Original Article

Association of Small Dense LDL with Coronary Artery Disease and Diabetes in Urban Asian Indians - The Chennai Urban Rural Epidemiology Study (CURES-8)

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Abstract

Objective: Earlier studies in Europeans have identified small dense LDL to be associated with coronary artery disease and diabetes. In this study we assessed the association of small dense LDL with diabetes and CAD in Asian Indians.

Methods: Study subjects were selected from the Chennai Urban Rural Epidemiology Study (CURES), a population based study on representative sample of Chennai city in southern India. Group 1:non-diabetic subjects (n=30); Group 2: diabetic subjects without CAD (n=30); Group 3:diabetic subjects with CAD (n=30). LDL subfractions were estimated using LipoPrint LDL system. LDL subfractions 3 and above, defined as small dense LDL was summed up to determine the overall small LDL. 75th percentile of the overall small dense LDL in non-diabetic subjects was used as a cut-off for defining elevated levels of small dense LDL.

Results: The mean age of the study subjects was not significantly different among groups. Overall small dense LDL was significantly higher in diabetic subjects with CAD (16.7 ± 11.1 mg / dl, $p<0.05$) and without CAD (11.1 ± 8.0 mg / dl, $p<0.05$) compared to non-diabetic subjects without CAD (7.2 ± 6.8 mg / dl). Small dense LDL showed a positive correlation with fasting plasma glucose ($r=0.252$, $p=0.023$), HbA1c ($r=0.281$, $p=0.012$), total cholesterol ($r=0.443$, $p<0.001$), triglycerides($r=0.685$, $p<0.001$), LDL($r=0.342$, $p=0.002$), total cholesterol / HDL ratio ($r=0.660$, $p=<0.001$) and triglycerides / HDL ratio($r=0.728$, $p<0.001$) and a negative correlation with HDL cholesterol ($r= -0.341$, $p=0.002$) and QUICKI values ($r= -0.260$, $p=0.019$). ROC curves constructed to predict elevated small dense LDL ((9.0 mg / dl) revealed that triglycerides / HDL ratio and total cholesterol / HDL ratio had higher AUC values compared to other parameters. A triglycerides / HDL ratio of 3.0 had the optimum sensitivity (80.0%) and specificity (78.0%) for detecting elevated small dense LDL.

Conclusion: This data suggests that in Asian Indians, small dense LDL is associated with both diabetes and CAD and that a triglycerides / HDL ratio (3.0 could serve a surrogate marker of small dense LDL. ©

Asian Indians have been consistently shown to have higher prevalence of premature coronary artery disease compared to Europeans (CAD).[1] Within the Indian subcontinent, the prevalence of CAD has increased by a factor of 10 within the last 40 years.[2] It has been reported that migrant Asian Indians have a typical dyslipidemia characterized by high triglycerides and low HDL levels with near normal LDL cholesterol levels.[3] However recent studies suggest that LDL cholesterol levels are strongly linked to coronary artery disease in Indians within the subcontinent.[2]

Two earlier studies[4,5] have compared small dense LDL distribution in migrant Indians and Europeans, but these produced contradictory results with one showing a higher frequency of small dense LDL in Indians[4] while the other reported that Indians have larger LDL size.[5] However, both these studies merely looked at the frequency of LDL subfractions in Indians and not specifically at its association with CAD.[4,5] Further, migrant Indians differ from native Indians in being more affluent and consequently they have higher BMI, waist circumference and increased triglycerides levels.[6] There are no studies to our knowledge on small dense LDL in native Indians.

Earlier studies have shown small dense LDL levels to be increased in diabetic compared to non-diabetic subjects.[7,8] India has the largest number of diabetic subjects in the world.[9] Moreover Asian Indians have been shown to have a greater degree of insulin resistance[3] and insulin resistance is associated with small dense LDL. Hence we felt it was useful to look at the association of small dense LDL with diabetes and coronary artery

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disease in a native Indian population and this was the basis of the present study.

Materials And Methods

Sample selection: The Chennai Urban Rural Epidemiology Study (CURES) is an ongoing epidemiological study conducted on a representative population (aged ≥ 20 years) of Chennai (formerly Madras) the fourth largest city in India with a population of about 4.2 million. The methodology of the study has been published elsewhere.[10] Briefly, in Phase 1 of the urban component of CURES, 26,001 individuals were recruited based on a systematic random sampling technique. Self reported diabetic subjects were classified as 'known diabetic subjects'. In Phase 2 of CURES, all known diabetic subjects (n=1529) were invited to our centre for detailed studies on vascular complications. In addition, age and sex matched non-diabetic subjects underwent oral glucose tolerance tests (OGTT) using 75 grams of glucose load. Those who were confirmed by OGTT to have fasting plasma glucose < 110mg / dl and 2 hr plasma glucose value <140 mg / dl were categorized as normal glucose tolerance (NGT).[11] For known diabetic subjects fasting and postprandial plasma glucose after a standard breakfast was measured. All study subjects underwent a 12 lead ECG.

