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# **Lead Exposure, B Vitamins, and Plasma Homocysteine in Men 55 Years of Age and Older: The VA Normative Aging Study**

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**Running title:** Lead exposure and B-vitamins influence homocysteine

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## **Abstract**

**Background:** Lead exposure may influence plasma concentration of homocysteine, a one-carbon metabolite associated with cardiovascular and neurodegenerative diseases. Little is known about associations between lead and homocysteine over time, or the potential influence of dietary factors.

**Objectives:** To examine the longitudinal association of recent and cumulative lead exposure with homocysteine concentrations and the potential modifying effect of dietary nutrients involved in one-carbon metabolism.

**Methods:** In a subcohort of the VA Normative Aging Study (1,056 men with 2,301 total observations between 1993 and 2011), we used mixed effects models to estimate differences in repeated measures of total plasma homocysteine across concentrations of lead in blood and tibia bone, assessing recent and cumulative lead exposure, respectively. We also assessed effect modification by dietary intake and plasma concentrations of folate, vitamin B6, and vitamin B12.

**Results:** An interquartile range (IQR) increment in blood lead (3- $\mu\text{g}/\text{dl}$ ) was associated with 6.3% higher homocysteine concentration (95% CI: 4.8, 7.8%). An IQR increment in tibia bone lead (14- $\mu\text{g}/\text{g}$ ) was associated with 3.7% higher homocysteine (95% CI: 1.6, 5.6%), which was attenuated to 1.5% (95% CI: -0.5, 3.6%) with adjustment for blood lead. For comparison, a 5-year increase in time from baseline was associated with a 5.7% increase in homocysteine (95% CI: 4.3, 7.1%). The association between blood lead and homocysteine was significantly stronger among participants with estimated dietary intakes of vitamin B6 and folate below (versus above) the study population medians, which were similar to the U.S. Recommended Dietary Allowance intakes.

**Conclusions:** Lead exposure was positively associated with plasma homocysteine concentration. This association was stronger among men with below median dietary intakes of vitamins B6 and folate. These findings suggest that increasing intake of folate and B6 might reduce lead-associated increases in homocysteine, a risk factor for cardiovascular disease and neurodegeneration.

## Introduction

Elevated homocysteine is a risk factor shared by neurodegenerative conditions, such as cognitive decline (Haan et al. 2007; Tucker et al. 2005) and Alzheimer's disease (Seshadri et al. 2002), and cardiovascular diseases (CVD), such as ischemic heart disease and stroke (Collaboration 2002; Wald et al. 2002). Increasing numbers of older adults are at risk for these conditions because of the unprecedented growth in the population of older adults worldwide. Thus, understanding and intervening on the determinants of elevated homocysteine may lessen the public health burden of these conditions.

Accumulating epidemiologic and experimental research has demonstrated that exposure to lead increases the risk of hypertension (Navas-Acien et al. 2007) and recent research has provided strong evidence that lead exposure is a risk factor for ischemic heart disease (Jain et al. 2007), and overall cardiovascular morbidity and mortality (Navas-Acien et al. 2007). More limited evidence suggests that exposure to lead may increase plasma homocysteine concentration. This is an important consideration for the many older adults who sustained long-term exposures to high levels of lead earlier in their lives from occupational sources, lead-based paint, and especially widespread combustion of leaded gasoline. Although regulatory actions have dramatically reduced these exposures over the last two decades, past exposures cannot be “undone.” Moreover, as bone turnover increases with age, lead that has accrued in bone over decades may re-enter the circulation, resulting in re-exposure (Hu et al. 2007).

Schafer et al. (2005) proposed that lead may increase homocysteine concentration by reacting with sulfhydryl groups on several proteins in the homocysteine-processing one-carbon metabolism cycle (Figure 1). Furthermore, in an *in vitro* study, methionine, an amino acid synthesized from the metabolism of homocysteine (in which folate and vitamin B12 are co-

factors) reduced oxidative stress and related physiological damage in lead-exposed neuroblastoma cells (Chen et al. 2011). Previous epidemiologic studies have reported higher blood lead concentration, an indicator of recent exposure to lead (half-life of lead in blood≈30 days) (Hu et al. 1998), in association with higher plasma homocysteine (Chia et al. 2007; Lee et al. 2012; Schafer et al. 2005; Yakub and Iqbal 2010) Little is known, however, about the potential influence of past and cumulative lead exposures on homocysteine and changes in homocysteine over time. Further, to our knowledge, there have not been any longitudinal studies of the potential influence of the methyl donors, folate and B vitamins, on the association between lead and plasma homocysteine. If diet can modify the association between lead exposure and homocysteine, as suggested by findings from an analysis of cross-sectional data from the US National Health and Nutrition Examination Survey (NHANES) (Lee et al. 2012), this could be a critical means for reducing deleterious effects of lead exposure, even after exogenous exposure has ended.

Therefore, using an established longitudinal study of older men, we evaluated the association between lead exposure and circulating levels of homocysteine, and the degree to which this association is modified by estimated intakes of methyl-donor nutrients/co-factors (folic acid, vitamin B12, and vitamin B6). We estimated the association between recent exposure to lead, measured by blood lead concentration, and concurrent homocysteine concentration. Using concentrations of lead measured in blood and bone, respectively, we compared associations of recent versus cumulative lead exposures with homocysteine concentration. Finally, we tested whether associations between lead exposure and homocysteine were increased among participants whose diets were low in methyl-donor nutrients compared with other men.

## **Materials and methods**

### **Study population**

From 1961 to 1970, 2,280 men in the greater Boston area, ages 21 to 81, were enrolled in the Veteran's Affairs Normative Aging Study (NAS) (Bell et al. 1972). All men were free of known chronic medical conditions at enrollment and were invited to participate in health assessments every three to five years. Blood lead measurements began in 1977 and bone lead measurements began in 1991. Homocysteine assessment began in 1993. Nearly all homocysteine measurements (>99%) were made within 30 days of a blood lead measurement. At each study visit, self-reported smoking status, medication use, physical activity, and dietary intake were assessed.

Of the 1,218 men with blood lead measurements, 1,056 (99.4%) had at least one homocysteine measurement and data on key covariates (2,301 repeated observations in total). Our analysis of change in blood lead in relation to change in homocysteine included the 747 men who had at least two repeated measures of blood lead and concurrent homocysteine (1,830 observations). Of the 851 individuals with bone lead measurements, 777 (91.3%) had homocysteine and key covariate data (2,158 observations) and were included in our analyses of baseline bone lead in relation to concurrent and subsequent homocysteine. Loss to follow up did not vary by baseline lead level or homocysteine (data not shown).

