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A STUDY ON THE RELATIONSHIP BETWEEN SMALL DENSE LDL AND AN EXTENDED LIPID PROFILE, INCLUDING NON-HDL, APO-LIPOPROTEINS, AND THEIR RATIO IN THE DIAGNOSIS OF CARDIOVASCULAR RISK

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ABSTRACT

Coronary Heart Disease (CHD) is one of the most prevalent metabolic disorders linked with dyslipidemia, with Asian Indians having a greater incidence. According to reports, migratory Asian Indians have dyslipidemia, which is characterised by high Triglycerides (TG), low High Density Lipoprotein (HDL), and near normal Low Density Lipoprotein (LDL) cholesterol levels. Elevated LDL, which is also the primary goal of lipid lowering treatment, is one of the key risk factors for CHD. LDL is classified into many subclasses based on its atherogenic propensity, with Small dense (sd) LDL being a highly important risk biomarker for early CHD. All atherogenic Apo-lipoprotein B-containing lipoproteins, including LDL-C, VLDL-C, IDL-C, lipoprotein(a), chylomicrons, and chylomicron remnants, are included in non-HDLc. Apo-lipoproteins, which include Apo-lipoprotein B and Apo-lipoprotein A_1, are cholesterol transporters that play a key role in lipid metabolism. Furthermore, Apo-B and Apo- A_1 indicate total atherogenic and non-atherogenic particles, respectively. As a result, their Apo-B/Apo- A_1 ratio has been significantly linked to a variety of cardiovascular problems. The study was conducted to assess the role of small dense LDL, Non-HDL and Apo-lipoprotein Ratios as an early clinical marker for cardiovascular risk. This Case-Control study was conducted in SRM Medical College Hospital and Research center on subjects attending the cardiology and medicine OP. The study was conducted on 546 subjects in the age group of 30 to 55 years. After overnight fasting blood samples were collected for analysis of Apo-lipoprotein- B, Apo-lipoprotein-A_1 were measured by the ELISA method. And sd-LDL, Non-HDL and Apo-B/Apo-A_1 ratio were obtained by Calculation. Statistical analysis was done using the Student 't' test and Pearson correlation analysis for the comparison between two groups. sd-LDL, Non-HDL, Apo-lipoproteins and Apo-B/Apo-A_1 ratio were significantly increased in the CHD group when compared to controls. Whereas The mean of Low-Density Lipoprotein level in CHD was elevated and statistically significant (p-value <0.001) compared to the normal healthy controls. The study concludes that a strong association exist between sd-LDL, non-HDL-C, apo-lipoproteins and its Apo-B/Apo-A_1 ratio. The estimation of all these parameters appears to be a better marker in predicting the early risk of cardiovascular disease when compare to conventional lipid parameters in both diabetic and non-diabetic CHD subjects, which could be effectively used in clinical practice.

KEYWORDS: Coronary Heart Disease, non- High Density Lipoprotein, Low Density Lipoprotein, Apo-lipoproteins

INTRODUCTION

Coronary heart disease (CHD) is the most common cause of death not only in the developed but also the developing countries. Biochemical marker which can pick up the risk or becoming important nowadays and an assessment of these risk factors is the need of the hour. There has been an explosive increase in coronary

heart disease (CHD) in Indians which affects a population of below 45 years of age. [1] Lipids play a key role in the development of atherosclerosis. For nearly 20 years, LDL‑C has been regarded as a major lipid risk factor and target of lipid-lowering therapy. Large meta-analyses of interventional studies with statins have shown that reduced levels of LDL‑C can be beneficial in lowering the risk of developing coronary heart disease in non-diabetic and diabetic populations. [2] The LDL which varies in structure size and density is divided into two fraction using gel electrophoresis. First fraction is small and dense it is called as sd-LDL and other one is large which is buyont. This sd-LDL has higher tendency to get oxidized leading to their deposition in macrophages to form foam cells resulting in the formation of atheromateous plaque. Higher the proportion of sd-LDL, more the risk of CHD. [3]

Sd-LDL and their atherogenic effect is due to the following mechanisms: remain longer in the plasma, do not bind very well to the LDL receptors, bind well with the scavenger receptors, show increased susceptibility to oxidation and enter the arterial wall easily. Also, sd-LDL may promote endothelial dysfunction, induce plasminogen activator inhibitor-1 (PAI-1) and thromboxane secretion in endothelial cells, and lead to calcium increase in the arterial smooth muscles. Hence, patients with a higher sd-LDL fraction are more prone to the severe form of CHD. [4] Numerous other studies have also observed that sd-LDL can predict CHD occurrence independent of traditional lipid parameters. [5–7].