The following groups of subjects were randomly selected from CURES for this study.

Group 1 comprised of 30 selected healthy non-diabetic subjects without CAD. The inclusion criteria for this group were normal glucose tolerance, non-smokers with normal resting 12 lead ECG and absence of history of angina or myocardial infarction.

Group 2 consisted of 30 Type 2 diabetic patients without CAD. Diabetes was defined according to WHO consulting group criteria, but the other inclusion criteria were similar to Group 1.

Group 3 consisted of 30 Type 2 diabetic patients as defined above but additionally had CAD. CAD was diagnosed based on a past history of documented myocardial infarction and / or medical therapy (nitrates) or revascularization for CAD and / or electrocardiographic (ECG) changes suggestive of Q wave changes (Minnesota codes 1-1-1 to 1-1-7) and / or ST segment depression (Minnesota codes 4-1 to 4-2).

Institutional ethical committee approval was obtained for the study and informed consent was obtained from all study subjects.

Anthropometric measurements

Physical examination included height and weight measurements and the body mass index (BMI) was calculated. Waist measurements were done in the standing position as described elsewhere.[10] Blood pressure (BP) was recorded to the nearest 2 mmHg in the sitting position in the right arm with a mercury sphygmomanometer (Diamond Deluxe BP apparatus, Industrial Electronic and allied products, Electronic Co-op Estate, Pune, India). Two readings were taken 5 minutes apart and the mean of the two was calculated.

Biochemical parameters

Biochemical analyses were done on Hitachi - 912 Autoanalyser (Hitachi, Mannheim, Germany) using kits supplied by Roche Diagnostics, (Mannheim, Germany). Fasting plasma glucose (GOD - POD method), serum cholesterol (CHOD-PAP method), serum triglycerides (GPO-PAP method) and HDL cholesterol (direct method - polyethylene glycol-pretreated enzymes) were measured. Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula.[12] Glycated hemoglobin (HbA1C) was estimated by high-performance liquid chromatography using the Variant machine (Bio-Rad, Hercules, Calif., USA). Serum insulin concentration was estimated using Dako kits (Dako, Glostrup, Denmark). Insulin sensitivity was calculated using the QUICKI formula: QUICKI = $1 / [(\log (I_0) + \log (G_0)]$, where I_0 is the fasting plasma insulin level (μIU / ml), and G_0 is the fasting plasma glucose level (mg / dl).[13]

Estimation of small dense LDL

Small dense LDL was determined using electrophoresis with high-resolution 3% poly-acrylamide tube gel with LipoPrint LDL System (Quantimetrix Corp., Redondo Beach, CA, USA). LipoPrint LDL system was approved by US FDA and uses polyacrylamide gel electrophoresis for separation of LDL subfractions. In brief, 25 μl of sample mixed with 200 μl of LipoPrint loading gel, was loaded on 3% polyacrylamide gel. Lipid specific dye was used to stain the lipoproteins. After 30 minutes of photo-polymerization at room temperature, electrophoresis was done for 60 minutes. The gel tubes were scanned after 30 minutes of completion of electrophoresis. For quantification, scanning was done at 610 nm with Artixscan 1100 scanner (Microtek Co., USA) and iMac personal computer (Apple Computer Inc., USA). After scanning, electrophoretic mobility (Rf) and area under the curve (AUC) were analyzed quantitatively with NIH image program vs. 1.62 (US National Institute of Health, USA). LDL subfractions were calculated with Rf between VLDL fraction, whose Rf was 0.0 and HDL fraction, whose Rf was 1.0. LDL is distributed from Rf 0.30 to Rf 0.61 as 7 bands, whose Rf's are 0.30, 0.36, 0.41, 0.46, 0.51, 058, and 0.61 and they are termed as LDL 1 to LDL 7. LDL 1 and LDL 2 are defined as the large LDL and LDL 3 and above as small LDL.[7] The subfractions of lipoprotein were expressed as mg / dl.

Six samples were repeated for determining the reproducibility of the assay. The intra-assay co-efficient of variation (CV) ranged from 1.6% to 4.2% for the LDL subfractions, while the interassay CV ranged from 2.5% to 4.9%.

