ORIGINAL RESEARCH PAPER

Clinical Biochemistry

BIOMARKERS OF CORONARY ARTERY DISEASE IN INDIANS – SHOULD WE IGNORE HOMOCYSTEINE?

KEY WORDS: CAD risk factors, cholesterols, triglycerides, homocysteine, lipoprotein(a)

Seema Bhargava* Dept of Biochemistry, Sir Ganga Ram Hospital, New Delhi-110060, India *Corresponding Author

Parul Chugh Dept of Research, Sir Ganga Ram Hospital, New Delhi, India

Ashwani Mehta Dept of Cardiology, Sir Ganga Ram Hospital, New Delhi, India

ABSTRACT

The global burden of cardiovascular disease (CVD/CAD) is still rising, more so in the developing countries with a consequent loss of DALYs. Addressing the traditional risk factors has reduced ischemic events, but prevalence continues to increase. It, hence, becomes imperative to identify and address all contributing risk factors. Management includes reduction of the cholesterols and triglycerides, but does not address homocysteinemia. Hence, we conducted a retrospective study of data from 1st January, 2021 to 31st December, 2021, to compare the significance and odds ratio conferred by traditional biomarkers and homocysteine for CAD. From amongst a North Indian urban population, 257 patients admitted for first episode of coronary artery disease and 4130 healthy controls were assessed for their circulating risk factors – the cholesterols, triglycerides, lipoprotein(a) and homocysteine. All subjects were within the age group of 18-80 years of age. The data was statistically analysed to elucidate significance of and odds ratio conferred by each of these risk factors. Multivariate regression analysis assigned significance to only HDL-cholesterol($p=0.006$), triglycerides($p<0.001$) and homocysteine($p=0.002$) with odds ratio of 0.893, 1.297 and 1.36, respectively. Current guidelines of management of CAD include lifestyle and pharmaceutical measures to reduce circulating lipoproteins [the cholesterols and Lp(a)] and triglycerides. It would benefit the Indian population to include homocysteine-reducing measures towards reduction of prevalence of CAD through adjuvant management with B vitamin supplements (including folate, B12 and B6) and/or food fortification/diversification.

Introduction

In the last three decades, epidemiological transition has caused a doubling of prevalence of non-communicable diseases from 27% in 1990 to 54% in 2017. In 2019, deaths due to cardiovascular diseases (CVDs) represented 32% of the global deaths (17.9 million) and 38% of deaths due to premature non-communicable diseases under the age of 70 years. 80% of these deaths occur in developing countries. Statistics reveal that the Indian population is 20 times more likely to suffer CVD than the Japanese and 6 times more likely than Caucasian Americans. In addition, the mean age of prevalence of CVD in Indians is 10 years younger than other populations, with the younger patients (<50 years of age) constituting 12-16% of the CVD patients as opposed to 5% in other populations.[1-5]

The markers of acute coronary syndrome have evolved over the preceding decades and now the troponins are the mainstay of diagnosis. However, ever since the cholesterols along with its constituent fractions and triglycerides were implicated in the deposition of atheromatous plaques in the coronary vessels, little has been studied about other possible risk factors. Also, it was observed by Enas et al that despite the higher rates of prevalence of coronary artery disease (CAD) and its occurrence at a younger age in the Asian Indian population, there was a low rate of conventional risk factors like smoking (3%), obesity (3%), hypertension (14%), high cholesterol (17%) – what they termed as the Asian Indian paradox. This would indicate that there is a need to identify newer markers of CAD and institute corrective measures to address them along with addressing the dyslipidemias prevalent in our population.[6]

Traditionally, the circulating cholesterols have been implicated in progression of CADs. To address this, dietary and lifestyle modification as well as lipid lowering drugs have been used. Yet, the incidence and prevalence of CAD has been on the rise.[7]