The Human Subjects Institutional Review Boards at the Harvard School of Public Health, the Department of Veterans Affairs Healthcare System in Boston, the Brigham and Women's Hospital, and the University of Michigan Health Sciences approved this study. All participants provided written informed consent.

### **Assessment of lead exposure**

We assessed participants' recent exposure to lead using concentration of lead in whole blood, measured via graphite furnace atomic absorption with Zeeman background correction. For this study, we used blood leads from 1993-2011 (up to six measures per participant). To assess participants' cumulative exposure to lead, we measured baseline lead concentrations in two bone sites (tibia and patella) via Cd<sup>109</sup> K-shell x-ray fluorescence (KXRF) spectroscopy using methods previously described (Hu et al. 1996). Lead's half-life in cortical tibia bone is approximately 49 years in the study population (Wilker et al. 2011). Its half-life in trabecular patella bone is shorter, 10-15 years, depending on the study population (Hu et al. 1998).

### **Assessment of dietary nutrients**

Annual average diet was assessed with the semi-quantitative Willett Food Frequency Questionnaire (FFQ) (van de Rest et al. 2009). The questionnaire assesses usual frequency of consumption of 126 foods (on a scale ranging from never to  $\geq 2$  times per day) and supplements (no/yes, plus the amount). We estimated participants' dietary and supplement intakes of three one-carbon metabolism nutrients (folate, vitamin B6, and vitamin B12). Intakes of vitamins B6 and B12 were available from all study waves, while estimates of folate intake were only available from the second study wave and onward.

### **Assessment of plasma homocysteine and B-vitamins**

Fasting blood plasma was collected during each clinic visit and frozen (-80°C). Samples were analyzed at the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging (HNRCA). Plasma concentrations of total homocysteine and B-vitamins (folate, vitamin B12 and pyridoxal 5 phosphate (PLP), the activated form of vitamin B6) were measured in

fasting blood plasma samples via fluorescence detection with high-performance liquid chromatography (HPLC) at the HNRCA (Araki and Sako 1987). Homocysteine detection methods have been previously described (Tucker et al. 2005). The coefficients of variation (CV) for the biochemical assays of homocysteine, folate, PLP, and vitamin B12 were 4.0%, 4.3%, 5.0%, and 4.7%, respectively.

### **Statistical analysis**

All analyses were performed using R Statistical Software (version 2.15.0). Means (SD) of lead markers and geometric means (geometric standard deviations) of homocysteine concentrations by key covariates were computed. Tests for linear trend across categories were assessed by assigning integer scores to each ordinal covariate category and modeling as an ordinal variable. Analysis of variance test was used to compare the mean differences of lead and homocysteine concentrations by smoking status.

We performed cross-sectional analyses of lead exposure and homocysteine at each individual visit. Using the repeated measures of blood lead exposure and homocysteine, we estimated the percentage difference in homocysteine per increment in lead exposure measure using multivariable-adjusted linear mixed effects models, with random intercepts, of natural log-transformed homocysteine (Fitzmaurice et al. 2004). We analyzed each of the lead exposure measures separately, modeling repeated blood lead measurements in relation to concurrent homocysteine, and baseline bone lead measurements in relation to concurrent and subsequent homocysteine measurements, collectively. We tested for non-linear trends using generalized additive models with penalized splines in the *mgcv* R package, but the estimated degrees of freedom for blood and bone lead concentrations were equal to one, consistent with a linear association between lead and homocysteine, so linear models were used (Hastie and Tibshirani

1990; Wood 2006). All analyses were adjusted for a core set of covariates previously associated with homocysteine: baseline age (years), time since baseline, attained education ( $\leq$ high school,  $>$ high school degree), smoking status (current, former, never; time-varying), alcohol consumption ( $<2$  drinks per day,  $\geq 2$  drinks per day; time-varying), and body mass index (BMI;  $\text{kg}/\text{m}^2$ ; time-varying) (Schafer et al. 2005). We checked for interactions between covariates and time since baseline, but including these terms did not improve the model fit. We further adjusted for intake of methyl-donor nutrients using two approaches. First, we adjusted for continuous plasma concentrations of PLP, vitamin B12, and folate concurrent with homocysteine measures. In the second approach, we adjusted for continuous FFQ-based estimates of total energy intake and energy-adjusted dietary intakes (Willett et al. 1997) of vitamin B6, vitamin B12, and folate, as well as supplement use (quantity per day) of vitamin B6, vitamin B12, and folate, reflecting intake over the previous year. As a sensitivity analysis, we adjusted bone lead with long-term dietary trend, calculated by averaging FFQ estimates of daily intake over three or more visits for a subset of individuals ( $n=221$ ). For a secondary test, we restricted blood lead analyses to individuals who had both plasma and dietary nutrient data. These analyses contain only 1221 observations from 774 participants. As a sensitivity analysis, we tested to see if the association between blood lead and homocysteine was mediated by kidney function (as approximated by serum creatinine and estimated creatinine clearance). Creatinine clearance ( $\text{mL}/\text{min}$ ) was estimated using the equation  $[(140 - \text{age}) \times \text{weight}(\text{kg})]/[72 \times \text{serum creatinine}(\text{mg}/\text{dL})]$  (Wu et al. 2003). We compared models of core covariates with blood lead alone, creatinine alone, and blood lead and creatinine together.

To explore the possibility that the association of cumulative lead dose with homocysteine might be mediated through recent exposure (e.g., from lead released into circulation via bone

resorption), we compared results from models of baseline tibia lead in relation to concurrently assessed homocysteine concentration with and without further adjustment for blood lead concentration. We performed similar analyses for patella lead. Although this simple approach for assessing mediation is problematic in many settings, it can generate valid estimates under the following conditions: (1) both the mediator (in the present study, blood lead) and outcome (homocysteine) are continuous and can be modeled using linear regression—a condition that is clearly met; (2) there is no confounding of the mediator-outcome association (e.g., no common causes of homocysteine and bone resorption, which increases blood lead); and (3) there is no interaction between exposure and mediator (i.e., at high bone lead concentrations, the effect per unit increase in blood lead is no larger/smaller than at low bone lead concentrations) (Kaufman et al. 2004). As an analysis of the sensitivity of this approach, we also applied a method with a formal basis in causal frameworks (Valeri and Vanderweele 2013) that estimates the total effect of bone lead on homocysteine along with the indirect effect of bone lead on homocysteine via blood lead concentration, and the direct effect of bone lead on homocysteine via pathways independent of blood lead concentration (Imai et al. 2010). This procedure, the Baron-Kenny procedure with interaction term, also quantitatively evaluates the impact of interaction (irrespective of magnitude) between the exposure and mediator on the findings.

