Discussion
4. DISCUSSION

4.1 General observations made in IHD and NIDDM

4.2 Cholesterol distribution in Plasma Lipoprotein

4.3 Phospholipids and Glycerides in Lipoproteins

4.4 Cholesterol Ester Transfer and Lipoprotein Metabolism

4.5 Role of Lipid Peroxidation and Free radical Damage in altered lipoprotein metabolism

4.6 Erythrocyte membrane and related changes in NIDDM and IHD

4.7 Uptake of cholesterol by WBC from plasma
4. DISCUSSION

4.1 GENERAL OBSERVATIONS MADE IN IHD AND NIDDM

4.1.1 Blood samplings made on the several patients and healthy volunteers fall into 5 distinct group with approximately 100 (80 - 118) numbers in each group. They are age and sex matched (65 - 75% males) as can be seen from Table 3.1. The difference among the groups DM$_1$, DM$_2$ and DM$_3$ mainly arise from the degree of glycaemic control and the duration of diabetes mellitus, while groups IHD and DM$_3$ differ on a single variable i.e. the absence or presence of glucose intolerance. Both the groups of patients had symptoms of coronary artery disease. No gross obesity is manifested in the subjects studied with the mean values for BMI ranging between 23 and 26 kg/m$^2$. Most of the patients with IHD and DM (68.8%) are classified as Non-vegetarians (NV) in their diet habits while only 55% of the healthy volunteers fall in this group. Many of the subjects in the IHD and DM groups had either stopped or had substituted part of the egg/meat/fish intake with vegetables, after the diagnosis of their respective clinical conditions. Similarly smokers, predominate in the IHD and DM groups, and many had since stopped smoking.

An interesting observation is that, among the healthy volunteers 30% had one parent with a history of diabetes mellitus, which is the frequency observed in our lab by earlier workers also. 75% of those with IHD (without DM) had atleast one parent as diabetic (Table 3.1).
4.2 CHOLESTEROL DISTRIBUTION IN PLASMA LIPOPROTEIN

4.2.1 Hypercholesterolemia, lowered HDL bound cholesterol and elevated LDL-cholesterol had been reported in IHD with and without diabetes mellitus by several workers (Gustafson et al., 1972, Hoff et al., 1975, Brunzell et al., 1984, Steinberg, 1987).

In this study, we have classified the cases with respect to their glucose homeostasis, and characteristic differences are seen in cholesterol distribution. Hypercholesterolemia is of a greater intensity in the diabetic subjects (compare $6.4 \pm 1.3$ and $6.7 \pm 0.5$ mmol/L in the IHD and DM$_3$ groups). The standard deviation in the IHD group is larger and denotes a much larger spread of normo and hypercholesterolemic cases in this group. Statistically significant differences are seen between the two groups. This difference (between IHD and DM$_3$) is further heightened by the very significantly lower HDL content making the coronary risk in the DM$_3$ group highest (at $6.6 \pm 0.5$). The average risk for incidence of myocardial infarction is 4.5 according to Framingham study (Castelli and Anderson, 1986), which was confirmed in many other epidemiological surveys. Shanmugasundaram et al. (1995) have reported a coronary risk index of $4.09 \pm 0.79$ for healthy adults (age $49.8 \pm 10.5$ years) in this region.

4.2.2 Carlson (1981) studied the lipoprotein patterns in patients with atherosclerotic disease and observed that the mean HDL bound cholesterol was significantly lowered in the patients. Protection conferred by HDL against IHD has been confirmed by observations in the Framingham study.
(Castelli et al, 1975). Low HDL cholesterol has been found to be associated with increased risk of CAD in both types of diabetes, and are used as predictor of CAD in adult diabetes (Gordon et al, 1977).

IHD is a very important problem in India, inspite of low levels of total cholesterol, dietary animal protein and abundance of dietary fibre in the people of the Indian subcontinent. The Standardised Mortality Rate (SMR) among Indians are reported to be higher than Europeans and their West Indian counterparts in studies made in Britain (Marmot et al, 1984), South Africa (Walker, 1963) and Trinidad (Miller et al, 1984). Miller et al. (1983) studied the population of Asian origin settled in London and observed high incidence of diabetes mellitus and hypertension when compared to those of African and Caucasian races.

4.2.3 The clinical, epidemiological and experimental evidence linking hyperlipidemia to atherosclerotic disease is well established (Keys, 1970 and several others). The risk with elevated serum cholesterol was associated with high levels of low density lipoprotein fraction (LDL) (Kannel et al, 1979). Brunzell et al (1984) observed high levels of serum apo-B the main apolipoprotein of LDL in patients with IHD.

HDL cholesterol in the patient groups, studied confirms the negative correlation that exists between HDL and the incidence of vascular disease first reported by Miller and Miller (1975). A number of clinical and epidemiological studies demonstrated a negative association of HDL with the incidence of atherosclerotic disease (Eisenberg, 1984).
4.2.4 LDL cholesterol is elevated significantly in IHD, while both LDL and VLDL cholesterol are significantly raised in DM₃ group (Table 3.2). In diabetes of shorter duration and/or better glycaemic control, (DM₁ and DM₂ groups), VLDL is elevated significantly when compared to healthy controls, suggesting that increased VLDL cholesterol is an important metabolic abnormality and may possibly be an initiator of secondary complications.

4.2.4.1 VLDL cholesterol is another atherogenic lipoprotein and has been identified as a risk factor for coronary heart disease (Castelli et al., 1975). Taskinen et al (1986) observed that during catabolism of VLDL and chylomicrons, HDL₃ is converted to HDL₂. In our study the HDL₂ is reduced, and VLDL cholesterol is raised suggesting that in these subjects VLDL catabolism may be slowed down. Hypertriglyceridemia in diabetes is associated with both increased VLDL synthesis and chylomicron catabolism. Kissebah et al (1982) observed increased VLDL synthesis in untreated patients with NIDDM, and the overproduction of VLDL has been shown to slow down with the improvement in glycaemic control.

4.2.4.2 The controls we have studied (Table 3.2) have VLDLC level 56 ± 17 mg/dl, while the triglycerides was 105 ± 7 mg/dl (Table 3.8) VLDL bound cholesterol had been found to be higher in our population, when compared to the North American and European studies (Shanmugasundaram et al., 1982). In LRCP clinics and in several labs in North America and Europe, VLDL-C is computed as Triglyceride/5 in mg according to Friedwald formula (Friedwald et al 1972).
4.2.5 In DM complicated with IHD, LDL cholesterol is also elevated. Laakso et al (1986) had compared DM with and without IHD, and had reported that LDL levels are higher in those with macrovascular complications. We did not find any correlation between HbA1C on one hand and the cholesterol content in LDL, VLDL and HDL (Table 3.2). However statistically significant correlation exists between LDL and total cholesterol in plasma, while LDL is inversely correlated to HDL-C, and HDL-C to total cholesterol. Dunn et al (1988) reported a direct correlation of LDL with both serum glucose and glycosylated haemoglobin, and Taskinen et al (1988) observed reduction in LDL with NIDDM when treated with insulin. In our studies we had taken up all cases (i.e. healthy, IHD and DM groups) for co-relation analysis, and this included cases of elevated LDL with normal HbA1C (as in the IHD group). Hence, we do not find a correlation between LDL-C and HbA1C. The absence of significant correlation between HbA1C and total cholesterol, HDL and VLDL in our studies (Table 3.2) may also be due to the inclusion of IHD cases in the study.