As evident as it is that the cholesterols, especially low density lipoprotein cholesterol, are the major constituents of a vascular plaque, it is also important to recognise that remodelling of the vascular endothelium, in the form of loss of its integrity, precedes plaque formation. In addition, in the healthy state, the vascular endothelium enables endothelium-dependent vasodilatation and also suppresses vascular inflammation and thrombosis. Risk factors responsible for a disruption of this normal functioning include several non-biochemical and biochemical factors. Amongst the non-biochemical factors, the predominant modulators of vascular health are diabetes, metabolic syndrome, hypertension, smoking, physical inactivity. Lifestyle modifications, therefore, help in reducing risk of CVD. Amongst the biochemical factors, in addition to the cholesterols and triglycerides, homocysteinemia is known to impact the endothelium through oxidative and non-oxidative mechanisms. It also promotes lipid peroxidation and platelet aggregation, thus, further enabling plaque formation.[8,9]

Over the past four decades, homocysteine has been extensively studied as a marker of vascular disease, especially CAD. Some studies claim evidence of association whereas some decline any significant role of this molecule in CAD. Also, there has been speculation as to whether it is a marker or factor and several studies have demonstrated its causative nature in CAD. Data from India, too, is inconclusive. Consequently, no therapeutic interventions target homocysteine in the management of patients of CAD.[7-9]

We have, therefore, assessed homocysteine vis-a-vis the cholesterols and triglycerides with respect to its association with CAD in a North Indian urban population.

Materials and Methods

This retrospective study included 257 patients (61 females; 196 males) above 18 years of age who were admitted for CAD. They had no history of previous episodes of CAD. As part of standard protocol, blood samples were drawn on the day of admission for estimation of the parameters of the lipid profile (total cholesterol [TC], HDL cholesterol [HDL-C], LDL cholesterol [LDL-C], triglycerides [TGS]), lipoprotein(a) [Lp(a)], and homocysteine [HCY]. All assays had been performed on fully automated analysers by the methods given in table 1. Data of 4130 normal subjects (1606 females;

2524 males) coming to our hospital for a routine health check-up served as control data.

Table 1: Methods used for estimation of the analytes

Analyte	Method of estimation
Total cholesterol	Enzymatic – cholesterol esterase
HDL cholesterol	Direct: Accelerator Selective detergent to separate non-esterified HDL cholesterol followed by estimation by enzymatic method– cholesterol esterase
LDL cholesterol	Direct: Liquid Selective detergent to separate non-esterified HDL cholesterol followed by estimation by enzymatic method– cholesterol esterase
Triglycerides	Glycerol Phosphate Oxidase
Lipoprotein(a)	Immunoturbidimetric assay
Homocysteine	Chemiluminescent immunoassay

Statistical analysis of the generated data was performed with SPSS version 20 (Chicago, Illinois, USA). Distribution of the data was not normal, so median (minimum, maximum values) were used for analysis. Categorical variables are expressed as frequencies. The Mann–Whitney U test was used for comparison of continuous variables between the controls and patients of cardiovascular disease. Odds ratio was calculated for all parameters by univariate analysis as well as multivariate logistic regression analysis.

Results

Demographic analysis showed that the mean (±SEM) age of the patients of CVD was significantly less than that of the controls. Similarly, the percentage of females was significantly less in the patient group as compared to the controls (Table 2).

Table 2: Age and sex distribution in controls and patients of CVD (Quality rating of evidence:3)

	Mean Age (in years)	Females (%)	Males
Controls N=4130	51.37 ± 0.20	1606 (38.8%)	2524 (61.2%)
CVD patients N=257	46.87 ± 0.59	61 (23.7%)	196 (76.3%)
Significance (p)	< 0.001	< 0.001	< 0.001

Age- and sex-wise data show that amongst the patients of CAD, the mean age was lower with a preponderance of males as compared to the control population.

The mean concentrations of all the analytes in the control population was observed to be near the upper limit of the biological reference interval, exemplifying an atherogenic millieu. This is shown in figure 1.