Non–high-density lipoprotein cholesterol (non-HDL-C) is the sum of all atherogenic cholesterol in serum, as proposed by the Third Report of The National Cholesterol Education Program. Decreasing the level of non-HDL-C is the secondary goal of treatment in the process of anti-atherosclerosis. [8] Previous studies have shown that non-HDL-C is associated with the risk of coronary heart disease. [9, 10] Non-high density lipoprotein cholesterol and Apo-lipoprotein are considered as a secondary objective for lipid-lowering therapy according to the European Society of Cardiology/European Atherosclerosis Society (ESC/EAS Guidelines) 2016. It has been suggested that non-HDL-c or Apo-lipoprotein may provide a more accurate estimation of CHD risk than LDL-c [11]. In 2019, the ESC/EAS Guidelines suggested that when evaluating patients with diabetes, metabolic syndrome, obesity, high triglyceride concentration, non-HDL, and Apo-lipoprotein could be chosen to assess cardiovascular risk. [12]

Apolipoproteins are important components of lipoprotein particles, and there is accumulating evidence that the measurement of various forms of apolipoproteins may improve the prediction of the risk of cardiovascular disease. [13] Apolipoprotein B (apoB) presents as a single molecule in all potentially atherogenic lipoprotein particles, i.e. very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), and LDL[5]. Thus, a plasma value of total apoB reflects the number of cholesterol and, to some degree, triglyceride-containing particles. [14, 15]

Apolipoprotein A_1 (apo A_1) is the major apolipoprotein associated with high-density lipoprotein (HDL), and a main initiator and “driver of the reverse cholesterol transport”. Apo- A_1 can also manifest anti-oxidant and anti-inflammatory effects, and it can stimulate both endothelial production of nitric oxide as well as release of prostacyclin from the endothelium. Thus, apo- A_1 manifests several anti-atherogenic effects. [16, 17] An accumulating body of data indicates that the apoB/apo- A_1 ratio is a powerful marker of risk for future cardiovascular disease, but the different relationships between the plasma apoB, apo- A_1, apoB/apo- A_1 ratio and the prevalence of CHD have not been consistently shown. [18] Therefore, adding a new dimension of our existing knowledge several studies have found independent and continuous association between these factors and cardiovascular disease

Materials and methods

Study design and population

This Cross-Sectional study was conducted from October 2020 to June 2021 at SRM Medical College Hospital and Research Centre, Chennai, Tamil Nadu, India on subjects attending the Cardiology and medicine outpatient. Totally 546 subjects were included in the age group 30-55 years.

Inclusion Criteria:

ISSN: 0975-3583, 0976-2833 VOL 12, ISSUE 03, 2021

The CHD Subjects including both males and females selected on the basis of coronary angiography. Patients with chest pain, ECG changes, increased cardiac markers such as creatinine phosphokinase (CPK-MB) and troponin level. chest pain lasting for > 30 min, elevated ST-elevation > 0.1 mV on at least 2 adjacent electrocardiographic leads, Increase of Creatine kinase (CK) to peak levels of at least 2-fold the upper limit of normal values

The control group consists of persons with no clinical and ECG evidence of CHD and negative history of the past event of CHD or stroke, Diabetes Mellitus, Hypertension, smoking, Dyslipidemia, and family history of CHD.

Exclusion Criteria

The subjects who were on treatment for renal failure, cancer, autoimmune diseases, surgery, fever, alcoholics, smokers, pregnancy, and patients with corticosteroids, estrogen, anti-retroviral drugs psychotropic medications. Thyroid, arthritis, rheumatoid arthritis, acute/chronic infection patients were excluded

Anthropometric Measurement

Anthropometric measurements including height (meters), Weight (Kg), Waist Circumference (cm) and Hip Circumference (cm), BMI, and waist-hip ratio were calculated.