4.2.5.1 Howard (1987) observed that glycosylation of apo-B occurs in diabetes because of their increased ambient glucose levels and this could result in altered LDL metabolism in vivo. Glycosylation of LDL appears to have altered its interaction with endothelial cells. Glycosylation of LDL in NIDDM may accelerate its deposition in vessel walls in a multifactorial manner. In vitro there is evidence of oxidative modifications of LDL with generation of lipid peroxides which leads to impaired catabolism of modified LDL along the
receptor mediated pathway while uptake and degradation of these lipoproteins by macrophages are enhanced (Morcel et al., 1984).

4.2.6 The distribution of free and esterified cholesterol in the plasma lipoproteins differ in IHD and in DM3 groups as can be seen from Table 3.4 to 3.7. In IHD alone, CE is increased by 20% in plasma, while the increase in FC is less than 10%. The CE/FC ratio is also elevated, while in DM, both FC and CE are elevated uniformly leaving the mean CE/FC ratio around 2.9, close to the normal level of 3.0. FC is present predominantly in the LDL accounting for about 50% of plasma FC (Table 3.5). In IHD, there is a major shift of FC from VLDL to LDL, making the LDL very rich in FC.

4.2.6.1 Fischer et al (1972), Shattil et al (1976), Pagnan et al (1977) and Fielding (1983) and others have reported higher levels of FC in VLDL and LDL in dysbetalipoproteinemia and other disorders of abnormal cholesterol transport. In DM, the distribution of FC remains fairly unaffected, the HDL:LDL:VLDL ratio remain at 1:2:1. The differences between IHD and DM3 is significant in the FC content in the VLDL fraction. Ester/Free cholesterol ratio in VLDL (Table 3.7) at 10.8 ± 0.4 is greater in IHD, while it is only 5.0 ± 0.4 in diabetes mellitus associated with IHD (DM3 group). While CE in HDL is progressively lowered in DM1, DM2 and DM3 groups (Table 3.6). CE in LDL and VLDL shows an upward trend, and this may be due to the increased action of LCAT seen in Table 3.13. CE is lowered in HDL in both IHD and the diabetic groups, possibly due to its transfer from HDL, in increased amounts.
4.2.7 Esterification of cholesterol in the plasma is mainly due to the action of LCAT and this enzyme activity is significantly elevated in diabetes mellitus (Table 3.13). The activity increases with increase in duration, HbA1C and complication as can be seen from the slow rise observed between DM1 and DM3, through the DM2 groups. In IHD group (without diabetes), LCAT is marginally lowered. Plasma LCAT activity, measured in IHD groups is lower, whereas in NIDDM and in NIDDM without and with IHD, the activity of the enzyme is significantly elevated. When patients with NIDDM alone (DM1, DM2 and DM3 groups) are taken into consideration (Table 3.22) LCAT is correlated to plasma CE and FC, PL, TG and TC/HDL ratio, while the index of glycaemic control (HbA1C) is inversely correlated to plasma CE (r = -0.321). But HbA1C is not correlated to LCAT, suggesting that increased LCAT seen in DM may not be the result of hyperglycaemia.

4.2.8 The unesterified cholesterol entering the LCAT reaction and HDL is mainly derived from surface constituents of catabolized VLDL or from cellular membranes (Glomset, 1980). The uptake of unesterified cholesterol from cellular membranes into HDL might be facilitated by cholesterol esterification and thereby the LCAT activity might be of importance for the removal of cholesterol from tissues (Ray et al, 1980). A high LCAT activity leads to an enhanced formation of cholesterol esters in VLDL and LDL. The increased Total cholesterol/HDL cholesterol ratio observed in IHD and reduced activity of LCAT acts as markers of disturbances of cholesterol ester metabolism, and may contribute to the development of coronary atheromatosi. Albers et al (1981) observed that the great majority of cholesterol ester molecules
generated in HDL by the LCAT reactions in human are not retained in HDL, but are transferred to VLDL and LDL.

4.2.8.1 The association between LCAT activity and atherosclerotic manifestations especially NIDDM is controversial. There are studies showing reduced LCAT (Wallentin 1982), similar activity (Wiklund et al, 1979) or increased (Wells, 1982) activity of LCAT in NIDDM when compared to controls. Haffner et al (1985) observed positive correlation between HDL₃ cholesterol and LCAT concentration. These findings supports the concept that LCAT is involved in the conversion of HDL₃ cholesterol to HDL₂ cholesterol. Miller et al (1988) suggested that LCAT is one of the metabolic determinants of the rate at which cholesterol concentration rises during ageing. The increase in lipids and LCAT activities observed in DM₁, DM₂ and DM₃ groups may be the result of the following possibilities: (a) that plasma cholesterol increases with age independently of LCAT, (b) that LCAT activity is secondarily increased in response to a greater demand for cholesterol esterification in plasma, and (c) increased LCAT activity in NIDDM may reflect a physiological adaptation to an altered need for the enzyme, with greater FC efflux from the cells.

Wallentin (1982) observed that the increased LCAT activity in diabetics could be related to the increased plasma phospholipid concentrations, as increased plasma phospholipids levels enhances LCAT activity by providing lecithin as substrate for the reaction. Mlekusch et al (1991) observed significant increase of the LCAT activity during the high fat feeding period, which correlated with the increase in plasma cholesterol. Mattock et al (1979)
reported increased LCAT in diabetes as a metabolic situation similar in many aspects to high fat feeding. It has been speculated that lysolecithin, the co-product of the LCAT activity may be a causal agent for arterial damage (Vidaver et al., 1985).

4.2.9 The distribution of FC and CE in the HDL fraction is a complex and dynamic phenomenon, due to the interconversion of HDL₂ and HDL₃, and the changes associated with these conversions (Bagdade, et al., 1993). Alaupovic (1980) and several others have reviewed this phenomenon, and have identified apo AI as the antiatherogenic component of HDL. apo AI is the ligand binding to the cells and mediates a dose-related efflux of cholesterol from cells (Falcone and Fielding, 1994).

4.2.9.1 HDL₃ is smaller (55 to 95 Å) in diameter and more dense, while HDL₂ is larger (95 to 120 Å) and less dense. Eisenberg (1984) studied the composition of HDL₂ and HDL₃. For every FC HDL₃ carries 4 molecules of CE and 8 to 9 molecules of phospholipid and their molar composition is 11,44 and 93. HDL₂ which is richer in lipids carries nearly 5 times FC (i.e. 52 moles/mole lipoprotein) as in HDL₃ particle, 2.5 times CE (109 moles/mole) and twice the quantity of phospholipids (180 moles/mole lipoprotein). HDL₃ is the dominant site of LCAT action and the esterification of FC is associated with conversion of HDL₃ to HDL₂. In healthy volunteers (Table 3.3) cholesterol bound to HDL₂ and HDL₃ are in the ratio 1.2 ± 0.5, while in IHD and the DM₁, DM₂ and DM₃ groups, HDL₂ is significantly lowered making the ratio HDL₂/HDL₃ cholesterol below 1. At the same time, CE content in the total HDL is also significantly
reduced (Table 3.6) in IHD and DM. This may be due to the significant reduction of (CE rich) HDL₃ particles in plasma, when compared to HDL₂. However, with increased LCAT activity, one would expect increased CE in the HDL in DM, if the reactions stated above are self limiting. Esterified cholesterol formed on the HDL is transferred to LDL and VLDL in the next step of RCT described by Glomset et al. (1973) and confirmed by later workers. In DM and in IHD, cholesterol transfer from HDL to apoB containing lipoproteins are also elevated (Table 3.14) and this may explain the reduction in HDL₂ bound cholesterol observed in these conditions.