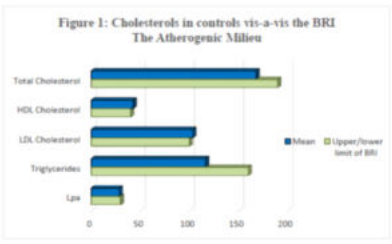


Figure 1: The mean circulating levels of cholesterols, triglycerides and lipoprotein(a) in the control population was observed to be close to the upper limit of the BRI (lower limit of BRI in case of HDL-cholesterol), constituting the atherogenic milieu in this population.

The mean (±SEM) circulating levels of the various parameters estimated are given in table 3. This also includes the calculated parameters, viz total cholesterol:HDL cholesterol and LDL cholesterol:HDL cholesterol ratios.

Amongst the controls, females had significantly lower ($p<0.001$) circulating levels of triglycerides, VLDL-C and homocysteine than males, but significantly higher ($p<0.001$) circulating total cholesterol and HDL-C. LDL-C was similar in both sexes.

Table 3: Mean(SEM) of all estimated parameters with 95% confidence intervals (Quality rating of evidence:3)

Analyte	Controls (n=4130)		CVD (n=257)	
	Males(n=2524) MeanSEM	Females(1606) MeanSEM	Males(n=196) MeanSEM	Females(n=61) MeanSEM
Total cholesterol (TC) (in mg/dL)	164.810.63	172.950.77	187.383.06	187.514.83
HDL cholesterol (HDL-C) (in mg/dL)	40.350.18	46.740.27	35.960.5	38.340.29
LDL cholesterol (LDL-C) (in mg/dL)	103.160.55	103.50.64	118.082.25	1224.35
TC:HDL-C	4.22±0.02	3.83±0.02	5.28±0.07	5.06±0.15
LDL-C:HDL-C	2.66±0.02	2.32±0.02	3.34±0.06	3.3±0.13
Triglycerides (in mg/dL)	120.240.76	110.670.99	202.869.04	160.1611.58
Lipoprotein(a) (in mg/dL)	28.891.83	27.741.41	34.451.88	29.782.16
Homocysteine (mol/L)	10.830.33	10.740.26	23.121.35	22.391.65
Age (years)	52.120.26	50.20.3	46.510.66	48.031.31

Table 3: The mean circulating levels of the cholesterols, triglycerides, lipoprotein(a) and homocysteine in controls and patients of CAD, along with the relevant ratios.

The ratios, TC:HDL-C ratio and LDL-C:HDL-C ratio, were consequently significantly lower in the females as compared to the males. Amongst patients of CAD, the only significant difference was in circulating triglycerides ($p=0.004$) and Lp(a) ($p=0.008$) which were lower in females.

Multivariate logistic regression analysis revealed similar results in both sexes and hence are presented here collectively. Only triglycerides, HDL-C and homocysteine were implicated as significant markers of CVD. The odds ratio conferred by all the parameters, thus derived, are tabulated in table 4.

DISCUSSION

In the current study, statistical analysis revealed that the population mean of the cholesterols, triglycerides and lipoprotein(a) were near the upper limit of the biological reference interval as shown in figure 1 (except HDL cholesterol which was near the lower limit of the BRI). Demonstration of this atherogenic milieu in the control population corroborates the observations of Enas et al and Joshi et al, who stated that Asian Indians had a higher risk of CAD, probably due to elevated levels of circulating triglycerides and lower levels of HDL-C. (6,7) Though

univariate analysis of the data assigns significance to all the parameters measured in the subjects, multivariate logistic regression shows an absence of significance of the total and LDL cholesterols, their ratios and lipoprotein(a) as markers of CAD. This emphasizes the probability that in the North Indian population, in addition to the predisposition to CAD due to expression of an atherogenic milieu, HDL-C along with other factors, like triglycerides and homocysteine, also contribute significantly to the high prevalence and mortality of CAD.