Baseline Measurement

Medical and demographic data were collected at the period of enrolment, and documents were de-identified before investigation. Basic info on age, gender, history of diabetes, and hypertension were collected by questionnaire during the clinical appointment. Arterial blood pressure was measured using standard methods. Information of laboratory reports was noted for all the subjects. The overnight fasting blood samples were collected from the antecubital vein. The blood was centrifuged for 15 minutes at 2500 RPM and serum was separated for the quantification of glucose and lipid profile includes plasma total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL), TC/HDL-C ratio, LDL-C/HDL-C ratio, and HbA1c were measured using (Beckmann Coulter AU480 Analyzer). Apo-B and Apo-A_I levels were estimated by the ELISA method

Measurement of Apo-lipoprotein- B 100

The Human ApoB ELISA Kit from Cell Biolabs is an enzyme immunoassay designed for the detection and quantification of human ApoB (ApoB-100) in blood samples. Before conducting the ELISA, a normal serum sample must be diluted 20,000 times with PBS containing 0.1 percent BSA. Using 450 nm as the principal wave length, measure absorbance with a spectrophotometer.

Measurement of Apo-lipoprotein- A_1

Precoated and blocked 96-well plates with an Apo-lipoprotein A_1 specific antibody. After adding test samples to the wells, an Apolipoprotein A_1 specific biotinylated detection antibody is added, and the wells are washed with wash buffer. Unbound conjugates are washed away with wash buffer after the Streptavidin-Peroxidase Complex is introduced. The Streptavidin-Peroxidase enzymatic reaction is then seen using TMB. TMB is catalysed by Streptavidin-Peroxidase to generate a blue color product that becomes yellow when an acidic stop solution is added. The amount of Apolipoprotein A_I collected in the plate is directly related to the amount of yellow colouring. Using a micro plate reader, absorbance is measured at 440-460 nm.

ISSN: 0975-3583, 0976-2833 VOL 12, ISSUE 03, 2021

Measurement of sd-LDL

SD-LDL was calculated indirectly by formulation assumed by Hattori et al., [19]

sd-LDL =

0.580 (non HDL-C) + 0.407 (LDL-C) -0.719 (Calculated LDL-C) - 12.05

Calculated LDL-C = Total Cholesterol – HDL- (TG/5)

Measurement of non-HDL

The sum of Very Low Density Lipoprotein (VLDL) +LDL cholesterol is called non-HDL cholesterol. It is calculated routinely as total cholesterol minus HDL cholesterol.

Statistical analysis

Data were analyzed using a Statistical Package for the Social Sciences (SPSS 21.0). The data collected from the study were shown as mean and standard deviation. Differences were considered significant if the p-value was <0.05. Student's t-test and ANOVA were used to analyze the difference between the mean levels of various parameters. Pearson's correlation coefficient was calculated to find out the correlation between different parameters.

RESULTS

Table 1: Demographics and baseline characteristics of Non-diabetic, Diabetic Coronary Heart Disease Subject and Healthy Controls

Parameters	**Group-I Controls (n=182)**	**Group-II Non Diabetic CHD patient (n=182)**	**Group-III Diabetic CHD patient (n=182)**	**P-Value**
Mean age (years, mean ± S.E.M.)	41.8 ± 9.7	42.3 ± 8.5	48.6 ± 6.4	<0.001
Male Sex (%)	114(62.6%)	124(68.13%)	148(81.31%)	-
Female Sex (%)	68 (37.3%)	58(31.8%)	34(18.68%)	-
Body mass index (kg/m^2)	21.91±0.37	23.47±0.35	25.03±0.19	<0.001
Waist circumference (cm)	90.9 ± 10.1	98.8 ± 9.6	98.8 ± 4.3	<0.001
Waist to hip ratio	0.94±0.02	1.01±0.01	1.05±0.03	< 0.001
Waist to height ratio	0.56±0.01	0.62±0.02	0.65±0.01	<0.001
FBG (mg/dl)	83.24±4.18	90.29±6.98	169.98±36.28	0.065
HbA1c (%)	4.9±0.17	4.41±0.28	8.49±2.32	< 0.001
Wt = weight (kg), Ht = height (metres), BMI = body mass index, WC = waist circumference, HC = hip circumference, WHR – waist hip ratio, WC/ht ratio – waist circumference / height ratio, FBG – Fasting Blood Glucose				
ANOVA	. The mean and standard deviation are used to express the values. P value of 0.05 is regarded as significant. **Very Significant ***Highly Significant NS-Not statistically significant			

ISSN: 0975-3583, 0976-2833 VOL 12, ISSUE 03, 2021

Table 2: Biochemical parameters of Non- Diabetic and Diabetic Coronary Heart Disease Subject and Healthy Controls