4.2.9.2 Lipoproteins occur as micelles in the aqueous plasma environment, and have characteristic structural arrangement. These spherical particles have on the outer surface phospholipids and free cholesterol arranged in a manner similar to the lipid bilayer of cell membrane interspersed with the apoproteins. The core of the lipoprotein is hydrophobic and carry CE and TG. Lipoproteins are undergoing dynamic metabolic changes in circulation (Reviewed by several workers including Siegrest et al., 1974, Eisenberg, 1984, Assman et al., 1986).

4.2.9.3 For the conversion of HDL₃ to HDL₂, the number of CE molecules should be increased by two to three fold and sufficient amounts of surface lipids (PL and FC) (as given by Eisenberg 1984) are needed. Nikkila and Kekki (1980) have shown that HDL cholesterol concentrations are generally proportional to lipoprotein lipase. This is linear in our studies (Table 3.13). The variation in plasma HDL-C is almost fully due to variation in HDL₂ (Table 3.3) in IHD and in DM groups, and such a correlation between HDL₂ and LPL have
been reported by Shepherd et al (1980). Patsch et al (1984) observed that during lipolysis of VLDL, FC, PL and apoC released are taken up by HDL₃ and the latter is converted to HDL₂.

4.2.9.4 Eisenberg (1984) has provided the composition of HDL₂ and HDL₃ subclasses as given in the table which gives the number of moles/mole lipoprotein.

<table>
<thead>
<tr>
<th></th>
<th>HDL₂</th>
<th>HDL₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mol. wt</td>
<td>400,000</td>
<td>200,000</td>
</tr>
<tr>
<td>Apo AI</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Apo AII</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Apo C</td>
<td>1</td>
<td>±</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>189</td>
<td>93</td>
</tr>
<tr>
<td>FC</td>
<td>52</td>
<td>11</td>
</tr>
<tr>
<td>CE</td>
<td>109</td>
<td>44</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>18</td>
<td>9</td>
</tr>
</tbody>
</table>

According to this HDL₂ carries three times as much cholesterol (FC and CE put together) 161 and 55 moles/mole of lipoprotein HDL levels in plasma is generally expressed in terms of mg cholesterol bound to HDL in 100 ml plasma, and in healthy adults HDL₂/HDL₃ cholesterol is approximately 1. Hence it may be inferred that for every one cholesterol rich HDL₂ particle, there will be approximately three HDL₃ complexes in plasma. Computing accordingly Eisenberg's (1984) data could be modified to state that for an HDL₂:HDL₃ (1:3 mixture), FC:CE:PL molar ratio will be 85:241:468 or
1:2.83:5.50. In our studies (Table 3.12) the molar ratio is 0.28:1.1:1.2 or 1:3.92:4.28 for HDL suggesting a close correlation to Eisenberg’s data in our population also.

4.2.9.5 The proportion of TG in HDL is higher in our studies (Table 3.12) than those reported by Eisenberg (1984). However, when total cholesterol/PL ratio is taken in HDL, it can be seen that in IHD and in DM₁ the relative proportion of cholesterol/phospholipid is marginally lowered. Both CE and FC content in LDL is increased in both IHD and in DM (Table 3.5 to 3.7). In IHD (uncomplicated with DM), FC is increased in LDL by 60%, while the increase is 20%, 35% and 60% in DM₁, DM₂ and DM₃ respectively. From Table 3.22 it is seen that CE content in plasma is inversely related to HbA₁C (r = -0.321). A linear increase in FC content in LDL is seen with increased duration and complexity of DM (Table 3.12). The CE increase in IHD is about 20% and 10 and 20% in DM₂ and DM₃ groups respectively. It is significant to note that the FC and CE content in LDL is identical in IHD and DM₃ groups suggesting that cholesterol ester content in IHD is unaffected by abnormal glucose tolerance. However the picture in VLDL is different. In IHD uncomplicated with DM, the CE is the predominant component (Table 3.7) making the mean CE/FC ratio 10.8. VLDL in diabetes mellitus is also associated with increased proportion of CE, although the ratio is only 4.9 ± 0.4 in DM₃ group. It may be suggested that the CE from HDL may be transferred predominantly to VLDL with the increased cholesterol ester transfer observed (Table 3.14).
4.3 PHOSPHOLIPIDS AND GLYCERIDES IN LIPOPROTEINS

4.3.1 Plasma phospholipids and triglycerides are significantly elevated by (40 and 60% respectively) in IHD as can be seen from Table 3.8. The plasma lipid increase in DM appears to be related to the duration and complexity of the disease as can be seen from the progressive rise from DM\(_1\) to DM\(_3\) with DM\(_2\) groups showing intermediate levels. It is interesting to note that both triglyceride and phospholipid levels in the DM\(_3\) groups is significantly greater than those found in IHD alone suggesting greater defect in the homeostasis of glycerides than in cholesterol homeostasis in DM\(_1\) (compare cholesterol levels in IHD and DM\(_3\) in Table 3.2). Diabetes itself leads to elevated levels of phospholipids, triglycerides and free fatty acid in circulation as can be seen in the DM\(_1\) group (Table 3.8) with no complication. Albrink (1974) observed that hyperlipaemia and hypertriglyceridemia are the most characteristic abnormality in NIDDM.

4.3.2 Pfeiffer et al (1983) reported a decreased clearance of plasma triglyceride in NIDDM. Serum triglyceride is an independent predictor of IHD under the age of 60 (Benfante et al, 1989). Nikkila (1984) reported that plasma triglyceride concentration is elevated in NIDDM. Mann et al (1978) reported that poor diabetic control is associated with hypercholesterolemia and hypertriglyceridemia. Chang and Schneider (1972) and Mellado and Lozoya (1984) observed that insulin deficiency leads to increased lipid metabolism from the adipose tissue leading to hypertriglyceridemia. As free fatty acid are the precursors for the synthesis of triglycerides for incorporation into VLDL in
the liver, a rise in the triglyceride synthesis and release in the form of VLDL appears in diabetes (Nikkila and Kekki, 1973). Increased total phospholipid levels was reported in atherosclerotic arteries both in man and experimental animals (Portman et al., 1970). Phospholipid synthesis was stimulated during the genesis of atherosclerosis (St. Clair, 1983). Tilvis and Miettinen (1985) observed significant correlation between the levels of linoleic acid in serum phospholipids and tissue phospholipids despite the fact that the fatty acid pattern showed rather large differences from tissue to tissue. The elevated levels of both PL and TG in plasma in uncomplicated diabetes as seen in DM₁ group, may be the fore runner for atherosclerotic changes which develop 15-20 years after the onset of hyperglycaemia.

4.3.3 Nestle et al., 1980 observed that elevated free fatty acids may predispose an individual to the development of atherosclerosis and may precipitate cardiac arrhythmias. Burstein et al (1978) proposed that increase in plasma free fatty acid levels increases the intensity of platelet aggregation which plays a major role in the evolution of atheroma and influences the basic disease process. In the population studied, ischaemic disease may be associated not only with abnormalities in cholesterol distribution in lipoproteins but also in the coagulation factors, aggravating the condition by hypercoagulability.

4.4 **CHOLESTEROL ESTER TRANSFER AND LIPOPROTEIN METABOLISM**

4.4.1 Accelerated cholesterol ester transfer from HDL to the apoB containing lipoproteins (LDL + VLDL) in NIDDM is observed (Table 3.14 and
Fig. 3.7), and the increase is correlated to the duration of DM and associated complications as can be seen by the progressive rise from DM₁ to DM₂ and DM₃ groups. CE transfer is accelerated in the plasma obtained from the IHD group without DM (Table 3.14). The CET is rapid in the first two hours and the slope becomes progressively low from two to four hours. The reaction does not reach a plateau at four hours.

