

Increased high-density lipoprotein-oxidant index in ischemic stroke patients

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Abstract. High-density lipoprotein (HDL) is known to have atheroprotective properties which could become dysfunctional under certain disease conditions, particularly during the atherosclerotic progression. The present study sought to assess HDL functionality in acute ischemic stroke (AIS) patients in comparison with controls and the functional alteration among the ischemic stroke subtypes. The HDL functionality was evaluated in 71 statin-naïve, adult patients of South Indian descent, admitted with AIS and were compared with that of 25 age- and sex-matched healthy volunteers. Functional assay of HDL was based on its antioxidant ability to inhibit low-density lipoproteins (LDL) oxidation by air using a dichlorodihydrofluorescein-based fluorescent assay and expressed as HDL oxidant index (HOI). The HOI was higher in ischemic stroke patients as compared with controls (1.07 ± 0.32 vs. 0.51 ± 0.12 ; $P < 0.001$) indicating high oxidative stress and dysfunctionality in HDL. Regarding the stroke subtypes, HOI was >1 in all stroke subtypes: 1.05 ± 0.301 for Cardioembolic subtype, 1.15 ± 0.41 for large vessel disease, and 1.01 ± 0.25 for small vessel disease when compared with controls. However, no significant difference was noted in HOI values among the three stroke subtypes in the post hoc analysis. It was found that HDL in all ischemic stroke subtypes had less antioxidant capacity, indicating dysfunctional HDL. Functional alteration occurred in HDL of patients even in the presence of normal HDL-cholesterol levels suggesting that dysfunctionality in HDL is unrelated to cholesterol content.

Introduction

Atherothrombosis is one of the important causes of ischemic strokes (1). High-density lipoproteins (HDL) are known to

display cardiovascular protective effects by their role in reverse cholesterol transport and cholesterol efflux (2). Apart from their known anti-inflammatory, anti-thrombotic and anti-platelet activation properties, HDL can prevent lipid oxidation, a major mechanism in atherogenesis attributed to its functionality (3). HDL functionality has been shown to be altered in several pathophysiological states such as acute phase response, obesity, and chronic inflammatory diseases (4,5).

We have previously identified HDL dysfunctionality in coronary artery disease (CAD) patients belonging to a South Indian cohort (6). Functional alteration in HDL may help explain why some individuals with advanced atherosclerosis have elevated levels of HDL (7). It was also observed that dysfunctional HDL induces inflammatory response in monocytes and macrophages with the evidence of enhanced production of $\text{TNF-}\alpha$, a proinflammatory cytokine, when compared with functional HDL derived from healthy subjects (6). This pro-inflammatory effect can potentially counteract the well-established anti-inflammatory capacity of HDL (8). It has also been shown that the inflammatory and anti-inflammatory activity of HDL distinguished patients with CAD from controls more effectively than serum HDL cholesterol levels, suggesting that functional HDL plays a greater role in the beneficial effects rendered by HDL than its cholesterol content (8). Even though CAD and stroke share common risk factors, stroke is a multifactorial disease that can be due to atherosclerosis, cardiac causes, small vessel disease, or rarer causes like dissection.

Our previous study observed a trend towards increased Apo B levels in intracranial atherosclerotic disease (ICAD) subtype of large vessel disease (LVD) despite showing no significant correlations between serum Apo B and A1 levels and the ischemic stroke subtypes (9). Using a subset of this sample population, the present study sought to investigate whether functionally impaired HDL particles were associated with ischemic stroke subtypes. Inflammation affecting the functional HDL components may influence the pathophysiology of LVD. We previously investigated the roles of High-sensitivity C-reactive Protein (Hs-CRP) and lipoprotein-associated phospholipase A2 (Lp-PLA2) as inflammatory markers in symptomatic ICAD which significantly correlated with severe stenoses (10). Hs-CRP, an inflammatory marker that may be associated with HDL functionality (11), was also significantly elevated in patients with early recurrence of

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stroke in our previous findings (10). Based on these findings, it was hypothesized that HDL functionality may vary among ischemic stroke subtypes with dysfunctionality.

