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Original article

Novel micronutrient associations in homocysteine metabolism

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Abstract. *Objective:* Elevated serum level of homocysteine is one of the most effective biomarker in diagnosing atherosclerotic cardiovascular disease and associated disorders. The elevated levels of homocysteine may result from mutations in genes coding vital enzymes or other environmental factors such as nutrition, medications, increased methionine intake, pregnancy, etc. The association of 36 vital micronutrients with circulating levels of homocysteine in a population of 336 free-living individuals was analyzed. *Methods:* Baseline serum homocysteine concentration was measured by enzyme-linked immunosorbent assay using Beckman Counter AU series Analyzers. Micronutrients were measured using LC-MS/MS Waters TQ-XS Tandem mass spectrometer. *Results:* Non-parametric analysis by Mann-Whitney U test as well as Pearson's correlation showed a strong association of vitamin B_9 (folate) and vitamin B_{12} with high serum levels of homocysteine. Interestingly several amino acids including asparagine, isoleucine, cysteine, citrulline, and carnitine exhibited a positive correlation with serum homocysteine. Among vitamins and minerals, vitamin A and selenium were positively correlated with circulating homocysteine. *Conclusion:* The study suggests that the serum levels of several micronutrients act as confounding variables in regulating the circulating levels of homocysteine. Several novel nutrient associations for modulating levels of homocysteine are highlighted. Given that homocysteine is a modifiable risk factor, these nutrients could play an important role in preventive care settings and should be further investigated clinically.

Key words: homocysteine, micronutrients, methionine, vitamins, amino acids, and cardiovascular diseases

Introduction

Cardiovascular disease (CVD) accounts for more than 25% of deaths in the United States and is also the major cause of morbidity. Diet and nutrition play a crucial role in the pathogenesis of CVD and are important factors that can be used to prevent or delay the onset of CVD (1-3). Predictive risk factors such as lipid concentrations, diabetes mellitus, and blood pressure are frequently used to measure the effect of diet on disease prevention (4). Studying these predictive biomarkers often measures a single factor, whereas the effect of dietary changes involves multiple factors. Several observational studies have reported elevated levels of circulating homocysteine and its association with an increased risk of CVD. It is also an independent marker for atherosclerotic cardiovascular disease. Elevated levels of homocysteine are associated with various other metabolic disorders such as Alzheimer's disease and Parkinson's disease (5, 6). McCully postulated the theory of homocysteine-derived atherosclerosis in 1969. He had observed 2 rare distinct genetic conditions, a disorder in vitamin B_{12} which inhibits the conversion of homocysteine to methionine in a vitamin

B_{12}-dependent reaction, and cystathionine β-synthase deficiency might result in homocystinuria, cystathioninuria, and methylmalonic aciduria (7).

Homocysteine is a sulfhydryl-containing essential amino acid and an intermediate metabolite in the biosynthesis of cysteine from methionine. Dietary methionine is the most common source of homocysteine which is produced via the demethylation of methionine. The circulating levels of homocysteine are regulated via various micronutrient-dependent pathways (8, 9). Free homocysteine can be converted to methionine by re-methylation of circulating homocysteine, this reaction is catalyzed by enzymes N5, N10-methyltetrahydrofolate reductase as a methyl donor with cobalamin (vitamin B_{12}) as a cofactor (10). Cystine, a semi-essential amino acid is synthesized from free homocysteine catalyzed by the enzyme cystathionine β-synthase and pyridoxine (Vit B_6) as a cofactor (11).

Numerous case-control, retrospective, and prospective studies have reported the correlation between various essential micronutrients and serum homocysteine (12-14). The elevated levels of serum homocysteine may result from mutations in genes coding vital enzymes of homocysteine metabolism or other environmental factors such as increased methionine intake, medications, pregnancy, etc. In addition to these factors' deficiency of vitamins and vitamin, cofactors are the most preventable cause of hyperhomocysteinemia. Several reports have shown that supplementing certain vitamins such as folate, vitamin B12, and B6 reduces serum levels of homocysteine (15, 16). An imbalance of methionine, which is mostly found in animal proteins, and a lack of vitamins B6, B12, and folate, may result in increasing the level of homocysteine in the body. Reports have suggested that higher consumption of total folate was linked to a decreased risk of glaucoma, but the protective effect was more significant when folate was obtained through supplementation in addition to dietary intake (17, 18). All those studies widely discuss only vitamins, the present study attempted to demonstrate the clinical importance of the diverse classes of micronutrients. Various micronutrients and amino acids such as folate, vitamin $B_{12,}$ and homocysteine are known to interact in one-carbon metabolism and are considered vital elements for cellular growth and differentiation (18). Hence, we hypothesized that the serum levels of micronutrients play a crucial role in regulating free homocysteine, and in the present study we investigated the association of 36 vital micronutrients with circulating levels of homocysteine in a population of 336 free-living individuals.