Where participants had at least two study visits, we examined changes in blood lead concentration over time in relation to concomitant changes in homocysteine, by regressing visit-to-visit change in homocysteine on change in blood lead concentration, with the general structure,  $E[\text{homocysteine}_t - \text{homocysteine}_{t-1}] = \beta_0 + \beta_1[\text{blood lead concentration}_t - \text{blood lead concentration}_{t-1}]$ , where  $t$  indexes a given visit,  $t-1$  indexes the visit prior to visit  $t$ , and the parameter of interest is  $\beta_1$ , the difference in visit-to-visit changes in homocysteine

corresponding to a 1- $\mu\text{g}/\text{dL}$  greater visit-to-visit change in blood lead concentration (e.g., comparing a visit-to-visit increase in blood lead concentration of 1  $\mu\text{g}/\text{dL}$  with no change). For these analyses, we used linear mixed models to account for correlations between repeated dyads of observations from the same individuals (e.g., change from wave 1 to wave 2, change from wave 2 to wave 3, etc.). The association between change in blood lead and change in homocysteine was not linear, thus we modeled increases and decreases in blood lead concentrations using separate terms (reference: no change in blood lead concentration), effectively a piece-wise regression model with a breakpoint at [*blood lead concentration*<sub>*t*</sub> – *blood lead concentration*<sub>*t-1*</sub>] = 0. We adjusted these analyses for time between visits, age at the start of the interval, and trajectory in smoking status over the interval (always a former smoker, quit smoking, always a smoker, began smoking, always a never smoker). Adjustment for additional covariates did not change the findings. These analyses involved data of 1245 visit-to-visit intervals from 747 participants.

To examine whether past or cumulative lead exposure might influence the long-term trajectory of homocysteine concentration with age, we compared trajectories in homocysteine (as percentage change from baseline homocysteine) by tibia bone lead concentration at baseline. To do so, we amended our mixed effects linear models for tibia lead concentration with a cross-product term between time since baseline and tibia lead concentration. This model also contained a cross-product term between time and baseline age. The inclusion of other time-covariate cross-products did not change the results (data not shown).

To compare the association of lead exposure with homocysteine by intake of methyl-donor nutrients, we fit models that incorporated all observations and included a cross-product term between each lead exposure measure and dichotomous nutrient measure (median or above versus

below). We also compared men who were below the median for all nutrient measures (n=517, plasma; n=383, diet) with men above the median for all nutrient measures (n=473, plasma; n=376, diet). In our primary analyses of the potential interaction of blood lead concentration with dietary nutrients, we fit models stratified according to intake from food and further adjusted for supplement use (yes, no), thus treating supplement use as a surrogate of confounding by socioeconomic position (SEP), especially in the stratum of high intake. Although dietary supplements make up part of the total nutrient intake, use of these supplements is also related to SEP, which predicts lead exposure, and can affect dietary choices in complex ways (Radimer et al. 2004; Rock 2007). Median estimated dietary intakes of folate and vitamins B6 and B12 were 343 µg/day, 2.24 mg/day, and 6.19 µg/day, respectively. Median plasma nutrient concentrations were 11.6 ng/mL folate, 64.1 nmol/L B6, and 449 pg/mL B12.

However, we tried multiple alternative analytical approaches, as well. We stratified analyses by supplement use (yes/no) among all participants; by supplement use (equal or above the median versus below) restricted to supplement users; and by total intake (dietary and supplement). Analyzing supplement data entails complex considerations. In addition to its patterning by SEP, particularly during the years of this study, supplement use dramatically increases total nutrient intakes (often by an order of magnitude or more), yet trials of supplement use in healthy populations have yielded equivocal findings with respect to several health effects (Fortmann et al. 2013). To circumvent these complexities, we also conducted sensitivity analyses that were stratified by dietary intake, restricted to non-supplement users.

## **Results**

Of the 1,056 men in our study, aged 55-97 at baseline, mean ( $\pm$  SD) concentrations of lead in blood and tibia bone were  $4.9 \pm 2.7$  µg/dl and  $21.4 \pm 13.5$  µg/g, respectively (Table 1). The mean

( $\pm$  SD) patella lead concentration was  $30.6 \pm 20.1$   $\mu\text{g/g}$  (data not shown). At baseline, the geometric mean homocysteine concentration was  $10.1$   $\mu\text{mol/L}$  (geometric SD, 1.3). Baseline plasma concentrations of vitamin B12 (98 to 4081  $\text{pg/mL}$ ) and folate (1 to 69  $\text{ng/mL}$ ) were also similar to US men over age 60 (Pfeiffer et al. 2005). Baseline plasma PLP (vitamin B6) nutrient concentrations ranged from 5 to 995  $\text{nmol/L}$ . The US Recommended Daily Allowance (RDA) for folate is 400  $\mu\text{g/day}$  and 279 (62.6%) participants consumed less than the RDA from estimated diet alone. RDAs of vitamin B6 and B12 are 1.7  $\text{mg/day}$  and 2.4  $\mu\text{g/day}$  respectively. In our population from estimated diet alone, 233 (22.1%) consumed less vitamin B6 and 47 (4.5%) consumed less vitamin B12 than the RDA.

At baseline, homocysteine was positively correlated with blood lead, tibia bone lead, and patella bone lead concentrations (respectively, Pearson's  $r=0.27$ , 0.15, and 0.15, all  $P<0.001$ ). Lead concentrations were lower among men who were younger, had more education, higher plasma concentrations of the three nutrients, and higher dietary intakes of vitamins B6 and folate (Table 1).

