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Original Article

Biochemistry Section

Identification of Predictable Biomarkers in Conjunction to Framingham Risk Score to Predict the Risk for Cardiovascular disease (CVD) in Non Cardiac Subjects

RAMA KRISHNA REDDY Y.V.[1], JAIDEEP MAHENDRA[2], PREMA GURUMURTHY[3], JAYAMATHI[4], SAI BABU[5]

ABSTRACT

Introduction: Although the cardiovascular disease (CVD) burden is rising in different countries, the morbidity and mortality rate is not reduced to much extent because of lack of application of the biomarkers for diagnosing CVD. Hence, we aimed to establish the predictable biomarkers in conjunction to framingham risk score in order to predict the risk for CVD in non cardiac patients.

Materials and Methods: Three hundred subjects were screened for the study who came for the master health checkup. Out of them 50 patients were excluded as they were under medication. 23 patients were excluded due to various systemic diseases like fever and infection etc. The remaining of 227 patients with age range of 30-80 y was randomly selected for investigation. These subjects were divided into four different groups: Group I – controls with age range: 30-60 y (n=50) these subjects were free from all the systemic ailments and risk factors. Study groups comprised of Group II - (n=44) with age range: 30-40 y, Group III - (n=50) with age range: 41-50 y and Group IV - (n=83) with age range: 51-80 y. Patients with different risk factors without medication participated as study groups. Routine biochemical parameters were analysed using fully automated analyser and atherosclerotic biomarkers was analysed using ELISA kit. In addition to this, framingham risk scores was calculated in all the groups, for 30 y risk prognosis for CVD.

Results: The atherosclerotic biomarkers such as E-selectin, Leptin, osteoprotegerin (OPG) and Ox-LDL were elevated among the study groups as compared to control group. Pearson correlation showed a significant association between the individual risk score (30 y framingham risk for CVD) of individuals, and the above biomarkers. The Receiver operating curve (ROC) analysis also showed a greater area under curve with higher sensitivity and specificity.

Conclusion: We conclude the application E-Selectin, leptin, OPG and Ox-LDL as biomarkers along with the framingham risk scores in prediction risk for CVD in the individuals with subclinical atherosclerosis. It is more reliable and predictable as compared to the individual biomarkers alone.

Keywords: Atherosclerotic biomarkers, Cardiovascular diseases, Framingham study, Osteoprotegerin, Subclinical atherosclerosis

INTRODUCTION

CVDs, currently are the leading cause of death in developed and developing countries, and have become the prominent health problem worldwide [1]. Atherosclerosis a progressive diseases, characterized by the accumulation of lipids and fibrous elements in the large arteries, constitutes the single most important contributor to the growing burden of CVD [2,3]. It is a multifactorial process which commences as early as childhood but clinically progress itself in later life, and is increasingly considered as an immune mediated process of the vascular system [4]. Frequently CVD such as myocardial infarction (MI) is a direct consequence of atherosclerosis. The prevalence of obesity, sedentary life style and the ageing of the population is a risk for CVD [5]. The clustering pathological factors like central obesity, glucose intolerance, hypertension, and dyslipidaemia are also underlying common threats of insulin resistance, its pathophysiological results form a complex genetic and environmental interactions that contributes to atherosclerosis [6].

Atherosclerosis is thought to be a response to a initial injury to the vascular wall [7]. Although several other theories have been postulated, that oxidative modification of structures contained in low density lipoprotein (LDL) particles renders them susceptible to phagocytosis by resident macrophages, lying the basis for the oxidative stress in atherosclerosis [8,9]. Oxidation of LDL to produce oxidized LDL (Ox-LDL) is also thought to be the initial step. Additionally, production of growth factors by foam cells triggers the proliferation and migration of smooth muscle cells (SMCs) to the intima, initiating the formation of a fibrous cap forming a more advanced lesion. Moreover, oxidative stress not only facilitates stable plaque formation, but also creates an environment that makes the plaque more vulnerable to rupture [10,11].