Materials and methods

Study population

The study included a total of 336 free-living general population who were tested for cardiovascular panel and micronutrient panel at Vibrant America Clinical Laboratory with no clinical indications of abnormalities. This was a retrospective study on deidentified individuals and hence exempted from formal ethical review by Western IRB (IRB # 1-1288754-1) (Washington USA).

Determination of serum micronutrients and homocysteine concentrations

Blood samples were processed for the separation of serum and further analyzed for serum concentrations of various micronutrients and homocysteine. Total serum homocysteine levels were estimated by in-vitro quantitative enzyme-linked immunosorbent assay using Beckman Counter AU series Analyzers. The serum level of homocysteine is measured as the inverse of the amount of NADH converted to NAD+ which can be measured at 340nm. Serum levels of micronutrients were determined using LC-MS/MS Waters TQ-XS Tandem mass spectrometer, Waters GC-MS, and Perkin Elmer NexION ICP-MS instrumentation using standard protocols.

Statistical analysis

Retrospective analysis of clinical data from deidentified subjects was performed using Java for Windows version 1.8.161. Homocysteine serum levels were stratified into four quartiles (Q1 to Q4) based on the cutoff points at 25^{th}, $50^{th,}$ and 75^{th} percentile values. The data was analyzed by a non-parametric Mann-Whitney U test for the evaluation of the association between serum levels of homocysteine and micronutrients. The univariant relationship between

serum levels of homocysteine and micronutrients was analyzed by Pearson's correlation with significance set at $p<0.05$. GraphPad Prism Version 7.00 was used to perform statistical analysis and descriptive statistics were used to express the continuous variables (mean ± SD, median, minimum, and maximum)

Results

The study comprised 336 individuals with a mean age of 48.7±15.0 and mean homocysteine was 9.9±3.0 μmol/L. The experimental population was stratified into quartiles with mean homocysteine of 6.9±0.9, 8.6±0.3, 10.2±0.6, and 14.0±3.1 for 25th, 50th, 75th, and >75th percentile values respectively. Age was found to have a positive trend and a strong significance with increasing levels of serum homocysteine (Table S1). Serum levels of cysteine and citrulline were also found to have a positive trend with an increase in serum levels of homocysteine. Vitamin B_9 (folate) and vitamin B_{12} were found to have strong significance and negative trends with increasing concentrations of homocysteine.

Non-parametric analysis by the Mann-Whitney U test showed a strong association between high serum levels of homocysteine with vitamin B_9 (folate) and vitamin B_{12} (Table 1). This result was further supported by Person's correlation analysis where vitamin

Table 1. Significant association of serum homocysteine with micronutrients.

		Greater than Reference Range (n=22)	Within range (n=314)	P ($P<0.05$)
		Baseline (X±SD)	Baseline (X±SD)	
Water-soluble vitamins	Vitamin B_1	17.2±9.4	21.8±15.3	0.2445
	Vitamin B_2	23.9±22.4	28.5±25.1	0.0682
	Vitamin B_3	18.5±6.9	19.1±8.7	0.8930
	Vitamin B_5	87.4±97.2	109.6±87.9	0.0635
	Vitamin B_6	13.8±14.2	16.5±14	0.1386
	Vitamin B_9	9.6±4.7	13.9±3.9	<0.0001
	Vitamin B_{12}	566.3±259.9	747.5±330.8	0.0032
	Vitamin C	0.44±0.28	0.52±0.52	0.8036
Fat-soluble vitamins	Vitamin A	81.0±30.8	77.1±23.9	0.8206
	Vitamin D_3	0.96±0.38	0.92±0.27	0.9322
	Vitamin D_{25} OH	43.6±20.6	48.2±23.2	0.3695
	Vitamin K_1	1.5±1.0	1.5±1.4	0.7364
	Vitamin K_2	0.66±0.84	0.72±1.2	0.5161
	Vitamin E	13.7±5.3	13.4±4.6	0.7837
Minerals	Selenium	147 ± 19.0	143.2±29.6	0.1309
	Sodium	140.3±3.0	141.7±3.4	0.0560
	Potassium	4.4±0.43	4.5±0.39	0.2598
	Calcium	9.7±0.3	9.6±0.4	0.0580
	Manganese	0.72±0.47	0.82±0.38	0.0841
	Zinc	0.68±0.12	0.71±0.16	0.2706
	Copper	1.0±0.3	1.0±0.3	0.2375
	Chromium	0.22±0.12	0.25±0.16	0.8063
	Iron	106.1±40.2	103.1±39.1	0.5325
	Magnesium	2.1±0.25	2.2±0.1	0.0855

Table 1 (*Continued*)