Table 4: Odds ratio (with 95% CI) for all parameters on univariate and multivariate regression analysis (Quality rating of evidence:3)

	Univariate				Multivariate			
	p value	Odds Ratio	95.0% C.I.		p value	Odds Ratio	95.0% C.I.	
			Lower	Upper			Lower	Upper
Age	<0.001	0.973	0.963	0.982				
Gen(F)	<0.001	0.489	0.364	0.656				
HDL Cholesterol	<0.001	0.925	0.911	0.940	0.006	0.893	0.824	0.969
CHOL:HDL Ratio	<0.001	2.820	2.482	3.203				
LDL Cholesterol	<0.001	1.023	1.018	1.029				
LDL:HDL Ratio	<0.001	2.610	2.265	3.007				
Total Cholesterol	<0.001	1.020	1.015	1.024				
Triglycerides	<0.001	1.023	1.020	1.026	<0.001	1.297	1.149	1.464
VLDL Cholesterol	<0.001	1.063	1.053	1.074				
Homocysteine	<0.001	1.282	1.216	1.351	0.002	1.36	1.115	1.657
Lp(a)	0.008	1.011	1.003	1.019				

Table 4: Multivariate regression analysis revealed significance of HDL-cholesterol, triglycerides and homocysteine as independent risk factors for CAD. HDL- high density lipoprotein; CHOL – cholesterol; LDL – low density lipoprotein; VLDL – very low density lipoprotein; Lp(a) – lipoprotein(a)

Till about two decades ago, there was so much focus on the cholesterols as the cause of CAD that the role of circulating triglycerides was overlooked. It is only when evidence built up that lowering the cholesterols was helpful in reducing risk of CAD, but did not totally prevent cardiovascular events, that attention was redirected to this forgotten biomarker.[10]

Data from our laboratory, earlier and in the current study, indicates that HDL cholesterol, triglycerides and homocysteine show a significant association with CAD. However, literature is equivocal about the role of triglycerides in CAD with most studies not finding any reduction in cardiovascular events after lowering triglycerides.[11,12]

Recently, Bhatt et al have conducted a multicentric randomized double-blind trial on 8179 subjects with established cardiovascular disease to elucidate whether reducing triglycerides actually reduced ischemic events. They concluded that the risk of ischemic events in the group receiving icosapent ethyl for reduction of triglycerides was significantly less than that in the group receiving only statins for correction of cholesterols.[13]

A systematic review and meta-regression analysis, including multiple lipid-lowering therapeutic classes, was conducted by Marston et al on 197270 subjects having established cardiovascular disease. They, too, concluded that lowering triglycerides is associated with lower risk of ischemic events even after adjustment for reduction in LDL-cholesterol.[14]

Hence, current management of CAD includes triglyceride lowering therapy along with the lipid-lowering regimen.
What about HDL-C and Lp(a)?

The cardioprotective actions of HDL-C are manifold. The major biological actions by which it protects the vascular endothelium are effluxing cellular cholesterol, diminishing cellular death, decreasing vascular constriction, reducing inflammatory response, protecting from pathological oxidation and lessening platelet activation. This functional heterogeneity reflects its compositional heterogeneity as HDL-C carries varied protein and lipid components. However, since its composition and production are genetically determined, it does not lend itself to modulation by therapeutic measures. Instead, lifestyle modifications are known to impact the circulating levels of this lipoprotein. Recently, HDL-replacement therapy is being assessed as a new strategy wherein acute administration of HDL is used to stabilize patients at imminent risk of myocardial infarction.[15-18]

Our current data reveals no significant difference between the circulating Lp(a) levels in controls as compared to CVD patients. This is probably due to the fact that even in the controls, the mean level of this parameter is at the upper limit of the BRI, adding to the atherogenicity of the circulating milieu. Therefore, it warrants recognition as a risk factor.