4.4.2 Earlier studies by Bagdade and coworkers (1993) have shown that the CE transfer curve in plasma reaches a plateau after 3 hours in NIDDM, while in the controls CE transfer is negligible in the first 2 hours (probably due to a lag phase), and then picks up rapidly so that at 4 hours, the CE transfer in normal and NIDDM are almost equal. However, in our studies (Fig. 3.7) we find that there is no lag period for the control plasma in the CE transfer reaction. While the reaction is significant in the first one hour in all cases, the reaction rate is faster in both IHD and in NIDDM when compared to controls. The CET rate of DM₁ (40 ± 5) observed by us is similar to the values reported by Bagdade et al. (1991).

4.4.3 Barter et al. (1983) had suggested the possibility of two pathways for the delivery of CE to VLDL and LDL; (a) an indirect pathway, a two step process in which cholesterol in HDL is esterified by LCAT, followed by its transport to LDL and VLDL and described by Pattnaick et al. (1978), Barter and Lally (1979), Barter et al. (1983) and (b) a direct pathway in which esterified cholesterol is delivered to the VLDL and LDL. While the direct pathway showed preference for LDL as the acceptor, the indirect pathway is
the greater contributor to CE transfer. It is possible that the CET activity (without a lag phase) seen in the healthy volunteers in our study may be related to the higher levels of the acceptor lipoproteins in plasma namely VLDL (discussed in Section 4.2.4.2) and this could be responsible for higher incidence and mortality from IHD in our population. The healthy population in Lipid Research Clinic Programme in North America (LRCP, 1979) VLDL-C is 24.2 ± 8.1 mg/dl. In the European cities (London, Naples, Uppsala, and Geneva), the VLDL-C reported by Lewis et al (1978) is in the range of 15-20 mg/dl, in Newzeland the levels are found to be about 12 mg/dl (Blackburn, 1980) and in Canada 24 mg/dl (Tann et al, 1980). In Japan the VLDL-C was reported to be 27 mg/dl (Hosaki et al, 1985). Earlier reports from our laboratory (Shanmugasundaram et al., 1995) in the age group of 30-65 years have reported 42.1 ± 16.5 in those with coronary risk index below 4.5 and 58.2 ± 24.1 for those with coronary risk above average (TC/HDL over 4.5). In our studies the value is 56.7 ± 17 (Table 3.2) for the combined population. They are older 52 ± 8 years and their CRI was 4.2 ± 0.3. If the Friedwald formula (VLDL-C = Total TG mg/5) is applied to our healthy population, mean VLDL-C will be computed as 21 mg/dl which is less than half the actual values.

4.4.4 In the South Asian population, we have reported higher levels of VLDL cholesterol (greater than the Triglyceride/5 ratio for VLDL-C) (Shanmugasundaram et al, 1982, Shanmugasundaram et al, 1986 and Shanmugasundaram, 1995) in healthy volunteers in South India numbering more than a thousand. Such a high level of VLDL-C may be attributed to the
dietary pattern with carbohydrates providing over 65% calories. Endogenous synthesis of cholesterol and secretion of VLDL is elevated, when bulk of the energy needs are provided by carbohydrates (Sirtori et al., 1977). The digestive products namely glucose enter the portal circulation and reach the liver before being redistributed to the systemic circulation, and the biosynthetic activity of the liver is enhanced leading to synthesis of lipids and secretion of VLDL. While dietary fats provide energy, the hydrolytic products enter the lymphatic system and reach the systemic circulation at the thoracic duct, bypassing liver. The energy needs of urban South Indians are met mainly from carbohydrates (67-71%) while 11-12% are provided by protein and 17-22% by fats (Shanmugasundaram et al., 1986). This diet pattern is associated with elevated levels of VLDL-C, which may be a contributing factor for increased risk of IHD by accelerating the CE transfer from HDL to VLDL, and rendering the latter rich in CE to be avidly taken up by the peripheral tissues including the arterial smooth muscle cells.

4.4.5 CET is a key step in the reverse cholesterol transport, involving a specialised protein cholesteryl ester transfer protein in plasma, which facilitates the transfer of nonpolar (neutral) lipids between the major lipoprotein classes (Tall, 1986). By this process there is a heteroexchange of CE from HDL and TG from VLDL, and leads to enrichment of the apo-B containing lipoproteins with CE, in exchange for TG transfer in the reverse direction. Exchange of CE and TG between HDL and VLDL was first reported by Nichols and Smith (1965). The shuttling of core/neutral lipids of lipoprotein is a central feature of RCT, which serves to redistribute the sterol from
peripheral tissues to the less dense lipoprotein in plasma from which it can be taken up by the liver for bile synthesis (Tall, 1986).

4.4.5.1 Atherogenesis is possible, either by the inhibition of cholesterol efflux from the arterial tissues or by increased CET, occurring after FC of HDL is esterified by LCAT. In the latter case, the VLDL and other apo-B containing lipoproteins accumulate in plasma which will be avidly taken up by arterial wall macrophages (Bagdade et al., 1993).

4.4.6 Bagdade et al. (1991) suggested that the increased CET in IDDM may be due to disturbances in the lipoprotein composition, which may enhance their interaction with CETP. HDL-cholesterol is significantly reduced in IHD, and also in DM (Table 3.2), with the diabetics showing significantly greater reduction in HDL-C in the DM₃ group (inspite of higher total cholesterol levels in plasma). It may be suggested that in IHD associated with NIDDM (as in DM₃ group), the relative lowering of HDL-C may arise from increased LCAT and CET activities, which quickly transfer the FC released from the cells into plasma HDL to the atherogenic lipoprotein LDL and VLDL. The higher incidence of MI in diabetes and higher mortality rate among diabetics after a myocardial infarction observed in epidemiological studies (Entmacher et al., 1985) has to be viewed in conjunction with the lowered HDL-C, and elevated VLDL-C levels in plasma.

CET in plasma are assayed by different methods. When assayed in the intact plasma, mass transfer of CE is measured with LCAT inhibited with DTNB, and the net loss from HDL is measured serially during incubation at
37°C. According to Bagdade et al (1993), CET in this system not only depends on the concentration of CETP, but is also influenced by the concentration and composition of VLDL as shown earlier by Morton (1988), Dullart et al (1989) and Mann et al (1991). In our studies, the very large increase in CE transferred observed in DM₃ may be due to the significantly higher triglyceride content in the VLDL fraction. A second method commonly employed is the isotopic method, in which isolated systems are employed and not the whole plasma. Radiolabelled CE bound to HDL is added to the patients serum fraction containing cholesterol ester transfer protein (CETP) and the isotope binding is studied (reviewed by Quig and Zilversmit, 1988). This system is unitary, and does not allow the modulating effect (if any) of the altered VLDL composition commonly encountered in diseases associated with elevated plasma triglycerides. Bagdade et al (1993) studied CET by a modified isotopic assay in which the patients whole plasma was used with exogenously labelled HDL-CE, and this led to their observation that the CET rate is accelerated with diabetic VLDL.

4.4.7 Earlier workers (Van Tol et al, 1991 and others) have noted a lag period of upto 2 hours in CET activity in the controls. A negative transfer of CE from LDL to HDL in the first instance, and this leads to poor net transfer of CE from HDL to apoB containing lipoproteins in the early stages. However, in the population studied in our lab (Table 3.17 and Fig. 3.7), and it may be noted that we did not find any lag phase in the CET in controls. Further the CET observed at the end of 4 hours was significantly higher in the DM₃ group, when compared to the controls and possibly the population we study may be
distinct from the Caucasian population studied by earlier authors. The people of South Asian origin (India, Pakistan, Bangladesh, and Srilanka) have very higher incidence of diabetes mellitus, hypertension (Miller et al., 1983). Standardised mortality rate for coronary heart disease, and the first episode of myocardial infarction is reported a decade earlier than those reported in the Caucasians and those of African origin, shown by reports and studies made in South Africa (Cosnett 1957), Britain (Tunstall et al., 1975), West Indian (Miller et al., 1982) and in USA (Thomas et al., 1986). Further, the diets consumed by Indians are rich in fibre, and low in fat, and 30% of dietary fat are derived from oleic acid (Shanmugasundaram, 1986). The genetic predisposition in our population may be manifested as the rapid CET activity in plasma.