		Greater than Reference Range (n=22)	Within range (n=314)	P (*P<0.05*)
		Baseline (X±SD)	Baseline (X±SD)	
Non-essential AA	Asparagine	57.6 ± 10.9	53.5±12	0.0642
	Serine	145.4 ± 32.7	147.8±34.4	0.8101
BCAA	Isoleucine	77.7±26.4	68.4±20.1	0.1392
	Valine	255.3±55.9	245.5±55.8	0.3863
	Leucine	170.8±34.0	163.3±34.9	0.3140
Conditionally essential AA	Cysteine	22.2 ± 14.6	18.1±8.8	0.4027
	Glutamine	500 ± 103.1	504.8 ± 80.2	0.8700
	Citrulline	32.3±13.2	30.0±8.1	0.9183
	Arginine	131.9±53.7	122.4±31.7	0.8612
	Coenzyme Q10	2.2±1.0	2.1±0.9	0.5241
	Choline	14±4.6	14.0±5.7	0.8070
	Inositol	36.5±9.3	37.1±10.1	0.9253
	Carnitine	30.0±6.0	27±7.2	0.0203
	Methylmalonic acid	0.3±0.11	0.3±0.17	0.5839

Baseline values are expressed as mean (X) ± standard deviation (SD). For intergroup p-value, an unpaired t-student test was used.

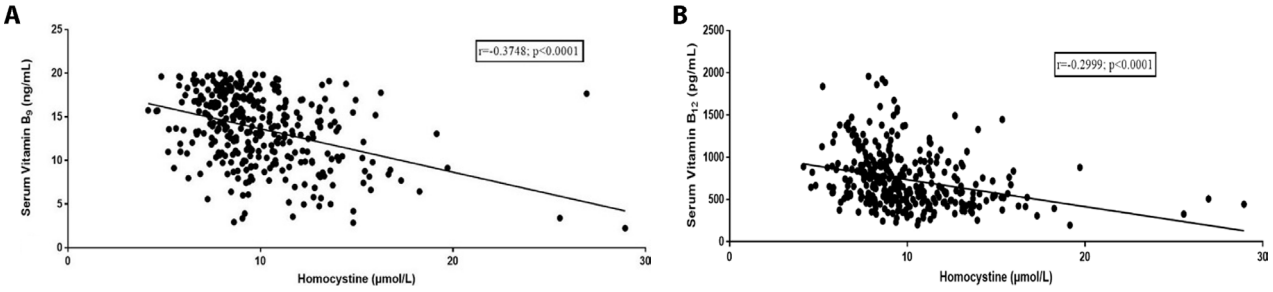

Figure 1. Pearson's correlation coefficients between homocysteine and micronutrients. (a) vitamin B_9. (b) vitamin B_{12}.

B_9 (r=-0.3748, p<0.0001) (Figure 1) and vitamin B_{12} (r=-0.2999, p<0.0001) (Figure 2) exhibited a strong negative correlation with serum concentrations of homocysteine (Table 2). Vitamin A was found to have a significant positive correlation with homocysteine (r=0.1724, p<0.0015). Among the minerals analyzed selenium (r=0.1255, p<0.0214) was found to have a positive correlation with circulating homocysteine. In the case of amino acids, BCAA isoleucine, and non-essential amino acid asparagine were found to have a positive correlation with serum homocysteine. Among other amino acids, Cystine was found to have a strong positive correlation (r=0.3216, p<0.0001) (Fig 3) whereas citrulline had a significant positive correlation with homocysteine.

Discussion

Homocysteine metabolism is regulated by several pathways depending on various metabolic enzymes and cofactors. Cystathionine β-synthase (CBS), methyltetrahydrofolate reductase (MTHFR), 5-methyltetrahydrofolate-homocysteine methyltransferase

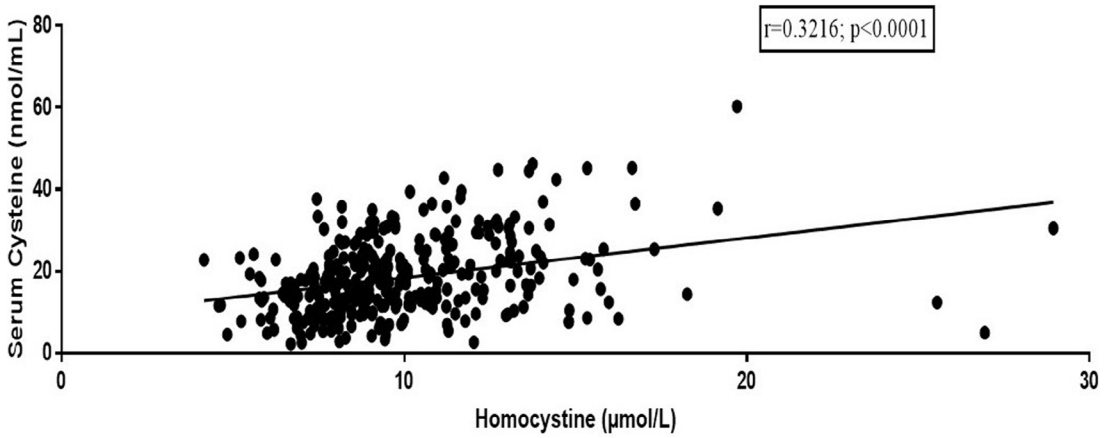

Figure 2. Pearson's correlation coefficients of homocysteine with cystine.

