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# Serum esterified cholesterol subfractions as a risk marker for coronary artery disease

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***Abstract*---**Objectives: The aim of this study was to evaluate the total esterified cholesterol and non-HDL esterified cholesterol as a risk marker for coronary artery disease and compare it with the classical risk factors. Methods: Fifty-six patients with proven coronary artery disease as assessed by angiography and 40 healthy controls were subjects for study. Total esterified cholesterol, Non-HDL esterified cholesterol and the classic lipid profile parameters were measured for both groups. Results: Total Esterified Cholesterol was not found to be significantly associated with CAD. However Non-HDL esterified cholesterol was found to be having statistically significant association. Using Receiver Operator Characteristics (ROC) Curve it was found that Non-HDL esterified cholesterol at a cut off value of 107mg/dl, has a sensitivity of 57% and specificity of 66.6%, as a risk marker for the coronary artery disease. Conclusion: Evaluation of coronary artery disease by measuring esterified cholesterol fraction of Non-HDL cholesterol may help in risk assessment and will provide more data to understand the mechanisms of cholesterol homeostasis.

***Keywords*---**Cholesterol, Non-HDL Esterified Cholesterol, Esterified Cholesterol, Coronary Artery Disease.

## Introduction

Coronary artery disease (CAD) is a modern epidemic causing more deaths, disability and economic costs than many other illnesses. Its etiology is atherosclerosis, which involves formation of plaques of foam cell macrophages whereby cholesterol is taken up by the monocyte-derived macrophages via scavenger receptors from the remnant lipoprotein (RLP) and the small dense low-density lipoprotein (LDL) particles [1]. The remnant lipoproteins and the LDL are relatively cholesterol ester rich [2]. Hence cholesterol ester, which is the main component of these lipoproteins, is atherogenic. The non-HDL Cholesterol component which consists of cholesterol from LDL, lipoprotein (a), intermediate density lipoprotein, chylomicron remnant and very low-density lipoprotein (VLDL) remnant, all of which have atherogenic potential according to the National Cholesterol Education Program (NCEP) guidelines [3]. Therefore, Non-HDL cholesterol could be used in the risk assessment of coronary artery disease as a better and more specific marker.

## Material and Methods

Consecutive patients of coronary artery disease not on lipid lowering therapy and found to have significant coronary artery stenosis on angiography attending the cardiology department were included in the study. Significant coronary artery stenosis was defined as having a stenosis of more than fifty percent in any one or more major coronary vessels [4]. Each of the patient's clinical and physical profile was noted. The biochemical parameters estimated for each were the classical lipid profile including total cholesterol, serum esterified cholesterol and free cholesterol; HDL cholesterol and triglycerides; and serum non-HDL esterified cholesterol. Serum Non-HDL Cholesterol was calculated by subtracting HDL cholesterol from total cholesterol. Total cholesterol, triglycerides and HDL cholesterol were estimated using enzymatic method using kits purchased from Bayer's Diagnostic, India. LDL cholesterol was derived from Freidwald's formula. Non-HDL Cholesterol was derived by subtracting HDL Cholesterol from Total Cholesterol. Free Cholesterol was measured directly by using the kit provided by Accurex, Biomedical, India, and esterified cholesterol was obtained by subtracting free cholesterol from total cholesterol. Free cholesterol fraction in Non-HDL Cholesterol was also measured directly. Cholesterol was removed from the precipitate obtained after using the HDL precipitating reagent by treating it to a solution of hexane and isopropanol (H/I) in a ratio of 3:2 at $37^0$C and mixing it well [5]. This was then centrifuged and the supernatant used to measure free cholesterol. Esterified cholesterol fraction of Non-HDL cholesterol was calculated by subtracting measured free cholesterol fraction of non-HDL cholesterol from total non-HDL cholesterol. All the readings were measured in a semiautomatic analyzer, Erba Chem 5.

A second group of forty individuals were taken as controls that were healthy based on a routine annual medical checkup, which compulsorily included electrocardiography. They were also subjected to similar measurement of biochemical parameters. Amongst the risk factors, smoking was categorized as present or absent; and alcohol intake was categorized as present when intake was more than or equal to 5g per day [6].

## Results

The distribution of risk factors namely smoking, alcohol intake, presence of diabetes mellitus and hypertension is given in Table 1. Odd's ratio and p value evaluated for each risk factor. Diabetes mellitus and smoking had statistically significant positive association with CAD. Various biochemical parameters were measured and calculated for cases and controls. The results are given in Table 2. These parameters were compared using Z statistical analysis test.