4.4.8 Lipoproteins are continuously remodelled during their transit through the plasma compartment, due to the action of enzymes and transfer protein which may affect the composition and distribution of lipids in the different lipoprotein complexes (Tall, 1986). Lipid transfer protein in plasma are analogous to the cellular lipid transfer proteins which mediate lipid transfer between organelles (Reviewed by Wirtz, 1991) or from the sites of their synthesis into the plasma as nascent lipoproteins (Welterau et al., 1984). Inherited defects of one or more of these transfer proteins have been identified in familial hypolipoproteinemia (Brown et al., 1990) and in abetalipoproteinemia (Welterau et al, 1984). CE can be transferred bidirectionally not only between HDL and VLDL, but also between VLDL and LDL and between LDL and HDL and these have been reported by Morcel et al 1980, Morton and Zilversmit 1981, Fielding et al 1983, Barter 1984, Noshida et al 1989 and others. Hence the CE lost from the HDL during
incubation of plasma with the LCAT inhibitor DTNB, may not be necessarily appear in the apo-B containing lipoprotein fractions. In our studies on the amount of FC and CE in the lipoproteins during the incubation of plasma (Table 3.14a) we observed no net change in total cholesterol, either in the HDL or in LDL + VLDL fractions. The CE that is lost by HDL was 8.5 mg/L/hour in controls and 21 mg/L/hour in NIDDM. These changes correspond to 21.9 and 54.3 μmoles/L/hour respectively in controls and in NIDDM. At the same time CE levels in the LDL + VLDL rose by 50 mg/L/hour in controls and 80 mg/L/hour in NIDDM. This corresponds to 129 and 207 μmoles/ L/hour. This was associated with a corresponding loss of FC in this fraction.

It would appear that CE transfer may not be a single step process as hitherto envisaged, but may be influenced or modulated by one or more cholesterol esterifying enzyme activity other than LCAT in plasma. Although LCAT is inhibited the net CE content in plasma did not remain constant during the incubation. This could happen under the two circumstances namely (a) DTNB may not be inhibiting the LCAT activity completely and (b) plasma may contain an alternate cholesterol esterifying activity possibly associated with the LDL and or VLDL. It may be postulated that LDL and or VLDL may be acted upon by this esterifying enzyme which accounts for the large increase in CE in this fraction at the expense of FC, while there could be transfer of FC from the larger and higher lipoproteins to the HDL to maintain the cholesterol (total cholesterol content in the fractions remained unaffected), while CE in the core of the HDL is lost by CE transport to LDL/VLDL, and this is replaced by FC from the LDL/VLDL which sits on the surface of the lipoprotein. Assuming that no phospholipid transfer takes place from HDL, one can envisage the
shrinking of HDL particle (by loss of CE), and possible shape modification, with the increased surface area available with the same phospholipid and elevated FC. At the same time the core lipids in LDL/VLDL is also increased making them larger. In NIDDM, the increase in CE is 60% higher (than in the controls) and this excessive increase in core lipids may lead to greater atherogenesis in this condition. This is further accentuated by the very reduced TG transfer from VLDL + LDL to the HDL fraction found in IHD and in the DM₁, DM₂ and DM₃ group (Table 3.14).

Diabetes *perse* even in the absence of active symptomatic IHD, the TG transfer is very limited, so that CE transfer from HDL leaves LDL + VLDL excessively rich in their core lipids and more atherogenic. Investigation on simultaneous phospholipid changes, the size, shape and heterogeneity of the lipoproteins are needed to fully comprehend the molecular mechanism of CE transfer and the resultant changes in the atherogenic potential of the apo-B containing lipoproteins. The various steps and facets of the development of atherosclerotic disease in NIDDM, may differ from that in a normoglycaemic individual.

4.5 **ROLE OF LIPID PEROXIDATION AND FREE RADICAL DAMAGE IN ALTERED LIPOPROTEIN METABOLISM**

4.5.1 Lipid peroxidation products in plasma (Table 3.15) is elevated more than two fold in DM₃ and IHD groups. In the erythrocyte membrane LPO is increased more than three fold in DM₃ and five fold in IHD. LPO products in
plasma is associated with the lipoproteins and are likely to affect the exchange of lipid among the lipoproteins and also the affinity of the donor and acceptor lipoproteins in the lipid transfer reactions.

4.5.1.1 Plasma LPO assayed is a measure of the net increase in oxidants and free radicals generated in the metabolism and antioxidant reserves available in the cells and tissues. While SOD, CAT and GPx together inactivates superoxide and hydrogen peroxide respectively, the antioxidant scavengers quenches the free radicals generated in the metabolism. SOD is significantly reduced in IHD and in DM (Table 3.16) with the greatest reductions seen in the DM₃ group. Lowering of catalase activity is parallel to SOD changes in the disease conditions, although with lesser intensity. However GPx is significantly and progressively elevated in DM, while 35 to 40% reduction is seen in IHD (Table 3.16) LPO in both plasma and erythrocyte membrane are directly correlated to their substrate namely phospholipids (Table 3.16) but is inversely correlated to SOD and CAT activity as can be seen from the Pearson's correlation coefficient.

4.5.2 Free radical scavengers or quenchers are also significantly lowered in DM and to a lesser extent in IHD. GSH which is the necessary cofactor for inactivating H₂O₂ by the enzyme GPx is also significantly lowered in the blood, and hence the effective H₂O₂ destruction is likely to be insufficient in DM₁ inspite of elevated GPx activity (it is to be remembered that enzyme activities are assayed at optimal conditions with added cofactor and does not represent the real life dynamics of metabolism in the cells). However, it should be noted
that the antioxidants and free radical scavengers (Table 3.16 and 3.17) are severely reduced in DM₃ when compared to the IHD group while LPO products in both plasma and erythrocyte membrane are significantly greater in the IHD group when compared to DM₃. Such an anomalous situation may rise due to one or more of the following reasons.

a) the oxidants and free radicals generated in IHD may be greater than those found in DM₃ group.

b) in DM₃, the free radicals and oxidants generated may be acting predominately on the proteins in the plasma and the cell membrane affecting the thiol groups, converting the amino group of lysine to a carbonyl group, cleavage of peptide bonds and glycosylation of proteins, and the intramolecular rearrangements leading to AGES.

4.5.2.1 Such changes in the different lipoprotein fractions needs a detailed investigation and may throw light on the differential expression of free radicals. Accumulation of free radicals and antioxidant depletion could occur under conditions of transient ischaemia. Ferrari et al. (1985) observed that in myocardial ischaemia and subsequent (recovery period) the sudden burst of O₂ radicals overcomes the protective enzymes present in the tissue. This leads to low defense potential and possible destruction of cell components due to accumulation of free radicals and exhaustion of antioxidants (Ferrari et al., 1985).
4.5.2.2 Packer (1992) states that feeding rats with antioxidants like vitamin E reduces the occurrence of atherosclerotic lesions. Takenaka et al. (1991) observed that the generation of radicals in the aqueous phase leads to the concurrent loss of membrane protein thiols and membrane tocopherols. On the other hand, generation of radicals within the lipid region, oxidation of thiols and formation of TBA reactive substances were suppressed during an induction period until tocopherol fell below a critical level.