### **Association of recent lead exposure with homocysteine**

Recent exposure to lead, measured by blood lead concentration, was significantly associated with concurrent plasma homocysteine concentration (Table 2). After adjustment for baseline age, time since baseline, education, alcohol, smoking, and BMI, an interquartile range (IQR) increment in blood lead (3  $\mu\text{g/dL}$ ) was associated with 6.3% higher homocysteine (95% CI: 4.8, 7.8%). This association diminished slightly with further adjustment for plasma concentrations of the three methyl-donor nutrients (5.5% higher; 95% CI: 4.0, 6.9%), but not with further adjustment for dietary intakes of these nutrients plus total energy consumption (6.5%; 95% CI: 4.3, 8.8%).

These associations may have varied at least in part because they were based on different subsets of observations due to missing nutrient exposure data. When we restricted the blood lead analyses to individuals with both plasma and dietary nutrient data, the estimated association between blood lead concentration and homocysteine varied by less than 10% across the core model (6.4%; 95% CI: 4.3, 8.5%), plasma model (5.8%; 95% CI: 3.7, 7.9%), and dietary model (5.9%; 95% CI: 3.8, 8.1%). For comparison, results from the model that included terms for the core covariates and current blood lead concentration indicated that homocysteine concentrations were 5.7% higher, on average, in association with a 5-year passage of time since baseline (95% CI: 4.3, 7.1%), and 13% higher (95% CI: -0.2, 28.1%) in current smokers compared with never smokers.

In additional sensitivity analyses that were further adjusted for creatinine clearance, an intermediate marker of kidney function, the association of an IQR increment in blood lead concentration with higher homocysteine remained significant (6.3%; 95% CI: 4.8, 7.8%).

### **Association of cumulative lead exposure with homocysteine**

Higher cumulative exposure to lead, measured by baseline tibia bone lead concentration, was also significantly associated with elevated plasma homocysteine (Table 2). After adjusting for the core group of covariates, an IQR increment in tibia bone lead concentration (14 µg/g) was associated with 3.7% higher homocysteine (95% CI: 1.7, 5.6%). Further adjustment for plasma and dietary methyl-donor nutrients did not alter this finding. The corresponding associations with homocysteine per IQR increase in baseline patella lead concentration (21 µg/g) were consistent with those estimated per IQR increase in tibia lead concentration (Table 2). For example, from the core covariate-adjusted model, homocysteine concentrations were 3.0% higher per IQR-increment in patella lead concentration (95% CI: 1.0, 5.0).

The association between an IQR increase in tibia lead at baseline (14  $\mu\text{g/g}$ ) and homocysteine (at baseline and follow-up) was substantially attenuated when adjusted for concurrent blood lead (1.5% higher; 95% CI: -0.5, 3.6%), while there was little change in the association between an IQR increase in concurrent blood lead and homocysteine (5.8% higher; 95% CI: 4.0, 7.6). These models were adjusted for the core covariates (time since baseline, baseline age, education level, smoking status, and BMI). Similarly, the association pertaining to patella lead was attenuated but the association pertaining to concurrent blood lead changed very little when both were included in the same model. These findings are consistent with the results generated by the Baron-Kenny mediation procedure, in which: the estimated effect of bone lead (per IQR increment) on homocysteine mediated through blood lead (i.e., the average causal mediation effect [ACME] via blood lead) was a 2.9% increase in homocysteine (95% CI: 2.0, 4.0); the average direct effect of bone lead (per IQR increment) on homocysteine via pathways other than blood lead was 1.7% (95% CI: -0.6, 4.3); and the estimated fraction of the total effect of bone lead on homocysteine mediated by blood lead was 0.63. Interaction between bone and blood lead did not alter these findings.

### **Longitudinal changes in blood lead in relation to changes in homocysteine**

Similar to the US population during the study period, blood lead concentration among study participants decreased over time, by 0.7- $\mu\text{g/dl}$ , on average, per 3.5-year interval (range, -14 to 8- $\mu\text{g/dL}$ ). Among individuals with at least two visits ( $n=747$ , 1245 observations), the average time interval between visits was 3.9 years. Although homocysteine concentration generally increased over time, the degree of change in homocysteine generally tracked changes in blood lead concentration. In particular, during intervals corresponding to the reference condition—i.e., never smoked, age 67.5 at the start of the interval, no change in blood lead concentration, 1-year

interval, —homocysteine increased by 0.19  $\mu\text{mol/L}$ , on average. By comparison, visit-to-visit intervals in which blood lead concentrations declined corresponded to concomitantly smaller increases (or steeper declines) in homocysteine ( $P = 0.02$ ). For each additional  $\mu\text{g/dL}$  decline in blood lead between visits, homocysteine concentration increased by 0.13  $\mu\text{mol/L}$  *less* (95% CI: -0.25, -0.01). Although visit-to-visit increases in blood lead concentration were associated greater increases in homocysteine (0.07  $\mu\text{mol/L}$  greater increase in homocysteine per each additional  $\mu\text{g/dL}$  increase in blood lead), this result was not statistically significant (95% CI: -0.11, 0.25).

### **Baseline tibia lead in relation to change in homocysteine**

Although cumulative exposure to lead, as measured by baseline tibia bone lead concentration, was associated with higher plasma homocysteine over the course of follow-up (as reported above), it was not associated with the degree to which plasma homocysteine *changed* over time, as estimated by the cross-product term between tibia lead and time since baseline ( $P_{\text{time} \times \text{bone Pb interaction}} = 0.9$ ). Specifically, the estimated percentage difference in homocysteine per IQR increase in baseline tibia lead (14  $\mu\text{g/g}$ ) was statistically consistent across visits: visit 1 (3.6% higher; 95% CI: 1.4, 6.0), visit 2 (3.8% higher; 95% CI: 1.2, 6.4), visit 3 (4.6% higher; 95% CI: 1.7, 7.5), and visit 4 (5.5% higher; 95% CI: 1.3, 9.9).