Variable	Controls (n=182)	Non Diabetic CHD patient (n=182)	Diabetic CHD patient (n=182)	p- Value
Total cholesterol (mg/dl)	158.8±16.3	199.46±21.42	204.55±22.33	< 0.001
Triglyceride (mg/dl)	102.6±30.5	169.7±13.4	188.86±12.08	0.391
HDL-C (mg/dl)	46±9	37.83±4.25	34±7	< 0.001
LDL-C (mg/dl)	107±12.71	161.68±31.15	186.4±23.68	< 0.001
VLDL (mg/dl)	17.26±8.77	28.06±12.14	34.08±14.29	<0.001
TC/HDL Ratio	3.71±0.70	6.17±1.14	6.50±1.36	<0.001
LDL/HDL Ratio	2.35±0.53	4.22±0.75	4.41±0.90	< 0.001
TC – Total Cholesterol, TG– Triglycerides, HDL–High Density Lipoprotein, LDL – Low Density Lipoprotein, VLDL – Very Low Density Lipoprotein, Non-HDL- Non- High Density Lipoprotein**,**				
ANOVA	. Values are expressed in Mean ± Standard Deviation. *P value < 0.05 is considered significant. **Highly Significant ***Very Highly significant NS-Not significant			

ISSN: 0975-3583, 0976-2833 VOL 12, ISSUE 03, 2021

TABLE 3: Comparison of Oxidative Atherogenic Risk Markers between diabetic and non-diabetic subjects with CHD and healthy controls

Variable	Controls (n=182)	Non Diabetic CHD patient (n=182)	Diabetic CHD patient (n=182)	p- Value
Non-HDL	17.77±3.56	35.80±4.36	36.38±4.96	<0.001**
sd-LDL	26.22±6.03	45.80±11.36	48.43±10.24	<0.001***
Apo-B 100 (mg/dl)	82.77±12	122.79±15.09	143.65±16.12	<0.001***
Apo-A_1 (mg/dl)	127.82±3.56	91.29±4.34	84.95±4.93	<0.001***
Non-HDL-C/ HDL	2.72±0.69	5.32±1.28	6.12±2.04	0.0993
Non-HDL-C/ TC	0.72±0.05	0.83 ±0.02	0.97 ±0.03	0.0882
Apo-B/Apo-A_1 Ratio	0.64±0.07	1.33±0.10	1.36±0.09	<0.001***
sd-LDL- small dense Low Density Lipoprotein**, Apo-B -** Apo-lipoprotein B 100, **Apo-A_1 -** Apo-lipoprotein A_1				
ANOVA	. Values are expressed in Mean ± Standard Deviation. *P value < 0.05 is considered significant. **Highly Significant ***Very Highly significant NS-Not significant			

ISSN: 0975-3583, 0976-2833 VOL 12, ISSUE 03, 2021

Table 4: The Pearson correlations analysis between sd-LDL with other biochemical parameters between diabetic and non-diabetic subjects with CHD

	Sd-LDLc			
	CHD Patients without Diabetes		CHD Patients with Diabetes	
	r-Value	p-Value	r-Value	p-Value
BMI	0.154 [A]	<0.001**	0.069 [A]	<0.001**
WAIST CIRCUMFERENCE	0.035 [A]	<0.001**	0.042 [A]	<0.001**
WAIST HIP RATIO	0.296 [A]	<0.001**	0.019 [A]	<0.001**
FBG	-0.024 [B]	<0.001**	0.163 [A]	<0.001**
TOTAL CHOLESTEROL	-0.051 [B]	<0.001**	0.084 [A]	<0.001**
TRIGLYCERIDE	0.221 [A]	<0.001**	0.131 [A]	<0.001**
HDL-C	-0.278 [B]	<0.001**	-0.158 [B]	<0.001**
LDL-C	0.905 [A]	<0.001**	0.809 [A]	<0.001**
VLDL-C	0.219 [A]	<0.001**	0.192 [A]	<0.001**
TC/HDL RATIO	0.568 [A]	<0.001**	0.831 [A]	<0.001**
LDL/HDL RATIO	0.045 [A]	<0.001**	0.254 [A]	<0.001**
non-HDL	0.905 [A]	<0.001**	0.809 [A]	<0.001**
non-HDL/TC	0.592 [A]	<0.001**	0.820 [A]	<0.001**
non-HDL/HDL	0.792 [A]	<0.001**	0.878 [A]	<0.001**
Apo-B 100	0.902 [A]	<0.001**	0.982 [A]	<0.001**
Apo-A_1	-0.906 [B]	<0.001**	-0.906 [B]	<0.001**
Apo-B/Apo-A_1 Ratio	0.037 [A]	<0.001**	0.037 [A]	<0.001**
HbA1c	-0.015 [B]	<0.001**	0.155 [A]	<0.001**

