table 4**). This effect appeared to associate with higher methionine consumption, as the upper two quartiles were significantly associated with increased cancer incidence when compared to the lowest methionine consuming group, and this effect was seen in both univariate (3rd: 1.17 95% CI:1.09-1.27; 4th: 1.27 95% CI:1.18-1.37) and multivariate analysis (3rd: 1.24 95% CI:1.13-1.37; 4th: 1.30 95% CI:1.19-1.44) (**Table 5**).

Unlike breast cancer, all cancer mortality was not significantly associated with methionine consumption in pooled analysis (univariate: 1.12 95% CI:0.94-1.33; multivariate: 1.24 95% CI:0.89-1.73) (**Table 4**). When stratified by quartile, the 4th quartile was borderline significantly associated with an increased risk of cancer mortality in the univariate analysis (1.11 95% CI:0.99-1.25) (**Table 5**). In the multivariate analysis, the 2nd quartile had a significantly decreased risk of all cancer mortality (0.76 95%

CI:0.60-0.95). Quartiles 4 had an increased risk relative to the other quartiles, but this increase was not significant (1.09 95% CI:0.88-1.35).

Discussion

Based on our analysis, it appears diets high in methionine may increase the risk of developing both breast cancer and all cancer in a pooled analysis of cancer types evaluated in the PLCO Cancer Screening Trial among post-menopausal women. Breast cancer related mortality also appeared to increase with methionine consumption, however no significant effect was found on all cancer related mortality. These findings suggest methyl group availability may play a greater role in the development of cancer than the progression. This finding is not surprising consider methylation is often altered early in carcinogenesis. Furthermore, considering dietary information was taken before any cancer cases were documented, it is possible patient diet changed upon diagnosis, and methionine values used in this study may not adequately reflect methionine consumption after cancer diagnosis.

Previous work found 80% methionine restriction in healthy adult mice increased global DNA methylation, with older methionine restricted mice appearing more similar to young mice[16]. This effect was attributed to an increased removal of SAH, preventing accumulation and inhibition of methyltransferases[16]. While human consumption is higher overall than the 80% methionine restriction in animal studies, the lower risk of both breast cancer and all cancer development in the lower methionine consuming quartile may indicate that lower levels of methionine within the diet may have a similar impact even at less extreme levels of methionine restriction.

While these results cannot confirm a causal association with methionine restriction, they do suggest further research to examine the impact of methionine restriction on epigenetic changes in global DNA methylation is warranted. A common feature of many, if not all, types of cancer is general loss of epigenetic stability, commonly leading to changes in histone methylation, global DNA hypomethylation with hypermethylation of specific CpGs, and loss of methylation boundaries between largely methylated and unmethylated DNA segments, leading to alterations in gene accessibility and expression[62-64]. Alterations in the methylation of breast CpG islands and global DNA hypomethylation have been found to be similar between inherited and sporadic tumors[65]. Alterations in chromatin, leading to increased euchromatic regions and areas of active transcription have also been found to play a role in the epithelial-to-mesenchymal transition used by cancer cells in the process of metastasizing[66], suggesting alterations in epigenetic stability influence the invasive capacity of a tumor. Furthermore, stem cells require high levels of methionine to maintain self-renewal and pluripotency, and when methionine is restricted stem cells decrease histone and global DNA methylation and lose their self-renewal capacity[67]. Many cancers contain cancer stem cell or tumor-initiating cell populations and it is hypothesized that many tumors originate from accumulating damage to stem cells leading to deregulated self-renewal and transformation[68]. Considering epigenetic changes occur early in tumorigenesis and impact stem cell self-renewal, methyl availability may also play a role in the development of cancer stem cells.

Findings of this study are consistent with the hypothesis that diets high in methionine may contribute to altered DNA methylation in a way that increases risk of breast cancer incidence and mortality. Experimental evidence across multiple species has shown a link between methionine consumption and DNA methylation. In breast cancer patients, a study evaluated normal breast tissue CpG methylation and found a significant increase in age-related methylation at specific CpG sites, in particular at CpG islands, and these sites showed partial overlap with hypermethylated CpGs in breast tumors[69]. Findings were also consistent for incident cancer for all types, but not related to all cancer mortality suggesting a potential association between methionine consumption, altered DNA methylation and overall cancer risk but further research is needed. A previous case-control study looking at site-specific hypermethylation of tumor suppressor genes in individuals from age 20-93 found tumor suppressor gene methylation increased with age and was found to a greater extent in cancer patients compared to healthy controls[70] The findings of both these studies suggest deviation from normal replication of DNA methylation signatures or loss of specific methylation marks in the DNA occur commonly with aging and may be a contributing factor in the development of cancer. It has been hypothesized that DNA hypomethylation in premalignant tissue results from unbalanced global hypomethylation due to overall methyl shortage resulting from the increased demand of highly replicative tissue for methyl groups[5]. Another study found areas of DNA hypomethylation in cancer cells were mainly in areas where parental cell methylation is copied late in replication, further suggesting a gradual loss of DNA methylation contributing to cancer may occur through shortage of methyl groups[64].

Strengths of this study include the large sample size and prospective design to minimize recall bias. The PLCO Cancer Screening Trial included a long follow-up period, resulting in 4,561 confirmed cases of breast cancer and 610 confirmed deaths from breast cancer. The DHQ used in the PLCO Trial included a large survey of foods used to calculate individual nutrient values. A limitation of this study is the DHQ was only administered before cancer incidence and does not reflect dietary changes with time or following cancer diagnosis. Future studies that re-administer a diet questionnaire at cancer acquisition may better characterize the role methionine consumption plays in breast cancer progression and breast cancer related mortality. Further limitations are associated with potential information biased linked to misclassification, a common challenge in nutritional assessments in epidemiologic studies. This study also only included post-menopausal women, so the translatability of the findings to pre-menopausal breast cancer would require evaluation in other cohorts.

In conclusion, high methionine consumption appeared to increase the risk of post-menopausal breast cancer incidence and mortality in the PLCO Cancer Screening Trial cohort. High methionine consumption also increased the incidence of all cancer types. Future study evaluating the role dietary methionine plays in epigenetic changes and regulation in cancer would better elucidate the mechanisms by which high methionine consumption may facilitate cancer development.

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Table 1 – Baseline characteristics of eligible women from the PLCO Cancer Screening Trial by breast cancer incidence

	Level	Overall	No Breast Cancer	Breast Cancer	p-value
Number of Patients		52243	49809	2434	
Age (mean (SD))		62.34 (5.32)	62.33 (5.32)	62.45 (5.24)	0.307

Age Level (%)	≤59	18433 (35.3)	17612 (35.4)	821 (33.7)	0.342
	60 - 64	15990 (30.6)	15217 (30.6)	773 (31.8)	
	65 - 69	11302 (21.6)	10761 (21.6)	541 (22.2)	
	≥70	6518 (12.5)	6219 (12.5)	299 (12.3)	
Age at Trial Exit (mean (SD))		74.00 (6.01)	74.21 (5.93)	69.88 (6.26)	<0.001
Days from Entry to Trial Exit (mean (SD))		4263.09 (866.39)	4338.84 (774.83)	2712.94 (1142.67)	<0.001
Confirmed Dead (%)	No	42877 (82.1)	40982 (82.3)	1895 (77.9)	<0.001
	Yes	9366 (17.9)	8827 (17.7)	539 (22.1)	
Race (%)	White	47641 (91.2)	45390 (91.1)	2251 (92.5)	0.206
	Black	2004 (3.8)	1932 (3.9)	72 (3.0)	
	Hispanic	631 (1.2)	602 (1.2)	29 (1.2)	
	Asian	1646 (3.2)	1576 (3.2)	70 (2.9)	
	Pacific Islander	202 (0.4)	193 (0.4)	9 (0.4)	
	American Indian	107 (0.2)	105 (0.2)	2 (0.1)	
	Missing	12 (0.0)	11 (0.0)	1 (0.0)	
BMI at Baseline (ln lbs/in2) (%)	0 - 18.5	545 (1.1)	524 (1.1)	21 (0.9)	0.129
	18.5 - 25	21010 (40.7)	20079 (40.8)	931 (38.7)	
	25 - 30	17879 (34.6)	17028 (34.6)	851 (35.4)	
	30+	12191 (23.6)	11590 (23.5)	601 (25.0)	
Family History of Breast Cancer (%)	No	44040 (84.9)	42124 (85.1)	1916 (79.4)	<0.001
	Yes, Immediate Female Relative	7304 (14.1)	6827 (13.8)	477 (19.8)	
	Male Relative	78 (0.2)	77 (0.2)	1 (0.0)	
	Possibly (Unclear)	478 (0.9)	460 (0.9)	18 (0.7)	
Age at Menopause (%)	<40	7071 (13.6)	6797 (13.7)	274 (11.4)	<0.001
	40-44	7247 (14.0)	6934 (14.0)	313 (13.0)	
	45-49	12284 (23.7)	11746 (23.7)	538 (22.3)	
	50-54	19284 (37.2)	18336 (37.1)	948 (39.3)	
	55+	5982 (11.5)	5645 (11.4)	337 (14.0)	
Number of Pregnancies (mean (SD))		2.79 (1.16)	2.80 (1.16)	2.72 (1.20)	0.001
Cigarette Smoking Status (%)	Never Smoked	29939 (57.3)	28560 (57.3)	1379 (56.7)	0.194
	Current Smoker	4405 (8.4)	4218 (8.5)	187 (7.7)	

