Original article

Evaluation of serum bilirubin and alkaline phosphatase levels in patients with cardiovascular disease

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Abstract:

Context: The enhanced inflammatory response and oxidative stress may play an important role, apart from classic risk factors like hypertension and diabetes mellitus, in the cardiovascular morbidity and mortality.

Aims: To evaluate the role of serum bilirubin and alkaline phosphatase levels in cardiovascular diseases.

Settings and Design: Study type is observational randomized controlled trials. Study group includes 100 CVD patients.

Methods and Material: All the parameters are analyzed on Olympus AU 400 fully automated random access analyzer.

Statistical analysis : We analysed the data using student t test and Pearson's correlation. Statistical software used was graphpad prism.

Results: Mean serum levels of bilirubin were lower in patients with cardiovascular diseases compared to control group. Serum alkaline phosphatase levels were comparatively higher in patients than in control group. These levels were statistically significant ($p<0.005$) and were corresponding to dyslipidemia and inflammatory status in these patients.

Conclusions: Lower levels of serum bilirubin indicate that there was an independent inverse association between serum total bilirubin and cardiovascular disease.Elevated alkalinephosphatase levels were associated with cardiovascular disease considering it as a cost effective diagnostic marker. Further research is needed to evaluate the effects of agents altering levels of serum bilirubin levels and alkalinephosphatase to decrease mortality.

Keywords : Bilirubin, alkalinephosphatase, lipid profile, cardiovascular diseases

Key Messages: Bilirubin and alkalinephosphatase levels can be used as cost effective markers for predicting cardiovascular diseases. Drugs can be designed accordingly.

Introduction:

Serum unconjugated bilirubin is the end product of heme degradation via the hemeoxygenase pathway. Bilirubin which is a water-insoluble compound can be made water soluble by glucuronidation using a microsomal enzyme , the uridinediphosphate glucuronyltransferase-1 A1 (UGT1A1). The *UGT1A1 locus* has been mapped to chrom-osome 2q37 [1] .One of the most common genetic variants that affects this gene locus in Caucasians is TA duplic-ation in the TATA box region of the promoter. Homozygosis for the TA duplication is associated with higher le-vels of unconjugated bilirubin. It is considered as the main cause of Gilbert syndrome in Caucasians [1] It is also the reason for some of the inter individual variations in bilirubin levels, even in the normal population account-ing for about 10% of the population.[2]The estimated frequency of this allele is 0.35 in Caucasians, but the freq-uency is highly variable in different ethnicities.[3]

High levels of bilirubin are associated with decreased risk of cardiovascular disease (CVD).[4] The exact mecha-nism by which bilirubin acts to

protect against CVD is not fully understood, it may protect against oxidative stre-ss by reducing reactive oxygen species and possibly having additional anti-atherogenic properties.[5] Alkaline ph-osphatase (ALP) is a hydrolysing enzyme which removes phosphorus from many types of molecules. ALP is produced by osteoblasts and helps in bone mineralization by hydrolyzing pyrophosphate in the extracellular matrix. [6]Three of four AP-encoding genes are tissue-specific (placental, embryonic and intestinal AP isoen-zymes) [7] the fourth AP gene is tissue non-specific and is especially abundant in bone, liver and kidney.

Subjects and Methods:

Study design and subjects:This study was conducted in the department of biochemistry. Study type was obser-vational randomized controlled trials.

Inclusion criteria: Study group includes 100 CVD patients who had survived a myocardial infarction, percuta-neous transluminal angioplasty, or coronary artery bypass grafting, stroke(ischemic or hemorrhagic), pulmonary embolism, cardiomyopathy and congestive heart failure before age 55 years for men and 65 for women. Demog-raphic characteristics and clinical features were collected from all the patients.

Exclusion criteria: Participants with known hemodynamic instability, autoimmune disease, neoplastic disease, impaired renal function (serum creatinine>2mg/dL), chronic kidney disease, hepatic disease or elevated transa-minases, malignancy, any bone diseases, chronic obstructive pulmonary disease, chronic or current infections, or the use of anti-inflammatory drugs in the past 30days were excluded.

Blood sample collection:

5ml of unhemolysed12hours overnight fasting blood samples were obtained by vene-puncture without adding anticoagulant. All the blood samples were kept away from bright light exposure. Sam-ples were centrifuged within 2 hours and serum was analyzed for total bilirubin, alkaline phosphatase ,C-React-ive Protein, phosphate and lipid profile.

Methods

All the analytes were analysed by Olympus AU 400 analyzer using standard operating procedures. All the enz-ymes ALP was analysed using IFCC kinetic method. Total cholesterol estimation was done by CHOD –POD method and triacylglycerol by GPO-POD method. HbA1c was done by immunoturbidimetric method. Serum total bilirubin was estimated by Diazo method.