Statistical Analysis

LDL subfractions 3 and above, representing small sized LDL were summed up to determine the overall small dense LDL fraction. One-way ANOVA or students "t" test as appropriate was used to compare groups for continuous variables. Mann-Whitney U or Kruskal-Wallis H test as appropriate was used for comparisons between groups for the parameters, which showed significance for normality with Kolmogorov- Smirnov test.

Chi-square test or Fisher's Exact test as appropriate was used to compare proportions. Spearman's correlation analysis was done to determine the relation of small dense LDL with other risk variables. Univariate regression analysis was done to determine the association of small dense LDL with diabetes and CAD. 75th percentile of the overall small dense LDL fractions in the non-diabetic group was taken as a cut-off for defining elevated small dense LDL. Receiver Operating Characteristic (ROC) Curve was constructed for diagnosis of small dense LDL with various lipid parameters. Cut-off values were selected based on optimum sensitivity and specificity. All analysis was done using Windows based SPSS statistical package (Version 10.0, Chicago) and p values <0.05 were taken as the level of significance.

Results

Table 1 shows the clinical characteristics of the study groups. HbA1c ($p < 0.001$), serum cholesterol ($p<0.01$), serum triglycerides ($p<0.01$), LDL ($p<0.05$), total cholesterol / HDL ratio ($p<0.05$) and triglycerides / HDL ratio ($p<0.01$) were significantly higher in diabetic subjects with CAD compared to non-diabetic subjects. QUICKI ($p < 0.001$) was significantly lower in diabetic subjects with CAD compared to non-diabetic subjects.

There was no difference in the anti-diabetic therapy between subjects with and without CAD (Diabetic subjects with CAD: 4 (13%) on di*et al*one, 21 (70%) on oral hypoglycemic drugs (OHA), 3 (10%) on insulin and 2 (7%) on a combination of insulin and OHA, Diabetic subjects without CAD: 3 (10%) on di*et al*one, 20 (67%) on oral hypoglycemic drugs (OHA), 3 (10%) on insulin and 4 (13%) on a combination of insulin and OHA). None of the study subjects were on statin or aspirin therapy.

LDL subfraction 3 was significantly higher in diabetic subjects with CAD compared to non-diabetic subjects (12.2 ± 9.6 vs 6.4 ± 6.6 mg / dl, $p < 0.05$), while LDL subfraction 4 was significantly higher in diabetic subjects with CAD compared to both healthy normals ($p<0.05$) and diabetic subjects without CAD ($p<0.05$) (normal: 0.7 ± 0.4 mg / dl, diabetes without CAD: 1.5 ± 2.1 mg / dl, diabetes with CAD: 3.7 ± 4.9 mg / dl). LDL subfraction 5 was also significantly higher in diabetic subjects with CAD compared to both healthy normals ($p<0.05$) and diabetic subjects without CAD ($p<0.05$) (normal: 0.07 ± 0.39 mg / dl, diabetes without CAD: 0.06 ± 0.34 mg / dl, diabetes with CAD: 0.79 ± 1.27 mg / dl). Overall small dense LDL fraction (LDL fractions 3 and above) was significantly higher in diabetic subjects with CAD ($p<0.001$) and without CAD ($p<0.05$) compared to healthy normals (Table 2).

The 75th percentile of the overall small dense LDL fractions in the non-diabetic group was 9.0 mg / dl. Using this cut off, the proportion of subjects with elevated small dense LDL in the study groups was computed. 58% of the diabetic subjects with CAD and 51% of the diabetic subjects without CAD had elevated levels of small dense LDL.

Small dense LDL showed a positive correlation with

Table 1 : Clinical and biochemical characterstics of study groups

Variables	Non-diabetic subjects without CAD (n=30)	Type 2 diabetic without CAD (n=30)	Type 2 diabetic with CAD (n=30)
Age (Yrs)	56 ± 11	57 ± 10	57 ± 8
Males n (%)	15(50)	15(50)	15(50)
Duration of diabetes (Yrs)	—	6 ± 6	9 ± 7
Body mass index (Kg / m2)	23.0 ± 5.4	24.9 ± 4.4	23.8 ± 3.1
Waist circumference (cms)	87 ± 16	92 ± 12	94 ± 13
Systolic BP (mm / Hg)	125 ± 14	133 ± 22	135 ± 28
Diastolic BP (mm / Hg)	78 ± 11	76 ± 9	79 ± 13
Fasting plasma glucose (mg / dl)	85 ± 7	167 ± 56 ***	171 ± 61 ***
HbA1c (%)	5.7 ± 0.6	8.7 ± 1.8 ***	9.6 ± 2.2 ***
Total cholesterol (mg / dl)	180 ± 31	200 ± 39*	215 ± 46 **
Triglycerides (mg / dl)	106 ± 27	159 ± 80**	177 ± 97 **
HDL cholesterol (mg / dl)	46 ± 12	42 ± 8	42 ± 8
LDL cholesterol (mg / dl)	115 ± 31	123 ± 35	139 ± 39*
Cholesterol / HDL ratio	4.2 ± 1.1	4.5 ± 1.0	5.2 ± 1.4 ** #
Triglycerides / HDL ratio	2.6 ± 1.3	3.6 ± 2.1*	4.3 ± 2.3 **
QUICKI	0.36 ± 0.04	0.32 ± 0.03 ***	0.32 ± 0.04 ***