	Former Smoker	17896 (34.3)	17029 (34.2)	867 (35.6)	
Years Smoked (median [IQR])		0.00 [0.00, 23.00]	0.00 [0.00, 23.00]	0.00 [0.00, 23.00]	0.735
Drinks Alcohol (%)	No	16122 (30.9)	15438 (31.0)	684 (28.1)	0.003
	Yes	36121 (69.1)	34371 (69.0)	1750 (71.9)	
Alcohol from Diet (g/day) (median [IQR])		0.89 [0.03, 4.72]	0.87 [0.03, 4.72]	1.09 [0.04, 5.36]	<0.001
Uses Ibuprofen Regularly (%)	No	34968 (67.3)	33364 (67.3)	1604 (66.2)	0.276
	Yes	17021 (32.7)	16203 (32.7)	818 (33.8)	
Uses Aspirin Regularly (%)	No	29984 (57.5)	28565 (57.5)	1419 (58.4)	0.394
	Yes	22148 (42.5)	21136 (42.5)	1012 (41.6)	
Caffeine from Diet (mg/day) (median [IQR])		200.13 [22.05, 599.21]	200.33 [22.04, 599.29]	196.81 [22.57, 570.85]	0.475
Strenuous Activity over the Last 12 Months (%)	0-1 day/week	22831 (56.0)	21671 (55.8)	1160 (59.6)	0.012
	2-3 days/week	12759 (31.3)	12204 (31.4)	555 (28.5)	
	4-5 days/week	3900 (9.6)	3726 (9.6)	174 (8.9)	
	6-7 days/week	1300 (3.2)	1242 (3.2)	58 (3.0)	
Duration of Strenuous Activity over the Last 12 Months (%)	0-15 minutes	20408 (50.0)	19378 (49.8)	1030 (53.0)	0.032
	16-19 minutes	4505 (11.0)	4281 (11.0)	224 (11.5)	
	20-29 minutes	4955 (12.1)	4736 (12.2)	219 (11.3)	
	30-39 minutes	5080 (12.4)	4862 (12.5)	218 (11.2)	
	40+ minutes	5879 (14.4)	5626 (14.5)	253 (13.0)	

Table 2 – Association between energy-adjusted methionine consumption and breast cancer incidence and mortality

	Person at-risk	**Cases**	**HR (95% CI)**	***p* value**
Breast Cancer Incidence				
Simple Model	52243	2434	1.65 (1.40-1.94)	1.22e-9
Multivariate[A]	39229	1868	1.53 (1.27-1.85)	1.14e-5
Breast Cancer Mortality				
Simple Model	9358	219	1.80 (1.06-3.06)	0.03
Multivariate[A]	4963	137	1.97 (0.97-3.98)	0.06

[A] HR adjusted by age at menopause, years of cigarette smoking, race, BMI, alcohol consumption, regular use of aspirin and ibuprofen, family history of breast cancer, number of pregnancies, caffeine consumption and frequency of strenuous activity

Table 3 – Association between energy-adjusted methionine consumption stratified by quartile and breast cancer incidence and mortality

	Person at-risk	Cases	Q1	Q2	Q3	Q4
Breast Cancer Incidence						
Simple Model	52243	2434	1.00 (reference)	1.24 (1.11-1.39)	1.27 (1.13-1.43)	1.39 (1.24-1.55)
p value				2.01e-4	4.20e-5	1.89e-8
HR (95% CI) [A]	39229	1868	1.00 (reference)	1.26 (1.11-1.44)	1.29 (1.13-1.47)	1.34 (1.17-1.53)
p value				5.33e-4	1.81e-4	1.67e-5
Breast Cancer Mortality						
Simple Model	9358	219	1.00 (reference)	1.16 (0.79-1.69)	1.40 (0.97-2.02)	1.38 (0.94-2.01)
p value				0.45	0.08	0.10
HR (95% CI) [A]	4963	137	1.00 (reference)	1.46 (0.89-2.38)	1.61 (0.99-2.64)	1.61 (0.97-2.68)
p value				0.13	0.06	0.07

[A] HR adjusted by age at menopause, years of cigarette smoking, race, BMI, alcohol consumption, regular use of aspirin and ibuprofen, family history of breast cancer, number of pregnancies, caffeine consumption and frequency of strenuous activity

Table 4 – Association between energy-adjusted methionine consumption and all cancer incidence and mortality

	Person at-risk	Cases	HR (95% CI)	*p* value
All Cancer Incidence				
Simple Model	52243	5322	1.46 (1.31-1.64)	1.18e-11
Multivariate[A]	39229	3521	1.46 (1.27-1.68)	6.91e-8
All Cancer Mortality				
Simple Model	52243	2278	1.12 (0.94-1.33)	0.20
Multivariate[A]	39229	631	1.24 (0.89-1.73)	0.19

[A] HR adjusted by age at menopause, years of cigarette smoking, race, BMI, alcohol consumption, regular use of aspirin and ibuprofen, family history of breast cancer, number of pregnancies, caffeine consumption and frequency of strenuous activity

Table 5 – Association between energy-adjusted methionine consumption stratified by quartile and all cancer incidence and mortality

	Person at-risk	Cases	Q1	Q2	Q3	Q4
All Cancer Incidence						
Simple Model	52243	5322	1.00 (reference)	1.07 (1.00-1.16)	1.17 (1.09-1.27)	1.27 (1.18-1.37)
p value				0.07	3.32e-5	4.77e-10

HR (95% CI) [A]	39229	3521	1.00 (reference)	1.14 (1.04-1.26)	1.24 (1.13-1.37)	1.30 (1.19-1.44)
p value				6.66e-3	8.43e-6	5.12e-8
All Cancer Mortality						
Simple Model	52243	2278	1.00 (reference)	0.98 (0.88-1.10)	1.02 (0.91-1.14)	1.11 (0.99-1.25)
p value				0.75	0.74	0.07
HR (95% CI) [A]	39229	631	1.00 (reference)	0.76 (0.60-0.95)	0.92 (0.74-1.14)	1.09 (0.88-1.35)
p value				0.01	0.44	0.42

[A] HR adjusted by age at menopause, years of cigarette smoking, race, BMI, alcohol consumption, regular use of aspirin and ibuprofen, family history of breast cancer, number of pregnancies, caffeine consumption and frequency of strenuous activity

Chapter 3: Dietary methionine consumption, cancer prevalence and global DNA methylation in the Survey of the Health of Wisconsin (SHOW) study population

Joni C. Sedillo, Vincent L. Cryns, Kristen M. Malecki

Abstract

Background: The availability of methionine cycle intermediates has previously been found to influence cellular methylation capacity through the availability of S-adenosylmethionine (SAM), the methyl donor for DNA, RNA and chromatin methylation. Both circulating and intracellular methionine concentration can be influence by dietary methionine consumption, suggesting dietary methionine may be capable of impacting cellular methylation capacity. Moreover, high methionine can lead to the accumulation of S-adenosylhomocysteine (SAH), which blocks DNA methyltransferase activity, potentially contributing to DNA hypomethylation. A common hallmark across multiple cancer types is a decrease in global DNA methylation while increasing the methylation at tumor suppressor gene transcriptional start sites. Based on this information, we hypothesize high dietary methionine intake will decrease the global level of CpG methylation and be associated with increased cancer prevalence among 548 of the Survey of the Health of Wisconsin (SHOW) participants. *Methods:* Dietary methionine was determined by a self-reported diet history questionnaire. Multivariable linear regression was used to determine the association between methionine intake and global CpG methylation. Logistic regression modeling was conducted to determine the association between methionine consumption and self-reported diagnosis of cancer. *Results:* Methionine consumption showed an increased odds ratio for all cancer prevalence, but this effect was not significant. Methionine consumption showed an inverse association with global CpG methylation; however, this association was only significant in univariate analysis. *Conclusion:* Methionine consumption showed a non-significant trend between high methionine consumption level and cancer cases within SHOW participants. Methionine consumption also inversely associated with

global CpG methylation, potentially impacting cancer development via reduced CpG methylation; however, more work is necessary to determine the relationship between high dietary methionine intake, global CpG methylation, and cancer prevalence.

Introduction

Methionine is used for various aspects of cell maintenance and growth including protein synthesis, synthesis of S-adenosylmethionine (SAM) for nucleotide and histone methylation, production of homocysteine for the generation of reduced glutathione for buffering oxidative stress, and for the synthesis of intermediates used in nucleotide biosynthesis[1]. Methionine can be synthesized, however due to its use in glutathione, nucleotide and SAM synthesis, mammalian cells cannot synthesize adequate amounts of methionine making it an essential amino acid[2]. The primary exogenous source of methionine in humans is diet.

Previous studies have found no association between dietary methionine intake and prostate cancer incidence[3], breast cancer incidence[4], or esophageal cancer[5]. A study evaluating colorectal cancer incidence found that in conjecture with high folate intake, high methionine intake increased cancer incidence compared to low methionine intake[6]. One study found reduced incidence of colon and rectal cancers when methionine intake was high[7]. Another study found higher methionine intake associated with lower risk of lung cancer, however no dose-response relationship was observed[8]. These findings suggest cancers of different origins may be differently impacted by dietary methionine level, however the variability in findings may also reflect study design, number of subjects, and methionine intake levels within the specific study population, making it difficult to determine the general role of methionine intake on cancer development. To date, no such studies have examined the potential mediating role of methionine associated with epigenetic changes as potential mediators between diet and cancer.