Table 2. Correlation of micronutrients with serum homocysteine.

		Correlation coefficient	
		r	p
Water-soluble vitamins	Vitamin B_1	-0.05311	0.3318
	Vitamin B_2	-0.07121	0.1929
	Vitamin B_3	0.0127	0.8165
	Vitamin B_5	-0.09271	0.0898
	Vitamin B_6	-0.03308	0.5457
	Vitamin B_9	-0.3748	<0.0001
	Vitamin B_{12}	-0.2999	<0.0001
	Vitamin C	0.04608	0.3999
Fat-soluble vitamins	Vitamin A	0.1724	0.0015
	Vitamin D_3	-0.00537	0.9219
	Vitamin D_{25} OH	-0.06897	0.2073
	Vitamin K_1	0.02252	0.6809
	Vitamin K_2	-0.01648	0.7635
	Vitamin E	-0.04578	0.4029
Minerals	Selenium	0.1255	0.0214
	Sodium	0.0395	0.4705
	Potassium	-0.06882	0.2083
	Calcium	0.05303	0.3325
	Manganese	0.007346	0.8933
	Zinc	-0.08034	0.1417
	Copper	-0.05889	0.2818
	Chromium	-0.0265	0.6283
	Iron	0.004576	0.9334
	Magnesium	0.03044	0.5781
Non-essential AA	Asparagine	0.1087	0.0464
	Serine	-0.01411	0.7967
BCAA	Isoleucine	0.1326	0.015
	Valine	0.04892	0.3714
	Leucine	0.07977	0.1445
Conditionally essential AA	Cysteine	0.3216	<0.0001
	Glutamine	0.05482	0.3164
	Citrulline	0.1346	0.0135
	Arginine	0.05025	0.3585
	Coenzyme Q10	0.07233	0.186
	Choline	0.08041	0.1413
	Inositol	-0.01533	0.7795
	Carnitine	0.1156	0.0342
	Methylmalonic acid	0.09457	0.0835

*Data are given as Pearson's correlation (r) coefficients with adjustment for serum homocysteine

reductase (MTRR), methionine synthase (MTR), betaine-homocysteine S-methyltransferase (BHMT) are the major enzymes involved in homocysteine metabolism (19). Vitamin B_6, vitamin B_9 (folate), and vitamin B_{12} are the vital cofactors for homocysteine metabolism. Upregulation of homocysteine can result

