Overall Discussion, Summary and Conclusion
Introduction

HDL was discovered in 1929 as one of the plasma lipoprotein. It is in the last 30 years, that it has gained major attention because it is the only component in the blood that shows an inverse relationship with atherosclerosis. However, there is an unresolved debate whether the HDL quantity (HDL-C) or HDL quality (functionality) is more important in the cardioprotection.

The antiatherogenic activities of HDL are summarized in table 5.1

Table 5.1 Antiatherogenic activities of HDL

<table>
<thead>
<tr>
<th>Activity</th>
<th>Protective</th>
<th>Reference</th>
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<tr>
<td>Reverse cholesterol transport</td>
<td>Efflux of cholesterol from foam cells</td>
<td>Zhang et al 2003</td>
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<tr>
<td>Anti oxidative</td>
<td>Protection of LDL from oxidation</td>
<td>Konthush et al 2003</td>
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<td>Anti inflammatory</td>
<td>Inhibition of PAF synthesis</td>
<td>Sugatani et al 1996</td>
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<td>Inhibition of cell adhesion molecule synthesis</td>
<td>Barter et al 2002</td>
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<td></td>
<td>Inhibition of expression of MCP-1</td>
<td>Mackness et al 2004</td>
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<tr>
<td>Anti thrombotic</td>
<td>Inhibition of procoagulant</td>
<td>Epand et al 1994</td>
</tr>
<tr>
<td>Anti infective</td>
<td>Reduction in pyrogenic activity of bacterial LPS</td>
<td>Ulevith et al 1979</td>
</tr>
<tr>
<td></td>
<td>Lysis of Trypanosoma Brucei</td>
<td>Hajduk et al 1989</td>
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In addition to these, HDL also has protective functions on the endothelium, like any other biological molecule, HDL is also subject to damage resulting in a loss of function. In vitro studies have shown that HDL, like LDL can undergo modified by oxidation and non-enzymatic glycation. These modified particles have been shown to have lost their specific function under study and have been called dysfunctional HDL.
The loss of function need not always imply that the individual is put at a severe risk of CVD. The HDL particles in humans are far more in number than the particles containing β lipoproteins (LDL, VLDL and IDL). While HDL concentration is in the micromolar range, of apo B particles are in the nanomolar range (Fogelman 2010).

Our results show that when HDL is oxidized invitro, although the phenyl acetate hydrolysing activity and paraoxonase activity decrease by over 80%, the thiolactonase activity had increased by over 2 fold (Chapter II). In our attempt to purify HDL we found that only a small proportion of the HDL-associated PON was held on the cibacron sepharose B column (<1%). The level of oxidized lipids in HDL was also high. These results suggest that the HDL could have been modified in such a way that either its PON is dissociated under the conditions of purification or that the HDL particles are not being held by the CBB column. Whatever the reason, this behaviour of HDL and its associated proteins is a cause of concern since Indians have a high predisposition to cardiovascular diseases.

The distribution of the three enzyme specificities in the sample of our population showed skewed distribution of paraoxonase activity with PON as substrate and the thiolactonase activity. The thiolactonase was positively skewed while paraoxonase was negatively skewed. This is consistent with our invitro experiments where we have shown a loss of activity of paraoxonase and increase in thiolactonase activity on oxidation. Hence it is possible that the skewed distribution may reflect an underlying oxidative stress.

Contrary to expectation the HDL in our study increased DCFH oxidation (Chapter III) and when mixed with LDL it showed a further increase in the fluorescence intensity, whereas oxidized LDL or HDL was not able to oxidize DCFH. We have found that the invitro oxidation actually decreased the 234nm absorption while the TBARS were increased. The decrease in 234nm absorption and decrease in the ability to oxidize DCFH are consistent with each other. If the HDL is mildly oxidized, it will be able to oxidize DCFH, since DCFH oxidation depends on the presence of oxidized lipids. Further oxidation would decrease the mildly oxidized lipids by degradation of the lipids to yield aldehydes, ketones and hydroxyls, which would not be able to oxidize DCFH.
We, therefore, wanted to quantitate the oxidized HDL in the serum and hence we raised antibodies to oxidized HDL and oxidized Apo A-I. Hen was used to raise antibodies because the human proteins would be highly immunogenic in the hen. The antibodies formed in the egg (IgY) (Chapter IV) was able to precipitate oxidized HDL. However we were not able to use this in the development of an ELISA since at higher antigen concentration we got lower antigen-antibody reaction. The reason for this anomalous behaviour of the antibody/antigen is not known. Moreover, the antibody cross reacted with all the proteins/lipoproteins in the native form and modified form, making it highly unreliable as a tool to assay oxidized HDL.