### **Association of blood lead concentration with homocysteine, by intake of methyl-donor nutrients**

The association between blood lead concentration and concurrent plasma homocysteine was significantly stronger among men whose dietary intakes of vitamin B6, folate, or vitamins B6, B12, and folate (combined) were below the median, even after adjusting for supplement use (Figure 2). For example, among men with lower vitamin B6 intake, an IQR increment in blood lead level was associated with 8.7% higher homocysteine concentration (95% CI: 6.0, 11.5%),

compared with 2.0% higher (95% CI: -0.8, 5.0%) among men with higher vitamin B6 intake ( $P_{interaction}=0.0004$ ). Associations between blood lead and homocysteine also were significantly stronger among men with lower dietary intakes of folate or of all three dietary nutrients combined compared with men whose estimated intakes were  $\geq$ median ( $P_{interaction}$ : 0.02 and 0.003, respectively); and among men with lower plasma folate, or men with lower plasma concentrations of all three plasma nutrients combined, compared with men whose plasma concentrations were  $\geq$ median ( $P_{interaction}$ : 0.002 and 0.006, respectively). In addition, a pattern in which low folate intake conferred greater lead-related homocysteine risk was present in the results of sensitivity analyses that (a) incorporated supplement intake into the total intake; (b) restricted to supplement users; and (c) compared users versus non-users of methyl-donor nutrient supplements (Figure 2). In these analyses, findings on other nutrients were less remarkable. Secondary models were restricted to dietary intake of non-supplement users: (1) to reduce confounding by supplement use and its correlates; (2) to reduce error in measurement of total intake; and (3) to limit the range of intakes to that which can be achieved via diet, especially in light of evidence-based scrutiny directed toward supplement use. We found evidence suggesting that the association between bone lead and homocysteine varied by plasma and long-term dietary intake of vitamin B12 ( $p_{interaction-plasma}=0.00002$ ;  $p_{interaction-diet}=0.1$ ), but results were less suggestive with respect to folate ( $p_{interaction-plasma}=0.1$ ;  $p_{interaction-diet}=0.8$ ) or vitamin B6 ( $p_{interaction-plasma}=0.5$ ;  $p_{interaction-diet}=0.1$ ).

## Discussion

In this study of older men with community-level exposure to lead and typical dietary intakes, recent exposure to lead, measured by blood lead concentration, was positively associated with concurrent plasma homocysteine concentration. This association was stronger among men with <

median dietary intakes of vitamins B6 and folate, and among men with < median plasma folate. Homocysteine concentration among men in our study was comparable to similarly aged men in the general population (Jacques et al. 1999). This study contains one of the richest available data collections on lead exposure, diet and homocysteine. It is strengthened by its use of both dietary and plasma measures of nutrient intake, and its repeated measures of homocysteine and blood lead concentrations, in addition to its measures of cumulative lead exposure, allowing detailed examination of differences and longitudinal changes in homocysteine with recent and cumulative lead exposure.

In light of other homocysteine predictors, the magnitude of the association of lead exposure with homocysteine is substantial. On average, homocysteine concentration was 6.3% higher per IQR (3- $\mu$ g/dl) increment in blood lead concentration, equivalent to the estimated increase in homocysteine over 5.5 years of follow up (i.e. 5.5 years of aging), and about half of the estimated difference in homocysteine between current and never smokers. In the Framingham Heart Study a 25% increase in plasma homocysteine was associated with a 14% increase in the relative risk of incident Alzheimer's disease over 8 years of follow up (95% CI: 6, 22%) (Seshadri et al. 2002), and a meta-analysis of 30 retrospective or prospective studies estimated that a 25% increase in homocysteine ( $\sim$ 3 $\mu$ mol/L) was associated with 11% increase in relative risk of ischemic heart disease (95% CI: 4, 17%) and a 19% increase in relative risk of stroke (95% CI: 5, 31%) (Homocysteine Studies 2002). Overall, these findings suggest that, if the association between lead and homocysteine is causal, a modest population increase in homocysteine due to lead exposure could dramatically increase the prevalence of cardiovascular and neurodegenerative diseases. Particularly in older adults, where bone turnover is relatively

high and current exogenous exposure is generally low, the source of blood lead may be bone resorption, which liberates historic lead exposure.

Intakes of methyl-donor nutrients below the median for the study population appeared to strengthen the association between blood lead and homocysteine. Among men with lower folate dietary intake, the estimated increase in homocysteine associated with an IQR increment of blood lead was similar to the estimated increase in homocysteine over 9 years of follow-up, whereas among men with higher folate intake, the estimated increase was comparable to that associated with about 3 years of follow-up. We observed this disparity in blood lead-homocysteine association by dietary nutrient intake, even after adjusting for supplement use or restricting to supplement non-users. These results are consistent with the possibility that the additional nutrients found in foods may allow vitamins to work optimally (Lichtenstein and Russell 2005), although we could not test this in our data. Thus, preventing lead exposure may not only reduce the risk of elevated homocysteine, but among those who have been exposed, higher consumption of methyl-donor nutrients may partially mitigate the influence of lead on homocysteine.

Higher cumulative exposure to lead, measured by concentrations in bone, also was associated with elevated homocysteine, but this association appeared to be attributable, in large part, to recent lead exposure. The problems specific to the analysis of discrete outcomes and/or mediators are not present in these analyses (Kaufman et al. 2004); assuming no interaction and unmeasured confounding of the estimated blood lead-homocysteine association (Cole and Hernan 2002; Kaufman et al. 2004), the results suggest that the influence of lead exposure on homocysteine is fairly immediate and, thus, that recent exposure to lead (measured in blood) may drive the association between lead and homocysteine. However, even with substantial

cumulative exposure, interventions that minimize acute elevations in blood lead concentration (e.g., from endogenous sources) may be an effective strategy to mitigate increases in homocysteine, though there is insufficient information to recommend such an intervention for this purpose at this time. Results from homocysteine-lowering dietary intervention trials among prior disease patients are mixed: the interventions effectively reduce circulating homocysteine, but perform inconsistently in affecting homocysteine-related health outcomes (Mei et al. 2010; Morris 2012). Among the explanations for these inconsistent findings are that: the study populations differed in their pre-intervention nutritional deficiencies, whereby supplementing deficiencies may help, but supplementing sufficient intake may have no effect or may cause harm (Morris 2012); the interventions may have begun too late to influence the health trajectory; or the observational associations of homocysteine with health outcomes may not be causal. Our study is among the first to evaluate whether the association between lead exposure and homocysteine might be influenced by intake of folate and other B-vitamins. In data from NHANES, the association between blood lead concentration and homocysteine was more pronounced among people with below-median plasma concentrations of vitamin B6 or folate than among people with higher plasma concentrations ( $p_{interaction}=0.002$  and  $0.06$ , respectively). However, results for plasma vitamin B12 followed the opposite pattern, with a significantly stronger adverse association between blood lead and homocysteine among those with *higher* vitamin B12 concentration ( $p_{interaction}=0.01$ ) (Lee et al. 2012). In contrast, although our findings on vitamin B6 and folate were consistent with those from the NHANES study, our analyses of vitamin B12, whether based on reported intake or plasma concentration, yielded stronger associations between blood lead and homocysteine among those with *lower* vitamin B12 than among those with higher intakes, but these interactions were not significant.