* $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to non-diabetic subjects without CAD, # $p<0.05$ compared to diabetic subjects without CAD

Table 2 : Lipoprotein levels measured using lipoprint machine in the study groups

Variables	Non-diabetic subjects without CAD (n=30)	Type 2 diabetic without CAD (n=30)	Type 2 diabetic with CAD (n=30)
Small dense LDL fraction 3 (mg/dl)	6.4 ± 6.6	9.6 ± 7.2*	12.2 ± 9.6 *
Small dense LDL fraction 4 (mg/dl)	0.7 ± 0.4	1.5 ± 2.1	3.7 ± 4.9 * #
Small dense LDL fraction 5 (mg/dl)	0.07 ± 0.39	0.06 ± 0.34	0.79 ± 1.27 * #
Overall small dense LDL (mg/dl)	7.2 ± 6.8	11.1 ± 8.0*	16.7 ± 11.1*#

* p<0.05 compared to non-diabetic subjects without CAD
# p<0.05 compared to type 2 diabetes without CAD

fasting plasma glucose (p=0.023), HbA1c (p=0.012), total cholesterol (p<0.001), triglycerides (p<0.001), LDL (p=0.002) and total cholesterol/HDL ratio (p=<0.001) and triglycerides/HDL ratio (p<0.001) and a negative correlation with HDL cholesterol (p=0.002) and QUICKI values (p=0.019). The highest correlation was found with triglycerides / HDL ratio (r=0.728) (Table 3).

The proportion of subjects with elevated small dense LDL was computed according to different tertiles of triglycerides/HDL ratio. 3.3% of the study subjects in the first tertile, 40.0% in the second tertile, and 90.0% in the third tertile had small dense LDL (trend chi square - 45.6, p<0.001).

Receiver Operating Characteristic (ROC) curve were constructed for predicting elevated small dense LDL using various lipid parameters. The area under the curve (AUC) for each lipid parameter is presented in Table 4. Triglycerides /HDL ratio and total cholesterol/HDL ratio had higher AUC values compared to other parameters. Total cholesterol/HDL ratio of 4.4 had the optimum sensitivity (77.0%) and specificity (78.0%) for detecting small dense LDL. The positive predictive value for total cholesterol/HDL ratio of 4.4 was 74% and negative predictive value was 81%. A triglycerides / HDL ratio of 3.0 had the optimum sensitivity (80.0%) and specificity (78.0%) for detecting elevated small dense LDL while the positive predictive value was 74% and negative predictive value, 83%.

Linear regression analysis was done to determine the association of triglycerides/HDL ratio and cholesterol/HDL with small dense LDL (Table 5). Even after adding HbA1c and QUICKI into the regression equation, the association of triglycerides/HDL ratio with small dense LDL persisted (p<0.001). Similarly total cholesterol/HDL ratio also showed a good association with small dense LDL even after adding HbA1c and QUICKI into the regression equation (p < 0.001).

Discussion

This study makes three important findings. First, small dense LDL has a strong association with CAD and diabetes in Asian Indians. Secondly, it shows a positive correlation with HbA1c and a negative correlation with insulin sensitivity (QUICKI) and HDL cholesterol. Finally, small dense LDL shows a strong

Table 3 : Correlation analysis of small dense LDL with other risk variables

Variables	Small dense LDL	
	r value	p value
Age	0.010	0.378
Body mass index	0.086	0.378
Systolic BP	0.124	0.270
Diastolic BP	0.110	0.330
Waist	0.033	0.785
Fasting plasma glucose	0.252	0.023
HbA_1c	0.281	0.012
QUICKI	- 0.260	0.019
Total cholesterol	0.443	<0.001
Triglycerides	0.685	<0.001
HDL cholesterol	-0.341	0.002
LDL cholesterol	0.342	0.002
Total cholesterol/HDL ratio	0.660	<0.001
Triglycerides/HDL ratio	0.728	<0.001