CVD is the life course disease that begins with the evolution of risk factors that in turn contributes to the development of subclinical atherosclerosis. The onset of CVD itself pretends and adverse prognosis with great risk of recurrent event, morbidity, and mortality. It is also increasingly clear that although clinical assessment is the key stone of patient management, such evaluation has its limitation. Clinicians have used additional tools to aid clinical assessment and to enhance their ability to identify the vulnerable patients at risk for CVD [12]. With the emergence of the newer novel biomarkers for atherosclerosis that reflect the diverse pathobiology for CVD, it has become possible to diagnose subclinical atherosclerosis in the subjects who are at risk for CVD. Biomarkers are one such tool to better identify high risk individuals to diagnose disease condition promptly and accurately. This has also led to effectively prognosticate and advice patients to change their life style in order to prevent the further progression of the disease. Framingham risk score is a widely used risk assessment method for prediction of CVD risk [5]. The score includes the established risk factors like

gender, age, smoking, Total cholesterol (TC), LDL cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), hypertension and diabetes however this method alone does not address family history of CVD (F.CVD), oxidative stress, inflammation and genetic susceptibility to CVD (Familial history of CVD) [5]. However when applied in conjunction with the oxidative stress markers, biomarkers of atherosclerosis, framinghan risk scores becomes a valuable tool in achieving the risk management for CVD. Hence the novelty of the study was to identify the atherosclerotic biomarkers namely E-Selectin, Leptin, OPG, and Ox-LDL in the serum of the subjects in conjunction to framingham risk score to identify the risk for CVD.

STUDY DESIGN

Three hundred subjects were screened for the study who came for the master health checkup. Out of them 50 patients were excluded as they were under medication. 23 patients were excluded due to various systemic diseases like fever and infection etc. The remaining of 227 patients with age range of 30-80 was randomly selected for investigation. These subjects are picked by computerized randomization (stratified method). The study protocol was approved by the Institutional Ethics Committee of frontier Lifeline (Ref: I.E.C/ No/79 Dated 07.12.2011). The written consent form was obtained from each subject.

Two hundred and twenty seven subjects were divided into four different groups: Group I – controls with age range: 30-60 y (n=50) these subjects were free from all the systemic ailments and risk factors. Study groups comprised of Group II - (n=44) with age range: 30-40 y, Group III - (n=50) with age range: 41-50 y and Group IV - (n=83) with age range: 51-80 y. Patients with different risk factors without medication participated as study groups.

Inclusion Criteria

Subjects with age range of 30-80 y. Controls were healthy subjects without any systemic complications. The study groups included subjects with the history of Obesity, Diabetes, Hypertension, Alcoholism, Hypothyroidism, and Smoking as risk factors.

Exclusion Criteria

Controlled diabetes with Insulin treatment, Kidney Diseases, HIV, MI, Inflammatory Diseases, gastro intestinal problems like Gastro-esophageal reflux disease (GERD). Patients on medication with Antihypertensive drugs or statins etc. were excluded from present investigation.

MATERIALS AND METHODS

Sample Collection

A total of 10 ml overnight fasting venous blood was collected from each subject, out of this 5 ml of blood sample was taken into plain tube and allowed to clot adequately for 15 min and centrifuged at 3000 rpm for 10 min for collecting serum, 2 ml of the blood was taken into fluoride tube for collecting plasma to estimate fasting blood glucose (FBS) and 3 ml of blood was collected into EDTA tube for hemoglobin estimation.

5 ml of postprandial blood was drawn into a plain tube and made to clot adequately for 15 min and centrifuged at 3000 rpm for 10 min for collecting serum. This was used for estimating (postprandial blood sugar) PBS and remaining sample was stored at -80°c to until analysis.

Estimation of Anthropometric Measurements

Anthropometric measurements such as height, weight, and Body Mass Index were measured. Weight was measured using a beam balance, to the nearest 0.1 kg and height to the nearest centimeter, using a tape stuck to the wall. Blood pressure levels were also recorded for all the individuals using mercury sphygmomanometer.

In order to screen the patients, routine biochemical parameters like blood glucose, TC, triglycerides, HDL-C, LDL-C were studied in serum using Randox Daytona - auto analyser. Hemoglobin levels were estimated using whole blood using Bio-Rad Affinity Chromatographic System. E-Selectin, Leptin, and OPG are estimated using ELISA kits from Ray Biotech. Whereas Ox-LDL is estimated using ELISA kit (QAYEE-Bio, China) as suggested by Zagura et al., 2010 [13].

Framingham Risk Score

The Framingham risk score was calculated using online framingham risk Calculator [14]. Cardiovascular risk Prognosis for 30 years was done using Framingham risk score by calculating individual scoring points from each risk factor for CVD. Based on the lipid and BMI values, the results were displayed as Hard CVD (Coronary death, MI, Stroke) and Full CVD (Coronary death, MI, Coronary insufficiency, angina, Ischemic stroke, Hemorrhagic stroke, Transient ischemic attack, Peripheral artery disease, Heart failure) [14].