It is seen that all parameters namely total cholesterol, LDL-C, HDL-C, NonHDL-C, triglycerides, total free cholesterol, esterified and free fraction of Non-HDL-C show statistically significant difference except total esterified cholesterol (FIG 1). As esterified cholesterol did not show statistically significance difference, it was not analysed further. The mean of esterified cholesterol in control patients is 136.26 ±28.72. The range is 72 to 196 mg/dl.

The difference in Non-HDL esterified cholesterol was found to be statistically significant and hence its cut off value for best sensitivity and specificity was established using Receiver Operator Characteristics (ROC) Curve (Fig 2). The cut off value was found to be 107mg/dl. Using this cut off value Non-HDL esterified cholesterol was compared with total cholesterol and LDL-C as risk marker. The cut off values of total cholesterol was taken as 200mg/dl and LDL-C was taken as 100mg/dl based on NCEP Guidelines [5].Sensitivity, specificity and odd's ratio were calculated for each parameter namely total cholesterol, LDL cholesterol and non-HDL cholesterol (Table 3, 4, 5).

Comparing the three parameters namely total cholesterol, LDL cholesterol and Non-HDL esterified cholesterol, LDL –cholesterol was found to be most sensitive with a sensitivity of 67.8% and total cholesterol the most specific with a specificity of 75%. However Non-HDL esterified cholesterol sensitivity of 57% is comparable to that of LDL-cholesterol and specificity of 66.6% is comparable to that of Total cholesterol. By odd's ratio method people with Non-HDL – esterified cholesterol more than or equal to 107 mg/dl have 2.6 times the risk of having coronary artery disease which is comparable to that of total cholesterol and LDL-cholesterol (Table 6).

## Discussion

The present study included fifty-six patients of coronary artery disease diagnosed on angiography. There were 40 controls who were included after a routine medical checkup including an electrocardiogram. The significance of smoking, alcohol intake, presence of diabetes mellitus and hypertension was evaluated and it was found that smoking and diabetes mellitus had statistically significant positive association with CAD. The significance of hypertension could not be defined as none of the controls had hypertension.

Cases were compared with controls and all parameters namely total cholesterol, total free cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, Non-HDL-esterified and free cholesterol fraction had statistically significant difference with

a p value of less than 0.05. The difference in total esterified cholesterol however was not statistically significant.

Under physiologic conditions sterol balance is maintained by influx, represented by the entry of lipoprotein free and ester cholesterol and efflux of free sterol to the plasma. Efflux normally proceeds at a rate, independent of plasma concentration. Apo A I containing fraction of HDL is in part responsible for increasing the efflux. Influx even at maximal rates need not and usually does not result in any permanent change in the sterol mass of cells. There are four mechanisms for influx. Out of this one type of uptake mechanism is described for endothelial cells, smooth muscle cells or fibroblasts in the presence of cholesterol rich lipoproteins. It involves the influx of sterol ester in proportion in excess of that present in the intact lipoprotein particle [7]. This mechanism may explain why esterified cholesterol was not significantly different in cases and controls, as esterified cholesterol enters the cell even in excess to that present and efflux is only in the form of free cholesterol. In human plasma, the free and ester cholesterol composition of the circulating lipoproteins is determined in large part by the activity of three coupled reactions. These are the transport of free cholesterol for esterification from either cell membranes or plasma lipoproteins; the esterification of cholesterol of either origin by the action of lecithin cholesterol: acyl transferase (LCAT) and the transfer of the cholesterol esters so formed to acceptor lipoproteins. In normal plasma a major part of LCAT derived cholesterol esters is transferred to the LDL and VLDL lipoproteins [8].

In humans, approximately 70% of plasma cholesterol is in the esterified form. This percentage may be lower in certain conditions such as hypertriglyceridemia. In the studies of Barter [9], there was no significant correlation between the production of esterified cholesterol and the concentrations of either free or esterified cholesterol. Hence even though there may be an increased production rate, it may not correlate with the serum concentrations of esterified cholesterol.
Esterified cholesterol fraction of non-HDL was found to be significantly different among controls and cases. Its cut off limit was established using ROC curve and was found to be 107mg/dl. Esterified cholesterol fraction of non-HDL was then compared with total cholesterol and LDL-cholesterol by odd's ratio and sensitivity and specificity as given in Table 3. Esterified cholesterol fraction of non-HDL was found to be comparable with total cholesterol in respect to specificity and LDL-cholesterol in respect to sensitivity.