Despite mixed results from epidemiological studies, methionine has recently received increased attention due to its potential as a target for cancer therapeutics. The uptake of methionine is frequently elevated in cancer cells due to the demands of rapid cell division and stress buffering along with the high rate of transmethylation reactions commonly seen[1,9-11]. This enhanced uptake has been found to correlate with the therapeutic response of tumors and overall patient survival[12]. L-Type Amino Acid Transporter 1 (LAT1), responsible for the uptake of essential amino acids including methionine, is overexpressed in various types of cancer[13,14]. Cells with high levels of LAT1 showed elevated SAM and the histone methylation mark H3K9me3[13], suggesting elevated uptake of methionine may play a role in a cells methylation potential.

There are around 28 million CpG residues in humans and roughly 60-80% are normally methylated, with most methylation marks relatively static throughout life[15-17]. Hypomethylated areas often stay hypomethylated through transcription factor binding and histone methylation marks that prevent DNA methylation; this is commonly observed at gene transcription start sites to maintain active gene expression[15]. These areas frequently become hypermethylated in cancer cells, possibly due to dysregulated histone methylation[15]. Epigenetic alterations have commonly been found in pre-cancerous cells and can precede or facilitate gene mutation[18]. Global hypomethylation is considered one measure of global instability associated with cancer.

Premalignant DNA hypomethylation is hypothesized to occur due to high levels of transmethylation reactions causing diversion of methyl groups to other areas for DNA methylation[19], however accumulation of S-adenosylhomocysteine (SAH) may also be partially responsible for this phenomenon as SAH can inhibit methyltransferase activity[20,21] leading to hypomethylation even in the presence of sufficient SAM[22]. Hepatic SAH has been found to increase with age and corresponds with DNA hypomethylation[23]. Chronic inflammation, through the production of reactive oxygen species (ROS), has been found to contribute to tumorigenesis[18] and, seeing as methionine cycle intermediates can be diverted to generate reduced glutathione, this increased oxidative stress may divert intermediates from the methionine cycle, possibly further contributing to epigenetic dysfunction due to reduced availability of SAM. Global DNA hypomethylation and promoter-specific hypermethylation are commonly seen in cancer cells[24,25]. One study comparing breast cancer cells to primary mammary epithelial cells found many of the domains hypo- or hypermethylated in cancer cells were partially methylated domains in primary epithelial cells[24]. Many of the regions of DNA hypomethylation were also enriched for repressive chromatin marks, such as H3K9me3 and H3K27me3[24].

Increased variability in methylated regions and methylation boundaries in phenotypically normal tissue samples correlates with the future development of cancer within that tissue[25]. This departure of a cell from its normal epigenetic signature can also help a cell acquire a hybrid stem-somatic state[25]. DNA methylation at Alu and LINE-1 repetitive DNA sequences in human blood samples is negatively associated with patient age and Alu methylation decreased when repeat samples were taken from the same patient 2-8 years later[26]. Lower LINE-1 methylation correlated with an increased risk in developing breast cancer in a prospective study of blood samples from women in the Sister Study[27]. LINE-1 hypomethylation was found in breast, colon, lung, head and neck, bladder, esophagus, liver, prostate, and stomach carcinoma samples compared to healthy tissue from the same location[17,28]. A case-control study of Greenlandic intuit women found significantly lower LINE-1 methylation in women who developed breast cancer compared to controls[29]. Another study found most of the hypomethylation associated with cancer was found in large hypomethylated blocks and not at repetitive elements[30], suggesting studies that only evaluate LINE-1 methylation may underestimate the impact of hypomethylation in cancer. A prospective study found genome-wide hypomethylation in white blood cells of patients that later developed breast cancer compared to those who did not[31]. All these findings suggest global DNA methylation and disturbances in epigenetic regulation appear to influence the development of cancer.

The methionine content of various types of food is reviewed elsewhere[1,12], however in general, meat, fish and dairy have the highest methionine content, legumes, nuts and grains have intermediate levels, and fruits and vegetables have the lowest amount. In one study human plasma methionine was found to correlate with fat intake while protein intake had no correlation[32], suggesting plasma methionine levels may be impacted by more than the raw amount of methionine in foods.

Given the variable findings in human studies evaluating methionine intake and cancer prevalence along with the evidence that global DNA methylation appears to play a role in cancer development, we hypothesize that dietary methionine consumption may influence cancer development in the Survey of the Health of Wisconsin (SHOW) cohort. Because high methionine diets have previously been implicated with elevated SAH, potentially contributing to global hypomethylation, we hypothesize high methionine content within the diet will lead to a decrease in global CpG methylation. We also hypothesize high methionine will increase the odds of having had cancer.

Materials and Methods

Subjects

The Survey of the Health of Wisconsin (SHOW) is a cross-sectional public health survey conducted in Wisconsin with the purpose of health monitoring, research and health-related education. The survey recruited over 6,500 people between 2008 and 2016, with follow-up beginning in 2017. The current study used a subset of the SHOW population that participated in DNA methylation analysis through the Center for Inherited Disease Research in 2016 and 2017. All participants within this subset were 18 years or older. All participants used in this study answered a health history questionnaire, a physical activity questionnaire, alcohol and smoking questionnaires, and a diet history questionnaire given to participants that consented to participate in both SHOW and the WARRIOR study. The final study population included 548 people who had complete values for both dietary methionine content and participated in DNA methylation analysis. A full description of study eligibility, design, consent, screening and follow-up can be found elsewhere[33].

Dietary Assessment

The diet questionnaire was administered by an interviewer using a computer-assisted personal interview at a SHOW study center. The diet history questionnaire (DHQ) version 2 from National Institute of Health was used[34] to collect dietary information on food consumption and frequency over the last 12 months. This information was used to determine individual nutrient compositions using the DHQ nutrient database[35] and Diet*Calc software program[36]. Methionine intake was adjusted to total caloric intake using the residuals method[37].

Cancer Ascertainment

Cancer prevalence was self-reported in a health history questionnaire as both ever having cancer and by specific cancer site. This analysis included cancer of the bladder, breast, cervix, colon, kidney, leukemia, lymphoma, melanoma, mouth, ovary, prostate, rectum, non-melanoma skin, testes, thyroid, and uterus. The most commonly observed cancer types were breast, prostate, melanoma and non-melanoma skin cancer.

Tissue Collection and DNA Methylation Analysis

Participants were scheduled for sample collection following initial completion of the health history and physical activity questionnaires. Participants fasted for at least 8 hours prior to blood draw. DNA methylation was measured using the Infinium MethylationEPIC Beadchip that covers over 800k CpG sites[38]. Illumina methylation microarray data was cleaned and beta values were determines using the R package minifi[39]. Intra-sample normalization was done using the R package RPMM[40]. From DNA methylation data a measure of global CpG methylation was determined as the percentage of methylated CpG sites with the cut point for methylated CpG as a normalized beta value greater than 0.3[41].

Statistical Analysis

Baseline characteristics were calculated comparing the 85 cancer cases to the remaining 463 study participants. Continuous variables were presented as mean and standard deviation if normally distributed and median and interquartile range if nonnormally distributed with p-values calculated by ANOVA or Kruskal-Wallis ANOVA respectively. Categorical variables were presented as percentages with p-values calculated by chi square test.

Logistic regression analysis was conducted to evaluate the association between dietary methionine intake and cancer cases seen within the SHOW dataset. Percent global CpG methylation was compared to dietary methionine intake using multivariable regression analysis. Covariates were included based on a significant association with cancer prevalence or literature suggesting a relationship between the given variable and cancer prevalence. Covariates included were race[42,43], gender[44], BMI[45,46], alcohol consumption[47], number of live birth pregnancies, diabetes status, physical activity, and poverty income ratio.

Statistical analyses were conducted in R (version 3.6.2 GUI 1.70 EL Capitan build)[48,49]. Packages used within R were tidyverse[50], tableone[51], modelr[52] and caret[53].

Results

Baseline characteristics of the participants evaluated from the SHOW study population comparing cancer cases to the remaining participants are presented in **Table 1**. People who reported having had cancer were older at study enrollment, had a lower percent of global CpG methylation and had a higher poverty to income ratio. No other variables were significantly different.

Methionine consumption did not significantly associate with cancer cases (**Table 2**). The odds ratios were elevated in both the simple and multivariate models, with the exception of the last model. Only age at trial consent was significantly associated with cancer cases in all models. The models predicted test data cancer cases with an accuracy of 86.2%, in general underestimating cancer cases.

Multivariable regression analysis was conducting to determine factors influencing global CpG methylation within this study (**Table 3**). When only methionine was considered there was a significant inverse relationship between methionine consumption and CpG methylation. When other factors were also considered, methionine consumption inversely associated with CpG methylation, but this trend became non-significant. In multivariable models age, gender and race were all significantly associated with CpG methylation, with age showing an inverse association, females showing higher methylation than males, and black people showing higher average methylation than white people. It is important to note that in all models evaluated the adjusted R-squared never went above 0.29, suggesting a lot of the variability in CpG methylation seen in unexplained by these models.

Discussion

Dietary methionine consumption within the SHOW study population did not significantly associate with cancer cases, however there was a trend towards more cancer cases with increasing methionine consumption. While susceptibility to epigenetic dysregulation may vary by type of cancer, there may be more general implications for methionine consumption in cancer development as many, if not all, types of cancer exhibit alterations in epigenetic signatures which can be greatly influenced by the availability of methionine cycle intermediates[18-21]. In line with this idea, global CpG methylation levels, measured from blood samples, showed an inverse association with dietary methionine consumption. This trend was only significant in univariate analysis. Evaluation of a larger sample may better determine the association between dietary methionine consumption and global CpG methylation as methylation can be influenced by a wide variety of factors and even in multivariable analysis the model only had limited utility in explaining the observed variation in global CpG methylation.