Our finding that lead exposure is associated with higher homocysteine concentration is consistent with prior epidemiological studies. Among workers occupationally exposed to lead in Vietnam, blood lead concentration and homocysteine were associated, although these analyses were not adjusted for any other factors (Chia et al. 2007). In a cross section analysis of Baltimore Memory Study data from men (mean age, 59) with blood lead and homocysteine concentrations comparable to those in our participants, a 1.0- $\mu\text{g}/\text{dL}$  increment in blood lead was associated with a 0.43- $\mu\text{mol}/\text{L}$  increase in homocysteine (Schafer et al. 2005). However, the investigators reported that there was no association between homocysteine and tibia lead. By comparison, in our population, a 1.0- $\mu\text{g}/\text{dL}$  increment in blood lead concentration was associated with a 0.30- $\mu\text{mol}/\text{L}$  increase in homocysteine (95% CI: 0.23, 0.37). In NHANES participants (mean age, 50), homocysteine was higher across progressively increasing quartiles of blood lead concentration, even though 95% of individuals had blood lead concentration below 5  $\mu\text{g}/\text{dL}$  (Lee et al. 2012). In our study, 55% of participants had blood lead concentration below 5  $\mu\text{g}/\text{dL}$  at baseline. Among adults in Pakistan (ages 18-60) with higher lead concentration (mean blood lead, 11.7- $\mu\text{g}/\text{dL}$ ), an IQR increase in blood lead (8.3- $\mu\text{g}/\text{dL}$ ) was associated with 4.6% higher homocysteine (95% CI: 2.6, 4.8%) (Yakub and Iqbal 2010). The association in our study was larger: an IQR (3- $\mu\text{g}/\text{dL}$ ) increase in blood lead corresponded to 6.3% higher homocysteine.

Our study has several limitations. Errors in the measurement of lead exposures and homocysteine may have biased and/or reduced the precision of the associations we observed between the two. For example, the time of day of blood draw for homocysteine measurement was not standardized, but homocysteine concentration follows a daily rhythm characterized by an evening peak and nighttime low (Bonsch et al. 2007). In addition, plasma measures of homocysteine assess the pool of homocysteine released after reduction of all disulfide bonds in

the sample. Total homocysteine does not include homocysteine thiolacetone (a product of misincorporation of homocysteine into proteins and subsequent error-editing) or homocysteine bound to protein by an amide bond (Perla-Kajan et al. 2007). These homocysteine groups are potentially toxic but might not be correlated with plasma homocysteine concentration (Perla-Kajan et al. 2007).

It is possible that our observations reflect effects of lead on kidney function. Lower kidney glomerular filtration rate (GFR) has been shown to increase homocysteine concentration in a prospective study of community-dwelling adults over age 70 (Lewerin et al. 2007). Chronic lead exposure appears to contribute to chronic kidney disease, indicated by low GFR (Batuman et al. 1983; Brewster and Perazella 2004; Muntner et al. 2003). In the NAS, for example, creatinine clearance was lower with higher long-term exposure to lead, estimated via patella lead concentration (Wu et al. 2003). Therefore, lead exposure may influence homocysteine through an effect on renal function. We performed sensitivity analyses of blood lead and homocysteine by further adjusting for creatinine clearance as a marker of kidney function, and as an intermediate in the above mechanism. Although the association of blood lead with homocysteine modestly decreased in magnitude (from a 6.3% increase in homocysteine per IQR increment in blood lead, to a 5.5% increase), it remained positive and significant, indicating that lead may elevate homocysteine independently of its renal effects. However, inferences about causal mechanisms should be interpreted with caution.

Some of our analyses had limited statistical power. Missing nutrient data meant that sample sizes were smaller for analyses of lead exposure, diet, and homocysteine. The change-change analyses also had limited power, as participants were lost to follow-up over time. Finally, generalizability of our study may be limited as the study population is older, non-Hispanic white men.

In conclusion, we report a significant positive association between blood lead concentration and plasma homocysteine in a cohort of older men. This association between blood lead and homocysteine was stronger than associations corresponding to baseline bone lead, and declines in blood lead concentration over time corresponded with smaller increases (or larger declines) in homocysteine over the same interval, suggesting that circulating lead may influence circulating homocysteine through lead's influence on homocysteine metabolism, even at very low levels of lead exposure. Our finding that the association between blood lead and homocysteine was stronger among men with lower estimated intakes of methyl-donor nutrients, especially folate and vitamin B6, suggests that nutrient intakes may influence susceptibility to effects of lead that might operate through effects on homocysteine. The associations of elevated homocysteine concentration with cardiovascular and neurodegenerative diseases, along with the high prevalence of cumulative and now endogenous exposures to lead in the population, suggest the potential public health value in researching joint interventions on diet and exposure to lead..

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**Table 1.** Blood lead, tibia bone lead, and plasma homocysteine concentrations, by key baseline participant characteristics among study participants with data on blood lead and homocysteine concentrations (N=1,056).