Most of the cholesterol received by HDL is unesterified cholesterol. The capacity to receive unesterified cholesterol is increased by the presence of an enzyme, LCAT that esterifies cholesterol. The cholesterol ester is more hydrophobic than free cholesterol and thus is more tightly packed into the HDL particle. The ability to convert cholesterol esters from free cholesterol thus allows the HDL to pick up relatively more free cholesterol. This cholesterol is transferred from the tissues to the liver thus exerting its anti-atherogenic effect [10]. If non-HDL fraction has more of esterified cholesterol, it would implicate that more of esterified cholesterol is available for uptake by macrophages, fibroblasts and vascular interstitium.

RLPs are depleted of triglycerides, phospholipids, and apolipoprotein C (apo C) and are enriched in cholesteryl esters and apolipoprotein E (apo E) and are

believed to be more atherogenic than the larger TRLs [11]. In fact, the prototypic disorder of remnant metabolism, type III dyslipidemia, is associated with accelerated atherosclerosis [12]. Increased intermediate-density lipoprotein (IDL) concentrations have been associated with an increased incidence or recurrence of CAD [11]. Kugiyama et al. [13] have shown that high RLP concentrations predict coronary events in CAD patients independently of traditional risk factors. Recently, Karpe et al [14] showed a significant correlation of RLP to carotid intimal medial thickness in a cohort of healthy 50-year-old men; this association was independent of plasma triglycerides and LDL-cholesterol**.** Masuoka et al [15] showed that RLP concentrations were strongly associated with coronary artery stenosis in normotriglyceridemic patients. Remnants include partially catabolized lipoproteins of chylomicrons and very low-density lipoproteins (VLDLs). Available data indicate that remnant lipoproteins probably equal LDL in their atherogenic potential. The atherogenic effects of triglyceride-rich lipoproteins may exceed the direct action of remnants to promote atherosclerosis. Cholesterol ester transfer protein (CETP) initiates a dynamic lipid exchange in which triglyceride-rich VLDL and chylomicrons lose much of their triglyceride to cholesterol-rich LDL and HDL, and cholesterol ester moves in the reverse direction. As triglyceride levels rise, this exchange accelerates. The resulting cholesterol-enriched VLDL or chylomicron remnants are probably the only known naturally occurring lipoproteins that mediate cholesterol uptake by macrophages. The frequent coexistence of elevated triglycerides, small LDLs and low HDLs has been called the 'lipid triad' or the 'atherogenic lipoprotein phenotype' [16].

An acceptable marker for remnant lipoproteins in patients with moderate hypertriglyceridemia is the cholesterol concentration in VLDL + IDL. Therefore, a simple way to estimate the risk engendered by atherogenic lipoproteins is to sum the cholesterol content of (VLDL + IDL) + LDL. This is expressed as non-HDL-cholesterol, and in contradiction to LDL cholesterol, can be determined in all patients regardless of the triglyceride concentration and the duration of fasting. This value is simply the difference between serum (or plasma) cholesterol and HDL cholesterol, as determined by standard clinical methods. This can be used in the risk assessment of coronary artery disease [3].

Atherosclerotic plaques are rich in esterified cholesterol which is plasma derived. In their study, Rapp et al [2] determined the site of esterification of cholesterol esters within the atherosclerotic plaque. They used the principle that the type of fatty acid esterified to cholesterol appears to be specific for the site of esterification. A predominance of cholesteryl oleate within plaque would most likely imply local esterification, while high levels of polyunsaturated cholesteryl ester (cholesteryl linoleate) within the plaque implies infiltration and deposition of cholesteryl ester from plasma. The lipid content of these advanced plaques as compared to those of xanthomas and earlier atherosclerotic lesions demonstrates an impressive increase of free cholesterol and cholesteryl ester, especially cholesteryl linoleate. The accumulation of both these substances may represent deterioration in the metabolic efficiency of the arterial wall, which apparently occurs with plaque progression. Plasma has been shown to be the major source of the cholesterol present in atherosclerotic lesions. Hence esterified cholesterol fraction in Non-HDL-cholesterol would be the principle source of cholesterol ester in atherosclerotic plaques. This correlates well with our study in which

cholesterol ester fraction of non-HDL - cholesterol is a comparable marker to total cholesterol and LDL-cholesterol.