Although several studies have reported the inverse association of HDL cholesterol with the risk of ischemic stroke (12-16), only two studies have investigated the functionality of HDL in acute ischemic stroke patients, which compared the distribution of subfractions of HDL and HDL inflammatory indices between patients and a healthy population to evaluate the anti-oxidant function of HDL (17,18). The present study employed the HDL Oxidant Index (HOI) metric to convey the HDL anti-oxidant capacity in stroke and if this may affect stroke subtypes differently, particularly in LVD. To date, HOI values for ischemic stroke subtypes have not been investigated and there are no studies conducted in the Asian population with regard to the correlation of HOI with ischemic stroke. The present study was thus aimed at investigating the functional alteration in HDL in ischemic stroke patients when compared with age and sex-matched controls and whether alterations in HDL functionality may vary among ischemic stroke subtypes.

Materials and methods

Study design and participants. The present cross-sectional study enrolled 75 consecutive, statin-naïve, first-ever ischemic stroke patients (age ≥ 18 years) of both sexes admitted to the Stroke unit of the Department of Neurology of Sree Chitra Tirunal Institute of Medical Science and Technology (SCTIMST), Trivandrum, India. Complete stroke etiologic workup was conducted to determine and classify stroke mechanism according to Trial of Org 10172 in Acute Stroke Treatment criteria (19). Clinical evaluation included neuroimaging (magnetic resonance imaging/brain computerized tomography), vessel imaging (magnetic resonance angiography/computed tomography angiography head/neck or carotid and transcranial Doppler ultrasound), cardiac assessment (echocardiography and electrocardiogram), and vascular risk factor workup (fasting blood sugar, total cholesterol, triglycerides, HDL, LDL). All relevant data including the demographics, vascular risk factors and medical history, and diagnostic workup (blood tests), were collected. The three-month post-stroke functional outcome was documented using modified Rankin Scale (20). The present study included ischemic stroke subtypes large vessel atherosclerotic disease (LVD), small vessel disease (SVD), and cardioembolic disease. Other stroke subtypes such as dissection, vasculitis and cryptogenic strokes were excluded. Patients with history of statin use, prior stroke, and those with intracerebral or subarachnoid hemorrhage were also excluded. Patients with diabetes mellitus and high blood glucose levels at the time of enrolment were excluded as this is known to affect HDL functionality (21).

The control group consisted of 25 age and sex-matched apparently healthy volunteers from the public and relatives of hospital staff without any history of co-morbidities, acute and chronic inflammatory disease, cancer, kidney or liver diseases and who were not on any anti-inflammatory medications or antioxidants. They were assessed based on a questionnaire and routine laboratory blood analysis for sugar and lipid profiles. The present study was approved by the institutional

ethics committee at SCTIMST. Written informed consent was obtained from all participants enrolled in the study.

Sample collection. Blood samples were collected in EDTA-containing tubes after a 12 h period of overnight fasting from both patients and healthy volunteers. Plasma was separated by immediate centrifugation at $665 \times g$ for 15 min at 25°C , divided into aliquots and stored at -80°C until further analysis.

Analytical methods. Glucose, total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides were measured using enzymatic methods with the Dimension RxL Max Integrated Chemistry System (Siemens Healthcare Diagnostics Inc.). HDL was isolated from plasma by polyethylene glycol (PEG) precipitation method as previously described (22). Equal amount of PEG 8000 in glycine NaOH buffer, pH 10 was added to plasma for the selective precipitation of all the apo-B containing lipoproteins-VLDL, LDL and Lp(a), and the supernatant containing HDL was collected by centrifugation at $956 \times g$ for 10 min at 25°C (22).

LDL was isolated from plasma of control subjects by standard sequential density gradient ultracentrifugation in Beckman Optima TLX 120 Ultracentrifuge with fixed angle rotor (Beckman Coulter, Inc.) (23). The isolated lipoproteins were desalted by passing through PD-10 columns (MilliporeSigma; cat no. GE17-0435-01) equilibrated with PBS, pH7.4, and subjected to the functional assay of HDL.

