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Vitamin B complex and homocysteine levels - A trend study among Asian Indians

Sandhya Iyer, Prachi Sinkar, Krishnakumar S, Kallathikumar K, Amruta V

Analytical Chemistry and Biochemistry Division, Thyrocare Technologies Limited, Plot No.D37/1,TTC Industrial area, MIDC, Turbhe, Navi Mumbai - 400703, India

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ABSTRACT

Background: Vitamin B complex levels have been strongly correlated with homocysteine (Hcy) status and many studies suggest supplementation with vitamin B to lower cardiovascular risk among adults. However, this relationship does not show a direct trend with many studies concluding otherwise. **Aims and Objective:** Our report is an attempt to study association between levels of vitamin B6, folate and B12 in relation to homocysteine in a Pan-India cohort of over 5000 individuals **Materials and Methods:** Data from a total of 5487 Asian Indians including 2942 males and 2545 females were considered for this study. Analysis for levels of vitamins B6 and folate was done using the technology of Liquid Chromatography - Mass Spectrometry (LC-MS), while that for vitamin B12 and homocysteine was done using the Chemiluminescence Immunoassay (CLIA). **Results:** Our analysis identified a clear correlation between vitamin B12 status and levels of homocysteine, while no such trend was observed with vitamin B6 and folate. The total frequency of vitamin B12 deficiency was detected to be 25% of which 50% exhibited clinically high homocysteine levels. The frequency among males was found to be high at 61.6% in comparison to females at 32.3% with the difference being statistically significant at $P<0.0001$. **Conclusion:** Our report is one of the first few to document levels of vitamin B6, folate and B12 in relation to homocysteine in a large Asian Indian cohort. Low levels of vitamin B12 was found to have a greater impact on Hcy levels in comparison to other B-vitamins studied.

Key words: Asian Indians; Vitamin B6; Folate; Vitamin B12; Homocysteine

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INTRODUCTION

The vitamin B group involving vitamin B2, B6, folate and B12 have been linked to levels of homocysteine (Hcy). Hcy is a metabolite of an essential amino acid methionine and high levels of the same have been linked with micronutrient deficiency, certain medications as well as genetic defects. Epidemiological studies indicate risk for vascular and thrombotic disease with elevated Hcy, though many early trials on B vitamin therapy did not indicate reduction of stroke with high doses of folate/B6 and cyanocobalamin. The findings led to the conclusion that folate alone is not an optimal solution to lower total Hcy, but inclusion with methylcobalamin or oxocobalamin should be considered.[1,2]

In case of vitamin B12 (cobalamin) deficiency indicator, elevated Hcy levels only account for a minor fraction, while other contributing factors include lifestyle choices like alcohol and drug abuse.[3] Traditionally studies have identified a cut-off of 200 ng/L to identify unequivocal cobalamin deficiency though many further studies have identified abnormalities in levels of total Hcy (tHcy) and/or methyl malonic acid (MMA), among individuals with cobalamin values of 300 ng/L to 350 ng/L.[4,5] Clinical deficiency of cobalamin has been classified as a serious medical condition and hence distinguishing clinical from subclinical deficiency due to malabsorption becomes very important.

Hcy is normally present in all healthy individuals at low plasma levels of between 5 - 50 μmol/L, the high

Address for Correspondence:
Dr. Sandhya Iyer, Thyrocare Technologies Limited, Plot No. D37/1, TTC Industrial area, MIDC, Turbhe, Navi Mumbai - 400703,India. **Phone:** +91- 9820423640. **E-mail:** sandhya.iyer@thyrocare.com

concentrations of which can be lowered through supplementation with folate, vitamin B6 and B12 combinations.[1,6] Hcy levels of over 50 µmol/l is clinically classified as severe hyperhomocysteinaemia and generally is seen among children homozygous for inborn errors of metabolism and adults with acquired nutritional deficiencies or renal disease which can also cause mild (15 - 20 µmol/L) to moderate (25 - 50 µmol/L) hyperhomocysteinaemia.[1] Studies have also highlighted an inverse correlation between serum Hcy levels and vitamin B6, B12 and folate even among those individuals with low-normal levels of the latter.[7,8]

Folate has also been studied with relation to Hcy status and a Nutrition Canada Survey identified a 69% increase towards relative risk of fatal coronary heart disease among individuals with lowest serum folate.[9] In case of vitamin B12 and hyperhomocysteinaemia, the concern remains active as B12 deficiency is a condition even among developed nations. Deficiency studies have recorded prevalence among children and young adults from India and Israel.[10, 11] Reports also indicate folic acid fortification to have actually accentuated deficiency of vitamin B12 as observations indicate folic acid to reverse megaloblastic anemia condition caused by B12 deficiency, but the neurological degeneration generally continues undiagnosed due to the underlying deficiency.[1]