Ethics

The purpose of the study was explained to all the eligible candidates and written informed consent was taken from all the patients involved in this study. The study was performed in conformance with the Declaration of Helsinki ethical guidelines.

Statistics

Statistical Analysis Data was expressed in Mean ± S.D. Comparison between patients and controls for all vari-ables was performed by student t-test and correlation between parameters was studied by Pearson's correlation coefficient using statistical software graphpad prism. $p<0.005$ was considered as statistically significant.

Results:

The study group included 100 CVD patients among whish 48 patients were with coronary artery disease, 36 pat-ients with stroke, 12 patients with congestive heart failure, 2 patients with pulmonary embolism and 2 patients with cardiomyopathy. Among 100 subjects, 63 were men with mean age group of 55 ± 8.0 and 37 were women with mean age group of 65 ± 3.0. Mean BMI levels in cases were high compared to controls. But the levels were not significantly different from control. From table

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1, it is seen that mean values of bilirubin were significantly lower in cases compared to control group. Similarly HDL levels were comparatively lower in cases compared to controls. Other lipid parameters were high in cases. There was significant increase in serum alkaline phosphatase and phosphate levels in cases compared to controls. CRP levels were also significantly elevated in cases. From table 2, it was shown that bilirubin levels were positively correlated with HDL levels and BMI, and negatively with CRP levels and LDL.From table 3, it can be seen that these high levels of alkaline phosphatase were posit-ively correlating with HbA1c and CRP levels.

Tables

Table 1showing mean ± S.D and p values of all the biochemical parameters in cases and controls

Parameters	Cases (n=100)	Controls (n=100)	p-value
BMI	23.15 ± 2.5	22.4 ± 1.01	0.12
Bilirubin	0.28 ± 0.06	0.76 ± 0.19	P 0.0001
Alkaline phosphatase	126.49 ± 7.80	61.54 ± 7.93	
Phosphate	5.07 ± 0.52	2.58 ± 0.54	
Cholesterol	222.65 ± 10.50	173.55 ± 9.50	
Triglycerides	196.05 ± 12.22	153.92 ± 11.10	
LDL	181.91± 6.46	138.73 ± 13.61	
HDL	38.10 ± 2.75	48.25 ± 7.88	
HbA1c	7.06 ± 0.32	5.91± 0.32	
CRP	2.42 ± 0.59	0.53 ± 0.27	

Table 2 showing Pearson correlation of different parameters in cardiovascular disease patients considering total bilirubin as the dependent variable

Parameter	r- value	p-value
LDL	-0.0677	0.96
HDL	.0044	0.01
CRP	-0.135	0.001
HbA1c	0.130	0.305
BMI	-0.0546	0.01

Table 3 showing Pearson correlation of different parameters in cardiovascular disease patients considering alkaline phosphatase as the dependent variable

Parameter	r-value	p-value
HbA1c	0.1794	0.03
CRP	0.0054	0.001

Discussion:

Bilirubin is considered to be a potent antioxidant under physiological conditions. [8] Multiple mechanisms had been there to explain the protective role of bilirubin, such as inhibition of lipid and protein peroxidation and anti-inflammatory pathways. 10nM of bilirubin is enough to protect cells against a10000-fold higher concentration of oxidants through rapid regeneration of bilirubin by biliverdin reductase, [9] that may protect against pathological processes occurring during cardiovascular disease. [10]Low levels of bilirubin in the cases in our study could exp-lain the cardiovascular risk status in this study group.

In our study high bilirubin levels were associated with low BMI but not to a significant extent. A range of socio demographic and lifestyle variables, including younger age, male sex, lower body mass index, and non smoking status, have also been associated with moderately higher bilirubin levels. [11] Unconjugated bilirubin causes red-uction in inflammatory status. This can be shown with lower levels of IL-6.[12]Previous studies showed that unc-onjugated bilirubin is negatively associated with CRP [13] which is a biomarker for both

inflammatory status and CVD risk. IL-6 induces production of CRP by liver. This could explain the reason for lower CRP levels with re-duced IL-6 levels seen in CVD patients. A high level of CRP in the study group shows that there is role of infla-mmation in the pathogenesis of cardiovascular disease. There is drawback in our study in this aspect also that we have not evaluated IL-6 levels.