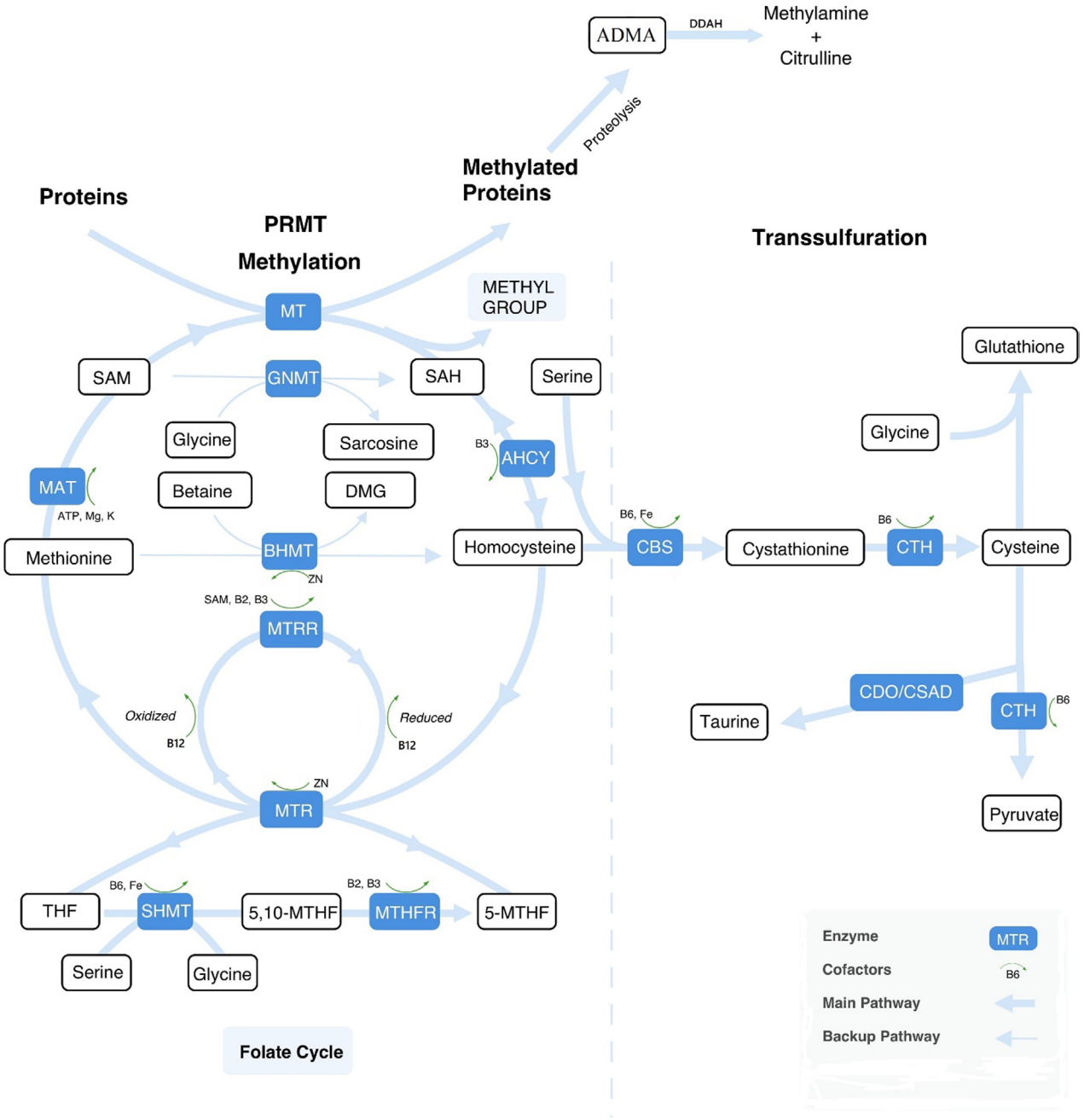

Figure 3. Diagrammatic representation of homocysteine metabolism detailing the interactions of cofactors vitamin B_6, vitamin B_9, and vitamin B_{12}. Abbreviations: PRMT: Protein Arginine Methyltransferase; ADMA: asymmetric dimethylarginine; MT: Methyl transferase; SAM: S-adenosyl methionine; SAH: S-adenosyl homocysteine; ACHY: Adenosylhomocysteinase; MAT: methionine adenosyltransferase; BHMT: Betaine-homocysteine methyltransferase; MTRR: methionine synthase reductase; SHMT: serine hydroxymethyltransferase; THF: Tetrahydrofolate; 5,10-MTHF: 5,10-methylene tetrahydrofolate; CBS: Cystathionine beta-synthase; CTH: cystathionine γ-lyase; CDO: Cysteine dioxygenase; CSAD: Cysteine Sulfinic Acid Decarboxylase; DDAH: Dimethylarginine Dimethylminohydrolase; DMG: Dimethylglycine; MTHFR: Methylenetetrahydrofolate reductase.

from two major factors, genetic enzymatic disorders, and inadequate cofactors (20). Several novel nutrient associations were found in our study.

Our population-based retrospective study of 336 individuals showed a linear relationship between serum levels of homocysteine with an increase in the age of the individuals and vital nutrients such as vitamin A, B_9, B_{12}, selenium, and amino acids such as asparagine, isoleucine, cysteine, and citrulline. These clinical variables particularly cysteine, vitamin B_9 (folate), and vitamin B_{12} are known to be regular harbingers of various metabolic disorders including chronic cardiovascular diseases while our study has found new associations due to the extensive testing of nutrients (21,22). Age is one of the prime causes of the upregulation of serum homocysteine resulting in various age-associated disorders particularly poor wound healing, loss of regenerative ability, decreased renal and cognitive functioning, endothelial dysfunction, etc (23,24). Although the mechanism between the association of homocysteine with aging and associated disorders remains unclear, the current study clearly shows that the increase in the serum levels of homocysteine has a significant linear association with aging (Table 1). Ostrakhovitch et al. 2019 (23) have reported the association of elevated serum levels of homocysteine with aging. Age is associated with several other compilations such as reduction in absorption of proteins and other nutrients resulting in dietary restriction of vital vitamin cofactors such as vitamin B_6, B_9, and B_{12} these, in turn, lead to increased levels of serum homocysteine (25).