In the Indian scenario, prevalence of coronary artery disease (CAD) continues to be high and several studies have linked it towards high Hcy levels as an independent risk factor.[12] In establishing link between vitamin intake and stroke risk, though observational studies identified high Hcy levels with adverse cerebrovascular and cardiovascular outcomes, but in prospective studies, the association was found to be weak.[13]

Our report is an attempt to present status of vitamins B12, folate and B6 along with Hcy levels studied in a large cohort of Asian Indians of both the genders to identify prevalence.

MATERIALS AND METHODS

Study cohort

Data from a total of 5487 pan-India individuals were considered for analysis, including 2942 males and 2545 females respectively. The need for informed consent was deviated from in this retrospective study as the data was analyzed from tests done in a reference lab and not a hospital setting. Apart from gender and age no other patient identifiers were included in this analysis. The entire cohort characteristics have been highlighted in Table 1.

Analysis of Vitamins B12, B6, folate and Hcy

Vitamin B12 estimation from serum was done using the technology of CLIA on the platform Advia Centaur (Siemens, USA). The ADVIA Centaur® VB12 assay kit was used for the competitive immunoassay using direct chemiluminescent technology. The test has an assay range of 45 – 2000 pg/mL. For quality control (QC) analysis, two different levels of serum controls were run with each run. Biorad Immunoassay Controls were used and the observations were plotted and analyzed for outliers using the Levey-Jennings chart.

Hcy analysis from serum was done using CLIA chemistry on the platform of Advia Centaur (Siemens, USA). The ADVIA Centaur® HCY assay competitive immunoassay test involves reduction of free Hcy in the serum, followed by conversion to SAH (S-adenosylhomocysteine) which is then coupled to the paramagnetic particles in phosphate buffer with BSA. Monoclonal mouse anti SAH antibody labeled with acridinium ester in PBS was used as the lite reagent and the relative light units (RLUs) measured by the system. The assay range was <0.50 – 65 µmol/L. Quality control analysis involved running two different levels of serum controls twice a day and for Hcy, BioRad Homocysteine Controls were put to use. Trend analysis and outlier detection was done using LJ plots.

Estimation of vitamin B6 and folate was done using the analytical platform of Liquid Chromatography Mass Spectrometry (LC-MS, Shimadzu Corp., Japan). Serum samples were treated and subjected to LC analysis using Kinetix 2.6 µm C18 column and a gradient mobile phase involving 0.1% formic acid deionized water and 0.1% formic acid methanol, with a run time of 6 minutes. The MS analysis involved an ESI (Electrospray ionization) source. Spiking analysis was done as part of quality control procedure as commercial certified reference material for vitamin B complex for LC-MS technology remains unavailable. Appropriate statistical analysis and bias was calculated with run each day to assess %CV to detect outliers.

Table 1 : Study cohort features

Cohort	N	Average age (Years)	Mean Hcy levels (µmol/L)	Mean vitamin B12 levels (pg/mL)	Mean vitamin B6 levels (ng/mL)	Mean vitamin folate levels (ng/mL)
Males	2942	44 +/- 14	32 +/- 14	343 +/- 266	18.8 +/- 12	0.8 +/- 2.6
Females	2545	45 +/- 15	23 +/- 10	380 +/- 312	16.5 +/- 12	1.0 +/- 3.2
Total	5487	45 +/- 14	28 +/- 13	360 +/- 289	17.8 +/- 12	0.9 +/- 2.9

RESULTS

Level of vitamins B12, folate, B6 and homocysteine was analyzed in a cohort of 5487 adults inclusive of 2942 males (44 +/- 14 years) and 2545 females (45 +/- 15 years) respectively. Estimation of Hcy done by competitive direct CLIA methodology in serum, levels of <30 µmol/L was considered to be low risk for reporting.

In case of vitamin B12 estimation in serum by CLIA technology, levels of 211 - 911 pg/mL was the normal reporting range as per manufacturer instruction. Analysis for low vitamin B12 detected the frequency to be 25.0%, and further among males it was higher at 28.1% in comparison to females at 21.5% respectively, This difference was also extremely statistically significant at $P < 0.0001$ by the two-tailed Fishers exact test. In this cohort of low vitamin B12, the mean Hcy levels were also found to be borderline high at 35 +/- 14 µmol/L.