High methionine diets generally lead to an accumulation of homocysteine[12,54] and subsequently SAH through the reverse reaction of S-adenosylhomocysteine hydrolase (SAHH or AHCY)[55-57]. Interestingly, rats on a high methionine diet increase the concentration of SAM and SAH in the liver, but only SAH increases in the kidneys and skeletal muscle[56], suggesting extrahepatic tissues may show a greater deficit in methylation potential due to a lower SAM:SAH ratio[57]. SAH can also inhibit histone methyltransferases[58], however less work has been done evaluating changes in histone methylation in response to SAH accumulation.

High methionine diets in humans exacerbate the symptoms of schizophrenia due to increased brain SAH levels and site-specific CpG hypermethylation[56], suggestive of alterations in brain epigenetic stability. Elevated homocysteine levels, due to dietary methionine loading, can also impair multiple vascular functions, including decreasing dilation and increasing coagulation, contributing to aortic lesion development and cardiovascular disease risk[2]. Greatly increased methionine intake in guinea pigs led to death within as little as 60 hours with symptoms of fatty liver, hypoglycemia and aminoacidemia[2]. Interestingly, despite the high availability of methionine cycle intermediates, animals on high methionine diets showed increased measures of oxidative stress and peroxide levels[59], while animals on low methionine diets showed improved stress resistance[60]. Methionine restriction in aging mice has also been found to increase global methylation of healthy cells similar to young mice, an effect likely attributable to the observed decreased in SAH up to 50% of control-fed animals[23].

The methionine and folate cycles exert regulation over each other to maintain methylation capacity when methionine levels fluctuate[61], however, cancer cells appear to uncouple the methionine and folate cycle making methionine cycle metabolites more sensitive to exogenous methionine input. The methionine and folate cycle have also been found to be uncoupled in cells during methionine restriction, allowing them to increase flux through the transsulfuration pathway to buffer oxidative stress, potentially making cells more sensitive to exogenous methionine levels depending on the cells specific need for methionine[32]. This is especially apparent during methionine restriction when intracellular SAM greatly decreases in methionine-dependent cancer cells but is maintained in healthy cells[10,62].

In vitro methionine deprivation in stem cells decreased available SAM, global DNA methylation, and the histone methylation mark H3K4me3 within 5 hours and poised cells for differentiation[63]. This heightened dependency on methionine metabolism and exogenous methionine is also observed in cancer stem cells (also called tumor initiating cells) and SAM depletion, either pharmaceutical or via methionine deprivation, of cancer stem cells leads to loss of tumorigenicity, and this effect was attributed to loss of various histone methylation marks necessary for stem cell function[55,64]. Methionine deprivation has also been found to reduce the histone methylation marks H3K4me2, H3K9me2 and H3K27me3 in adherent cancer cell lines of various tissue origins, altering specific gene expression[32,65]. It was recently discovered that during SAM depletion when di- and tri-methylation marks are lost, H3K9me1 is preferentially maintained to allow re-establishment of the cells epigenetic signature upon return to sufficient SAM supply, allowing for epigenetic persistence not seen when H3K9me1 marks are removed[66]. In stem cells changes in DNA methylation are generally a response to changes in histone methylation[67], suggesting histone methylation changes may precede DNA methylation changes in cancer development. While much work has been done evaluating the effects of methionine restriction on cancer stem cells, more work is needed looking at high methionine diets and the development of cancer stem cells.

Previous work found normal breast tissue showed an age-related increase in methylation at 197 CpG sites, predominantly in CpG islands, and a subset of these CpG sites were hypermethylated in breast tumors compared to normal breast tissue[68]. Another case-control study evaluating tumor suppressor gene methylation found much high methylation in cancer tissue compared to control as well as increased methylation with increasing age[69]. Promoter CpG island methylation increased when comparing benign to localized to metastatic prostate cancer cells and this methylation associated with gene repression[70]. CpG promoter methylation has also been observed in hereditary tumors to inactivate the remaining gene copy[71]. Hereditary tumors also commonly exhibit global hypomethylation[71]. Interestingly, many of the same CpG sites show altered methylation when compared to matched normal tissue in cancer samples from a variety of different tissue origins[30], suggesting similar regions are susceptible to epigenetic dysregulation regardless of the site of cancer development. Considering excess methionine consumption has previously been associated with site-specific promoter hypermethylation[56], future study evaluating specific CpG marks may better elucidate the role high methionine consumption plays in cancer development.

Strengths of this study include the enrollment of people only in the state of Wisconsin to reduce demographic variability in dietary intake and the methionine content of specific food types, follow-up to account for cancer cases diagnosed after trial entry, and repeat administration of surveys to account for changes in other lifestyle factors. Shortcomings include a limited number of cancer diagnoses within the subpopulation evaluated, the use of only survey-based methods for evaluation of diet and lifestyle factors with the potential for recall bias, and the recording of cancer cases both before and after trial entry, making it more difficult to associate diet with cancer cases, particularly if diet changed over time or after cancer diagnosis. Considering the diet history questionnaire and blood samples for methylation analysis were taken at more similar time points, dietary recall may better reflect dietary methionine intake at the time of blood draw. Another shortcoming is that cancer cases largely occurred before trial entry potentially leading to the inclusion of less fatal cancers within this study. Considering the role methionine plays within stem cells and cancer stem cells, the analysis of mainly survivable cancers may underestimate the role methionine plays in cancer prevalence and cancer outcomes.

In conclusion, methionine consumption within the SHOW population showed no significant association with cancer cases, however there was a trend between increasing methionine consumption and cancer cases. Dietary methionine intake was inversely associated with global CpG methylation; however, this association was only significant in the univariate analysis and only explained a small amount of the variability in global CpG methylation, indicating many factors influence global CpG methylation levels. Future studies using a larger sample size and prospective design may better determine the role of dietary methionine intake on both cancer prevalence and global CpG methylation measures. Evaluation of blood CpG methylation before cancer diagnosis may better represent the influence of dietary methionine on global CpG methylation, as cancer is commonly found to influence global methylation levels of cancer and cancer-adjacent cells and the presence of circulating tumor cells within blood could influence apparent CpG methylation within these samples. The use of blood methionine level in conjecture with self-reported dietary methionine consumption would also aid to strengthen the association between dietary methionine and global CpG methylation due to the possible influence metabolism and lifestyle factors may play into the utilization and absorption of dietary methionine. Overall, it appears high dietary methionine consumption may increase cancer prevalence, possible by lowering global markers of CpG methylation, however further work into this association is necessary.

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Table 1 – Baseline characteristics from the Survey of the Health of Wisconsin (SHOW) dataset by cancer prevalence

	Level	Overall	No Cancer	Cancer	p-value
Number		548	463	85	
Age at Consent (mean (SD))		54.89 (16.45)	53.04 (16.44)	64.98 (12.45)	<0.001
5+ Alcohol Days Last Year (median [IQR])		11.64 (35.57)	11.71 (33.82)	11.29 (44.14)	0.923
BMI (median [IQR])		29.24 [24.98, 34.15]	29.17 [24.92, 34.39]	29.30 [25.52, 32.99]	0.759
Diabetes (%)	Yes	76 (14.2)	63 (13.9)	13 (15.5)	0.841
	No	460 (85.8)	389 (86.1)	71 (84.5)	
Gender (%)	Male	235 (42.9)	194 (41.9)	41 (48.2)	0.334
	Female	313 (57.1)	269 (58.1)	44 (51.8)	
Dietary Methionine (g) (median [IQR])		1.37 [0.98, 1.97]	1.35 [0.98, 1.96]	1.47 [0.97, 2.04]	0.728
Total Dietary Energy (kcal) (median [IQR])		1660.12 [1220.85, 2359.57]	1636.88 [1217.53, 2316.84]	1772.12 [1236.34, 2568.27]	0.491
Percent Methylated CpGs (mean (SD))		71.67 (0.79)	71.70 (0.77)	71.50 (0.91)	0.034
Physically Active (%)	Yes	383 (69.9)	325 (70.2)	58 (68.2)	0.816
	No	165 (30.1)	138 (29.8)	27 (31.8)	
Poverty Income Ratio (median [IQR])		3.35 [1.89, 5.46]	3.16 [1.72, 5.39]	3.58 [2.33, 5.52]	0.034

Race (%)	White alone	466 (85.0)	387 (83.6)	79 (92.9)	0.312
	Black or African American alone	46 (8.4)	43 (9.3)	3 (3.5)	
	Asian alone	3 (0.5)	3 (0.6)	0 (0.0)	
	American Indian or Alaska Native alone	3 (0.5)	3 (0.6)	0 (0.0)	
	More than one race	24 (4.4)	21 (4.5)	3 (3.5)	
	Unknown or not reported	6 (1.1)	6 (1.3)	0 (0.0)	
Age at First Menstrual Period (mean (SD))		12.63 (1.58)	12.61 (1.57)	12.73 (1.63)	0.653
Number of Pregnancies Resulting in Live Birth (median [IQR])		1.16 (1.57)	1.14 (1.54)	1.29 (1.75)	0.395
Age at Last Menstrual Period (median [IQR])		49.00 [43.00, 52.00]	49.00 [42.00, 52.00]	50.00 [45.00, 51.50]	0.72
Cigarettes Smoked Per Day (median [IQR])		10.00 [7.00, 18.00]	10.00 [7.00, 18.00]	10.00 [7.75, 10.00]	0.516
Years Smoked (mean (SD))		21.41 (16.77)	22.31 (16.85)	11.17 (12.86)	0.119

