

# Small Dense LDL Particles in Relation to LDL Oxidation in Normolipidemic CAD Patients

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## Abstract

**Aims:** Lipid and lipoprotein cholesterol levels are not predictive of coronary artery disease (CAD) risk in all the subjects. The heterogeneity of lipoprotein particles plays an important role in this respect. This fact needs to be studied in normolipidemic CAD patients. The aim of the present study was to evaluate the role of small dense LDL particles in relation to LDL oxidation in normolipidemic coronary artery disease patients. **Methods and Results:** One hundred and twenty eight CAD patients and 200 age and sex matched normal individuals were studied. Subjects were screened for various biochemical investigations such as lipid profile, serum apoB, LDL apoB carbonyl content, MDA-LDL levels. Log (TG/HDL-C) ratio was taken as an index of LDL particle size. In the present study, lipid levels could not discriminate well between patients and normal subjects. However normolipidemic CAD patients had increased positive values of log (TG/HDL-C) as compared to controls indicating the predominance of small dense LDL particles. This was further supported from the raised serum apoB levels. LDL apoB carbonyl content (LDL protein oxidation) and MDA-LDL levels (LDL lipid peroxidation) were raised in normolipidemic CAD patients as compared to normal subjects. **Conclusions:** Assessment of LDL particle size and LDL oxidation status might be more predictive of CAD risk even in subjects with normal lipid and lipoprotein cholesterol levels.

## INTRODUCTION

The relationship between plasma LDL cholesterol levels and atherosclerosis has evolved over the past fifty years. The National Cholesterol Education Programme Adult Treatment Panel formulated recommendations for therapy to prevent CAD on the basis of LDL cholesterol levels<sup>1</sup>. However, with subsequent research it was revealed that LDL particles are heterogeneous in nature. LDL particles containing less cholesterol and increased triglyceride content are small and dense in nature. Small dense LDL particles are highly atherogenic. These particles possess an increased readiness for oxidation due to their lower antioxidant content, stimulate the endothelial dysfunction and are easily recognized by scavenger receptors leading to foam cell formation<sup>2</sup>. Several studies reported heterogeneity of LDL particles with apoB enrichment in hypertriglyceridemic individuals<sup>3,4</sup>. Elevated apoB levels together with predominance of small dense LDL particles have been reported in patients suffering from coronary artery disease<sup>5</sup>. In the Quebec study (13 year follow-up study), small dense LDL particles were observed to be a strong independent predictor of CAD<sup>7</sup>. The available data clearly suggests that small dense LDL particles increase the risk of coronary

artery disease. Many of the studies reported the importance of small dense LDL particles in certain conditions associated with CAD such as hypertriglyceridemia, diabetes mellitus, metabolic syndrome<sup>3,4,6</sup>.

The significance of small dense LDL particles in CAD patients with normal lipid and lipoprotein cholesterol levels is not well covered in the literature even though many individuals suffering from coronary artery disease do have a normal lipid profile. Hence, it becomes important to look for the heterogeneity of LDL particles and their relationship with LDL oxidation in such individuals. In the light of these facts, the present study was aimed to investigate the role of small dense LDL particles in relation to LDL oxidation in normolipidemic CAD patients.

## METHODS

The present study was conducted on 128 CAD patients and 200 age and sex matched normal subjects. Diagnosis of CAD was carried out by the clinician on the basis of clinical symptoms, ECG changes and/or stress test and angiography (if required). The clinical condition of the normal subjects was confirmed by physical examination, ECG and/or stress

test. The selection of CAD patients was framed on the basis of their lipid profile. CAD patients having normal lipid and lipoprotein cholesterol levels as per ATP III guidelines were selected to meet the objective of the present study. It was ensured that none of the patients were on lipid lowering therapy. No subject either patient or normal individual selected were taking any antioxidant supplements. A detailed questionnaire was prepared to obtain the necessary details of the subjects. Informed consent was obtained from all the individuals for participating in the study.

### EXCLUSION CRITERIA

Subjects suffering from diabetes mellitus, renal disease, thyroid disorder, rheumatoid arthritis, any acute infection and women on oral contraceptives or undergone hysterectomy were excluded from the present study.

Blood samples were taken after a 12 hour overnight fast from healthy individuals and CAD patients. Serum was used for various biochemical investigations. ApoB carbonyl content (LDL protein oxidation), LDL-MDA levels (LDL lipid peroxidation), serum apoB and lipid levels of all the subjects were estimated. Value of Log (TG/HDL-C) has been used as an index of LDL particle size. We tried to determine the oxidation parameters on the same day the blood samples were drawn to avoid spontaneous oxidation of LDL. However, where storage was needed to be done, butylated hydroxytoluene (BHT) was added to check the spontaneous oxidation of LDL.

