

## ORIGINAL PAPER

doi: 10.5455/medarch.2018.72.103-107

MED ARCH. 2018 APR; 72(2): 103-107

RECEIVED: FEB 10, 2018 | ACCEPTED: MAR 16, 2018

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# The Ratio of Unesterified/esterified Cholesterol is the Major Determinant of Atherogenicity of Lipoprotein Fractions

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## ABSTRACT

**Background:** The hypothesis is proposed that the atherogenicity of lipoprotein fractions is correlated with the content of unesterified cholesterol. **Objectives:** To evaluate the role and prognostic values of unesterified and esterified cholesterol in lipoprotein fractions for coronary artery disease (CAD). **Design and methods:** The study population consisted of 400 patients who were divided to CAD controls and cases according to the data of coronary angiography. Fractional cholesterol esterification (FCE) as well as the complete profile of lipids and (apo)lipoproteins were determined. **Results:** Total cholesterol was increased significantly in CAD patients ( $196.3 \pm 52.3$  mg/dL vs.  $185.7 \pm 48.0$ ,  $p \leq 0.049$ ) and the increment occurred totally in unesterified portion ( $77.2 \pm 28.4$  mg/dL vs.  $71.1 \pm 24.4$ ,  $p \leq 0.031$ ). HDL cholesterol showed a significant decrease in CAD group ( $39.9 \pm 9.5$  mg/dL vs.  $44.6 \pm 10.5$ ,  $p \leq 0.001$ ), but the decrement occurred wholly in the esterified portion ( $26.2 \pm 9.2$  mg/dL vs.  $31.1 \pm 8.1$ ,  $p \leq 0.001$ ). NonHDL cholesterol was increased significantly in CAD group ( $156.8 \pm 48.3$  mg/dL vs.  $140.3 \pm 43.6$ ,  $p \leq 0.001$ ), and the changes occurred in both un- and esterified portions. FCE in HDL was diminished significantly in CAD patients ( $64.8 \pm 13.9\%$  vs.  $69.3 \pm 7.9$ ,  $p \leq 0.01$ ). In multivariate logistic regression analysis, unesterified cholesterol in NonHDL (UeNonHDLc) and esterified cholesterol in HDL (EsHDLc) excluded total cholesterol and HDLc respectively from the regression equation. In ROC analysis, the ratio of UeNonHDLc/EsHDLc was the strongest predictor for CAD among cholesterol subfractions. **Conclusions:** The results confirm that UeNonHDLc is atherogenic and EsHDLc is antiatherogenic and are independent risk factors for CAD.

**Keywords:** Cholesterol, CAD, Unesterified, Esterification, Lipoproteins, LDL.

## 1. INTRODUCTION

The role of cholesterol and its sub-fractions is well known in the pathogenesis of atherosclerosis. LDL is the main catabolic product of VLDL in vascular space and delivers cholesterol into the tissues (1). The level of plasma LDL-cholesterol (LDLc) is related directly to the incidence of Coronary Artery Disease (CAD) (2). LDL particles are atherogenic because they can pass via the intima layer of vascular beds and be taken up by macrophages to make foam cells (2). HDL particles participate in the removal of excess free cholesterol from the peripheral tissues and deliver it into the liver for excretion, the process known as 'cholesterol reverse transport' (3, 4). The esterification of cholesterol is important in this process. HDL is a repository for the apo C and E (4), and also contains the enzymes with antioxidant activity (3). The level of plasma HDLc is

related inversely to the incidence of CAD (5). The antiatherogenic activity of HDL is attributed primarily to cholesterol reverse transport and also to antioxidant property (5, 6).

In vascular bed of tissues, the free cholesterol is transferred to HDL in the side of concentration gradient (6, 7). Unesterified cholesterol is rapidly esterified by lecithin: cholesterol acyl transferase (LCAT) (8). It has been reported that the deficiency of LCAT associates with the increase in the occurrence of CAD (8-10). Finally, cholesteryl esters (CE) are transferred to lighter fractions i.e. VLDL and chylomicrons remnant by cholesteryl ester transfer protein (CETP) (11-14). It is reported that these processes have been altered in diabetes mellitus (6, 9), CAD (11-15) and affected by statins consumption (16-9).

We proposed the hypothesis that the atherogenic and antiatherogenic characteristics of LDL and HDL

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fractions are attributed partly to their contents of unesterified and esterified cholesterol respectively. In the current study, we evaluated the validity of this hypothesis by examining the role and prognostic values of unesterified and esterified cholesterol in lipoprotein fractions for discriminating CAD patients.