4.5.3 Reduced levels and altered metabolic turnover of ascorbic acid had been reported in several tissues in experimentally induced diabetes (Yu et al., 1989) and in human diabetes (Som et al., 1981). We observed a small reduction in the vitamin C levels (ascorbic acid) in the IHD groups, and these levels are drastically reduced in NIDDM with progressively lower values in DM₁, DM₂ and DM₃ groups (Table 3.17).

4.5.3.1 Mirreisova (1976) observed that in patients with ischaemia, the blood concentration of vitamin C was below normal. Knox (1973) and Ginter (1979) have inversely correlated vitamin C with IHD mortality. Deficiency of ascorbic acid may be important in the pathogenesis of some diabetic complications like microangiopathy (Kapeghian and Verlangieri, 1984). Sinclair et al. (1991) showed that low levels of ascorbic acid concentration in diabetes were not secondary to inadequate intake of the vitamin but due to diabetes itself. Studies using human lymphocytes suggested that competitive inhibition between glucose and ascorbic acid (which have structural similarities) for
transport across the cell membrane may be an important factor in the abnormalities associated with diabetes mellitus (Davis et al., 1983).

4.5.4 The increased level of lipid peroxide has been considered as a cause of the degeneration of organs or tissues (Hiramitsu et al., 1976). The increased level of MDA is an evidence of intensification of lipid peroxidation process in patients with atherosclerotic lesions and in turn contribute to a state of chronic stress on endothelial cells (Warson and Land, 1984). It is also considered that lipid peroxide formed at the primary site would be transferred via blood to other organs or tissue where the damage would be provoked by the propagation of lipid peroxidation (Yagi, 1987).

4.5.4.1 Significant changes in lipid metabolism and structural changes observed in diabetes with vascular complications are oxidative in nature and oxidation of lipids in plasma lipoproteins and in cellular membranes is associated with the development of vascular disease (Baynes, 1991). Nishigaki et al. (1981) observed that in diabetes with symptomatic angiopathy increase in MDA in serum is mainly due to the elevation of lipid peroxide level in HDL fraction.

4.5.4.2 Excessive lipid peroxidation accelerates the atherosclerotic process through (a) initiating endothelial lesion in the artery (b) increased uptake of LDL by the macrophages and (c) enhancing platelet aggregation and thrombus formation. Attention on lipid peroxides as one of the factors inspiring the function of the arterial endothelial cells and causing primary lesions in the vascular wall was reported by Chang and Tai, (1983). Oberley (1988) reported
that patients with type II diabetes and angiopathy had 90% more MDA than controls, while patients without angiopathy had no such increase and this led to the suggestion that the increased TBA-reactive material originated from the intima of the blood vessels and might be related to the development of atherosclerosis.

4.5.4.3 Peroxidation takes place on polyunsaturated fatty acids to give rise to free radicals and endogenous peroxides which are highly reactive and have chemotactic and cytotoxic properties (Loeper, 1991). He observed that peroxidation involved in cardiac diseases, especially myocardial infarction and unstable angina are accompanied by an increase in the production of MDA and a transient inhibition of protective enzymes especially SOD. Ledwozyow et al. (1986) demonstrated that there was statistically significant correlation between the levels of lipid peroxides in plasma and arterial wall of patients with atherosclerosis and that the level of MDA was correlated with the severity of lesions.

4.5.5 Free radical intermediates have been postulated to occur during auto oxidation of carbohydrates and glycosylated protein under physiological conditions. Free radical induced oxidative damage had been proposed to amount for fluorescent changes in proteins with short half life perind in patients with diabetic microangiopathy. Evidence of lipid peroxidation have also been observed in a number of complications accompanying diabetes such as retinopathy, cataract formation and atherosclerosis (Zigler and Hess, 1985).
4.5.5.1 Increase membrane MDA levels and membrane oxidation in human erythrocyte may result in hypercoagulability (McDonald and Hathaway, 1983). Ito et al. (1989) observed that LPO is a trigger in the pathogenesis of myocardial changes in spontaneously hypertensive rats and its membrane associated enzyme changes are secondary responses to progressive cardiac damage. Peroxidative lipid damage in the membrane play a role in the increased coagulability, altered phospholipid organisation and reduced cell survival of erythrocytes and cellular damage known to occur in other tissues of diabetic patients (Jain et al., 1989). Mezzatti (1995) observed that the increased peroxidation of lipids may be due to release of catecholamines during emotional stress. It is quite possible that the repeated cycles of oxidant stress with concomitant release of radicals, may have contributed to hypercholesterolemia, elevated FFA, and significant reduction in HDL bound cholesterol in addition to increase in LDL.

4.5.6 Esterbauer (1992) has described the role of free radical induced modification of plasma lipoprotein in their incorporation into atherosclerotic plaque. Fogelman et al. (1980) reported that cholesteryl ester accumulates in human monocyte derived macrophages is from oxidised LDL. Oxidatively modified LDL was found to be avidly taken up by the scavenger receptors (Parthasarathy et al., 1989). Oxidative modification by free radicals are mediated through their action on PUFA present in the phospholipids on the surface of the LDL (Morel et al., 1984; Cathcart et al., 1985) and several Others. Steinbrecher et al. (1984) reported that lipid peroxidation products derivatives the apolipoprotein B, and this makes them bound to the scavenger
receptors on the macrophages. Uptake of LDL by the cells are mediated through the cell surface apoB receptors which are tightly regulated (Goldstein and Brown, 1978). But the oxidised LDL enters the cells by the different scavenger pathway, which has no such control (Goldstein et al., 1980).

4.5.6.1 Esterbauer (1992) has reviewed the oxidation of LDL and the role of LPO and antioxidants. While native LDL is a complex of FC, CE, TG, PL, apo-B and vitamin E, ubiquinol and other antioxidants accounting to 14.2nmole/LDL protein (Salter et al., 1991; Esterbauer et al., 1991), the oxidised LDL contain little vitamin E, and lesser amounts of the lipids/mg protein (several reports, brought together was reviewed by Esterbauer, 1992).

4.5.6.2 Lipophilic antioxidants like vitamin E protect the PUFAS in LDL against free radical attack. In the patients with IHD and DM (Table 3.17) vitamin E and other antioxidants are significantly lowered, and the bulk of LDL is likely to be present in the oxidised form which is distinctly atherogenic. In addition to the LPO increase seen in these subjects (Table 3.15), there will be several protein modifications affecting their affinity to the cell surface receptors. The oxidative modification of lipoprotein may be accentuated by significant reduction in the SOD activity leading to elevated levels of superoxide anion which can modify LDL in more than one way.