The mechanisms of cholesterol homeostasis are complex. Interpretation of the plasma kinetics of isotopically labeled cholesterol is greatly limited by the rapid and reversible exchanges of unesterified (free) cholesterol between the plasma and many tissue pools. Influx, synthesis and efflux are normally in balance. The major factor catalyzing sterol ester synthesis in plasma is LCAT, which is stimulated by apo A-I and inhibited by apolipoprotein A-II. Efflux is unaffected when LCAT is inhibited but there is no net transport of sterol. When LCAT is active most of the sterol is trapped in plasma in its ester form [7]. Finally, even though CETP, LCAT, and HDL mediate reverse cholesterol transport, genetic deficiency states of each of these components show no clear increase in atherosclerosis. The relationship of CETP and reverse cholesterol transport to atherosclerosis is complex [17]. When remnant lipoproteins accumulate in plasma (as in dysbetalipoproteinemia), CETP may promote decreases in HDL and cholesterol ester enrichment of remnants. The inhibition of CETP activity or synthesis by drugs would be an interesting experimental approach to the treatment of dyslipidemia and atherosclerosis. Although this strategy would increase HDL and decrease VLDL, IDL, and LDL cholesterol ester, consequences for atherosclerosis are presently unpredictable [17]. Thus, evaluation of coronary artery disease by measuring esterified cholesterol fraction of Non-HDL-cholesterol would not only help in risk assessment but provide more data to understand the mechanisms of cholesterol homeostasis.

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## Tables and Figures

Table 1
Distribution of risk factors among CAD patients (n=56) and controls (n=40)

| Parameter | CAD patients (%) | Controls (%) | Odd's Ratio | p value |
|---|---|---|---|---|
| Diabetes Mellitus | 26.7 | 2.5 | 14.27 | <0.001 |
| Hypertension | 37.5 | 0 | - | - |
| Alcohol | 64.2 | 55 | 1.47 | >0.05 |
| Smoking | 62.5 | 27.5 | 5.91 | <0.001 |

Table 2
Mean and Standard Deviation of Biochemical Parameters
in CAD patients (n=56) and Controls (n=40)

| Parameter | CAD patients (mg/dl) Mean ± S.D | Controls (mg/dl) Mean ± S.D |
|---|---|---|
| Total Cholesterol | 200±54.6 | 179.61±32.49 |
| Total Esterified Cholesterol | 131.62±37.15 | 136.26±28.72 |
| Total Free Cholesterol | 68.4±29.52 | 43.34±11.52 |
| HDL-C | 36.56±8.5 | 43.54±5.8 |
| Non-HDL-C | 163.46±54.8 | 136.06±37.27 |
| Non-HDL esterified cholesterol | 117.79±35.27 | 88.9± 31.37 |
| Non-HDL free cholesterol | 32.02±15.04 | 45.16± 9.06 |
| LDL-C | 132.24±48.23 | 112.62±50.72 |
| Triglycerides | 156.14±64.7 | 117.21±65.82 |

Table 3
Relationship between coronary artery disease and total cholesterol levels

| | CAD | Controls |
|---|---|---|
| Total Cholesterol (≥ 200mg/dl) | 26 | 10 |
| Total Cholesterol (< 200mg/dl) | 30 | 30 |

Table 4
Relationship between coronary artery disease and LDL cholesterol levels

| | CAD | Controls |
|---|---|---|
| LDL-C (≥ 100mg/dl) | 38 | 24 |
| LDL-C ( < 100mg/dl) | 18 | 16 |

Table 5
Relationship between coronary artery disease and Non High Density Lipoprotein Esterified Cholesterol (NHDL EC)

| | CAD | Controls |
|---|---|---|
| NHDL EC (≥ 107mg/dl) | 28 | 13 |
| NHDL EC (< 107mg/dl) | 21 | 26 |

Table 6
Comparison of Total cholesterol, LDL-Cholesterol and Non-HDL esterified Cholesterol as a risk marker of coronary artery disease

| | Sensitivity | Specificity | Odd's Ratio |
|---|---|---|---|
| Total Cholesterol | 46.4% | 75% | 2.6 |
| LDL -Cholesterol | 67.8% | 40% | 1.4 |
| Non-HDL Esterified Cholesterol | 57% | 66.6% | 2.6 |