Table 4 : Area under the curve (AUC) for ROC's to determine the presence of small dense LDL

Variables	Area
Triglycerides /HDL ratio	0.885
Total cholesterol/HDL ratio	0.857
Serum triglycerides	0.868
Total cholesterol	0.732
HDL- cholesterol	0.672

Table 5 : Linear regression analysis using small dense LDL as a dependent variable

Variables	Beta	p value
Unadjusted		
Triglycerides/HDL ratio	0.676	< 0.001
Adjusted for HbA_1c		
Triglycerides/HDL ratio	0.624	0.185
HbA_1c	< 0.001	0.033
Adjusted for HbA_1c and QUICKI		
Triglycerides/HDL ratio	0.618	< 0.001
HbA_1c	0.160	0.104
QUICKI	- 0.052	0.590
Unadjusted		
Total cholesterol/HDL ratio	0.636	< 0.001
Adjusted for HbA_1c		
Total cholesterol/HDL ratio	0.586	< 0.001
HbA_1c	0.163	0.077
Adjusted for HbA_1c and QUICKI		
Total cholesterol/HDL ratio	0.579	< 0.001
HbA1c	0.129	0.213
QUICKI	- 0.070	0.489

association with triglycerides / HDL ratio and a value of 3.0 had the optimum sensitivity and specificity to detect elevated small dense LDL.

The pattern of LDL subfraction change with diseases and subjects with diabetes[7,8] and CAD have been shown to have higher levels of small dense LDL fractions.[4] Indeed prospective studies have shown small dense LDL to be a predictor of both diabetes and CAD.[14,15] The link of small dense LDL to diabetes is considered to be through insulin resistance.[15,16] In our study, small dense LDL showed a negative correlation with QUICKI, an indication of decreased insulin sensitivity. This result corroborates earlier studies, which have shown insulin resistance and plasma insulin levels to be associated with small dense LDL.[16]

Small dense LDL is considered to be an atherogenic moiety. While LDL cholesterol is a strong risk factor for CAD, more than 50% of the subjects with CAD have normal LDL cholesterol levels.[17] The increased prevalence of CAD among subjects with normal LDL can be explained by LDL particle size. Studies have shown small dense LDL to be more prone to oxidation and conformational changes.[18] This results in the reduction of LDL clearance by its receptors, with increased production of scavengers, which triggers immunological changes resulting in atherosclerosis. An atherogenic lipoprotein pattern characterized by a predominance of small dense LDL, moderately elevated plasma triglycerides and low HDL levels, is the most powerful risk factor for CAD.[19] Thus the role of small dense LDL as an important cardiovascular risk factor is very well established among Europeans. In this we confirm that even in Asian Indians who have one of the highest prevalence rates of premature CAD, small dense LDL is an important risk factor for CAD.

However, estimating small dense LDL still remains a challenge, as methods such as (quantification, nuclear magnetic resonance or use of non-denaturing polyacrylamide gel electrophoresis methods are required. Moreover all these procedures are time-consuming, labor intensive and expensive and these factors restrict the use of small dense LDL as a cardiovascular marker. In this context, an easy clinical tool to determine the elevated small dense LDL would be of great significance.

One of the interesting observations in this study is that small dense LDL showed a strong correlation with total triglycerides / HDL ratio and cholesterol / HDL ratio. Although both total cholesterol / HDL ratio and triglycerides / HDL ratio had a more or less same AUC in the ROC, triglycerides / HDL ratio value of 3.0 had the optimum sensitivity and specificity to predict elevated small dense LDL. Surprisingly, this ratio is not often used in clinical settings. An earlier study by Maruyama *et al*[20] showed that 75% of subjects with small dense LDL had triglycerides / HDL ratio above 2.0. A very recent editorial on atherogenic index of plasma suggest the practical usefulness of log transformed triglycerides / HDL ratio.[21] In this study both triglycerides / HDL ratio and their log-transformed values had similar AUC for elevated small dense LDL suggesting that both parameters could be used as surrogates for elevated small dense LDL. As routine estimation of small dense LDL is difficult and expensive, triglycerides / HDL ratio could be used as a surrogate marker for small dense LDL for epidemiology studies.

In conclusion, our study shows that small dense LDL is associated with both diabetes and CAD in Asian Indians and that a triglycerides / HDL ratio ≥ 3.0 could serve a surrogate marker of small dense LDL in this ethnic group.

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