Characteristic	N (%) or Mean ± SD	Blood lead (µg/dL) Mean ± SD	P value <sup>a</sup>	Tibia bone lead (µg/g) Mean ± SD	P value <sup>a</sup>	Plasma homocysteine (µmol/L) GM (GSD)	P value <sup>a</sup>
<b>Overall value</b>		4.9 ± 2.7		21.4 ± 13.5		10.1 (1.3)	
<b>Age</b>	69 ± 7.4						
50-65 years	355 (34)	4.6 ± 2.6		16.5 ± 9.3		9.8 (1.4)	
66-71 years	327 (31)	4.9 ± 2.8	0.03	21.8 ± 12.3	<0.0001	9.9 (1.3)	<0.0001
72-97 years	374 (35)	5 ± 2.8		26 ± 16.3		10.7 (1.3)	
<b>Education</b>							
less than high school	80 (8)	5.8 ± 3		30.2 ± 18.6		9.8 (1.3)	
high school	303 (29)	5.2 ± 2.9		24.7 ± 15.9		10.2 (1.4)	
some college	288 (27)	4.8 ± 2.7	<0.0001	20.3 ± 11	<0.0001	10.1 (1.3)	0.9
college degree	202 (19)	4.5 ± 2.3		18.9 ± 11.3		9.9 (1.3)	
> college degree	183 (17)	4.4 ± 2.8		17 ± 9.4		10.2 (1.3)	
<b>Smoking status</b>							
never	296 (28)	4.8 ± 3		20 ± 13.7		9.8 (1.3)	
former	698 (66)	4.8 ± 2.6	0.1	22 ± 13.6	0.002	10.1 (1.3)	0.005
current	62 (6)	5.8 ± 3.2		22.1 ± 11.5		11.2 (1.3)	
<b>Alcohol consumption</b>							
< 2 drinks/day	834 (79)	4.6 ± 2.7	<0.0001	21.4 ± 13.9	0.5	9.9 (1.3)	<0.0001
≥ 2 drinks/day	222 (21)	5.7 ± 2.9		21.2 ± 12.1		10.8 (1.4)	
<b>BMI</b>	28 ± 3.9						
< 25 kg/m <sup>2</sup>	216 (21)	4.9 ± 2.8		21.4 ± 11.5		10.1 (1.3)	
25 to < 30 kg/m <sup>2</sup>	574 (54)	4.8 ± 2.7	0.5	21.8 ± 14.3	0.3	10.1 (1.3)	0.9
≥ 30 kg/m <sup>2</sup>	266 (25)	4.8 ± 2.8		20.4 ± 13.3		10.1 (1.3)	
<b>Plasma vitamin B6</b>	87.5 ± 87.2						
< 61.8 nmol/L	561 (53.1)	5.2 ± 2.9	<0.0001	23.0 ± 14.2	<0.0001	10.8 (1.3)	<0.0001
≥ 61.8 nmol/L	483 (45.7)	4.4 ± 2.5		19.3 ± 12.4		9.4 (1.3)	
<b>Plasma vitamin B12</b>	466.4 ± 222						
<431 pg/mL	566 (53.6)	5.1 ± 2.8	<0.0001	21.5 ± 12.0	0.5	10.8 (1.3)	<0.0001
≥ 431 pg/mL	469 (44.4)	4.5 ± 2.6		21.1 ± 14.9		9.3 (1.3)	

Characteristic	N (%) or Mean $\pm$ SD	Blood lead ( $\mu\text{g/dL}$ ) Mean $\pm$ SD	P value <sup>a</sup>	Tibia bone lead ( $\mu\text{g/g}$ ) Mean $\pm$ SD	P value <sup>a</sup>	Plasma homocysteine ( $\mu\text{mol/L}$ ) GM (GSD)	P value <sup>a</sup>
<b>Plasma folate</b>	11 $\pm$ 7						
< 9.8 ng/mL	621 (58.8)	5.2 $\pm$ 2.8	<0.0001	21.5 $\pm$ 12.9	0.1	10.7 (1.3)	<0.0001
$\geq$ 9.8 ng/mL	405 (38.4)	4.4 $\pm$ 2.6		20.9 $\pm$ 13.8		9.3 (1.3)	
<b>Dietary vitamin B6</b>	2.3 $\pm$ 0.9						
<1.8 mg/day	254 (24)	5.4 $\pm$ 2.8		22.8 $\pm$ 14.7		10.6 (1.3)	
1.8 to <2.2 mg/day	255 (24)	4.9 $\pm$ 2.8	<0.0001	21 $\pm$ 12.4	0.2	10.1 (1.3)	0.0002
2.2 to 2.8 mg/day	246 (23)	4.7 $\pm$ 2.7		21 $\pm$ 13.5		10.2 (1.3)	
$\geq$ 2.8 mg/day	252 (24)	4.4 $\pm$ 2.6		21 $\pm$ 13.9		9.6 (1.3)	
<b>Dietary vitamin B12</b>	7.9 $\pm$ 5.5						
< 4.4 $\mu\text{g/day}$	252 (24)	5 $\pm$ 2.6		22.1 $\pm$ 13.8		10.4 (1.3)	
4.4 to 6.2 $\mu\text{g/day}$	253 (24)	4.7 $\pm$ 2.8	0.9	20.1 $\pm$ 12.8	0.4	10.1 (1.3)	0.2
6.2 to 9.0 $\mu\text{g/day}$	250 (24)	4.8 $\pm$ 2.8		20 $\pm$ 12.6		9.8 (1.3)	
$\geq$ 9.0 $\mu\text{g/day}$	252 (24)	5 $\pm$ 2.8		23.4 $\pm$ 14.9		10.2 (1.3)	
<b>Dietary folate<sup>b</sup></b>	374.8 $\pm$ 178.1						
< 248.5 $\mu\text{g/day}$	135 (18)	4.5 $\pm$ 2.8		21.7 $\pm$ 11.7		9.9 (1.3)	
248.5 to 327.3 $\mu\text{g/day}$	134 (18)	4.0 $\pm$ 2.4	0.001	21.1 $\pm$ 13.1	0.2	10.3 (1.3)	0.7
327.3 to 440 $\mu\text{g/day}$	134 (18)	4.0 $\pm$ 2.1		19.3 $\pm$ 19.3		10.1 (1.4)	
$\geq$ 440 $\mu\text{g/day}$	134 (18)	3.6 $\pm$ 1.7		19.6 $\pm$ 19.6		9.9 (1.3)	

Abbreviations: Pb, lead; GM, geometric mean; GSD, geometric standard deviation; SD, standard deviation; BMI, body mass index; N, number of individuals.

<sup>a</sup>Calculated using a test for linear trend across ordinal categories of nonmissing data. <sup>b</sup>Calculated at visit 2 due to missing data at visit.

**Table 2.** Adjusted percentage difference (95% confidence interval [CI]) in plasma homocysteine (Hcy) per interquartile range (IQR) increment in lead exposure biomarker.