The inability to regulate the methionine pathway results in the accumulation of homocysteine which can be attributed to various endogenous and exogenous factors (26). The endogenous factors include SNPs in the genes coding the enzymes involved in methionine metabolism (Fig 3), which can be regulated by enzyme therapy or nutritional support (27). Nutritional support in the form of vital cofactors such as vitamin B_6, B_9, and B_{12} regulates the supply of methyl group and ensures the biochemical pathway for methylation of homocysteine to methionine or induces the transsulfuration pathway with the help of serine to convert the serum homocysteine into cysteine (28). The current data demonstrated a negative trend between increasing serum levels of homocysteine with vitamins B_9 (folate) and B_{12}. Non-parametric analysis by Man Whitney and univariant analysis by Pearson's exhibited a significant association and a strong negative correlation of vitamins B_9 and B_{12} with circulating levels of homocysteine. These results signify that a decrease in the serum level of vitamin B_9 and B_{12} increases the circulating levels of homocysteine by limiting the availability of methyl groups and inhibiting methylation. Numerous studies have reported the protective effect of vitamins by downregulating homocysteine. It's also been reported that dietary supplementation of vitamin B_1, vitamin B_2, vitamin B_9, and vitamin C was inversely associated with the development of hyperhomocysteinemia (29).

Our data exhibited a positive correlation between vitamin A and homocysteine. This association might result from the altered inflammatory and lipid metabolism at the hepatic level. Olsen et al. (2018) (30) observed a similar association where the patients with high total homocysteine were reported to have elevated levels of vitamin A. Inflammatory markers such as oxysterols produced during inflammatory conditions such as non-alcoholic fatty liver disease induces the hepatic expression of genes responsible for the synthesis of all-trans-Retinoic acid, a potential activator of glycine N-methyltransferase (GNMT). The activation of GNMT accelerates the synthesis of SAH (S-Adenosyl-L-homocysteine) and homocysteine (Fig 3) (31).

Minerals are the most vital nutrients with diverse functionalities in maintaining body homeostasis and metabolism. The present study included various vital minerals, among all the minerals selenium was noted with a positive correlation with serum homocysteine. Selenium is a vital trace mineral with wide interindividual variability in metabolic activities (32). Various reports have demonstrated selenium as a potential factor in altering total homocysteine. Considerable reports have described the role of selenoproteins in various metabolic pathways, particularly thyroid

synthesis, and homocysteine, but the detailed mechanism remains unclear (33). Stranges et al. (2010) (34) in their study on the association of high selenium with blood lipids observed a positive correlation between plasma selenium and total cholesterol and non-HDL cholesterol. This was one of the notable negative effects of elevated serum selenium.

Increased citrulline levels with elevated homocysteine is a novel finding in our study. The increase in the serum levels of homocysteine causes decreased nitric oxide (NO) bioavailability by inhibiting endothelial nitric oxide synthase (eNOS) induced by asymmetric dimethylarginine (ADMA) in hyperhomocysteinemia. Plasma levels of asymmetric dimethylarginine (ADMA) increase rapidly after acute methionine intake, which increases intracellular levels of S-adenosylmethionine (SAM). This accelerates the production of ADMA by inducing SAM-dependent protein arginine methyltransferase activity (PRMT) (35). Homocysteine synthesized by hydrolysis of SAH remethylates into methionine (Figure 3), where the increase in the intake of methionine and high serum levels of homocysteine result in the conversion of arginine residues in the dietary protein into methylated protein. This results in the increase of ADMA by proteolysis of methylated protein, which inhibits eNOS resulting in the decrease of NO production driving endothelial dysfunction. Homocysteine may also elevate the serum ADMA alternatively by inducing endoplasmic reticulum stress and apoptosis, which leads to protein degradation resulting in methylarginine residues. Dimethylaminohydrolase (DDAH) is the major enzyme involved in the metabolism of ADMA, which produces citrulline and methylamine by hydrolysis of ADMA. The increase in serum levels of homocysteine or citrulline indicates NO-mediated endothelial dysfunction (36). The parallel increasing trend of homocysteine and citrulline ($p<0.05$) in the present study might be associated with ADMA-mediated citrulline synthesis.

Increased levels of homocysteine can be associated with toxicity during arthritis treatment with methotrexate. Initial homocysteine levels were predictive of toxicities such as gastrointestinal intolerance and elevations of liver enzymes (37). Methotrexate treatment causes increased serum levels of homocysteine in rheumatoid arthritis and other diseases. However, toxicity-related discontinuation of methotrexate treatment was not associated with the change in homocysteine concentration. Methotrexate works by inhibiting an enzyme involved in the metabolism of folate, which can lead to an accumulation of homocysteine in the blood. One strategy to manage increased homocysteine levels in arthritis patients receiving methotrexate is to supplement with folic acid, which can help to lower homocysteine levels. Other strategies may include dietary changes, such as increasing intake of foods rich in folate and other B vitamins, or adding additional vitamin supplements (38, 39).