A- Positive Correlation B- Negative Correlation

Out of 546 Subjects, 124(68.13%) of male and 58(31.8%) of female subject have Non-Diabetic CHD. 148(91.31%) of male and 34(18.68%) of female subject have Diabetic CHD. And 114(62.6%) of male and 68(37.3%) of female subject were selected as control (Table-1). Majority of Non-Diabetic CHD subjects fall in the age group of 40-50 years and in Diabetic Subjects with CHD were in the age group of 40-50 years. While most of the control subjects fall in the age group of 30-45 years. In both Diabetic and Non-Diabetic CHD patients, the mean level of BMI, Waist Circumference, Waist Hip Ratio, systolic blood pressure were significantly elevated when compared to controls as depicted in [Table 1].

The study shows FBG, Total cholesterol, Triglyceride, LDL-C, VLDL-C, LDL/HDL ratio, Total Cholesterol/HDL ratio, HbA1c are significantly elevated in these patients compared to control depicted in

the [Table 2]. The mean levels of HDL-C levels did not differ significantly among the two groups in diabetic and non-diabetic subject with CHD. In non-diabetic CHD subjects and CHD subjects with Diabetes, The mean level of sd-LDL, non-HDL, Apo-B and Apo-B/ Apo- A_1 ratio values show a statistically significantly elevated when compared to controls ($P < 0.001$). And the mean level of Apo-A_1 were decreased significantly in diabetic subjects with CHD when compared to CHD subjects without Diabetes and control ($P < 0.001$).

In non-Diabetic subjects with CHD, Small Dense Low density lipoprotein cholesterol were positively correlated with BMI, Waist Circumference and Waist Hip Ratio, Triglyceride, HDL-C, LDL-C, VLDL-C, Total Cholesterol/HDL ratio, LDL/HDL ratio, non-HDL, Apo—B, Apo-B/Apo-A_1 Ratio. And sd-LDL are negatively correlated with FBG, HDL-C, Apo-A_1 [Table 3].

In Diabetic subjects with CHD, Small Dense Low density lipoprotein cholesterol were positively correlated with BMI, Waist Circumference and Waist Hip Ratio, FBG, Triglyceride, LDL-C, VLDL-C, Total Cholesterol/HDL ratio, LDL/HDL ratio, non-HDL, Apo—B, Apo-B/Apo-A_1 Ratio. And sd-LDL are negatively correlated with HDL-C, Apo-A_1 [Table 4].

Discussion:

Predominance of sd-LDL-C is a major component of an atherogenic lipoprotein phenotype, and a source of increased risk for coronary heart disease. Various case control and prospective studies have shown that sd-LDL levels are high in patients at high risk of cardiovascular events, including those with Type 2 diabetes. [20]

Relative risk of all lipoproteins in CVD has been extensively studied and the principle target for cardiovascular preventive strategies has been the low density lipoprotein cholesterol. Based on the mechanism by which sd-LDL induces atherosclerosis, it can be concluded that sd-LDL carries a higher risk of coronary heart disease than LDL-C [21]. Several large studies have evaluated the relationship between LDL-C particle size and coronary heart disease [22].

This study demonstrated a significant correlation of sd-LDL with non-HDL which was stronger than the correlation with LDL as well as with apo B/apo A_1. The sd-LDL is found to be elevated, in conditions like dyslipidemia is a feature seen in DM and metabolic syndrome. This sd-LDL has maximum capacity to get oxidized and form foam cells that leads to atherosclerosis. The atherogenic nature of sd-LDL is particular significance because of the high incidence of CHD in Indians. Sd-LDL helps to penetrate the arterial walls very easily and also their longer circulation timeincreases the probability of atherogenic modificationswhile their pro-inflammatory properties make them proneto aggregation and formation of complexes, furtherincreasing their atherogenicity. [23, 24]

Small dense LDL is associated with a threefold higher risk of myocardial infarction and its predominance forms a major component of an atherogenic lipoprotein phenotype. Both non-HDL and Apo-lipoprotein are also recommended for screening, diagnosis, and management alternatively when compared to LDL-c, regarding the progression of CHD. [25]