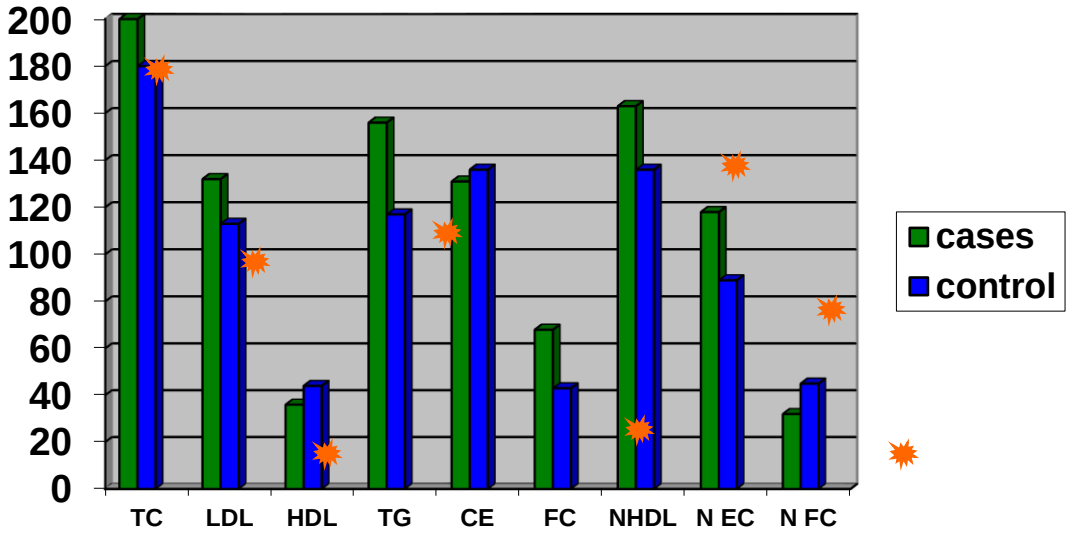


FIG 1. Comparison between Cases and controls using Z statistical test - significant statistically with $p<0.05$
TC- Total Cholesterol; LDL – Low Density Lipoprotein; HDL- High Density Lipoprotein; TG – Triglycerides; CE – Esterified Cholesterol; FC – Free Cholesterol; N HDL- Non High Density Lipoprotein; NEC – Esterified cholesterol fraction in non high density lipoprotein; N FC – Free cholesterol fraction in non high density lipoprotein

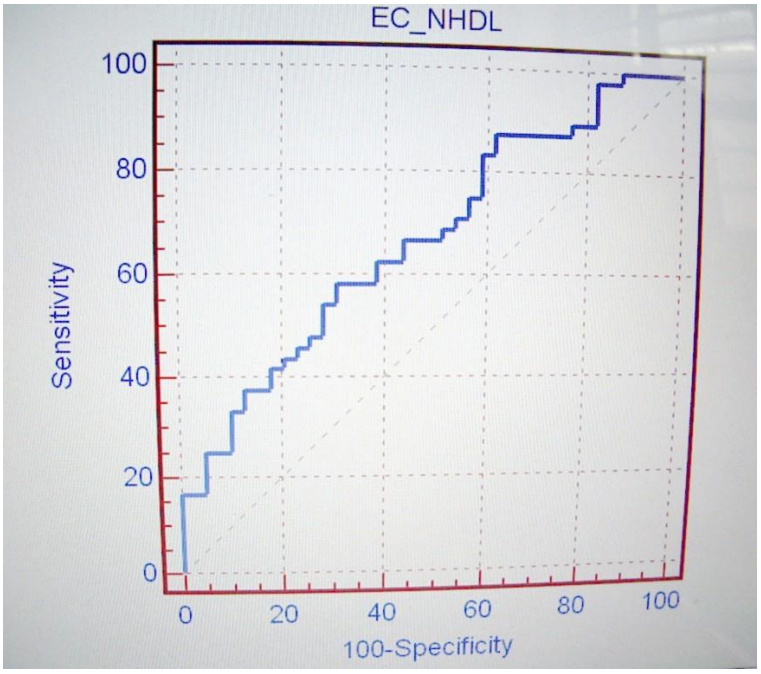


FIG 2. Receiver Operator Characteristics Curve for Esterified Fraction of Non-HDL cholesterol