Functional assay of HDL. HDL function can be measured by several assays including measuring its antioxidant capacity to prevent *in vitro* oxidation of LDL (24,25). The present study used an HOI to express the HDL functionality. HDL isolated from both patients and control subjects were evaluated for the functionality of HDL using a dichlorodihydrofluorescein (DCF)-based cell-free fluorescent assay consisting of a widely used fluorescent probe for the measurement of oxidation (26). The HDL anti-oxidant capacity which measured the ability of HDL to inhibit oxidation of LDL by air was assessed. LDL oxidation activates 2',7'-dichlorofluorescein diacetate fluorescence (MilliporeSigma; cat no. 35848). An aliquot of human LDL ($50 \mu\text{g}$ LDL cholesterol/ml) was subjected to air oxidation in the presence and absence of test HDL ($50 \mu\text{g}$ HDL cholesterol/ml) for 1 h at 37°C by mixing in black polystyrene microtiter plates and make up to $100 \mu\text{l}$ with Tris-HCL buffer (pH7.4). A volume of $25 \mu\text{l}$ of DCFH solution ($50 \mu\text{g}/\text{ml}$) was added to each well, mixed, and incubated at 37°C for 2 h. The resultant fluorescence was measured (EX:485/EM:530 nm) using a fluorescence microplate reader (BioTek Instruments, Inc.; cat. no. FLX 800).

For evaluating the HOI of the samples, LDL oxidation was performed in the presence and absence of test HDL and the change in fluorescence due to oxidation of DCFH was measured. The DCF fluorescence data was converted into an HOI that equaled the ratio of fluorescence of LDL in the presence of HDL divided by the fluorescence of LDL in the absence of HDL. An index <1.0 denoted protective anti-oxidant HDL, while an index >1.0 denoted pro-oxidant dysfunctional HDL. The assay distinguished the antioxidative potential of HDL taken from different participants. Each of the HOI values

Table I. Baseline and biochemical characteristics of study population.

| Characteristic | Patients (n=71) | Control (n=25) | P-value |
|--|--------------------|----------------|---------|
| Age, median (interquartile range), years | 57 (23-85) | 53 (34-70) | 0.04 |
| Male, n (%) | 47 (66) | 15 (60) | 0.60 |
| BMI, (mean \pm standard deviation) | 23.85 \pm 1.17 | 24.1 \pm 2.5 | 0.50 |
| Smoking, (yes/no) | 27/44 | 0/25 | <0.0001 |
| High blood pressure, (yes/no) | 39/32 | 0/25 | <0.0001 |
| Coronary artery disease, (yes/no) | 6/65 | 0/25 | 0.30 |
| Atrial fibrillation, (yes/no) | 12/59 | 0/25 | 0.03 |
| Fasting glucose, (mg/dl) | 76.69 \pm 40.35 | 83 \pm 10 | 0.48 |
| Total cholesterol, (mg/dl) | 195.2 \pm 46.81 | 197 \pm 31.1 | 0.86 |
| HDL cholesterol, (mg/dl) | 45.56 \pm 12.22 | 49 \pm 10.78 | 0.22 |
| LDL cholesterol, (mg/dl) | 129.19 \pm 43.68 | 131 \pm 26.5 | 0.85 |
| Triglyceride, (mg/dl) | 100.6 \pm 60.01 | 89 \pm 31.5 | 0.36 |

Data are expressed as mean \pm standard deviation or median (interquartile range). P-values are from Student's t-test for continuous variables, Mann-Whitney U test and Fischer's exact test for categorical variables. BMI, body-mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table II. HOI values in stroke patients, each ischemic stroke subtypes and in healthy controls.

| Parameter | HOI |
|-----------------------------|-----------------|
| Total patients (n=71) | 1.07 \pm 0.32 |
| Cardioembolism (n=25) | 1.05 \pm 0.30 |
| Large vessel disease (n=22) | 1.15 \pm 0.41 |
| Small vessel disease (n=24) | 1.01 \pm 0.25 |
| Healthy controls (n=25) | 0.51 \pm 0.12 |

Data are given as mean \pm standard deviation. HOI, high-density lipoprotein oxidant index.

was given as the mean of duplicate measurements and the experiment was repeated twice to ensure its reproducibility.