### ISOLATION AND PRECIPITATION OF LDL

Serum LDL of all the samples was isolated by the method of <sup>8</sup>. The precipitation buffer consisted of 0.064M trisodium citrate adjusted to pH 5.05 and contained 50,000IU/L heparin. The insoluble lipoproteins were sedimented by centrifugation at 1,000g for 10 minutes. The pellet was resuspended in 1 ml of 0.1M sodium phosphate buffer pH 7.4 containing 0.9% normal saline.

### ESTIMATION OF APOB CARBONYL CONTENT

ApoB carbonyl content was estimated by the method of <sup>9</sup>. Ten millimolar concentration of dinitrophenylhydrazine(DNPH) in 2M HCl was added to isolated LDL solution in a centrifuge tube. The contents were incubated for 1 hour at room temperature. Then 0.6 ml of denaturing buffer (0.15M sodium phosphate buffer, pH 6.8 containing 3% sodium dodecyl sulphate) was added and tubes were allowed to vortex for 1minute. After mixing, an equal volume of ethanol and heptane mixture was added and

the contents were mixed again. The tubes were centrifuged at 1,000g for 5 minutes. LDL was recovered from the interface and was washed three times with ethanol ethyl acetate (1:1v/v) mixture. Each DNPH sample was dissolved in denaturing buffer and was scanned from 320nm to 410nm. The peak absorbance was used to calculate protein carbonyls with an extinction coefficient of 22,000 M<sup>-1</sup>cm<sup>-1</sup>. Results were expressed as nmoles carbonyl/mgLDL protein.

LDL-MDA levels were estimated by the method of <sup>10</sup>. The protein concentration of each LDL sample was determined by the protocol of <sup>11</sup>. Total cholesterol, HDL cholesterol and triglyceride levels were estimated by commercially available kits (Biotech India Pvt Ltd). LDL cholesterol levels were calculated by Friedwald formula <sup>12</sup>. Serum apoB levels were estimated by procuring kits from Diasys India Pvt Ltd using immunoturbidimetric method. The reagents and calibrators were claimed to be in accordance with WHO International reference material. Results were expressed as mg/dl.

### STATISTICAL ANALYSIS

Statistical analysis was done using student's t test. Level of significance was determined at p<0.05

### RESULTS

Table 1 illustrates the lipid levels of normal subjects and CAD patients. The CAD patients were normolipidemic. Their lipid and lipoprotein cholesterol levels were in a normal range as per ATPIII guidelines. Insignificant difference (p>0.05) was observed in the lipid levels of normal subjects and CAD patients. This clearly indicates that lipid levels could not predict the risk of CAD successfully in all the subjects and there is a need to look for other robust risk indicators. It may be noticed that we have taken total cholesterol <200mg/dl; LDL-C 100-129mg/dl; triglycerides <150mg/dl and HDL-C >40mg/dl as normal levels for comparison.

**Figure 1**

Table 1: Lipid and lipoprotein cholesterol levels in CAD patients and normal subjects

Subjects	Total Cholesterol	Triglycerides	VLDL-C	LDL-C	HDL-C
<b>Normal subjects</b> (n=200)	215±12.6	170±12.5	34.2±10.2	115±12.6	44±10.2
<b>CAD patients</b> (n=128)	192±12.8* (t=1.88)	155±12.3* (t=1.42)	31±10.1* (t=1.62)	112±12.3* (t=1.47)	42±10.4* (t=1.44)

\*p>0.05 (insignificant difference in lipid levels between normolipidemic CAD patients and normal subjects); Results were express as Mean±S.D mg/dl; n=number of subjects

Lipid levels were biased as only normolipidemic CAD patients were included. However, at the same time, it revealed the weak predictive risk power of lipid and lipoprotein cholesterol levels. Hence we tried to estimate serum apoB levels in normolipidemic CAD patients and normal subjects (Table 2). ApoB is a constituent component of LDL. Serum apoB levels of normolipidemic CAD patients were significantly raised ( $p<0.001$ ) in comparison to normal subjects. This observation was based on the individual apoB levels included in the present study. It is pertinent to notice that LDL cholesterol levels in CAD patients were in the normal range i.e. 100-129mg/dl. Increased serum apoB levels are an indicative of predominance of small dense LDL particles. This was further supported from the observation that normolipidemic CAD patients had significantly increased positive values of log (TG/HDL-C) as compared to normal subjects. This ratio has been taken as an index of LDL particle size.

**Figure 2**

Table 2: LDL particle size and serum apoB levels in normolipidemic CAD patients and normal subjects.

Subjects	Serum apoB levels (mg/dl)	Log (TG/HDL-C) ratio
<b>Normal subjects</b>	108±12.6	0.20±0.06
<b>Normolipidemic CAD patients</b>	130±12.2* (t=3.36)	0.56±0.13* (t=2.93)

\*  $p<0.01$  (significant difference)  
Log (TG/HDL-C) is taken as an index of LDL particle size

Small dense LDL particles are more prone to oxidation. We evaluated this fact by assessing the status of LDL oxidation in normolipidemic CAD patients (Table 3). ApoB carbonyl content of LDL was significantly higher in normolipidemic CAD patients as compared to healthy subjects. ApoB carbonyl content is taken as an index of LDL protein oxidation. MDA-LDL levels were also higher in these patients as compared to controls. On the whole, LDL was oxidized in normolipidemic patients suffering from coronary artery disease.