<b>Estimate</b>	<b>Blood lead concentration (IQR: 3 µg/dL)</b>	<b>Tibia lead concentration (IQR: 14 µg/g)</b>	<b>Patella lead concentration (IQR: 21 µg/g)</b>
<b>Core model<sup>a</sup></b>			
N (Observations)	1056 (2301)	777 (2158)	770 (2133)
Percentage difference in Hcy per IQR biomarker (95% CI)	6.3 (4.8, 7.8)	3.7 (1.7, 5.6)	3.0 (1.0, 5.0)
<i>P</i> value	<0.0001	0.0002	0.003
<b>Core model plus plasma nutrients<sup>b</sup></b>			
N (Observations)	1033 (2240)	767 (2106)	760 (2082)
Percentage difference in Hcy per IQR biomarker (95% CI)	5.5 (4.0, 6.9)	3.6 (1.7, 5.5)	3.0 (1.0, 5.0)
<i>P</i> value	<0.0001	0.0002	0.003
<b>Core model plus dietary and supplement nutrients<sup>c</sup></b>			
N (Observations)	779 (1241)	634 (1328)	627 (1314)
Percentage difference in Hcy per IQR biomarker (95% CI)	6.5 (4.3, 8.8)	3.0 (0.8, 5.2)	2.1 (-0.2, 4.4)
<i>P</i> value	<0.0001	0.008	0.07

<sup>a</sup>Core covariates were baseline age, time since baseline, education, smoking status, alcohol consumption, and BMI. Mixed effects models use random intercepts and unstructured correlation structures. <sup>b</sup>Analyses further adjusted for plasma nutrients including core covariates and plasma PLP (B6), B12 and folate. <sup>c</sup>Analyses further adjusted for dietary nutrients including core covariates, total energy, and total energy-adjusted dietary intakes of vitamin B6, vitamin B12, and folate, as well as supplement use (yes/no) of vitamin B6, vitamin B12, and folate, based on FFQ responses.

## Figure legends

**Figure 1.** One-Carbon Metabolism Pathway. Homocysteine can be elevated in conditions of low folate, low B6, or low B12. The sulfhydryl groups on several proteins, including Cystathionine  $\beta$ -synthase (*CBS*), in the one-carbon metabolism pathway are potential sites for lead's interferences. Abbreviations Used: Adenosine (*Ado*), S-adenosylmethionine (*SAM*), S-adenosylhomocysteine (*SAH*), Glutathione (*GSH*), Glutamate (*Glu*), Glycine (*Gly*), Tetrahydrofolate (*THF*), Methyltransferase (*MT*), Betaine-Homocysteine S-Methyltransferase (*BHMT*), Dimethylglycine (*DMG*), Methylenetetrahydrofolate Reductase (*MTHFR*), Methionine Synthase (*MS*).

\*Indicates putative locations for interference by lead. Adapted from Bistulfi et al. (2010).

**Figure 2.** Adjusted percentage difference in homocysteine per interquartile (IQR) increment in blood lead concentration, within nutrient status level. All analyses were adjusted for age, education, alcohol consumption, smoking status, and BMI. Low corresponds to individuals below the population median and high represents equal to or greater than the population median.

Figure 1

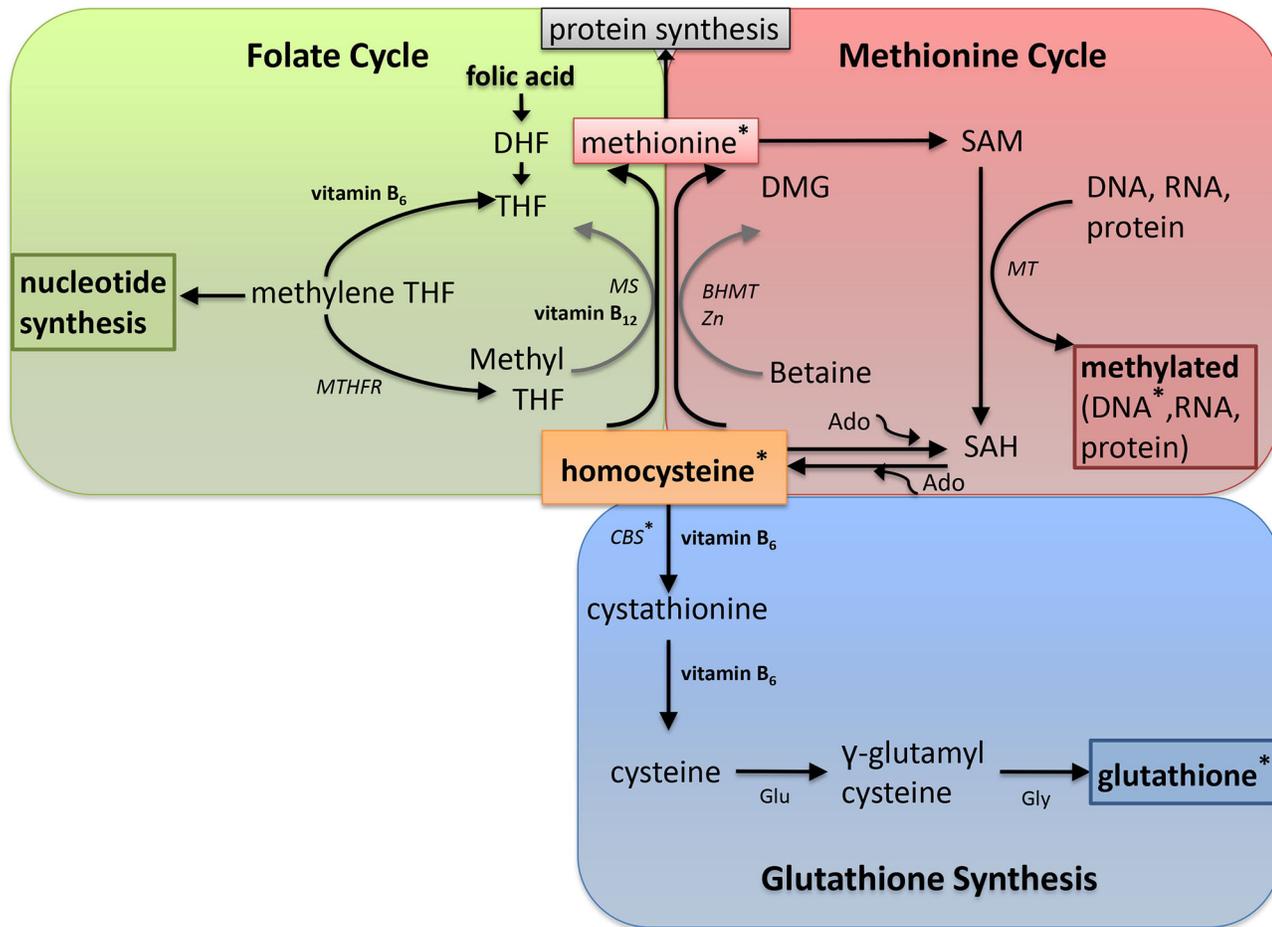


Figure 2