Table 2 – Association between energy-adjusted methionine consumption and all cancer prevalence

	Regression Coefficient	**Standard Error**	***p* value**	**Odds Ratio**	**AIC***
Simple Model	0.08	0.48	0.87	1.08	389.20
Multivariate[A]	0.41	0.54	0.44	1.51	359.31
Multivariate[B]	0.27	0.56	0.63	1.31	366.45
Multivariate[C]	-0.13	0.62	0.84	0.88	338.65

[A] Adjusted by age
[B] Adjusted by age, race, and gender
[C] Adjusted by age, race, gender, BMI, alcohol consumption, number of live birth pregnancies, diabetes status, physical activity, and poverty status
*Akaike Information Criterion – estimate of information loss in model

Table 3 – Regression results for global CpG methylation

		A	**B**	**C**	**D**
Constant		72.55*** (0.28)	71.76*** (0.28)	71.61*** (0.32)	71.63*** (0.37)
Energy-Adjusted Methionine		-0.39** (0.12)	-0.12 (0.11)	-0.04 (0.11)	-0.05 (0.12)
Age			-0.005** (0.002)	-0.004* (0.002)	-0.004* (0.002)
Gender	Female		0.80*** (0.06)	0.82*** (0.06)	0.84*** (0.08)

Race	Black		0.35*** (0.11)	0.37*** (0.11)	0.39*** (0.11)
	Asian		0.03 (0.39)	-0.008 (0.39)	-0.02 (0.39)
	American Indian or Alaska Native		-0.23 (0.39)	-0.19 (0.39)	-0.20 (0.39)
	More than one race		-0.03 (0.14)	-0.06 (0.14)	-0.04 (0.15)
	Unknown or unreported		-0.21 (0.28)	-0.18 (0.28)	0.05 (0.31)
BMI				-0.004 (0.004)	-0.005 (0.004)
Physically Active	No			-0.03 (0.07)	-0.04 (0.07)
Alcohol consumption				0.0006 (0.0008)	0.0006 (0.0008)
Diabetes status	No				-0.06 (0.09)
Cancer status	No				0.03 (0.09)
Poverty income ratio					0.008 (0.01)
Number of live births					0.0004 (0.03)
R-squared		0.018	0.29	0.30	0.30
Adjusted R-squared		0.016	0.28	0.29	0.28
No. observations		548	548	526	502

Standard Error in parenthesis
Significance codes: ≤0.001*** ; 0.01** ; 0.05*

Chapter 4: Methionine Restriction to prime triple negative breast cancer cells to the Phosphoglycerate Dehydrogenase inhibitor, NCT-503

Abstract

Background: Methionine dependence is commonly seen in many types of cancer including breast cancer. This metabolic vulnerability specific to transformed cells can be targeted to impair cancer growth as well as alter metabolic enzymes within cancer cells to make them viable drug targets, a concept we call metabolic priming. The phosphoglycerate dehydrogenase (PHGDH) inhibitor NCT-503 has recently gained attention as PHGDH plays numerous roles in cancer progression, however the utility of NCT-503 has been limited to cancer cells with high expression of PHGDH. We hypothesize methionine restriction could be used as a way to upregulate PHGDH to increase the response and utility of NCT-503. *Methods:* Breast cancer cell lines both with (MDA-MB-468 and BT-20) and without (MDA-MB-231 and MCF7) PHGDH copy number amplification were subjected to methionine restriction to determine if PHGDH is upregulated in response. These cell lines were also subjected to methionine restriction followed by NCT-503 to determine the impact of combine treatment on cell death. *Results:* Methionine restriction caused an increase in PHGDH expression at both the protein and RNA level in all cell lines evaluated. Combine methionine restriction and NCT-503 increased cell death in MDA-MB-231 cells at a dose of NCT-503 found to have no effect on cell death when used as a single agent. *Conclusion:* Metabolic priming using methionine restriction may be a useful way to increase PHGDH expression to make it a viable drug target in cell lines with low or no basal expression of PHGDH. Preliminary data showed combined methionine restriction and NCT-503 caused substantial cell death in a cell line with low basal PHGDH expression that is normally non-responsive to NCT-503, potentially broadening the utility of NCT-503 in cancer treatment.

Introduction

Methionine dependence *in vitro* is classified as the inability of cells to grow in media when methionine is replaced with homocysteine, which under normal metabolic conditions can be re-methylated to form methionine in the methionine cycle[1,2]. Methionine-dependent cancer cells exhibit late S/G2 phase cell cycle arrest when methionine is removed from media, which is reversible upon growth in full media[3,4]. The exact nature of how cancer cells become methionine dependent is still unclear and methods to determine the status of a tumor in terms of methionine dependence are still being investigated, however the frequency at which methionine dependent cancer cells have been found suggests methionine restriction could offer therapeutic value to a variety of tumor types. One study evaluated 23 tumor cell lines and of those 11 were fully methionine dependent, 3 were partially dependent, and 9 grew regardless of having homocysteine replace methionine in the media, with tumor cell lines spanning a variety of cancer types, including breast cancer[2]. Another study similarly found 13 of 20 different tumor cell lines to be methionine dependent[3]. A variety of untransformed cell lines have also been evaluated and all were found to be methionine independent, indicating at least partial selectivity for the use of methionine restriction as a cancer therapeutic[2]. This metabolic vulnerability specific to certain cancer cell types allows for a selective toxicity when exogenous methionine is restricted; however, the full utility of methionine restriction in breast cancer therapeutics is still under investigation due to the implications of long-term methionine restriction and the variability in methionine dependent status seen in different tumor cell lines.

Methionine-dependence has been associated with excess transmethylation and use of S-adenosylmethionine (SAM), the universal methyl donor[3,4]. SAM has been implicated as playing a key role in pluripotent stem cell maintenance, with reduction in SAM levels leading to differentiation and if long term, apoptosis[5]. Methionine-dependence has also been associated with an increased dependence on the transsulfuration pathway, possibly to combat the high levels of reactive oxygen species generated by rapid growth and proliferation seen in cancer cells. A recent study evaluating methionine-dependent status found that enhanced transsulfuration pathway utilization of homocysteine to generate cysteine correlates with methionine-dependent status in breast cancer cell lines by depleting homocysteine available to be used to generate methionine[6]. Considering transsulfuration pathway activity is only present in a few normal tissues: the liver, pancreas, and kidney, and is highly utilized in various cancers including breast cancer, off-target toxicity to methionine restriction may be limited to tissues with high transsulfuration pathway activity[6].

As part of the cellular response to methionine restriction, the integrated stress response is activated resulting in cellular up-regulation and translocation of Activating transcription factor 4 (ATF4)[7]. ATF4 is a transcription factor that leads to the upregulation of various genes involved in amino acid metabolism. AFT4 binding sites have been found in the promoters of Phosphoglycerate Dehydrogenase (PHGDH), Phosphoserine Aminotransferase 1 (PSAT1), and Serine Hydroxymethyltransferase 2 (SHMT2), directly linking amino acid limitation with expression of serine biosynthesis pathway and folate cycle genes[8]. Furthermore, silencing either *phgdh* or *aft4* has previously been found to decrease reduced glutathione (GSH), cystathionine, and homocysteine levels as well as the NADPH/NADP+ ration and glutamate-derived α-ketoglutarate, which are all thought to aid in the survival of cancer cells under stress conditions[8].These findings suggest that in cancer cells methionine restriction may upregulate *phgdh* expression via ATF4 as a response to the stress imposed on the cell by methionine restriction.

PHGDH is an enzyme that catalyzes the rate-limiting step in the serine biosynthesis pathway and it has been found to be overexpressed at the protein level in 70% of estrogen receptor negative breast cancer cases[9,10]. This upregulation of PHGDH and the entire serine synthesis pathway results from cancer cells preferentially shunting 3-phosphoglycerate away from the citric acid cycle and into serine biosynthesis[11] to promote protein and lipid synthesis, to generate one-carbon units for nucleotide synthesis, and to increase the production of NADPH to generate reduced glutathione for redox maintenance[12,13]. A copy number gain of chromosome 1p12, containing PHGDH, is found in 16% of all cancers[14], and PHGDH overexpression has been observed in breast cancer, colorectal cancer, gastric cancer, cervical cancer, pancreatic cancer, thyroid cancer, colon cancer, non-small-cell lung cancer, lung adenocarcinoma, melanoma, and Ewing's sarcoma[13]. PHGDH expression has also been implicated in luminal polarity and anchorage-independent survival, suggesting a possible role in metastasis[14].

NCT-503 decreases the stability of PHGDH and results in the wasting of serine-derived one-carbon units and a reduction of glucose-derived serine[10]. Since PHGDH is not highly expressed in untransformed cells, NCT-503 offers good selectivity and safety and is minimally toxic to PHGDH-independent cell lines[10]. Pharmacokinetic studies of NCT-503 *in vivo* found significant partitioning to the brain, suggesting the potential utility of NCT-503 in treating primary tumors as well as brain metastasis, a common metastatic site in breast cancer with few effective treatments due to the lack of therapeutics that cross the blood-brain barrier. Interestingly, PHGDH expression has also been found to be elevated in brain metastasis relative to primary tumors for both breast cancer and melanoma[13,15], and inhibition of PHGDH reduced the burden and incidence of brain metastases[15]. PHGDH induction has also been found to protect Glioma cells from hypoxic-induced cell death by increasing the NADPH:$NADP^+$ ratio[16]. PHGDH inhibitors, including NCT-503, have garnered success as a treatment option in cancer cell lines exhibiting high basal levels of PHGDH with little to no effect in cell lines exhibiting low basal PHGDH. While this gives them a relatively high safety profile, it also limits the utility of PHGDH inhibitors as a cancer therapeutic.