In case of high vitamin B12 levels, the frequency was detected to be 4.4% and among females was found to be high at 5.5% in comparison to males at 3.5%. This difference was also found to be statistically significant at $P = 0.0003$ by the two-tailed Fishers exact test. In this cohort, levels of Hcy remained normal as expected with mean values at 19 +/- 9 µmol/L. Mean levels of vitamins B6 and folate remained within normal range.

Analysis for low vitamin B12 along with high Hcy levels, detected a total of 50.0% of the low vitamin B12 cases to show this trend. Independently they accounted for 12.5% of our study cohort. In this cohort as well, the frequency among males was found to be high at 61.6% in comparison to females at 32.3% with the difference being statistically significant at $P <0.0001$ by the two-tailed Fishers exact test. The vitamin B12 analysis outcome has been represented in Tables 2 (a, b, c).

In case of vitamin B6 estimated by the technology of LC-MS, the reporting range was set at 5 - 50 ng/mL for normal. Analysis for low vitamin B6 detected a total frequency of 0.6% and no statistically significant difference in frequency among males and females in this case. The mean Hcy levels in this cohort remained around borderline high levels at 29 +/- 15 µmol/L. In case of high vitamin B6, the total frequency was detected to be 3.1% and no statistically significant difference among males and females. The mean levels of Hcy and folate remained within normal ranges, while for vitamin B12 the mean levels were detected to be 663 +/- 480 pg/mL.

In case of folate estimation by LC-MS technology, levels of 0.2 - 20 ng/mL was considered normal. Analysis of low folate levels detected the frequency to be 0.1% while the levels of Hcy remained well within normal averaging at 13 +/- 4 µmol/L. Similarly in case of high folate cases, frequency of 1.1% in our cohort, Hcy levels remained within normal ranges at 21 +/- 9.2 µmol/L. In both cases of low and high, mean levels of vitamin B6 remained within normal ranges. In case of high folate, mean levels of vitamin B12 was detected to be 828 +/- 653 pg/mL.

DISCUSSION

The association analysis between reduction in levels of Hcy in relation to levels of B vitamins have been recorded by many studies and one of the first few large scale analysis in this regards involves the Vitamin Intervention for Stroke Prevention Study (VISP). This study involved 3680 ischaemic stroke patients among whom reduction in Hcy levels were studied in correlation to high-dose vs. low-dose formulation of vitamins B6, B12 and folate. Though the study did not record any significant treatment benefit, a consistent association between Hcy baseline concentration and probability of stroke was detected. Thus hyperhomocysteinaemia is continued to be considered as a marker rather than as a cause for vascular diseases.[14]

Our report documents analysis of serum Hcy levels in relation to levels of vitamins B6, B12 and folate in a large adult Asian Indian cohort. Among all the vitamins analyzed in conjunction with Hcy levels, our study detected strongest association between vitamin B12 and Hcy levels, wherein of the 25% with clinically low vitamin B12 levels, 50% were detected to have average levels of Hcy at 46 +/- 11 µmol/L. The frequency as well as average levels among males was found to be higher in this cohort at 61.6% and 48 +/- 11 µmol/L in comparison to females at 32.3% and 42 +/- 10 µmol/L. This difference was also statistically significant at $p < 0.0001$. Few early studies among Asian Indians detected mean plasma

Table 2(a) : Low vitamin B12 analysis

Cohort	N	Frequency (%)	Age (Years)	Vit B12 (pg/mL)	Hcy (µmol/L)	Vit B6 (ng/mL)	Folate (ng/mL)
Total	1373	25.0	42 +/- 13	173 +/- 26	35 +/- 14	14 +/- 8	0.5 +/- 1.4
Male	826	28.1	42 +/- 13	172 +/- 26			
Female	547	21.5	43 +/- 13	176 +/- 25			

Table 2(b) : High vitamin B12 analysis

Cohort	N	Frequency (%)	Age (Years)	Vit B12 (pg/mL)	Hcy (µmol/L)	Vit B6 (ng/mL)	Folate (ng/mL)
Total	241	4.4	56 +/- 15	1468 +/- 401	19 +/- 9	28 +/- 19	3.6 +/- 7.2
Male	102	3.5	55 +/- 17	1466 +/- 403			
Female	139	5.5	57 +/- 15	1469 +/- 401			

Table 2(c) : Summary of high Hcy cases in low vitamin B12 cohort

Cohort	N	Frequency (%)	Age (Years)	Hcy (µmol/L)	Vit B12 (pg/mL)	Vit B6 (ng/mL)	Folate (ng/nL)
Total	686	50.0	43 +/- 14	46 +/- 11	169 +/- 27	14.4 +/- 8	0.4 +/- 0.5
Male	509	61.6	42 +/- 14	48 +/- 11	168 +/- 27		
Female	177	32.3	45 +/- 15	42 +/- 10	170 +/- 26		