## 2. METHODS AND SUBJECTS

### Experimental design, subjects and angiographic assessment

The project was approved by the research and ethical committee at university of Mazandaran (IR.MAZUMS.REC.94-1188) and all subjects signed the informed consent. The experimental design and angiographic assessment were as described previously (20). In brief, the study population consisted of 190 men and 210 women aged 30-75 years who referred consecutively and had abnormal sport-test and introduced to have their first coronary angiography at Zahra hospital of university of Mazandaran. The subjects were excluded from the study who had a recent history of acute myocardial infarction, percutaneous transluminal coronary angioplasty, infectious or inflammatory disease, severe liver or renal disease, neoplasm and hematologic disorders. Subjects with one or more lesions that narrowed the lumen of any coronary artery significantly (>70%) were considered to be CAD cases, whereas those without any narrowing (<10%) were taken as controls (20).

### Biochemical and hematological measurements

Blood samples collection and serum preparations are described in reference 20. All samples were stored at -70°C before analysis for maximum of six months. Serum total cholesterol and triglycerides (TG) were measured enzymatically by the CHOD-PAP and GPO-PAP methods respectively. LDL- and HDL-cholesterol were determined by using new homogenous assays (22). Un-esterified total cholesterol and unesterified HDLc were measure by the same kits but lacked the enzyme of cholesterol esterase (Pars-Azmon Inc., Tehran). Esterified total cholesterol and esterified HDLc were calculated by subtractions of unesterified fractions from total cholesterol and HDLc. NonHDL-cholesterol and its fractions (unesterified and esterified) were calculated by total cholesterol minus HDLc and their fractions. ApoB100 and apoAI were assayed by immunoturbidometric methods (20). Inter- and intra-assay coefficients of variance were less than 5% for all measurements. All other biochemical and hematological parameters were measured by routine laboratory methods.

### Statistical analysis

The results are presented as the means  $\pm$  SD and median (25th-75th percentiles) for normal and skewed distributed variables respectively. Proportions and means (or median) were calculated for baseline risk factors. The significance of any differences in proportions or medians was tested with Mann-Whitney test, and in means using student's t-test. All p-values are two-tailed and differences were considered significant if p-values were  $\leq 0.05$ . A multivariate logistic regression analysis with condi-

tional forward approach was carried out to find out the independency of the correlations (SPSS version 21).

## 3. RESULTS

### Demographic and clinical parameters of the study population

The prevalence of physical inactivity and diabetes mellitus was more in CAD cases than control subjects (Table 1). There were not significant differences in BMI, cigarette smoking, hypertension and consuming statins between two groups. Patients with CAD compared with the controls had increased levels of serum glucose, BUN and creatinine. The levels of hemoglobin concentration and leukocytes counts as the markers of dehydration and inflammation were not changed significantly between two groups.

Total cholesterol was consisted of about 38% unesterified and 62% esterified fraction in control group. Total cholesterol was increased significantly in CAD patients ( $196.3 \pm 52.3$  mg/dL vs.  $185.7 \pm 48.0$ ,  $p \leq 0.049$ ) and the