STATISTICAL ANALYSIS

Statistics was done using SPSS 17.0 software. Results were expressed as Mean±SD. p-value ≤0.05 was considered significant. Group comparison is done by using ANNOVA (Post-hoc Sheffe's alpha test). Pearson correlation was used for correlating the values of atherosclerotic biomarkers with 30 y framingham risk scores. Receiver operating characteristic (ROC) Curve analysis was done for testing sensitivity and specificity.

RESULTS

BMI, were found to be significantly ($p<0.05$) elevated in Group II, III and IV when compared to Group I. The Systolic and diastolic Blood pressure were found to be significantly ($p<0.05$) elevated in Group II, III when compared to Group I. FBS and PPBS were found to be significantly ($p<0.05$) elevated in Group IV when compared to Group I. The lipid profile values were not significant when compared to Group I [Table/Fig-1].

[Table/Fig-2] shows risk factors distribution which emphasis that the likelihood ratio of getting CVD for diabetes and hypertension is greater when compared to other risk factors like smoking, alcoholism, hypothyroidism, obesity, F.CVD.

E-Selectin, OPG and Ox-LDL, the markers of atherosclerosis were found to be significantly ($p<0.05$) elevated in Group III and IV where as for leptin, it was found to be significantly ($p<0.05$) elevated in Group II, III and IV when compared to Group I [Table/Fig-3].

From the ROC Curve analysis it is clear that E-Selectin, Leptin, OPG, and OxLDL (Markers of Atherosclerosis) has shown a greater area under the curve (>0.600) with high sensitivity and specificity which proves to be a good biomarkers of subclinical atherosclerosis [Table/Fig-4,5].

The pearson correlation was done between atherosclerotic biomarkers and framingham risk score for individual study groups [Table/Fig-6]. In Group-III and IV, E-selectin showed a significant correlation ($p<0.01$) for both lipid and BMI based full and hard CVD where as in Group II, E-Selectin did not shown any significant correlation. Leptin has shown a significant correlation ($p<0.01$) for BMI based Full and hard CVD in Group II, III and IV except for lipid based full CVD in group II. In Group II, III and IV, OPG showed significant correlation ($p<0.05$) with lipid and BMI based full CVD. Ox-LDL has shown significant correlation ($p<0.01$) for lipid and BMI based full and hard CVD only in Group IV, where as in Group III, Ox-LDL showed a significant correlation ($p<0.05$) for BMI based full and hard CVD and Lipid based full CVD., however in Group II, no correlation was shown. It is clear from the results that leptin and OPG are markers for CVD risk prediction in individuals with age above 30 y. and leptin and OPG is a markers for CVD risk prediction in individuals with age above 40 y [Table/Fig-6].

Study Groups	Group I Age=30- 60	Group II Age=30-40	Group III Age= 41-50	Group IV Age=51- 80	Significance P≤0.05 (between control and cases)
Age (years)	44.08±11.47	35.84±4.05	46.12±2.93	59.72±5.96	S(Group IV)
Height (Cms)	163.15±12.47	165.02±7.97	164.58±7.82	160.97±8.37	NS
Weight (Kgs)	71.80±10.23	77.81±10.27	78.34±12.74	71.33±10.76	NS
BMI	25.46±3.52	28.65±3.99	28.85±3.74	28.12±4.41	S(Group II, III, IV)
SBP mm Hg	123.78±13.00	109.27±31.44	100.46±28.00	104.21±33.99	S(Group II, III, IV)
DBP mm Hg	76.95±9.22	108.45±26.84	105.20±25.37	107.61±29.19	S(Group II, III, IV)
FBS mg/dl	93.72±21.88	100.0023.55	112.12±34.40	126.85±54.30	S(Group IV)
PPBS mg/dl	125.26±33.03	168.06±171.146	156.10±66.34	187.87±100.45	S(Group IV)
TC mg/dl	164.23±25.78	171.59±31.18	182.28±34.81	166.75±38.07	NS
LDL mg/dl	100.84±21.89	101.20±29.87	108.50±27.18	101.31±28.10	NS
HDL mg/dl	40.89±4.98	40.38±4.07	44.26±13.33	40.36±4.60	NS
TG mg/dl	137.71±62.88	158.31±113.63	175.50±65.66	143.68±58.42	NS
Hb G%	13.89±1.99	13.94±1.67	14.94±1.22	13.26±1.64	NS

[Table/Fig- 1]: Distribution of anthropometric and biochemical parameters in different groups.