Hcy levels to be 19.8 mmol/L with 77% exhibiting hyperhomocysteinemia and over 50% having vitamin B12 deficiency.[10]

Elevated levels of vitamin B12 have also been studied as a sign for liver damage and few studies have assessed its significance among chronic stable heart failure (HF) cases. One such study carried out in 129 HF patients identified baseline vitamin B12 levels to be higher in comparison to controls with median levels of 311 pg/mL and 235 pg/mL among those with and without right sided HF. Median B12 levels was also detected to be high among those patients who subsequently died in comparison to survivors. This study identified increased vitamin B12 among stable HF to be associated with high direct bilirubin levels indicating a cardiohepatic syndrome.[15] In our analysis, of the 4.4% detected with high vitamin B12 levels, the levels of Hcy, vitamin B6 and folate were within normal ranges; 19 +/- 9 µmol/L, 28 +/- 19 ng/mL and 3.6 +/- 7.2 ng/mL respectively. We did not assess serum bilirubin levels among these cases.

Levels of vitamin B6 have also been inversely associated with total Hcy levels, indicating low blood levels to lead to hyperhomocysteinemia albeit to a lesser extent in comparison to vitamin B12.[16] Effects of high-dose vitamin B6 on Hcy levels among schizophrenic patients have also detected statistically significant benefit to be more pronounced among male patients with schizophrenia or schizoaffective disorders.[17] Another study among Indians which focused on reporting Hcy levels in relation to folate and vitamin B6 status in 40 apparently normal males detected no significant relationship between fasting Hcy levels and vitamin B6 status.[18] In our analysis, the frequency of low vitamin B6 was detected to be just 0.6% and this cohort has Hcy levels of 29 +/- 15 µmol/L, indicating presence of borderline high Hcy. In this cohort, levels of vitamin B12 and folate were found to be in the normal range.

Folate has also been studied in relation to hyperhomocysteinemia and premature CAD. One such study from Tehran among 294 individuals of which 43.1% has CAD identified 10.7% to have folate deficiency, while 26.6% exhibited vitamin B12 deficiency. This study concluded male gender with vitamin B12 deficiency exhibited hyperhomocysteinemia which was an independent risk factor for CAD among young patients, below 45 years of age.[19] Our analysis identified the frequency of low folate to be just 0.1% and the cohort exhibited normal mean levels of Hcy, vitamins B12 and B6 at 13 +/- 4 µmol/L, 373 +/- 112 pg/mL and 7.5 +/- 2.4 ng/mL respectively.

In our study, a high profound effect of Hcy levels was found to bear a greater association with vitamin B12 status rather than status of vitamin B6 or folate. This large cohort analysis highlights the status of all three crucial B vitamins like B6, folate and B12 in conjunction with Hcy levels and highlights trend among Asian Indians.

CONCLUSION

Many studies have evaluated relation between Hcy-lowering interventions involving Vitamin B complex therapy for preventing cardiovascular events detected no positive effects of the same.[20] However, the hypothesis on the relation still remains and is actively studied. Our study though did not analyze the effect of any intervention, was focused on delivering analysis outcome of trend between Hcy levels in relation to mean levels of vitamin B6, folate and B12. Our large scale cohort analysis indicates vitamin B12 deficiency to have a high bearing on elevated Hcy levels.

Ethical approval

Not required. This study involved data analysis from a reference laboratory and only details pertaining to gender and age was taken for analysis. No other patient identifiers were noted or mentioned in the manuscript to the extent of compromising patient confidentiality.

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Authors Contribution:
SI - Study design, Data analysis and interpretation, drafting of manuscript; **PS** - Study design, Proof reading, Manuscript finalization; **KK** - Collection of data, Proof reading; **KKK** - Collection of data, Interpretation, Proof reading; **AMV** - Study design, Proof reading.

Work attributed to:
Department of Analytical Chemistry, Thyrocare Technologies Limited

Orcid ID:
Dr. Sandhya Iyer- https://orcid.org/0000-0002-8872-0322
Dr. Prachi Sinkar- https://orcid.org/0000-0002-3336-5140
Mr. Krishnakumar- https://orcid.org/0000-0002-0669-3675
Mr. Kallathikumar- https://orcid.org/0000-0001-8219-7182
Ms. Amruta Velumani- https://orcid.org/0000-0002-8337-247

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