Metabolic priming involves altering a cells nutrient status leading to the upregulation of various metabolic and stress response proteins to make them viable drug targets[17,18]. Our lab has focused on limiting the essential amino acid methionine to increase expression of metabolic enzymes normally expressed at low levels[17,18]. Metabolic priming of cancer cells is gaining attention as a way to improve the efficacy of many currently used cancer therapeutics. Considering the dependencies of many cancer cell types to both exogenous methionine and PHGDH activity, potentially as stress adaptation pathways to combat oxidative stress during transformation, we hypothesize that priming cells using methionine deprivation will increase cellular PHGDH, making it a viable drug target in cancer cell lines with low basal expression of *phgdh* (**Figure 1**). Furthermore, we hypothesize that combined treatment with methionine deprivation and NCT-503 will lead to synergistic cell death in breast cancer cell lines both with and without copy number amplification of *phgdh*.

Materials and Methods

To investigate the use of NCT-503, methionine deprivation, or the combination as a possible breast cancer therapeutic, breast cancer cell lines with high basal PHGDH due to copy number amplification (MDA-MB-468 and BT20) and cells with low basal PHGDH (MDA-MB-231 and MCF7) were used. Cell lines with low basal PHGDH expression are normally insensitive to cell death induced by NCT-503 and were used to determine if methionine deprivation can upregulate PHGDH to induce sensitivity to NCT-503. Cell lines were subjected to methionine deprivation for the following times: 0, 4, 8, 16, 24, 48, and 72 hours and pellets were collected. Immunoblotting of whole-cell lysates was conducted as described[19] for PHGDH, ATF4 and β-actin (Sigma-Aldrich). RNA samples were subjected to the same treatment regimen as described above. Total RNA was isolated using the SpinSmart RNA complete kit (Denville Scientific) and cDNA synthesized using iScript cDNA Synthesis Kit (Bio-Rad). qRT-PCR was conducted with primers for PHGDH and GAPDH. qRT-PCR was conducted using iQ SYBR Green supermix (Bio-Rad) and the CFX96 Real Time PCR sequence detection system (Bio-rad). Relative changes in gene expression were determined using the $2^{-\Delta\Delta Ct}$ method[20] normalized to GAPDH and experimental controls.

To determine possible synergistic effects of NCT-503 and methionine deprivation in combination, cells were grown in complete media or methionine deprivation media for 24 hours then treated with 2.5 μM NCT-503 in combination with methionine deprivation for another 24 hours. Cell death was assessed using the Caspase Glo assay according to manufacturer's instructions (Promega).

Results

We found that PHGDH expression is upregulated starting around 24 hours of incubation in methionine-free media in cell lines both with (MDA-MB-468 and BT-20) and without (MDA-MB-231 and MCF7) the focal amplification of the *phgdh* gene (**Figure 2**). This upregulation was seen at both the protein and mRNA level.

Preliminary investigation into the role methionine restriction plays in metabolically priming breast cancer cell lines to respond to NCT-503 found a potential synergy between methionine deprivation and NCT-503 in a cell line (MDA-MB-231) that normally has low basal expression of PHGDH. Using crystal violet staining assays[21], MDA-MB-231 cells have shown the greatest combined effect when subjected to 24 hours of methionine deprivation followed by 24 hours with both methionine restriction and NCT-503 at 2.5 μM; a level well below that previously found to have any effect in MDA-MB-231 cells[10]. This dosing regimen showed potential synergy in apoptosis induction in MDA-MB-231 cells, as measured by Caspase Glo (**Figure 3**).

Discussion

Based off preliminary data there appears to be enhanced efficacy of NCT-503 in cells with low basal expression of PHGDH when used in combination with methionine restriction. Given these findings, we predict that the degree of induction of *phgdh* by methionine restriction may predict the response of a cell line to PHGDH inhibitors, including NCT-503. In all cell lines tested there is also preliminary evidence indicating a synergistic relationship between methionine restriction and NCT-503 in inducing cell death in breast cancer cell lines.

The concept of metabolic priming developed by our lab[17,18] has gained a lot of attention recently as a therapeutic strategy for cancer treatment. Previous work from our lab found that methionine deprivation in triple negative breast cancer cell lines leads to the up-regulation of TNF-related apoptosis-inducing ligand receptor-2 (TRAIL-R2), sensitizing cells to caspase activation in response to lexatumumab, an agonistic TRAIL-R2 monoclonal antibody[17]. An orthotopic xenograft model found that methionine deprivation enhances the effects of lexatumumab in vivo as well[17]. Breast cancer stem cells (BCSCs) were found to upregulate Methionine Adenosyltransferase 2A (MAT2A) in response to methionine depletion and combine methionine deprivation with MAT2A inhibition impaired mammosphere formation[18]. This combination in a rodent model suppressed both primary and lung metastatic tumor burden[18]. Furthermore, untransformed cells showed little response to methionine deprivation when compared to transformed cells, allowing methionine deprivation to be used to selectively prime and kill tumor cells. Due to the increased incidence of chemotherapy and radiation resistance, alternative treatment approaches are greatly needed. Combining methionine restriction with current therapeutics to enhance drug efficacy and widen utility is a relatively new concept and has never been tried with PHGDH inhibitors, including NCT-503. Using methionine restriction to metabolically prime breast cancer cells to respond to PHGDH inhibitors allows NCT-503 to be used at doses lower than normally used when NCT-503 is administered as a single-agent and could potentially allow for NCT-503 to be used in a wider panel of tumors and tumor types.

NCT-503 is an enticing therapeutic option due to its relatively high safety profile and ability to partition to the brain[10]. It has previously been found that loss of PHGDH through gene mutation or pharmacologic inhibition is toxic to breast cancer cells over-expressing PHGDH, regardless of exogenous serine status[10]. The EC_{50} for PHGDH dependent cell lines has been found to be as low as 8-16 μM in vitro, however that value is up to 10 fold higher for lowly-expressing cells and no drug-induced toxicity was observed in PHGDH-independent cell lines[10]. While this offers a nice selectivity profile for PHGDH dependent tumor cell lines, its utility in tumor cells with low or no expression of PHGDH has been minimal. PHGDH and the serine biosynthesis pathway play a major role in glutathione synthesis, through the production of NADPH[22], and nucleotide production[8]. Serine synthesis pathway activity has been found to be upregulated by the activity of both ATF4 and NRF2 in response to various stressors, including hypoxia, metabolic and oxidative stress. NRF2 is a regulator of various genes involved in anabolic processes through its interaction as a heterodimer with AFT4, and this heterodimer is thought to play a role in serine biosynthesis through an AFT4 binding site that has been identified at the promoter of *phgdh*[8]. It has also previously been found that ATF4 transcriptionally activates various serine biosynthesis genes as a result of nutrient deprivation[8].

Certain stressors, including hypoxia and cytotoxic chemotherapies, have been found to contribute to enrichment for BCSCs, mediated by HIF-1 and downstream targets[23]. During chronic hypoxia HIF-1 induces a switch from oxidative to glycolytic metabolism as a way to combat reactive oxygen species[22,23]. Hypoxia induces PHGDH expression in a HIF-1 dependent manner to aid in this glycolytic transition by shunting 3-phospoglycerate away from the TCA cycle and by increasing NADPH and GSH production through one-carbon metabolism[23]. Furthermore, PHGDH overexpression appears to play a key role in enrichment of BCSCs, as PHGDH knockdown impaired both hypoxic and chemotherapy-induced BCSC enrichment[12,14]. Previous work looking at mouse embryonic stem cells has found that under methionine deprivation conditions cells become poised for differentiation and eventually undergo apoptosis, at least in part due to a reduction in enzyme activity in the methionine cycle and salvage pathway[5]. Similar work in our lab using BCSCs indicate that methionine restriction can target BCSCs and lead to apoptosis or differentiation, a finding with large clinical implications since many BCSCs are radiation and chemotherapy resistant and help drive metastasis and relapse. PHGDH has also been implicated in the survival of BCSCs, as PHGDH silencing alters NADPH production, mitochondrial redox homeostasis, and can lead to apoptosis of BCSCs[23]. Since both treatments individually contribute to reducing the prevalence of BCSCs, future work targeting both PHGDH and cellular SAM availability, using methionine restriction, may be a useful way to reduce BCSC enrichment and promote differentiation or apoptosis of existing BCSCs.

In conclusion, preliminary investigation into the use of methionine deprivation to enhance the utility and efficacy of PHGDH inhibitors has found methionine deprivation increased PHGDH expression and showed anti-cancer activity in combination with doses of NCT-503 lower than what is normally effective in breast cancer cell lines. These effects were apparent in a cell line with low basal expression of PHGDH, indicating methionine deprivation broadens the utility of NCT-503 to cell lines normally unaffected by treatment.