Variables	Group I Age=30-60		Group II Age=30-40		Group III Age= 41-50		Group IV Age=51-80		Likelihood Ratio	Significant groups at 5% Level
	No	Percentile	No	Percentile	No	Percentile	No	Percentile		
Smoking	0	0%	07	15.6%	14	28.0%	07	8.4%	22.65	Sig 0.001
Alcoholic	0	0%	11	24.4%	09	18.0%	06	7.2%	20.89	Sig 0.001
Hypertension	0	0%	21	46.7%	24	48.0%	49	59.0%	61.15	Sig 0.001
Diabetes	0	0%	13	28.9%	18	36.0%	50	60.2%	61.93	Sig 0.001
Dyslipidaemia	0	0%	16	35.6%	15	30.0%	29	34.9%	33.53	Sig 0.001
Obesity	0	0%	07	15.6%	17	34.0%	12	14.5%	25.90	Sig 0.001
Familial CAD	0	0%	08	17.8%	13	26.0%	10	12.0%	19.71	Sig 0.001
CAD	0	0%	04	8.9%	07	14.0%	22	26.5%	23.66	Sig 0.001
Hypothyroidism	0	0%	02	4.4%	05	10.0%	07	8.4%	7.81	Sig 0.001
LVH	0	0%	01	2.2%	02	4.0%	04	4.8%	3.83	Sig 0.001

[Table/Fig-2]: Risk factors distribution in different groups

Study Groups	Group I Age=30-60	Group II Age=30-40	Group III Age= 41-50	Group IV Age=51-80	Significance p≤0.05 (Between control and cases)
E-Selectin ng/ml	47.63±23.47	54.24±18.91	65.27±18.17	67.28±20.11	S(Group III, IV)
Leptin ng/ml	12.08±5.72	17.32±7.36	18.36±8.79	22.99±10.17	S(Group II, III, IV)
OPG ng/ml	1.72±0.511	2.06±0.418	2.29±0.616	2.86±0.78	S(Group III, IV)
Ox-LDL ng/ml	83.93±50.86	114.53±71.59	156.76±54.90	236.84±85.24	S(Group III, IV)

[Table/Fig-3]: Distribution of Atherosclerotic Biomarkers in different groups
S = Significant, NS = Non Significant

Test Result Variable(s)	Sensitivity	Specificity	Area Under Curve	Asymptotic 95% Confidence Interval		Cut-off Value
				Lower Bound	Upper Bound	
E-Selectin	65.1	62.1	0.647	0.574	0.719	41.43
Leptin	73.5	57.1	0.702	0.628	0.776	15.55
Osteoprotegerin	56.6	89.3	0.791	0.729	0.854	2.71
Ox-LDL	69.9	90.0	0.859	0.806	0.912	198.65

[Table/Fig-4]: ROC Curve analysis data

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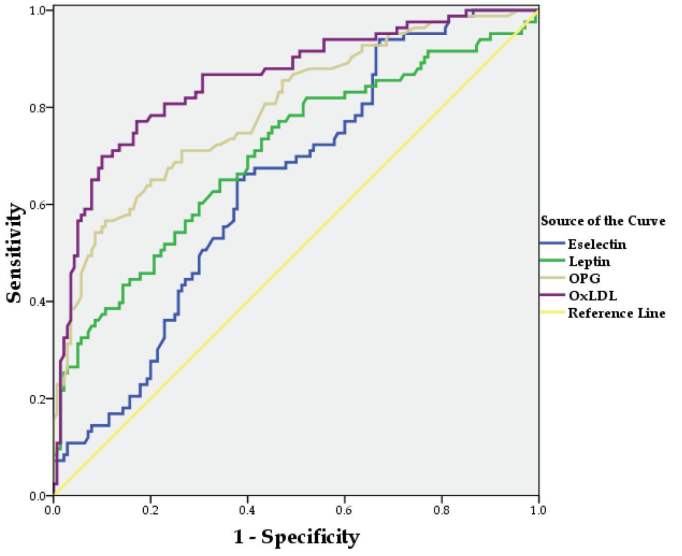
DISCUSSION

From the results it is clear that BMI is significantly increased in study subjects on comparison with controls [Table/Fig-3]. Freedman et al., [15] have suggested that cardiovascular risk factors have a stronger association with BMI. Metabolic risk assessment, regardless of BMI, can also predict the development of type 2 diabetes, CVD, and death. FBS and PPBS were found to be significantly elevated in Group IV when compared to Group I. It is stated that hyperglycaemia is thought to induce oxidative stress interfere with normal endothelial function by over production of reactive oxygen species, which influence atherosclerosis through several mechanisms. In addition glucose variability might contribute to the process of atherosclerosis as well [16]. However Gong su et al., [17] stated that the role of glucose variability in pathogenesis of atherosclerosis is not clear. The lipid profile values like TC, HDL-C, LDL-C, and Triglycerides, were found to be non significant when compared to study groups to control group as the patients were in subclinical atherosclerosis, and the present study is in accordance with Bugge et al., [18]. He also stated that TC, HDL-C, LDL-C, and Triglycerides levels were independent predictors of subclinical atherosclerosis.