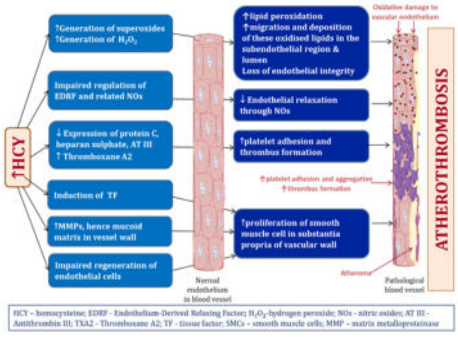
Lipoprotein(a) has been associated with CVD not only on account of its content of LDL-cholesterol but also due to the atherogenicity of the apoprotein(a) attached to the apolipoprotein B100 of the LDL particle. Apoprotein(a) binds to the thromboplastin activating factor (t-PA) at its plasminogen binding sites and also stimulates the production of plasminogen activator inhibitor (PAI 1), thus reducing clot dissolution through dual mechanisms. PAI 1 is also proinflammatory as its increase results in adhesion of monocytes to the vessel wall. Furthermore, it inhibits endothelial repair by interfering with the interaction of platelets with exposed collagen fibers at the site of injury. Lipoprotein(a) also inhibits TGF-□, causing smooth muscle cell proliferation and migration and accelerating stenosis. Thus, the smaller protein fraction of this Lp(a) molecule deleteriously impacts cardiac vasculature.[19]

The data of this study shows the mean lipoprotein(a) in the control population to be very high with 70.45% having levels above the mean of the BRI (15mg/dL). Hence, it is important to identify individuals with high levels of this protein as they are at a high risk for development of CAD. Since this is genetically determined, it does not lend itself to easy therapeutic amelioration. However, recently, Hardy et al in their review on the therapeutic use of pelacarsen in atherosclerotic cardiovascular disease, observed that this drug significantly reduces circulating lipoprotein(a) levels. Currently, there is an ongoing international multicentric double blind case-control trial – The Horizon Study – which is evaluating the role of pelacarsen (TQJ230) in reducing major cardiovascular events on basis of its lipoprotein(a)- reducing action.[20,21]

Homocysteine, the last marker under consideration in this study, is a sulphur containing amino acid which lies at the

metabolic crossroads of methylation reactions and folate metabolism. It is metabolised by two pathways – the remethylation cycle and the transsulfuration pathway – which are predominantly governed by the enzymes methylene tetrahydrofolate reductase, methionine synthase and cystathionine-beta-synthase and the vitamin cofactors of these enzymes folate, B12 and pyridoxine, respectively. Polymorphisms of the genes encoding these enzymes and/or deficiency of any of these vitamins, therefore, leads to high circulating concentrations of homocysteine. The mechanisms by which homocysteine promotes atherothrombosis and remodelling of vascular endothelium are manifold and are schematically represented in figure 2.[9,22]

Our data analysis reveals a highly significant association of homocysteine with CAD. Boushey et al (1995) in their metanalysis of 27 studies, including over 70000 subjects, elucidated that homocysteine above the population mean confers an increased risk of CAD. A 5 µmol/L increase in homocysteine confers the same risk as an increase in total cholesterol of 20 mg/dL. They also observed that decreasing homocysteine by 3-4 µmol/L reduced the risk of CAD by 30-40% even within its BRI. Interestingly, even when the cause for high circulating homocysteine is a genetic polymorphism, administration of the vitamins folate, B12 and B6 lowers the circulating concentration of homocysteine.[23]



Reducing circulating homocysteine, therefore, allows a mode of reducing risk of CAD by a simple therapeutic intervention – administration of the B vitamins, specifically folate, B12 and B6. Yet current therapeutic interventions for CVD do not include homocysteine lowering measures.