	Without CAD (n≈150)	With CAD (n≈250)	P
Clinical characteristics:			
Age, year	54.0 $\pm$ 10.5	59.4 $\pm$ 9.7	0.001
Gender, male%(n)	35.6 (52)	53.6 (133)	0.001
BMI, kg/m <sup>2</sup>	27.7 $\pm$ 4.7	27.2 $\pm$ 4.1	0.273
Physical activity, %(n)	52.7 (77)	43.1 (107)	0.036
Smoking, %(n)	17.1 (25)	20.2 (50)	0.531
Diabetes mellitus, %(n)	22.6 (33)	36.2 (90)	0.007
Hypertension, %(n)	50.0 (73)	60.5 (150)	0.067
Statins, % (n)	27.4 (40)	32.7 (81)	0.318
Biochemicals:			
Glucose, mg/dL	107.9 $\pm$ 31.1	123.1 $\pm$ 56.6	0.012
BUN, mg/dL	16.4 $\pm$ 5.1	17.8 $\pm$ 7.9	0.043
Creatinine, mg/dL	0.95 $\pm$ 0.21	1.07 $\pm$ 0.50	0.008
Hemoglobin, g/dL	12.9 $\pm$ 1.7	13.1 $\pm$ 1.6	0.193
Leukocyte counts (cells/ nL)	8.2 $\pm$ 2.0	8.3 $\pm$ 2.3	0.435
Lipids profile:			
Triglycerides, mg/dL	156(113–243)	159(113–400)	0.044*
Total cholesterol, mg/dL	185.7 $\pm$ 48.0	196.3 $\pm$ 52.3	0.049
Unesterified	71.1 $\pm$ 24.4	77.2 $\pm$ 28.4	0.031
Esterified	115.5 $\pm$ 35.7	118.0 $\pm$ 36.8	0.520
HDLc, mg/dL	44.6 $\pm$ 10.5	39.9 $\pm$ 9.5	0.001
Unesterified	13.6 $\pm$ 4.1	13.9 $\pm$ 5.7	0.578
Esterified	31.1 $\pm$ 8.1	26.2 $\pm$ 9.2	0.001
LDLc, mg/dL	98.7 $\pm$ 31.8	103.2 $\pm$ 35.0	0.206
NonHDLc	140.3 $\pm$ 43.6	156.8 $\pm$ 48.3	0.001
Unesterified	57.7 $\pm$ 23.3	63.3 $\pm$ 26.5	0.037
Esterified	83.8 $\pm$ 33.6	92.0 $\pm$ 35.5	0.030
ApoAI, mg/dL	176.4 $\pm$ 45.9	170.8 $\pm$ 50.4	0.347
ApoB100, mg/dL	112.7 $\pm$ 39.6	119.0 $\pm$ 37.6	0.186
Log(triglyceride)/HDLc	0.051 $\pm$ 0.013	0.060 $\pm$ 0.016	0.001

**Table 1.** Demographic and clinical characteristics in CAD controls and patients. The continuous and categorical variables were compared by t- and  $\theta^2$ -tests, respectively. The number in each group has shown in parentheses. The results are presented as the means  $\pm$  SD and median (range). Mann-Whitney test (\*). BMI; body mass index, CAD; coronary artery disease

Model	Included variables	OR Exp(B)	95% CI	Cox-Snell R- square	Predictive value, %	-2LL	P
1	Male sex	2.715	1.621–4.548	0.103	64	454	0.001
2	+ Age	1.045	1.029–1.061	0.203	69	411	0.001
3	+ EsHDLc	0.922	0.897–0.947	0.226	71	401	0.001
4	+ UeNonHDLc	1.010	1.001–1.020	0.241	72	394	0.035
5	+ Hypertension	2.127	1.280–3.536	0.257	73	386	0.004
6	+ Diabetes mellitus	2.205	1.253–3.881	0.267	73	382	0.006

Table 2. Multivariate regression analysis. In each model a new variable was added to the previous variables and the data of the last model with six parameters (without a constant value) has been presented. R: multiple correlation coefficient of each model, OR: odds ratio, CI: confidence interval and LL: Log of likelihood, EsHDLc: esterified cholesterol in HDL, UeNonHDLc: unesterified cholesterol in NonHDL

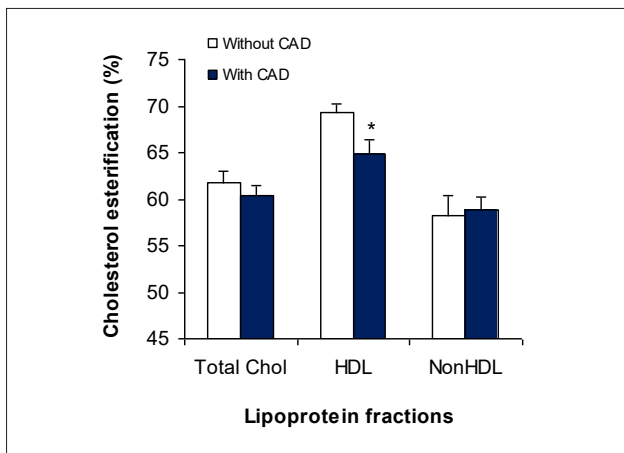


Figure 1. The changes of fractional cholesterol esterification (FCE) in CAD. FCE was calculated as the ratio of esterified- per total- cholesterol in each fraction of lipoproteins. The study population was divided into two groups according to have CAD. The number of subjects in each group was 150 and 250 respectively. \* Indicates  $p < 0.01$

increment occurred totally in unesterified portion ( $77.2 \pm 28.4$  mg/dL vs.  $71.1 \pm 24.4$ ,  $p \leq 0.031$ ). HDL cholesterol showed a significant decrease in CAD group ( $39.9 \pm 9.5$  mg/dL vs.  $44.6 \pm 10.5$ ,  $p \leq 0.001$ ), but the decrement occurred wholly in the esterified portion ( $26.2 \pm 9.2$  mg/dL vs.  $31.1 \pm 8.1$ ,  $p \leq 0.001$ ). NonHDL cholesterol was increased significantly in CAD group ( $156.8 \pm 48.3$  mg/dL vs.  $140.3 \pm 43.6$ ,  $p \leq 0.001$ ), and the changes occurred in both unesterified and esterified portions. The ratio of log (triglyceride)/HDLc as atherogenic index also had significant increase in CAD group ( $0.060 \pm 0.016$  vs.  $0.051 \pm 0.013$ ,  $p \leq 0.001$ ).