The atherosclerotic biomarkers like E-Selectin, Leptin, OPG, and Ox-LDL were found significant elevated in the subjects within the age of 40-80 year on comparison with controls. This observation shows that the subclinical atherosclerosis was significantly elevated from the age group of 41-50 year and 51-80 year but not with the age group of 30-40 year (Group-I). The reason could be that this early form of atherosclerosis may not be strongly associated with our biomarker panel, which has been associated with atheroma in the coronaries and carotid arteries. Furthermore, these biomarkers also may be closely associated with clinical states leading to



[Table/Fig-5]: ROC curve for atherosclerotic biomarkers

the development of atherosclerosis [19] (e.g.,: obesity, elevated cholesterol, diabetes) in age group of 30-40 year.

In our study, ROC curve analysis of atherosclerotic biomarkers like OPG, Leptin, E-Selectin and Ox-LDL showed a greater area under curve with a good sensitivity and specificity values, which explain the applicability of these biomarkers in subclinical atherosclerosis [Table/Fig-4&5]. Recently, many investigators have focused on the determination of circulating – blood markers of atherosclerosis. There are a number of markers found in blood that indicate the presence of the atherosclerotic process and represent risk for the development of cardio vascular events. Endothelial dysfunction is the first step of atherosclerosis and precedes macroscopic morphologic alteration [20]. E-selectin is found only on endothelial cells stimulated by inflammatory cytokines involved in the recruitment of leukocytes on the activated vessel wall during inflammation and play an important role in the early stages of atherosclerosis and its complications [21] whereas Leptin is secreted by adipocytes, a hormone, which is increased in obese subjects. Leptin is involved in the regulation of the energy expenditure and appetite via hypothalamic receptors [22]. In our study leptin showed a significant rise in the study groups [Table/Fig-4] and the results were in accordance to the Soederberg et al., [23] who stated that leptin were found to be significantly associated with established cardiovascular risk factors such as elevated blood pressure and obesity [23]. Francisco Leyva et al., [24], has also demonstrated that inter individual variations in plasma leptin concentrations are strongly related to the components of a metabolic syndrome of cardiovascular risk [24]. Osteoprotegerin, a key factor in bone remodeling is a member of the tumor necrosis factor receptor family and a decoy receptor for the receptor activator of nuclear factor-B ligand and tumor necrosis factor–related apoptosis-inducing ligand [25]. According to Abedin et al., [26] OPG was an independent predictor of angiographically diagnosed but asymptomatic CVD in type 2 diabetic patients. Elevated OPG levels were also found to be associated with the degree of coronary calcification in the general population as a marker of coronary atherosclerosis [25]. In our study there was a significant correlation between OPG and framingham risk score in the study group and the findings are in accordance with the Avignon et al., [25] who showed that a close correlation between OPG levels and surrogate measures of cardiovascular risk [26]. Number of studies suggested that OxLDL is a more potent pro-atherosclerotic motivator than the native unmodified LDL [27].