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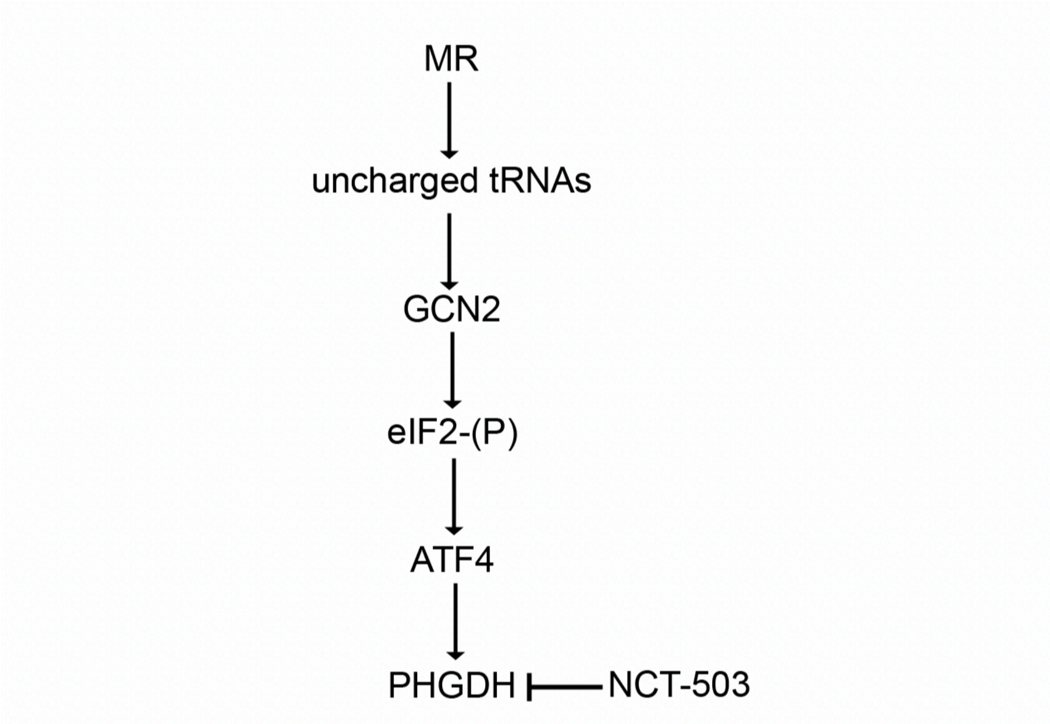

Figure 1. Proposed mechanism for PHGDH up-regulation in response to methionine restriction (MR). MR senses uncharged transfer RNAs (tRNAs), activating General Control Nonderepressible 2 (GCN2), which phosphorylated eukaryotic initiation factor 2 (eIF2), leading to Activating transcription factor 4 (ATF4) activation and preferential translation of genes involved in amino acid metabolism, including Phosphoglycerate Dehydrogenase (PHGDH), the enzyme catalyzing the rate-limiting step in the serine synthesis pathway. NCT-503 is a PHGDH inhibitor found to be effective in cells with high basal expression of PHGDH. The efficacy of NCT-503 could potentially be enhanced by MR priming cancer cells both with and without elevated basal PHGDH to increase expression of PHGDH.

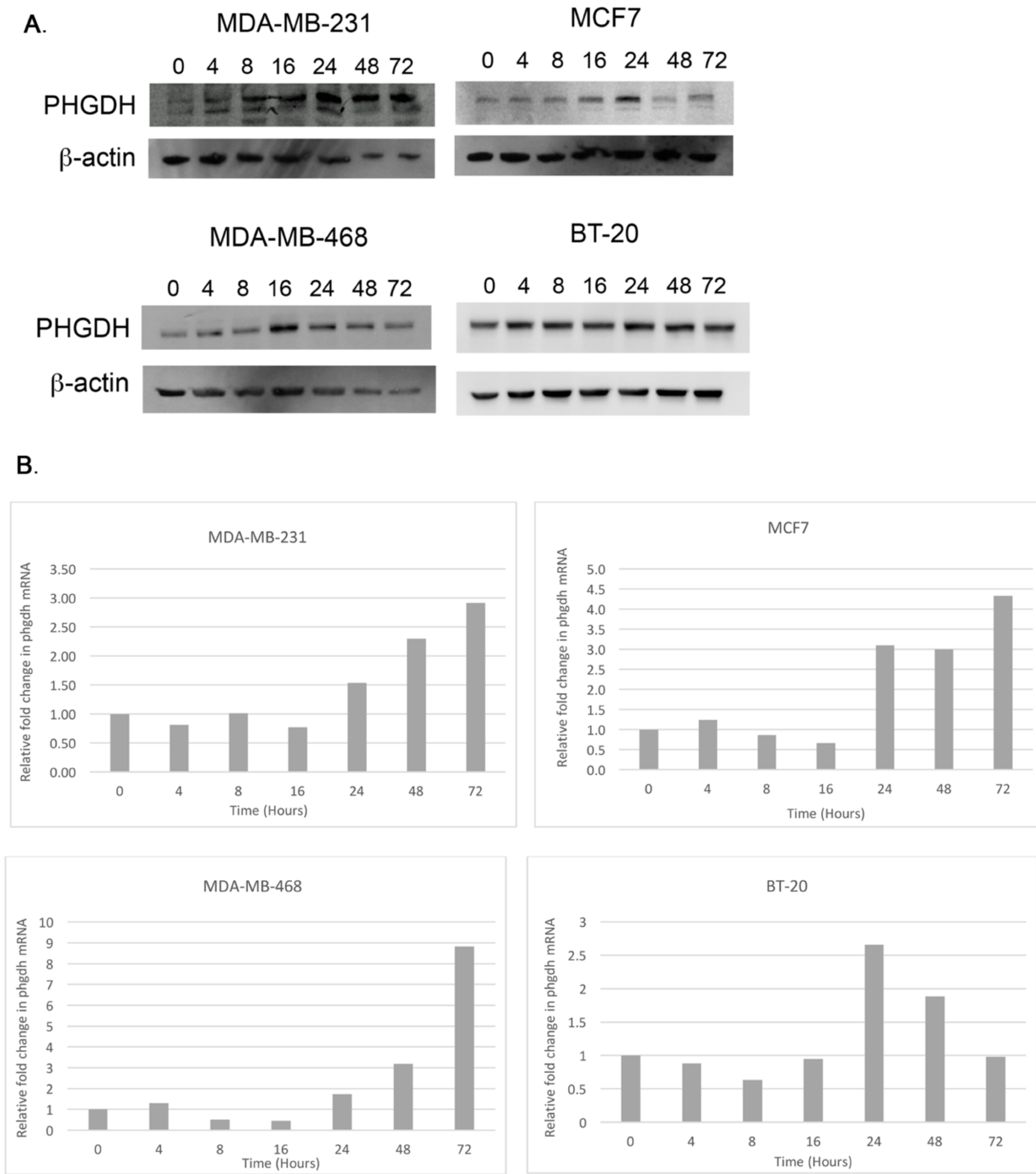

Figure 2. MR induced expression of PHGDH in cell lines with both low and high basal expression of PHGDH.

A) Immunoblots for PHGDH expression following methionine deprivation for 0, 4, 8, 16, 24, 48, and 72 hours in cell lines with low (MDA-MB-231 and MCF7) and high (MDA-MB-468 and BT-20) basal PHGDH expression.

B) mRNA levels of PHGDH normalized to GAPDH measured by qRT-PCR in cell lines with low (MDA-MB-231 and MCF7) and high (MDA-MB-468 and BT-20) basal PHGDH expression.

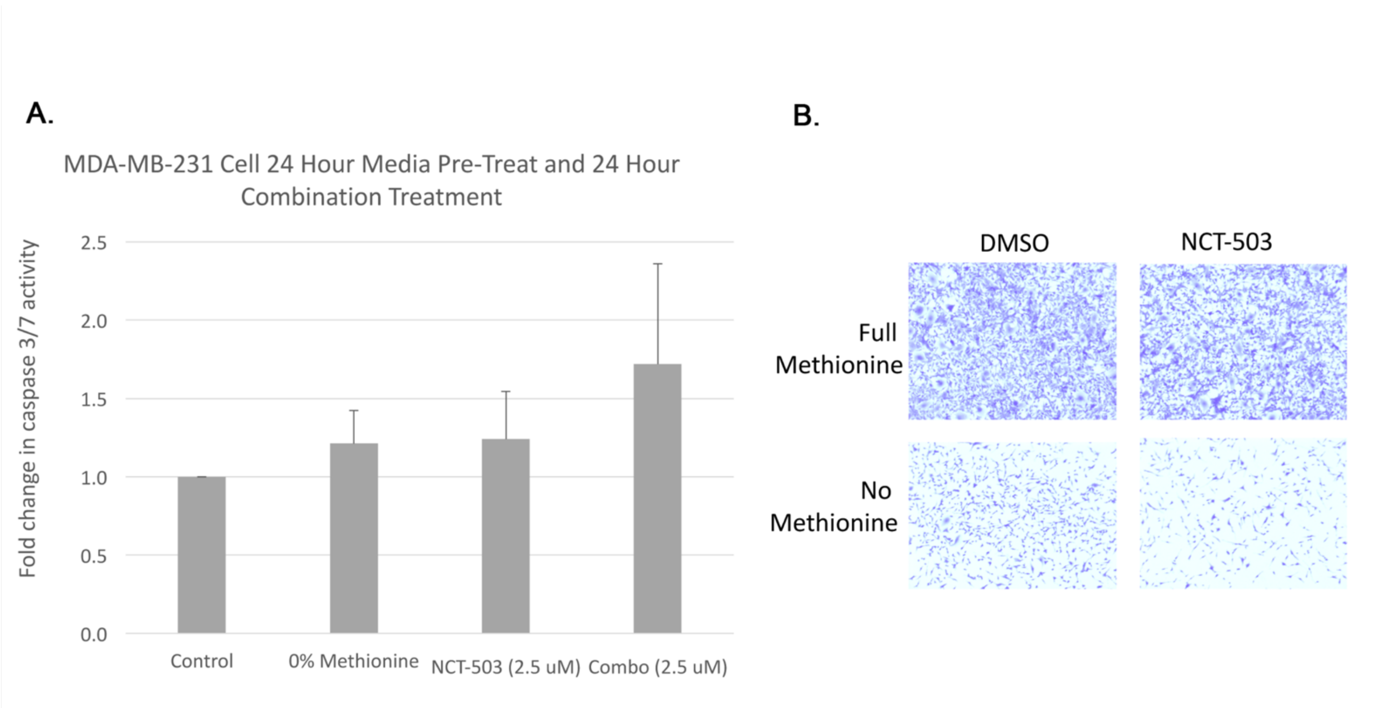

Figure 3. MR primes MDA-MB-231 cells to respond to the PHGDH inhibitor, NCT-503.