4.5.7 While earlier studies on oxidised LDL involved measurement of TBARS, lipid hydroperoxides, conjugated dienes, fluorescence emission at 430nm (Cominacini et al., 1991), the kinetics of the process under experimental oxidation set up has been measured in terms of the
disappearance of antioxidants or PUFAS, the fragmentation of apo-B and the increase in electrophoretic mobility (Esterbauer et al., 1992). These studies have revealed that the oxidation is a complex process, with a lag phase, propagative phase, and decomposition phase. Oxidation of PUFAs with isolated double bonds gives PUFA hydroperoxides with conjugated double bonds (dienes) with a UV absorption maxima at 234 nm. During the lag phase LDL becomes progressively depleted of antioxidants α-tocopherol getting exhausted first followed by β-carotenes. This is the lag phase in which Vitamin E scavenges lipid peroxy radicals. Lipid peroxidation sets in subsequentially. Brown and Goldstein (1990) described the vitamin E depleted LDL as being "left to the mercy of oxygen", and is subjected to autocatalytic chain reaction of lipid peroxidation and this LDL is the propagative phase. When 70 to 80% of the PUFAs are oxidised the decomposition reactions set in. Decomposition of LOOH to aldehydes is generally observed in biological systems (Reviewed by Esterbauer et al., 1989) and these aldehydes are toxic second messengers. These aldehydes reacts with aminoacid residues in apoB, such as the ε amino group of lysine which is lost, and similar loss of the basically charged amino group makes apoB more negatively charged. These changes make the LDL recognisable by the macrophage scavenger receptors. At the sametime carbonyl groups are also produced by free radicals (Lo and Loo) mediated degradation of amino acids, and apoB breaks down to two large peptides of 260 and 232 kD (Salter et al., 1991).

4.5.7.1 Oxidative modification of LDL includes cholesterol oxidation from 7 keto cholesterol (Bhadra et al., 1991, Jialal et al., 1991 and Zawadski et al.,
1991). LDL oxidation has been studied extensively by scientists working in the field of Atherosclerosis and Free Radicals and Antioxidants. In North America and Europe, LDL is the main atherogenic lipoprotein and is the chief carrier of cholesterol ester. Detailed studies on the oxidised form of VLDL is essential in population like ours, in which VLDL content is high, and can be an equal contributor to atherosclerosis. In view of the fact that antioxidant and free radical scavenger deficiency is very severe in NIDDM (reports from our lab includes Table 3.17) in this study and (Sundaram et al., 1996), and in IGT (Vijayalingam et al., 1996) it will be interesting to isolate the VLDL and measure the oxidative changes in the formation of LO*, LOO*, LOOH, aldehydes, carbonyl groups in protein, protein fragmentation etc. The study of the apoproteins will be interesting and more complex since unlike LDL, VLDL carries several proteins other than apoB.

4.6 ERYTHROCYTE MEMBRANE AND RELATED CHANGES IN NIDDM AND IHD

4.6.1 Erythrocyte membrane composition is altered in NIDDM and in IHD in different directions (Table 3.18 and 3.19). Mean cholesterol/PL molar ratio is 0.66 ± 0.03 in healthy controls, while it is elevated to 0.73 ± 0.03 in IHD and reduced progressively (0.46 ± 0.04, 0.41 ± 0.04, 0.31 ± 0.04) in NIDDM with longer duration, greater complexity and poorer glycaemic control (Table 3.19). While conventionally membrane composition is expressed in terms of mg protein, we set about arriving at the component mass in terms of the number of cells, because in our labs, earlier workers have observed that when 1 ml of
packed cells containing identical number of RBC's are lysed and the membrane collected by sedimentation the mass of the pellet was higher in IHD, and lower in DM (when compared to controls), suggesting that the membrane mass/cell is varied in the diseases. The protein content also (Table 3.18) show similar variation with cells from IHD patients showing 30% higher levels and in severe DM (as in the DM₃ group) mass of protein/10¹² cells is lower by nearly 40%.

4.6.1.1 It is relevant to bring here the following observation already discussed earlier in (section 4.5.2). Mean LPO products on the erythrocyte membrane (Table 3.15) is 1320 nmoles/10¹² cells in IHD group, but only 945 nmoles/10¹² cells in the DM₃ group, in spite of the fact that the antioxidant enzymes (Table 3.17) and free radical scavengers (Table 3.16) are more severely reduced in DM₃ group when compared to IHD group. Hence, we would except greater LPO product on the erythrocyte membrane in the DM₃ group than in the IHD group. LPO is a process acting on the polyunsaturated fatty acids esterified in the phospholipids of the cell membrane. In the DM₃ group, PL content/10¹² cells is 261 µmoles, while in IHD group, this accounts for 460 µmoles and hence LPO production is greater on the latter.

4.6.2 The excess of free radicals and oxidants formed in the RBCs in diabetes mellitus must be acting on the proteins on the cell membrane and destroying their structure and function affecting both integral and peripheral proteins. Oxidative modification of protein include carbonyl formation. Carbonyl groups are formed when the ε amino group of lysine in the proteins are oxidised (with deamination), and this has been suggested as a biomarker
for protein oxidation (Stadtman et al., 1991). Protein fragmentation by oxidative process also been described (Fong et al., 1973). Earlier workers in our laboratory have demonstrated that healthy RBC's when incubated in phosphate buffered saline containing increased quantities of H₂O₂ undergo oxidative changes in the cell membrane composition characterised by lowering of both lipid and protein content (Vijayalingam, 1997 Ph.D. thesis). By incubation for 10 to 30 minutes in 4mM H₂O₂, normal healthy RBCs underwent changes such that the cell membrane isolated had a composition similar to those found in poorly controlled diabetes mellitus confirming that the cell membrane changes we report in DM are likely to arise from the oxidative changes on the membrane.

4.6.3 Poorly controlled diabetes mellitus as in DM₂ and DM₃ groups are characterised by condition favoring a) glycosylation of protein and b) oxidative damages arising from free radical scavengers found in hyperglycaemic states (Wolff, 1987) and antioxidant deficiencies encountered Table 3.15 and 3.16. When protein bound hexose is expressed as µg/mg protein (Table 3.18), in diabetes, the values are higher than controls confirming greater proportion of glycoconjugate in the membrane protein. This is also associated with a reduction in hexosamine and increase in sialic acid bound to membrane protein (Table 3.18). While protein bound hexose reduces the hydrophobicity of the integral proteins and affect their binding to the phospholipid bilayer of the cell membrane, loss of hexosamine is associated with a reduction in the cationic charges of the protein(s). Sialic acid provides the negative charges to the glycoconjugates. In diabetes, the changes observed in hexosamine and sialic
acid content of the membrane proteins render them less cationic and more anionic, with increased net negative charge. The integral proteins are held to the centre of the phospholipid bilayer, through the hydrophobic bonds while the polar ends of the phospholipid molecules have negative charges. The cations on the glycoprotein may be stabilising the linkage through electrostatic attractions between the phosphate anion and the hexosamine groups. With loss of hexosamine (as in DM) the linkage may be slackened, so that the cell membrane components may be less strongly held making the membrane susceptible to rupture. At the same time the membranes will have greater permeability to polar substances. The reduced deformability of RBCs in DM reported by (McDonald and Hathaway, 1983) may be due to this aberration in the cell membrane composition and structure.

4.6.4 The cell membrane in DM appears to shed lipid and proteins. Cholesterol is stacked between the phospholipid bilayer and lower number of cholesterol molecules on the RBC membrane (as seen in DM₁, DM₂ and DM₀ group in Table 3.18) is likely to arise as a sequence to reduced phospholipid. Bryszewska and Leyko (1983) reported changes in physical properties of erythrocyte membrane occurring in diabetes, such as decreased membrane fluidity, decreased cell deformability, diminished oxygen releasing capacity. These physiological changes may occur as a result of broad compositional change in the erythrocyte membrane and plasma of patients suffering from diabetes mellitus. Miyahara et al (1984) showed that there is a greater turnover of erythrocytes in diabetes leading to early loss of membrane constituents with cholesterol loss and osmotic fragility. Composition and
structural changes in erythrocyte membranes and compositional changes in plasma lipids may contribute to the development of secondary complications in diabetes (Bryszewka et al 1986). They also observed that the increased microviscosity of diabetic erythrocyte membranes provide unambiguous proof of the structural deterioration of erythrocyte membrane. Lipid composition in the erythrocyte membrane undergoes significant changes in diabetes mellitus, and such changes affect the deformability of RBCs which is essential for effective capillary action (Jain and Shohet, 1982). One of the earlier events in development of atherosclerosis is the endothelial lesion occurring in the arteries. The deficiency of protein and lipids found in the diabetic cell membrane is likely to make the endothelial cells weak and easily amenable to lesions such as peroxidative damage.