The combined detection of sd-LDL along with non-HDL and Apo-lipoprotein is shown to be very effective in pinpointing the risk of CHD. In accordance, the 2019 guidelines update have also highlighted the importance of Apo-lipoprotein and non-HDL-C determination in patients with diabetes. [26]

Suguna et al., showed that coronary heart disease risk in patients with diabetes was significantly associated with increasing non-HDL-C. They concluded that among patients with diabetes, non-HDL-C was a stronger predictor of coronary heart disease than LDL-C. [27]

ApoB is the major apolipoprotein in all potentially atherogenic lipoprotein particles, i.e. VLDL, IDL, and LDL particles. There is only one apoB per particle. Thus, plasma value of the total apoB reflects the number of cholesterol and, to some degree, triglyceride containing particles. The most abundant apoB particle is the small dense LDL, which constitutes about 90% of the whole apoB population. [28]

Apo A_1 is the major apolipoprotein in the HDL particles. Apo A_1 is a main initiator and “driver of the reverse cholesterol transport”. Apo A_1 can also manifest anti-oxidant and anti-inflammatory effects, so it can stimulate both endothelial production of nitric oxide as well as release of prostacyclin from the endothelium. Thus, apo A_1 manifests several anti-atherogenic effects. [29]

Therefore, the apoB/ A_1 ratio reflects the balance between pro-atherogenic IDL, VLDL, LDL particles and anti-atherogenic HDL particles. The mean level of Apo-B and Apo- A_1 were shown to be

significantly associated with coronary heart disease. The mechanisms by which increased Apo-A_1 clearance in CHD are largely unknown, however, the increased triglycerides fraction on HDL and the reduced adiponectin plasma levels have been suggested to accelerate Apo-A_1 degradation. [30]

It is also unclear whether hyperglycemia itself is linked to the increased clearance and may contribute to impaired HDL function. Wu et al., found Low levels of Apo- A_1 also seem to independently associate with the new onset of T2DM. [31]

The apoB/apoA1 ratio was the single best lipid-related risk variable, also considering the other conventional lipids and lipid ratios. The higher the level of the apoB/apoA_1 ratio is more likely cholesterol is to be deposited in the arterial wall, thereby provoking atherogenesis. In our study, the comparison between CHD patients and healthy subjects was performed and TG and apo-B/apo-A_1 ratio levels were found higher and HDL-C and apoA_1 levels in CHD patients were lower.

Therefore, we believe that apoB/apoA1 levels were better than LDL-C levels in predicting the risk of CHD. ApoB/ Apo- A_1 ratio is a simple and accurate measurement to estimate the risk of CHD. In our study, the ratio of Apo-B/Apo- A_1 was significantly increased in diabetic subjects with CHD when compared to diabetic CHD subjects. A low Apo-B/Apo- A_1 ratio reflects a less atherogenic lipid profile, regardless of LDL-C. [32]

Several studies have also suggested that an elevated Apo-B/ Apo- A_1 ratio is a more powerful predictor than other lipid fractions for metabolic disorders, including T2DM. [33, 34]

Recently, it has been demonstrated that Apo-B/Apo- A_1 ratio is independently associated with carotid atherosclerosis in T2DM patients with controlled LDL-c levels. [35]

More attention to sd-LDL, non-HDL-C, Apo-lipoproteins and its ratio Apo-B/ Apo- A_1 which are more persuaded for predicting the severity of coronary heart disease particularly in diabetic subjects with CHD.

CONCLUSION: sd-LDL, non-HDL-C and Apo-lipoprotein are easy to obtain biomarkers of inflammatory response, and the Apo-B/ Apo- A_1 ratio are closely related to the severity of CHD. Early detection can improve the diagnosis and prevention of CHD.

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ETHICAL APPROVAL: All procedures performed in studies involving human participants were in accordance with the ethical standards. The study protocol was approved by the institutional ethical committee (ECN: 1513/ICE/2018).

INFORMED CONSENT: Informed consent was obtained from all individual participants included in the study.

AUTHOR'S CONTRIBUTIONS

Thirunavukkarasu Jaishankar- Data collection and Data analysis and Critical revision

Karini Keerthi- Designing and Drafting the study

Kasthuri Natarajan - Finishing inputs and authorization of the article to publish.

Shantha Kannamma- Management of the article

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