**Figure 3**

Table 3: LDL oxidation status in normolipidemic CAD patients and Normal subjects

Subjects	MDA-LDL levels	ApoB carbonyl content
<b>Normal subjects</b>	0.19±0.08	1.4±0.91
<b>Normolipidemic CAD patients</b>	0.32±0.07* (t=2.68)	3.4±0.61* (t=2.62)

Results were expressed as Mean±S.D. nmoles/mgLDL protein

\*  $p<0.01$  (significant difference in the given parameters between normal subjects and normolipidemic CAD patients)

## DISCUSSION

Lipid profile is currently the clinical test to evaluate the risk of coronary artery disease. However, cardiac risk is not necessarily related to lipid levels but is more complex. Many individuals have normal lipid levels but still suffer from

coronary artery disease. Lack of robust CAD risk indicators increase the difficulty in timely identification of people at risk and lessen treatment effectiveness and disease management.

The heterogeneity of lipoprotein particles especially LDL plays an important role in modifying the susceptibility of an individual towards the risk of CAD. Normolipidemic CAD patients are of special concern. The main objective of the present study was to evaluate the role of small dense LDL particles in relation to LDL oxidation in normolipidemic CAD patients. CAD patients in the present study were labeled normolipidemic because their lipid and lipoprotein cholesterol levels were within a normal range as per ATPIII guidelines. When lipid levels of these patients were compared with that of normal individuals, insignificant difference was observed. It may be noticed that only normolipidemic CAD patients were included in the present study to meet the objective. The observation was biased in this direction but at the same time indicates the weak predictive power of lipid profile and also reveal that a significant population does exist that have normal lipid levels but suffer from coronary artery disease. Such individuals are at increased risk of fatal coronary events in the absence of better risk indicators. In our study, lipid levels could not efficiently distinguish patients from disease free subjects as other factors may play a hidden role and hence need to be explored.

We evaluated the nature of LDL particles in CAD patients with normal LDL cholesterol levels. Normolipidemic CAD patients had higher positive values of  $\log(\text{TG}/\text{HDL-C})$  as compared to healthy subjects. This ratio is taken as an index of LDL particle size.  $\log(\text{TG}/\text{HDL-C})$  ratio has been reported to range from negative to positive with a zero value corresponding to LDL particle diameter of 25.5nm. This LDL particle size is used as a cut-off between LDL pattern A and LDL pattern B<sup>13</sup>. LDL pattern A refers to normal LDL particles while pattern B refers to small dense cholesterol depleted LDL particles which are more atherogenic. Dobiasov and Frohlich (2001) proposed  $\log(\text{TG}/\text{HDL-C})$  as an atherogenic index of plasma (AIP)<sup>14</sup>. Small dense LDL particles can penetrate the arterial wall easily and are more prone to oxidation than normal LDL particles. Increased predominance of small dense LDL particles in CAD patients has also been reported by other researchers<sup>2,4,7</sup>. In the present study using normolipidemic CAD patients, the presence of small dense LDL particles was further supported by significantly raised levels of serum

apoB. As there is one molecule of apoB per LDL particle, hence apoB levels indicate the number of atherogenic particles in the body. Raised apoB levels together with high positive values of  $\log(\text{TG}/\text{HDL-C})$  indicate the presence of small dense LDL particles in normolipidemic CAD patients. This vital information is not revealed by their LDL cholesterol levels.

It is a known fact that small dense LDL particles are more easily recognized by the CD36 scavenger receptors and hence are more prone to oxidation. Oxidized LDL is highly atherogenic. There are several studies available supporting this finding<sup>14,15</sup>. Our purpose was to investigate the levels of small dense LDL particles in normolipidemic CAD patients. Both LDL lipid and protein oxidation was taken into account. MDA-LDL levels and apoB carbonyl content was significantly high in normolipidemic CAD patients as compared to healthy subjects. The cascade of atherosclerosis commences with the formation of oxidized LDL, leading to coronary artery disease and indicates the importance of estimating LDL oxidation status in patients with normal lipid and lipoprotein cholesterol levels.

The evaluation of the risk of CAD at an initial stage is a key requirement to check the fatal consequences. In the present study, lipid profile does not prove to be a robust clinical test as the patients had normal lipid levels but still were suffering from coronary artery disease. There was insignificant difference in lipid levels of CAD patients and healthy subjects. Hence, it may be concluded from the observations that lipid and lipoprotein cholesterol levels do not successfully predict the risk of CAD in all the subjects and in such cases, other parameters such as apoB, heterogeneity of LDL particles and their oxidation could provide significant information.  $\log(\text{TG}/\text{HDL-C})$  ratio is a simple index which could efficiently predict the nature of LDL particles even in normolipidemics.

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