Statistical analysis. Results of the demographic and baseline characteristics of the patient population were expressed as mean \pm standard deviation along with frequency tabulation for variables with proportion or categorical distribution. The P-values were computed using Student's t-test for continuous variables, Mann-Whitney U Test, and Fischer's Exact test for categorical variables. Multiple comparisons of HOI were assessed in the patients, subtypes, and control groups using the analysis of variance and Tukey's honestly significantly difference post hoc test. Statistical analysis was performed with GraphPad Prism version 7.0 (GraphPad Software Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Baseline and biochemical characteristics of the study population. A total of 75 patients were enrolled for the present study, of which four patients were excluded due to the

Table III. Post-hoc analysis of the HOI values of the total patients, ischemic subtypes, and healthy controls.

| Pairwise comparisons | HSD.05=0.2152, HSD.01=0.2581 | P-value |
|-------------------------|---------------------------------|---------|
| CE:LVD | 0.15 | 0.3 |
| CE:SVD | 0.03 | 0.9 |
| LVD:SVD | 0.17 | 0.1 |
| CE:Total patients | 0.04 | 0.9 |
| LVD:Total patients | 0.11 | 0.6 |
| SVD:Total patients | 0.07 | 0.9 |
| SVD:Controls | 0.49 | <0.001 |
| CE:Controls | 0.52 | <0.001 |
| Controls:Total patients | 0.56 | <0.001 |
| LVD:Controls | 0.67 | <0.001 |

Data are given as mean \pm standard deviation. P-values are from one-way analysis of variance followed by Tukey's HSD post hoc test. HSD, honestly significantly difference. HOI, high-density lipoprotein oxidant index; CE, cardioembolic disease; LVD, Large vessel disease; SVD, small vessel disease.

presence of high blood glucose levels at the time of enrollment. A total of 25 age- and sex-matched healthy volunteers were also included in the study for comparison. The baseline and biochemical characteristics of the study population are summarized in Table I. The median age of the population was 57 years (interquartile range 23-85 years). Among the patients, 47 were male and 24 were female. Hypertension was reported in 39 patients, whereas six patients had a history of CAD and 12 patients were diagnosed with atrial fibrillation at the time of recruitment. There was no significant difference in the biochemical parameters, such as fasting glucose and lipid profile, between the patients and controls.

HOI. HOI values of controls, total patients, and ischemic subtypes are given in Table II, which were >1 for stroke patients compared with controls indicating high oxidative stress and hence dysfunctionality in HDL. In the post hoc analysis, as shown in Table III, it was found that the mean HOI values were elevated in total patients (1.07 ± 0.32) and in all stroke etiological subtypes (1.05 ± 0.301 in cardioembolic subtype, 1.15 ± 0.41 in LVD, 1.01 ± 0.25 in SVD) when compared with controls (0.51 ± 0.12 ; $P < 0.001$). However, there was no statistical significance when a pairwise comparison of HOI was made between the subtypes.

Discussion

The main finding of the present study was that HDL in stroke patients including the LVD, SVD and cardioembolic subtypes had a noticeably increased oxidant capacity of HDL than that isolated from healthy subjects, thereby indicating the pathophysiological role of dysfunctional HDL in stroke. By measuring its antioxidant capacity in terms of its ability to inhibit LDL oxidation, which is the key mechanism in atherogenesis, it was found that functional alteration of HDL occurred in stroke patients, even in the presence of normal HDL cholesterol levels, suggesting that dysfunctionality in HDL is not related to its cholesterol content in stroke.

The association of dysfunctional HDL with stroke has been previously demonstrated in three other studies (17,18,27). One study showed the presence of large HDL particles in ischemic stroke patients, indicative of dysfunctional antioxidant properties, as compared with that of controls, and its inability to prevent TNF- α thereby leading to endothelial dysfunction (17). This may affect its functional and atheroprotective properties (7). HDL particles are highly modifiable and alterations in their protein components suggest changes in their antioxidant abilities (21). In a pooled cohort analysis, Singh *et al* (27) examined the HDL particles in participants of three major cohort studies and found them to be inversely correlated with myocardial infarction and stroke, albeit displaying racial disparities. Another study showed that impaired antioxidant capacity of HDL is associated with poorer outcome in ischemic stroke (18). The ability of HDL to prevent LDL oxidation is an important antiatherogenic property because LDL oxidation in the artery wall is believed to be the primary event leading to the initiation of atherosclerosis (28). Generation of excess levels of reactive oxygen species may increase the levels of oxidized lipids pertaining to the existence of other co-morbidities such as diabetes mellitus (29).