There was a negative correlation between bilirubin levels and LDL level in our study but the significance was w-eak. We might have measured oxidised LDL levels which might have given better result in our case. It has been suggested that bilirubin is associated with lower CRP levels via reduction of blood lipid concentrations and not by direct inhibition of inflammation. [14] Recently, it was described that elevated serum unconjugated bilirubin le-vels could delay atherosclerotic plaque progress. The possible pathological process behind this could be that it prevents thrombus formation through the prevention of collagen induced platelet aggregation.[15] The immune-odulatory effects of unconjugated bilirubin may also explain the delayed plaque formation.[16]There are eviden-ces that support a role for bilirubin in protecting lipids from various oxygen radical species particularly by copp-er. [17] Lipoproteins, particularly LDL-c, are highly susceptible to oxidation. The atherogenic process involves an uptake of oxidized LDL by intimal macrophages leading to accumulation of lipid-rich foam cells. [18] A recent clinical study reported acute MI is associated with 60 % raise in bilirubin levels. Patients with elevated bilirubin levels had better collateral flow into the ischemic myocardium. [19] Bilirubin levels were positively correlated with HDL levels in our study group that as bilirubin levels are decreased, HDL levels are also reduced. Causal relationship between bilirubin and CVD risk could be established by studying the effect of agents causing mode-rate increases in serum bilirubin on the risk of CVD. Niacin (vitamin B3) can increase bilirubin levels by stimu-lating hemeoxygenase activity. It is also used as a provocation test for the Gilbert syndrome.[20] A study showed that 1 to 3 g/d niacin can reduce CVD events by 25%.[21] The mechanism underlying could be increase in high-density lipoprotein.In addition, it was proved that by applying bilirubin directly to vascular endothelial tissue, there was an improvement in the markers of oxidative stress and cellular dysfunction.[22] The present research is going on drugs that specifically inhibit or downregulate UGT1A1 activity. But this increased bilirubin levels co-uld be a potential risk of gallstones or adverse drug reactions. [23] Statins have also been used to increase heme oxygenase levels in human endothelial tissues which increase serum bilirubin levels. But this also causes chan-ges in liver enzymes of 10% to 20% after statin treatment. [24]Nowadays UGT1A1*28 genotype has been used to more precisely assess bilirubin - CVD risk factor associations as there is a strong causal relationship between them. The effect of *UGT1A1* gene polymorphism in the outcome of CVD in the general population has been studied by only few people. This is one of the limitations even in our study that we have not done any genetic analysis.

There were studies revealing the relationships between bilirubin and peripheral artery disease (PAD) and carotid intima-media thickness(IMT) which prove that bilirubin acts against plaque formation and atherosclerosis.[25,26]. Another drawback for this study. The therapeutic effect of bilirubin is that it inhibits cell growth without causing cell death, preventing cellular debris, inflammation and rupture of the lesions. Stents can be coated with bilirubin so that growth of smooth muscle cells can be arrested and prevent blocking of stent. [27]High ALP levels in the cases could be explained by that ALP is released following smooth

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muscle cell osteoblastic transformation by tumour necrosis factor α following vascular injury. [28]ALP is considered to be an indicator of accelerated vascul-ar calcification. Vascular calcification is the preliminary stage for vascular hardening and aging, which contribu-tes to atherosclerosis. [29] Smooth muscle cells can be changed phenotypically into osteoblast-like cells by abnor-mally raised serum phosphate levels. [30] They can also cause degradation of the extracellular matrix and increase the production of reactive oxygen species. These free radicals generated can stimulate an osteoblastic transcript-tional program in the vasculature [31] in vascular smooth muscle. Hypovitaminosis D is also associated with elev-ated serum ALP and phosphate levels. There are other changes in hypovitaminosis D like greater plasma renin activity, inflammation, and higher blood pressure, and FGF-responsive hormones. [32] This association could not be established in our study as we have not evaluated vitamin D levels.

There were significantly higher phosphate levels in cases when compared to controls. Vascular calcification has been considered an active process. There are multiple circulating promoters and inhibitors involved in this proc-ess. Phosphate is one such promoter which helps in osteogenic and chondrogenic differentiation resulting in va-scular calcification. Pyrophosphate (PPi) homeostasis is indicative of soft tissue mineralisation. ALP is an enzy-me involved in hydrolysis of PPi. So an increase in ALP indicates decreased inhibition of vascular calcification as PPi is an inhibitor of vascular calcification. [33] ALP levels in our study are positively correlated with CRP le-vels as ALP is an acute phase reactant.[34] It is an indicator of poor nutritional status and increased susceptibility to infection. ALP level is positively associated with neutrophil to lymphocyte ratio (N/ L), [35] and is elevated in vessels with medial calcification. [36] This could explain that inflammation and calcification could play a role in the association of ALP with CVD deaths.

It is reported that 38% of diabetic patients have elevated serum alkaline phosphatase levels. Mean HbA1c levels in our study were positively associated with ALP. But bone ALP is the predominant type, which may be caused by diabetic bone diseases which we have not assessed in our study.[37] Some studies showed that oral phosphorus binders fail to lower serum phosphorus concentration and some studies showed they can be used which are still in trials. One proposal was that better choice to reduce it may be to reduce dietary phosphorus intake [38] which needs to be confirmed further. Genetic ablation of tissue-nonspecific ALP leads to decreased soft tissue calcify-cation in animal studies. [39] Future studies to be concentrated on genetic studies on humans in this view.

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