#### The changes of FCE in CAD

Fractional cholesterol esterification (FCE) was calculated as the ratio of esterified- per total- cholesterol in each fraction of lipoproteins. Figure 1 shows that about 62% of total cholesterol was esterified in the control group and was diminished in CAD group slightly but not significantly ( $60.4 \pm 9.5\%$  vs.  $61.8 \pm 10.3$ ,  $p \leq 0.153$ ). The changes of cholesterol esterification were occurred totally and significantly in HDL but not in nonHDL fraction. About 69% of HDL- cholesterol was esterified in the control group and was decreased in CAD group significantly ( $64.8 \pm 13.9\%$  vs.  $69.3 \pm 7.9$ ,  $p \leq 0.01$ ).

#### Association of variables with the incidence of CAD

Multi-variate logistic regression analysis with conditional forward approach was performed to test the independency of the correlations between risk factors and

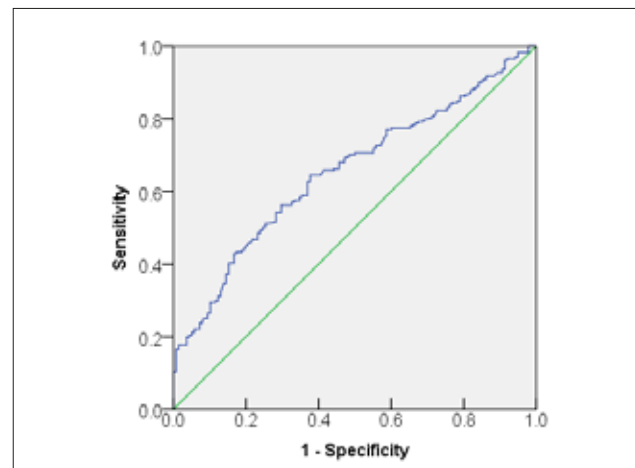


Figure 2. ROC analysis for the ratio of UeNonHDLc/EsHDLc. The AUC was  $0.654 \pm 0.028$ ,  $p < 0.001$

CAD. The criteria for entrance and removal of the variables into regression equation were 0.05 and 0.1 respectively. Major classical risk factors as well as unesterified and esterified cholesterol in lipoprotein fractions were included in the analysis. Finally, male sex, age, unesterified cholesterol in NonHDL (UeNonHDLc), esterified cholesterol in HDL (EsHDLc), diabetes and hypertension were kept in the model significantly (Table 2). This means that UeNonHDLc and EsHDLc excluded total

Parameters	AUC $\pm$ SE	P
Total cholesterol	$0.562 \pm 0.030$	0.042
Unesterified	$0.560 \pm 0.030$	0.048
Esterified	$0.516 \pm 0.031$	0.617
HDLc	$0.630 \pm 0.029$	0.000
Unesterified	$0.503 \pm 0.029$	0.921
Esterified	$0.658 \pm 0.028$	0.001
LDLc	$0.540 \pm 0.030$	0.184
NonHDLc	$0.596 \pm 0.030$	0.002
Unesterified	$0.569 \pm 0.031$	0.027
Esterified	$0.561 \pm 0.030$	0.050
ApoA1	$0.543 \pm 0.034$	0.221
ApoB100	$0.553 \pm 0.036$	0.142
LDLc/HDLc	$0.634 \pm 0.029$	0.001
UeNonHDLc/EsHDLc	$0.654 \pm 0.028$	0.001
Log(TG)/HDLc	$0.659 \pm 0.029$	0.001

Table 3. ROC analysis for cholesterol subfractions. The presence of CAD and biochemicals were entered in the analysis as state and test variables respectively. AUC; area under the curve

cholesterol and HDLc from the regression equation respectively and were independent risk factors for CAD.