Studies conducted in CAD patients found dysfunctional HDL to be associated with poorer outcome and trigger inflammatory pathways in macrophages leading to atheroma formation (5,29). Previous studies by our colleagues reported HDL dysfunctionality in CAD patients and functional alterations even in the presence of normal HDL-C levels, suggesting that the functional HDL levels are responsible for the beneficial effects of HDL which far outweighs its cholesterol content (6,30). More studies are needed to convincingly validate this hypothesis. Functional deficiency in HDL may reflect a greater risk for atherosclerotic stroke subtypes (24). However, the present study did not show any significant differences in HOI values between stroke subtypes, probably owing to the limited sample size in each of the groups.

Inflammation is a key mechanism causing structural variations and functional alterations in HDL (31). The role of inflammatory mediators involved in the progression of stenosis during atherothrombotic events in stroke was confirmed in our previous study which reported the association of the proinflammatory markers (10). Hs-CRP and Lp-PLA2 correlate with high-grade stenoses in patients diagnosed with symptomatic ICAD and Hs-CRP is associated with long-term recurrence in these patients (10). Dysfunctional HDL hinders reverse cholesterol transport and may not protect LDL from oxidation.

In animal studies, on the other hand, HDL has been shown to exert a direct vascular and neuroprotective effect when administered during the acute phase of embolic stroke in rats, possibly by antioxidant or anti-inflammatory mechanisms (32). However, the pathophysiology of dysfunctional HDL in ischemic stroke remains to be elucidated. The present study suggested a need to examine the functional characteristics of HDL while assessing stroke risk, as HDL is a modifiable risk factor. Improving the functionality of HDL is essential to lower the risk of vascular events including stroke. Using the same study population, we had previously reported that the Apo B/A1 ratio was not found to be significantly different among ischemic stroke subtypes. Patients with ICAD had higher serum Apo B levels albeit not statistically significant (9).

The major strengths of the present study were thorough classification of the stroke subtypes and detailed ischemic stroke etiological evaluation with the documentation of clinical and imaging risk factors for all patients enrolled. The present study was the first to assess HDL function. However, it was not without limitations. Due to the small sample size, the present study may have been not strong enough, hence limiting the interpretation of the true difference in HDL oxidation across the stroke subtypes. The male ratio was higher because age-specific stroke rates are higher in men. The Hyderabad Stroke registry also found an increased incidence of stroke in men (78.3%) in India (33). The present study included the distribution of sex (male percentage) in both the groups and there was no difference in the frequency of the sexes between the groups. In addition, this was a single-center study conducted in South India which limits the extrapolation of the results to a wider population. Nevertheless, the loss of antioxidant activity of HDL in ischemic stroke and its subtypes underscores the role of functional HDL in the pathophysiology of ischemic stroke and the importance of incorporating HDL functional assay in the diagnosis of stroke. Prospective work may involve large multicentric studies to fully establish the link between HOI and stroke. In addition, the importance of monitoring the benefits of exercise to improve the functional outcome after stroke and whether this may correlate with the improvement of the atheroprotective functions of HDL using the HOI metric will provide information on its role in stroke rehabilitation.

In conclusion, decreased antioxidant capacity in ischemic stroke subtypes, indicating dysfunctional HDL, may affect its protective functions in stroke.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to compromising individual privacy but are available from the corresponding author on reasonable request.

Authors' contributions

DD, SK, PNS and SG contributed to the conception and design of the present study. Material preparation, data collection, and analysis were performed by DD and SK. The first draft of the manuscript was written by DD and all authors commented on previous versions of the manuscript. SG and DD confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was performed in line with the principles of the Declaration of Helsinki. It was approved by the Institutional Ethics Committee of Sree Chitra Tirunal Institute for Medical Sciences and Technology (approval number IEC/1017). Informed consent was obtained from all individual participants included in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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