A) Relative change in caspase 3/7 activation measured by Caspase Glo (Promega) in MDA-MB-231 cells following 24-hour pre-treatment with methionine-free or control media followed by 24-hour treatment with the PHGDH inhibitor, NCT-503 in methionine-free or control media.

B) MDA-MB-231 cells stained by crystal violet following the same dosing scheme as in A.

Chapter 5: Implications of altering dietary methionine for breast cancer prevention and treatment

Abstract

Methionine is used as an intermediate to support many aspects of cell maintenance, growth and proliferation. Due to its high uptake into cancer cells and the prevalence of cancer cell methionine dependence, much work has been done to investigate the role methionine plays in cancer development and progression. Here we review conclusions from our work contributing to this field of study as well as future directions that warrant investigation. We also briefly review sources of dietary methionine and methods for implementing both low methionine diets for cancer prevention and methionine restricted diets for use in cancer treatment.

Methionine Intake and Cancer Incidence

Previous studies into the relationship of dietary methionine and breast cancer risk have been inconsistent in their findings. Many studies have found no association between methionine consumption and breast cancer incidence[1-12]. A few studies have found high methionine led to a lower risk of breast cancer development[13,14], and one study found a heightened risk of breast cancer with high methionine consumption[15]. Considering these studies are a mixture of prospective, case-control and meta-analysis with variable sample sizes and differences in dietary methionine evaluation, it is hard to compare findings or draw general conclusions about the role high methionine consumption plays in breast cancer development. In an attempt to better understand this relationship, we evaluated 52,243 post-menopausal women within the Prostate, Lung, Colorectal, Ovarian (PLCO) screening trial population, looking at dietary methionine consumption level and the future development of breast cancer.

Our analysis found high methionine consumption showed a significantly increased risk for the development of breast cancer as well as cancer development in general. When evaluating quartiles of methionine consumption, there was a dose response relationship, with quartile four having the greatest increase in risk for cancer development. Breast cancer related mortality showed a trend towards increased risk of death with high dietary methionine; however, this effect was not significant. All cancer related deaths showed a slight, albeit not significant, increase in risk in quartile four. Interestingly quartile two showed a significantly decreased risk compared to quartile one, suggesting the dose response relationship was not present when evaluating mortality. The differing effects of dietary methionine level on cancer incidence versus mortality may suggest methionine plays a greater role in cancer development than it does in progression; however, dietary data was taken before cancer incidence for all participants examined in this study, meaning changes in diet after cancer diagnosis are not accounted for in this study and may impact dietary methionine level.

Future work to better elucidate the role high methionine consumption plays in the development of cancer should evaluate calculated dietary methionine and blood methionine levels, as previous work has found plasma methionine may correlate more strongly with dietary fat consumption than protein[16]. Re-administration of the diet history questionnaire following cancer diagnosis would allow for a more accurate evaluation of methionine consumption on cancer development and mortality, as cancer diagnosis may greatly impact dietary habits. Intracellular tumor methionine level may also strengthen the evaluation of methionine consumption and cancer mortality, however caution should be taken when evaluating tumor-specific methionine uptake, as different cell types within a tumor have been found to uptake methionine at different rates[17].

Methionine Consumption and Epigenetic Alterations

Alterations in DNA methylation have been considered for over 50 years as a possible contributing factor to cancer development[18], with a recent resurgence of interest in investigating this association. Because methionine cycle intermediates contribute the methyl group for all nucleotide and histone methylation and dietary methionine can influence a cells methylation capacity[16,17,19], interest in dietary methionine consumption on epigenetic regulation and cancer development has been increasing.

Evaluation of the Survey of the Health of Wisconsin (SHOW) study group showed a trend towards lower global CpG methylation with increasing methionine consumption. High methionine consumption has previously been associated with increased concentrations of S-adenosylhomocysteine (SAH) and SAH can inhibit both DNA and histone methyltransferases, contributing to global DNA hypomethylation[20-22].

Future study evaluating SAH, DNA hypomethylation and dietary methionine consumption may better elucidate the mechanism by which high methionine diets may promote DNA hypomethylation. A larger prospective study would help determine if the impact of high methionine on DNA methylation impacts the development of cancer.

Methionine Restriction and Cancer Treatment

Methionine restriction, while not a new concept, has gained attention lately as a way to treat cancer in combination with many commonly used chemotherapies and small molecule inhibitors[23]. Work from our lab has been used to develop the concept of metabolic priming, in this case using dietary methionine restriction as a way to expose metabolic vulnerabilities specific to cancer cells to make them more targetable for drug treatment[24,25].

Preliminary work in our lab indicates methionine restriction increases both the mRNA and protein expression of phosphoglycerate dehydrogenase (PHGDH), the first and rate-limiting step in the serine biosynthesis pathway, and this effect was seen in multiple different breast cancer cell lines. PHGDH is commonly overexpressed in cancers of various origins[26,27], which lead to the creation of small molecule inhibitors of PHGDH, including NCT-503; however, utility of these drugs has remained limited to cancers exhibiting this overexpression[28]. In this study we showed PHGDH can be up-regulated in cancer cells both with and without elevated basal PHGDH, potentially widening the utility of drugs such as NCT-503. Preliminary work showed enhanced cell death in a cell line normally expressing low basal *phgdh* when cells were pre-treated in methionine-free media followed by NCT-503 compared to cells treated with either agent alone.

Future work evaluating methionine restriction in combination with PHGDH inhibitors in *in vivo* systems would better determine the biological relevance of the ability of methionine restriction to enhance the targetability of PHGDH. Testing at various levels of methionine restriction and for different lengths of time would clarify potential treatment regiments for future clinical translation. The evaluation of methionine restriction and PHGDH inhibition in tumor-initiating cells (cancer stem cells) would help to determine the utility of combination treatment to prevent recurrence and on cancer metastatic potential and treatment of metastases.

Implications of Methionine Consumption on Cancer Development and Progression

The recommended daily intake for methionine is 12.6 mg/kg body weight[29]. Interestingly, in both the PLCO and SHOW datasets, even the average for the lowest quartile of methionine consumption was slightly above the recommended daily intake, suggesting most participants within both studies were consuming well above their recommended daily intake for methionine. Taken with the findings of both studies, consumption of the recommended daily intake may lower the risk of developing cancer compared to consumption above recommendation. This level of methionine intake is sufficient for cellular processes and well above the level of clinical methionine restriction, making it potentially more feasible for long-term use for cancer prevention. Certain diets also exhibit generally low levels of methionine, including Mediterranean and vegan diets[30,31], making them potential alternative approached to lowering dietary methionine content for long-term use; however, further investigation into use of Mediterranean and vegan diets to obtain a methionine deprivation-specific response is warranted, as previous study has found the all amino acid deprivation response seen in caloric or amino acid restriction lacks the methionine deprivation-specific response[32]. Meat, fish and dairy products in general contain the highest levels of methionine, grain, nuts and legumes have intermediate levels, and fruits and vegetables contain the lowest levels; a more complete list of specific foods and methionine content are reviewed elsewhere[33,34].

Clinical methionine restriction is generally considered to be reducing dietary methionine to 2 mg/kg body weight a day[35]. Clinical methionine restriction in combination with common chemotherapies and small molecule inhibitors is become increasingly utilized to improve the utility of drugs and target both tumor-initiating cells and metastases[17,24,25,36,37]. This level of methionine restriction is feasible for short-term use through the adaptation of a diet where 75% of total protein is provided via a methionine-free medicinal beverage (Homiex-2, Abbott Nutrition) and the remainder from low-methionine fruits, vegetables and grains[30,38]. Other mechanisms that have found success at depleting serum methionine include use of a methionine-free total parenteral nutrition (TPN) solution[39], where most nutrients are given directly via an intravenous solution lacking methionine for short-term methionine depletion, or use of the bacterially-derived enzyme L-methionine-γ-lyase (methioninase)[40,41], which degrades sulfur containing amino acids.

Conclusion

Current work suggests a role for methionine in both cancer development and progression, suggesting possible utility in altering dietary methionine for cancer prevention and treatment. Dietary methionine restriction has already shown much success in pre-clinical and clinical study in combination with many standard-of-care chemotherapies as well as newly investigated small molecule inhibitors. Considering methionine restriction is capable of targeting cancer stem cells (tumor-initiating cells), its use in preventing recurrence and metastases is being investigated. Here we also show that lowering dietary methionine at or below the recommended daily intake may also aid in preventing cancer development. This effect may be related to the inverse association seen between methionine intake and global CpG methylation, as hypomethylation is commonly found in cancer and pre-cancerous cells. Future evaluation of dietary methionine and DNA hypomethylation in conjecture with cancer development is warranted. It is also necessary to determine what level of dietary methionine is necessary to prevent CpG hypomethylation and the increase in oxidative stress seen under high methionine diets while still maintaining healthy cellular functions, as clinical methionine restriction is not safe for long-term use.

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