Additionally, in the present study, a diverse class of amino acids was evaluated for homocysteine-mediated cardiovascular disorders. Among the analyzed branched-chain amino acids (BCAA), isoleucine was found to have a considerable positive correlation with homocysteine. BCAAs have shown associations in the development of metabolic syndrome which involves increased levels of plasma homocysteine (40). A positive correlation was also observed between carnitine and serum homocysteine. Several studies have reported the clinical significance of carnitine when supplemented as Acetyl-L-Carnitine or Propionyl- L -Carnitine. However, being metabolites of exogenous amino acids both homocysteine and carnitine are independent indicators of abnormal amino acid metabolism (41). Hence the association of carnitine with homocysteine remains complicated and requires more research.

Homocysteine can be converted to methionine through a process called remethylation. This reaction is catalyzed by either methionine synthase or betaine-homocysteine methyltransferase (Fig 3). The conversion of homocysteine to methionine requires vitamin B12 and folic acid as cofactors. A deficiency of these vitamins may lead to increased homocysteine levels. Homocysteine and its precursor methionine have been found to decrease reactive oxygen species (ROS) production in erythrocytes and inhibit erythrocytes' osmotic fragility increase and methemoglobin formation (42). Homocysteine was also demonstrated to enhance ferryl Hb reduction. Methionine is a precursor of homocysteine, and it can regulate metabolic processes, the innate immune system, and digestive functioning in mammals (43). The methionine-homocysteine cycle

is a vital process that exists at a critical biochemical intersection in the methionine cycle. High blood levels of homocysteine signal a breakdown in this process, which can result in far-reaching biochemical and life consequences.

Gene mutations that could be responsible for the increased homocysteine levels could not be considered in the study. Apart from this limitation, the present study has several highlights such as understanding the complete nutrient profile including a diverse class of micronutrients - vitamins, minerals, and amino acids. The study also presents a clear statement on the association of vital cofactors involved in homocysteine metabolism. To the best of our knowledge, this is the first report that studied the association of a diverse class of micronutrients with serum homocysteine and reported several novel associations.

Conclusion

The present study confirms the association of various dietary nutrients with elevated levels of homocysteine. Apart from the conventional biomarkers vitamins B_9 and B_{12}, the current study signifies the association of other nutrients namely vitamin A, selenium, and amino acids asparagine, cystine, isoleucine, citrulline, and carnitine as vital biomarkers affecting the levels of homocysteine. The results motivate further research into the role of nutrition in modulating the levels of inflammatory markers such as homocysteine which is of vital importance in preventive care settings.

Competing Interests: Krishnamurthy, Jayaraman, Krishna, Wang, Bei, and Rajasekaran are employees of Vibrant Sciences LLC. Reddy, Song, and Rajasekaran are employees of Vibrant America LLC. Vibrant America is a commercial diagnostic lab that could benefit from increased testing of micronutrients and cardiovascular biomarkers.

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Availability of Data and Materials: To access supporting data, contact the corresponding author.

Authors' Contributions: Hari Krishnamurthy, Karthik Krishna, and Tianhao Wang performed the research. Hari Krishnamurthy, John J. Rajasekaran, Karenah Rajasekaran, and Vasanth Jayaraman designed the study. Qi Song, Kang Bei, and Swarnkumar Reddy analyzed the data. Hari Krishnamurthy and Swarnkumar Reddy wrote the article.

Institutional Review Board Statement: The study comprises retrospective analysis exempted by the Western Institutional Review Board.

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Appendix

Supplementary files

Table S1. General characteristics of the all-study subjects according to the categories of serum homocysteine