			E-selectin ng/ml	Leptin ng/ml	OPG ng/ml	Ox-LDL ng/ml
	GROUP II					
30 Years CVD	FULL CVD % Lipid	R-Value	0.117	0.309*	0.325*	0.254
		Sig.	0.454	0.044	0.034	0.100
	HARD CVD % Lipid	R-Value	0.039	0.300	0.226	0.304*
		Sig.	0.806	0.051	0.145	0.048
	FULL CVD % BMI	R-Value	0.143	0.416**	0.317*	0.239
		Sig.	0.359	0.006	0.038	0.123
	HARD CVD % BMI	R-Value	0.068	0.407**	0.249	0.260
		Sig.	0.663	0.007	0.107	0.092
	GROUP-III					
30 Years CVD	FULL CVD % Lipid	R-Value	0.561**	0.546**	0.315*	0.299*
		Sig.	0.000	0.000	0.026	0.035
	HARD CVD % Lipid	R-Value	0.521**	0.597**	0.259	0.242
		Sig.	0.000	0.000	0.069	0.090
	FULL CVD % BMI	R-Value	0.573**	0.453**	0.337*	0.347*
		Sig.	0.000	0.001	0.017	0.014
	HARD CVD % BMI	R-Value	0.510**	0.468**	0.276	0.307*
		Sig.	0.000	0.001	0.053	0.030
	GROUP-IV					
30 Years CVD	FULL CVD % Lipid	R-Value	0.274*	0.535**	0.234*	0.459**
		Sig.	0.013	0.000	0.035	0.000
	HARD CVD % Lipid	R-Value	0.185	0.563**	0.092	0.424**
		Sig.	0.096	0.000	0.409	0.000
	FULL CVD % BMI	R-Value	0.339**	0.541**	0.225*	0.521**
		Sig.	0.002	0.000	0.043	0.000
	HARD CVD % BMI	R-Value	0.262*	0.597**	0.119	0.493**
		Sig.	0.017	0.000	0.288	0.000

[Table/Fig-6]: Pearson correlation between framingham risk percentile to atherosclerosis biomarkers
**Significant at p<0.01 levels, *Significant at p<0.05 level

It was observed that E-Selectin, OPG and Ox-LDL are significant biomarkers for tracking the individual, with age above 40 years for diagnosing risk to CVD [Table/Fig-3]. ROC analysis has also shown that these are more precdicatable biomarkers with >0.650 AUC [Table/Fig-4]. The association between framingham risk scores to biomarkers of atherosclerosis was found to be significant [Table/Fig-6]. E-Selectin and Ox-LDL is well correlated in Group III and IV but not with Group II for lipid and BMI based Hard and Full CVD framingham risk scores which states that E-Selectin and Ox-LDL are good biomarkers for tracking the individual for risk to CVD with age above 40 year.

Our study showed that Leptin and OPG were significant biomarkers for tracking the individual for risk to CVD with age above 30 y and 40 y respectively [Table/Fig-3&4]. The correlation analysis [Table/Fig-6] also showed leptin to be a significant biomarker for tracking the individual for risk to CVD with age above 30 y. However, OPG found to be a significant biomarker for tracking the individual for risk to CVD with age above 30 y with more sensitivity and specificity when used with framingham risk score [Table/Fig-6].

From the Annova and ROC curve analysis it is observed that E-Selectin, OPG and Ox-LDL very significant biomarkers for tracking the individual for risk to CVD with age above 40 y [Table/Fig-5&6]. However, in correlation analysis, when the biomarkers were correlated with framingham risk scores [Table/Fig-6], we found out that E-Selectin and Ox-LDL was well correlated in Group III and IV which states that E-Selectin and Ox-LDL are good biomarkers for tracking the individual for risk to CVD with age above 40 y. On the other hand, leptin was also found to be a significant biomarker for tracking the individual for risk to CVD with age above 30 y [Table/Fig-6].

Hence framingham correlation study in conjunction with biomarkers has enhanced our findings with OPG, leptin and Ox-LDL to certain age in establishing the risk prediction for CVD. The application of E-Selectin, leptin, OPG and Ox-LDL as biomarkers along with framingham risk scores in prediction risk for CVD in the individuals with subclinical atherosclerosis is more reliable and predictable as compared to the individual biomarkers alone.

CONCLUSION

Hence we conclude the application of E-Selectin, leptin, OPG and Ox-LDL as biomarkers along with framingham risk scores in prediction risk for CVD in the individuals with subclinical atherosclerosis is more reliable and predictable as compared to the individual biomarkers alone.

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PARTICULARS OF CONTRIBUTORS:
1. Research Scholar, Department of Biochemistry, Frontier Lifeline Hospital, Mogappair, Chennai, India.
2. Professor, Department of Periodontics, Meenakshi Ammal Dental College, Madhuravoyal, Chennai. India.
3. Director - Research, Meenakshi Academy of Higher Education and Research, West K.K. Nagar, Chennai, India.
4. Professor, Department of Biochemistry, Meenakshi Ammal Dental College, Madhuravoyal, Chennai, India.
5. Head, Department of Biochemistry, Frontier Lifeline Hospital, Mogappair, Chennai, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Rama Krishna Reddy Y.V.,
Research Scholar, Department of Biochemistry, Frontier Lifeline Hospital, Mogappair, Chennai-600101, India.
E-mail: krishna262424@gmail.com

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