Hence we raised antibody in rabbit against oxidized HDL. This antibody showed a linear response with increasing antigen concentration. When this antibody was used to quantitatively estimate the amount of oxidized HDL, we found even the control subjects had oxidized HDL. This is consistent with our observation described in the earlier two chapters (Chapter II and III).

We then wanted to see the effect of HDL on LPS mediated TNF-α production in adipose tissue stimulated by LPS. Although these are preliminary results, and more controls are needed to find out whether this observation reflects a proinflammatory function of HDL. HDL clearly increased TNF-α production in the presence of LPS, but not oxidized HDL. At this point it is difficult to envisage a mechanism for the enhanced TNF-α production and also to speculate whether it is a beneficial effect or a harmful effect.
Summary and conclusion

- Oxidation of HDL caused a loss of phenyl acetate hydrolysing activity and paraoxon hydrolysing activity but increased thiolactonase activity.
- Purification of HDL by cibacron blue sepharose column gave a very small yield of enzyme activities bound to the column (<1%) while 99% of the activity was eluted in the wash.
- Paraoxon activity was negatively skewed and thiolactonase was positively skewed among subjects.
- HDL increased the oxidation of DCFH whereas oxidized HDL decreased the oxidation of DCFH.
- Oxidation of HDL decreased 234nm absorption.
- Oxidation of HDL increased TBARS.
- When antibodies to ox-HDL were added to oxidized serum, a precipitate was obtained. Precipitate did not cause DCFH oxidation. However the supernatant had greater ability to oxidize DCFH than the oxidized serum.
- The oxidizing ability of HDL or serum could be abolished by heating and trypsin treatment. It did not precipitate with γ-globulin fraction of the serum but was present in the β-globulin fraction.
- Oxidation of serum did not affect the amount of HDL-C determined by the clinical assay procedures.
- Oxidized HDL had greater electrophoretic mobility.
- Oxidized HDL and oxidized Apo A-I were antigenic and produced antibodies in hen’s egg (IgY)
- IgY gave a precipitin band with oxidized HDL and oxidized serum.
- Precipitated oxidized HDL when added to HDL caused an inhibition of PON1 activity.
The inhibition was competitive in nature.

IgY could not be used develop ELISA since it gave lower antigen-antibody reaction at higher antigen concentration.

IgY to ox-Apo A-I cross reacted with normal HDL, oxidized HDL, glycated HDL, normal LDL, oxidized LDL, glycated LDL, normal BSA and oxidized BSA.

Cross reactivity was highest with LDL and modified LDL.

Antibodies to ox-HDL raised in rabbit reacted with ox-HDL. With increasing concentration of ox-HDL there was an increase in antigen-antibody reaction.

ELISA was developed using rabbit antibody.

Serum of control subjects and patients with CVD were tested for the amount of ox-HDL.

Control subjects had 253±174 ng/ml ox-HDL. CVD patients had 379±141 ng/ml. The difference was statistically significant (P=0.015).

LPS and HDL simulated adipose tissue to produce TNF-α.

The TNF-α production reached a maximum in 16 hrs and remained high at 24 hrs.

When LPS was mixed with HDL the TNF-α production was enhanced 2.4 fold.

When LPS was mixed with ox-HDL there was no stimulation of TNF-α production.
Overall conclusion

Our results suggest that the HDL of our subjects is oxidized to a greater extent than the LDL. The HDL on account of its mild oxidation was a better oxidant of DCFH than LDL. The HDL enhanced LPS mediated TNF-α production in the adipose tissue in vitro.

Our results suggest that the higher predisposition of Indians to cardiovascular diseases may in part at least be because of the increased oxidative stress resulting in mildly oxidized HDL.

Future prospects

While genetics has been attributed to the increased incidence of cardiovascular diseases among Indians, the role of oxidative stress in the predisposition to CVD cannot be ignored. Indians are in an epidemiological transition resulting in a drastic change in life style. Added to this, the pollution, particularly caused by diesel fumes and smoke, has increased oxidative stress leading to the modification of the only negative risk factors, namely HDL.

Although antioxidant therapies have not shown beneficial effects on cardiovascular diseases in clinical trials, their role in reducing the oxidative stress among Indians needs to be investigated with proper control to eliminate influence confounding factors. In recent times the media has managed to change the public opinion concerning what is healthy and what is not. The role of recent switch to unhealthy life style in the name of healthy life style needs to be investigated critically. For example TV advertisements show that polyunsaturated oils are “heart healthy”. This could be the reason why many individuals would switch the use of their traditional source of fats to the so called “heart healthy” oils. Consumption of unsaturated oils would result in increased oxidative stress.

Consumption of free sugars has also increased particularly through the vigorous marketing of convenience foods and beverages. This could be another reason for the increased obesity which in turn is a risk factor for inflammatory disorders like CVD.

The need of the hour is to re look at what the marketing technology defines as “healthy” and to get back the people on the right track to health and well being.
References