#### Comparison the power of tests for diagnosis of CAD

The receiver operating characteristic (ROC) analysis was used to evaluate the efficiencies of the tests for discriminating CAD patients from control individuals. The ROC curve can be constructed by plotting sensitivity versus 1 minus specificity or the true positive rate versus the false-positive rate. The area under the ROC curve (AUC) represents a relative measure of the test's efficiency (Table 3). Total cholesterol showed significant power to diagnosis CAD and the ability was attributed totally to unesterified fraction. HDLc had higher prognostic power and its capability was attributed wholly to esterified fraction. Both fractions of nonHDLc showed significant power to distinguish CAD. Among cholesterol subfractions, the ratio of UeNonHDLc/EsHDLc had the highest efficiency to discriminate CAD (Figure 2).

## 4. DISCUSSION

The data of the current study shows that total cholesterol is increased in CAD patients and the increment occurred totally in unesterified fraction. HDL cholesterol was also decreased, but the decrement happened wholly in the esterified fraction. Fractional cholesterol esterification in HDL was diminished significantly in CAD group. The results also indicate that unesterified Non-HDLc and esterified HDLc were independent risk factors for CAD and were the strongest predictors for CAD among cholesterol subfractions.

Lipophilic compounds as well as trace elements and minerals are as free and protein bound in the body fluids. Free ligand acts as bioactive entity while protein bound serves as ligand reservoir (22). We extended this viewpoint for lipid portion of lipoproteins, especially for cholesterol. Cholesterol is present as unesterified (free) and esterified portions in the body fluids (1). Free cholesterol is biologically active and has cytotoxic effects whereas cholesteryl ester (CE) is protective form for storage in the cells and transporting in plasma (23). Unesterified cholesterol is shielded at two levels, at the first step it is converted to acyl ester (or CE) and then it binds to proteins by taking part in lipoproteins structure.

Total cholesterol (or LDLc) and HDLc are among seven independent classical risk factors for CAD (22). The LDL particles are atherogenic because they can pass through the intima layer of vascular beds and be taken up by macrophages to make foam cells (2). The level of plasma LDLc reflects the concentration of portion of cholesterol which is directed to the tissues. The anti-atherogenic activity of HDL is attributed primarily to its role in cholesterol reverse transport and also to its antioxidant property (3). The level of plasma HDLc reflects cholesterol efflux from peripheral tissues to the liver for excretion.

We proposed the hypothesis that the atherogenicity of lipoproteins may be related to their content of 'unshielded free cholesterol'. Free cholesterol is transferred from peripheral tissues and other lipoproteins to HDL, a process that is facilitated by transporters of ABC-A1,

ABC-G1 and receptors of SR-B1 (8). In HDL, free cholesterol is esterified rapidly by LCAT and cofactor of apo AI (8). Esterification causes a concentration gradient and draws in cholesterol from tissues or other lipoproteins. Finally, cholesteryl esters are transferred to lighter fractions by CETP to deliver to the liver for excretion. These processes have been altered in diabetes (6, 9), CAD (10-14) and modified by treatment with statins (16-19). It has been shown that the deficiency of LCAT associates with the increase in the occurrence of CAD (8-10). We showed that cholesterol esterification in HDL fraction is decreased in diabetes and CAD and enhanced following treatment with statins (unpublished data).

## 5. CONCLUSION

The present results confirm clearly that unesterified cholesterol in NonHDL has direct and esterified cholesterol in HDL fraction has inverse significant and independent correlation with CAD. Furthermore, ROC analysis demonstrated that the ratio of UeNonHDLc/EsHDLc is a more powerful index than the conventional cholesterol ratios to diagnosis CAD patients. The ratio could be adopted as the best test to identify subjects at risk for CAD in clinical practice.

- **Study limitations:** The efficiency of total cholesterol and its subfractions is influenced and weakened highly by statins consumption. So the predictive values of cholesterol subfractions may vary in different studies based on the extent of statins consumption by the patients.
- **Acknowledgments:** The authors thank Mal Haysom, Australia for proof-reading this manuscript. Prof Rasouli designed and conducted the study, analyzed the data, interpreted the findings and wrote the manuscript. Prof Bagheri performed coronary angiography and analyzed its results. Alikhani, and Mokhtari were PhD students and performed the experiments. All authors have read and approved the final article.
- **Abbreviations:** CAD; Coronary Artery Disease, CE; Cholesteryl Esters, CETP; Cholesteryl Esters transfer proteins, HDL; High Density Lipoproteins, LDL; Low Density Lipoproteins, LCAT; Lecithin: Cholesterol Acyl Transferase, VLDL; Very Low Density Lipoproteins.

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