	Characteristics	Quartiles of serum homocysteine levels					
		Total (n=336)	Q1 (n=84)	Q2 (n=84)	Q3 (n=85)	Q4 (n=83)	p
	Homocysteine	9.9±3.0 (4.1-28.9)	6.9±0.9 (4.1-8.0)	8.6±0.3 (8.0-9.3)	10.2±0.6 (9.3-11.3)	14.0±3.1 (11.3-28.9)	
	Age	48.7±15.0	41.9±15.0	48.6±14.1	50.1±14.7	54.3±13.6	<0.0001
Water- soluble vitamins	Vitamin B1	21.6±15.0	19.8±12.0	24.8±19.3	21.6±14.3	20.0±13.2	0.1113
	Vitamin B2	28.2±25.0	26.3±18.3	34.1±35.7	28.5±22.7	23.9±18.3	0.0531
	Vitamin B3	19.1±88.6	17.6±7.0	19.0±7.3	21.4±11.9	18.3±6.9	0.0232
	Vitamin B5	108.1±88.6	117.6±85.0	103.9±82.4	113.9±100.1	96.9±85.5	0.4184
	Vitamin B6	17.0±18.5	15.3±12.5	17.7±13.0	18.7±17.3	16.3±27.4	0.6603
	Vitamin B9	13.7±4.1	15.3±3.3	14.4±4.2	13.7±3.5	11.1±4.2	<0.0001
	Vitamin B12	735.6±329.4	866.8±326.0	811.0±355.7	659.6±300.5	604.6±261.5	<0.0001
	Vitamin C	0.5±0.3	0.5±0.4	0.4±0.2	0.5±0.2	0.4±0.3	0.6345
Fat – soluble vitamins	Vitamin A	77.5±24.4	72.9±24.3	77.2±24.5	78.3±21.0	81.4±27.2	0.1556
	Vitamin D3	0.9±0.3	0.9±0.3	1.0±0.3	0.9±0.3	0.9±0.3	0.1804
	Vitamin D 25 OH	47.9±23.0	48.5±21.1	47.9±22.5	51.2±26.5	43.9±21.4	0.2329
	Vitamin K1	1.6±1.4	1.5±1.1	1.8±1.8	1.5±1.0	1.6±1.4	0.5231
	Vitamin K2	0.7±1.2	0.8±1.6	0.6±0.7	0.8±1.6	0.6±0.8	0.5561
	Vitamin E	13.4±4.7	13.7±5.4	13.4±4.4	13.9±4.8	12.8±4.2	0.4778
Minerals	Selenium	143.5±29.1	139.1±22.6	138.2±24.6	150.1±40.5	146.5±23.3	0.0179
	Sodium	141.6±3.4	141.0±3.0	141.8±3.2	141.9±3.4	141.8±4.0	0.2324
	Potassium	4.5±0.4	4.6±0.4	4.5±0.4	4.6±0.4	4.5±0.4	0.3445
	Calcium	9.7±0.4	9.6±0.4	9.7±0.4	9.6±0.4	9.7±0.5	0.4712
	Manganese	0.7±0.5	0.8±0.7	0.7±0.3	0.7±0.4	0.7±0.3	0.5526
	Zinc	0.7±0.2	0.7±0.2	0.7±0.2	0.7±0.2	0.7±0.1	0.3060
	Copper	1.1±0.3	1.1±0.3	1.1±0.4	1.0±0.3	1.0±0.3	0.8545
	Chromium	0.3±0.2	0.2±0.1	0.3±0.1	0.3±0.2	0.2±0.2	0.2969
	Iron	103.3±39.1	101.5±42.3	104. 9±35.6	108.4±43.2	98.2±34.5	0.3682
	Magnesium	2.2±0.2	2.2±0.2	2.2±0.2	2.2±0.2	2.2±0.2	0.0751
Non-essential AA	Asparagine	53.8±12.0	52.4±11.9	53.7±13.3	54.1±12.0	55.1±10.5	0.5552
	Serine	147.7±34.3	145.3±32.8	151.7±36.7	149.9±36.6	143.7±30.4	0.3894

	Characteristics	Quartiles of serum homocysteine levels					
		Total (n=336)	Q1 (n=84)	Q2 (n=84)	Q3 (n=85)	Q4 (n=83)	p
BCAA	Isoleucine	69.1±20.7	63.7±15.2	70.3±21.5	69.3±22.6	73.1±21.8	0.0271
	Valine	246.2±55.8	235.9±51.8	252.5±59.7	243.8±54.3	252.5±56.4	0.1604
	Leucine	163.8±34.9	155.2±33.9	164.6±36.1	167.3±34.0	168.0±34.5	0.0634
Conditionally essential AA	Cysteine	18.4±9.3	14.0±6.8	17.5±7.7	18.7±8.4	23.5±11.3	<0.0001
	Glutamine	504.5±81.8	494.9±87.0	501.8±78.8	511.5±78.9	509.7±82.6	0.5305
	Citrulline	30.2±8.6	28.3±9.1	29.7±7.5	30.3±7.6	32.6±9.5	0.0121
	Arginine	123.0±33.6	122.5±32.4	121.7±33.0	123.7±33.5	124.3±36.0	0.9596
	Coenzyme Q10	2.1±0.9	1.9±0.9	2.1±0.9	2.2±0.9	2.2±1.1	0.1563
	Choline	14.0±5.7	13.0±5.5	13.9±5.8	14.4±5.5	14.7±5.8	0.2055
	Inositol	37.1±10.1	36.1±10.4	38.6±9.9	37.4±10.0	36.3±10.1	0.3716
	Carnitine	27.2±7.2	25.9±7.5	27.1±6.7	27.8±6.9	28.0±7.8	0.2253
	MMA	0.3±0.2	0.3±0.1	0.3±0.1	0.3±0.2	0.3±0.3	0.0337