This gains more significance in a population deficient in many of these vitamins. Data published from our laboratory including nearly 50000 subjects from an urban population showed a prevalence of vitamin B12 deficiency in 75% of this population, whereas prevalence of deficiency of folate was minimal (2.9%). Hence, in addition to targeted therapy in patients of CAD, it would be beneficial to institute homocysteine lowering measures for the population as a whole. This could be addressed by food fortification with these vitamins, a measure which has been shown to decrease the prevalence of CAD in addition to reducing neural tube defects (for which it was initially instituted). In addition, food diversification should be part of school curriculum as it focusses on available seasonal foods in the right combinations.[24,25]

Conclusion

Despite lifestyle modifications that have gained momentum in all populations, prevalence and mortality of CAD is on the rise, especially in the younger age group and in the developing countries like India, where nutritional deficiencies of the B group vitamins is still high. Current management includes therapeutics to reduce circulating lipoproteins and triglycerides, and increase HDL cholesterol. However, it is equally important to recognise other risk factors, like homocysteinemia, that are prevalent in our population and address them adequately, by individual specific measures and/or population based methods like food fortification.

Acknowledgements: The authors would like to acknowledge the contribution of Mr. Virender Singh, Ms. Jyoti Punjabi and Ms. Radhika for their assistance in data extraction. Funding: No funding was procured for this retrospective study.

REFERENCES:

1. India State-Level Disease Burden Initiative Malnutrition Collaborators. The burden of child and maternal malnutrition and trends in its indicators in the states of India: the Global Burden of Disease Study 1990–2017.The Lancet Child and Adolescent Health 2019; 3(12):855-870. DOI:https://doi.org/10.1016/S2352-4642(19)30273-1
2. Nutrition transition in India, 1947-2007; Ministry of Women and Child Welfare
3. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds) (accessed on 14th September, 2021)
4. Yusuf, S., Reddy, S., Ounpuu, S., Anand, S. (2001) Global burden of cardiovascular diseases: part I: general considerations, the epidemiological transition, risk factors, and impact of urbanization. Circulation; 104(22):2746-2753. DOI: 10.1161/hc4601.099487.
5. Enas, E.A., Mehta, J. (1995) Malignant coronary artery disease in young Asian Indians: thoughts on pathogenesis, prevention and treatment. Clin Cardiol; 18:131-135. DOI: 10.1002/clc.4960180305.
6. Joshi, P., Islam, S., Pais, P., Reddy, S., Dorairaj, P., Kazmi, K., et al. (2007) Risk factors for early myocardial infarction in South Asians compared with individuals in other countries. JAMA; 297:286–294. DOI: 10.1001/jama.297.3.286.
7. Enas, E.A., Garg, A., Davidson, M.A., Nair, V.M., Huet, B.A., Yusuf, S.(1996) Coronary heart disease and its risk factors in first-generation immigrant Asian Indians to the United States of America. Ind Heart J; 48(4):343-353.
8. Patel, S., Adams, M.R. (2008) Prevention of cardiac disease: lifestyle modification or pharmacotherapy? Int Med J 2008; 38:199-203. DOI: 10.1111/j.1445-5994.2007.01616.x.
9. Jacobsen, D.W. (2000) Hyperhomocysteinemia and Oxidative Stress-Time for a Reality Check? Editorial. Art Thromb Vasc Bio; 20(5):1182-1184. DOI: 10.1161/01.ATV.20.5.1182.
10. Nordestgaard, B.G., Varbo, A. (2014) Triglycerides and cardiovascular disease. Lancet; 384:626-635. DOI: 10.1016/S0140-6736(14)61177-6.
11. Bhargava, S., Ali, A., Manocha, A., Kankra, M., Das, S., Srivastava, L.M. (2012) Homocysteine in occlusive vascular disease – Risk Marker or Risk Factor? Indian J of Biochem and Biophys; 49(6):414-420. http://nopr.niscair.res.in/handle/123456789/15244
12. Pradhan, A., Bhandari, M., Vishwakarma, P., Sethi, R. (2020) Triglycerides and cardiovascular outcomes – can we REDUCE-IT? Int J Angiol; 29:2-11. DOI: 10.1055/s-0040-1708529.
13. Bhatt, D.L., Steg, P.G., Miller, M., Brinton, E.A., Jacobson, T.A., Ketchum, S.B. et al. (2019) Cardiovascular risk reduction with Icosapent Ethyl for hypertriglyceridemia. NEJM; 380(1):11-22. DOI: 10.1056/NEJMoa1812792.
14. Marston, N.A., Giugliano, R.P., Im, S.M.K., Silverman, M.G., O'Donoghue, M.L., Wiviott, S.D. et al. (2019) Association between triglyceride lowering and reduction of cardiovascular risk across multiple lipid-lowering therapeutic classes. Circulation; 140(16):1308-1317. DOI: 10.1161/CIRCULATIONAHA.119.041998.
15. Stein, O., Stein, Y. (1999) Atheroprotective mechanisms of HDL. Atherosclerosis; 144:285–301. DOI: 10.1016/s0021-9150(99)00065-9.
16. Assmann, G., Nofer, J.R. (2003) Atheroprotective effects of high-density lipoproteins. Annu Rev Med; 54:321–341. DOI: https: 10.1146/annurev.med.54.101601.152409.
17. Davidson, W.S., Silva, R.A., Chantepie, S., Lagor, W.R., Chapman, M.J., Kontush, A. (2009) Proteomic analysis of defined HDL subpopulations reveals particle-specific protein clusters: relevance to antioxidative function. Arterioscler Thromb Vasc Biol; 29:870–876. DOI: 10.1161/ATVBAHA.109.186031.
18. Remaley, A.T., Amar, M., Sviridov, D. (2008) HDL-replacement therapy: mechanism of action, types of agents and potential clinical indications. Expert Rev Cardiovasc Ther; 6(9):1203–1215. DOI: 10.1586/14779072.6.9.1203.
19. Enas, E.A., Varkey, B., Dharmarajan, T.S., Pare, G., Bahl, V.K. (2019) Lipoprotein(a): An underrecognized genetic risk factor for malignant coronary artery disease in young Indians. Ind Heart J; 71:184-198. DOI: 10.1016/j.ihj.2019.04.007.
20. Hardy, J., Niman, S., Goldfaden, R.F., Ashchi, M., Bisharat, M., Huston, J. et al. (2021) A Review of the Clinical Pharmacology of Pelacarsen: A Lipoprotein(a)-Lowering Agent. Am J Cardiovasc Drugs; DOI: 10.1007/s40256-021-00499-1.
21. ClinicalTrials.gov Identifier: NCT04023552. Assessing the Impact of Lipoprotein (a) Lowering With TQJ230 on Major Cardiovascular Events in Patients With CVD (Lp(a)HORIZON). https://clinicaltrials.gov/ct2/show/NCT04023552. Accessed on 30th November, 2021.
22. Bhargava, S. Clinical Application Of Homocysteine. Ed Springer Books International. 2018. ISBN 978-981-10-7632-9. Section II, Chapter A, pg no 26-30.
23. Boushey, C., Beresford, S.A., Omenn, G.S., Motulsky, A.G. (1995) A quantitative assessment of homocysteine as a risk factor for vascular disease: possible benefits from increasing folate intake. JAMA; 247:1049-1057. DOI: 10.1001/jama.1995.03530130055028.
24. Bhargava, S., Srivastava, L.M., Manocha, A., Kankra, M., Rawat, S. (2022) Micronutrient deficiencies and anemia in urban India – Do we need food fortification? Ind J Clin Biochem; 37(2):149-158. DOI 10.1007/s12291-021-00966-1.
25. https://www.who.int/publications/i/item/9241594012 (accessed on 16th November, 2021)