4.6.5. In nutritional hypercholesterolemia (without hyperglycaemia) red cell membrane has been found to be loaded with cholesterol (Pozhilenkova and Shrohov 1972, Butkas et al, 1974, Ferritti et al, 1988 and others). Increased cholesterol and phospholipid content and increased incorporation of acetate into lipids has been reported in the IHD (Burton et al, 1980). In our studies (Table 3.18) we find elevated levels of lipids and also proteins in RBC membrane in IHD.

4.6.5.1 Thorton et al (1990) experimented to verify whether the excess proteins found in IHD is of protective nature for preconditioning the heart and making it resistant to infarction from a subsequent ischemic insult. They confirmed by demonstrating that fresh rabbit developed larger infarct zone
than preconditioned rabbit and observed that the increase in the glycoprotein content of the red blood cell membrane may partially be a compensatory mechanism for increased cholesterol content in plasma. Goldstein and Brown (1982) showed that sialic acid acts as a binding ligand for LDL. The increased proportion of sialic acid observed with increasing CRI may be an adaptive mechanisms for internalising cholesterol in circulation into the cells.

4.7 UPTAKE OF CHOLESTEROL BY WHITE BLOOD CELLS FROM PLASMA

4.7.1 It is interesting to note that while the cell membranes in NIDDM is poor in lipids (while in IHD, they are significantly elevated), the lipid content in the whole cell are uniformly elevated (Table 3.20) both in IHD and diabetes mellitus. Other workers in our lab have observed elevated levels of cholesterol in RBC’s in DM (unpublished). DM3 group shows maximum lipid content in WBC’s when expressed in terms of protein content. Earlier report from our laboratory (Shanmugasundaram et al, 1995) have found higher WBC counts (8520 ± 182 cells/mm³) in IHD when compared to healthy age and sex matched controls (5320 ± 607). The WBC isolates were predominantly polymorphonuclear lymphocytes. In the healthy controls WBC isolate contained around 55% lymphocytes while in IHD sample they amounted to about 85%. WBC isolate from DM1 group shows 75 to 80% lymphocytes.

4.7.2 When WBC’s from normal DM1 and IHD are incubated in plasma from normal (healthy volunteers) (Table 3.21), the uptake of cholesterol (µg cholesterol/mg protein in the cell) is very similar (mean values being 2.5, 2.8,
and 2.5). Similar changes are seen, when cholesterol uptake is expressed in terms of mg cholesterol in the incubating medium. But when normal cells are incubated in either diabetic or IHD plasma, cholesterol uptake/mg protein is more than doubled (6.0 ± 0.3 and 5.5 ± 0.3 respectively). When diabetic cells are incubated in diabetic plasma and cells from patients with IHD, in plasma from similar source, the uptake of cholesterol is significantly greater (19 ± 1.1 and 24 ± 1.0 μg cholesterol/mg protein).

4.7.3 Factors that are known to affect the uptake of cholesterol from plasma by cells are (a) the apo-B containing lipoproteins in the plasma, (b) oxidised LDL and possibly Lp_{α}, (c) other LPO products in plasma which may alter the permeability of the cell membrane, (d) number of cell surface receptors for apo-B on the cells, (e) number of scavenger receptors binding oxidised LDL and (f) passage of cholesterol through exchange at the membrane. The number of apo-B receptors on the normal, diabetic and IHD blood samples appear to be nearly identical since incubation in control (normal) plasma leads to similar uptake of cholesterol (μg/mg protein). Since plasma LPO is elevated in diabetes and IHD (Table 3.15), they should be carrying significantly high levels of oxidised LDL (possibly VLDL also), and when normal cells are incubated in these plasma samples (combination 2 and 3) the cholesterol uptake is more than two fold. This increased uptake of cholesterol may be directly attributed to the oxidised LDL, in plasma, and the acetyl (or scavenger receptors) present in normal cells. It may be inferred that scavenger or acetyl receptors may be present on the normal lymphocyte surface also.
4.7.4 When blood cells from diabetics and IHD are incubated in the plasma rich in oxidised LDL (combination 5 and 7) the uptake of cholesterol is increased more than eight fold. It may be inferred that in the two disease conditions, the number of scavenger receptors on the leucocyte surface are significantly higher than those found in the normal cells. This postulate is also supported by our findings (Table 3.21) when cholesterol uptake is expressed in terms of plasma LPO also. When either plasma or cells are from normals (combination 1 to 4 and 6), mean cholesterol uptake is about 0.5 µg/mg protein/nmole LPO in plasma/hour. In combinations 5 and 7, the uptake of cholesterol is increased both due to higher levels oxidised lipoprotein in plasma and also by the possible presence of a larger number of scavenger receptors on the cell surface.

4.7.5 This is also borne out by the calculations of cholesterol uptake when expressed in terms of the cholesterol in the incubation medium (plasma). In combinations 1, 4 and 6 (normal plasma) uniform uptake of about 0.5 µg protein/mg cholesterol in the medium/hour is seen. Here the plasma LPO is 190 ± 30 nmoles/dl (Table 3.15) with a total cholesterol of 221 ± 19 mg/dl (Table 3.2). But when incubated in diabetic plasma, normal cells appear to be avidly taking up cholesterol (compare 2.31 ± 0.06 to 0.46 ± 0.03 uptake/mg cholesterol in the medium). Normal cells take up cholesterol at the rate of 0.88 µg cholesterol/mg cholesterol/hour when incubated in IHD plasma, in spite of the fact that LPO is greater in IHD than in DM. This difference in the rate of uptake (also seen in combinations 5 and 7) may not account for the oxidised LDL alone. The source of cholesterol other than oxidised LDL in the incubation
medium for example may be VLDL bound cholesterol. It has to be noted that VLDL bound cholesterol is significantly higher in DM than in IHD (Table 3.2). VLDL carries apo-B together with other apoproteins and their uptake by peripheral cells is not fully understood. More detailed study on the effect of oxidation of VLDL and the resultant alteration in their uptake through the scavenger receptor needs in depth investigation.

4.7.6 Gonen et al (1983) chemically modified LDL and studied their effect on the LDL entry into cultured fibroblasts. Carbamylation of up to 2% lysine residues on apo-B, resulted in 70 to 80% reduction in the ability to internalise LDL after binding on apo-B receptor. When up to 25% lysine residues are carbamylated, the resultant LDL binds rapidly to the scavenger receptor and loses the ability to bind to the apo B-E receptor. It may be extrapolated to our findings that in diabetes mellitus, the oxidative modification of one or more of the apoproteins in VLDL, namely apo AI, AII and apo C may be influencing its binding to the receptors, internalisation and the deposition of cholesterol in the cells.

4.7.7 Peripheral cells can take up cholesterol from plasma by mechanisms which include a) internalisation of LDL through binding at the apo B receptor described by Goldstein et al. (1995) b) selective uptake of FC from lipoproteins, particularly LDL and c) selective uptake of CE from both LDL and HDL (Reaven et al, 1995, Acton et al, 1996). Fielding and Fielding (1995a) observed that FC was rapidly internalised by human fibroblasts without simultaneous internalisation of the intact LDL. Similar observations have been reported by
the same authors in smooth muscle cells and endothelial cells also. The increased uptake of cholesterol by cells exposed to diabetic plasma (combination 2 and 5 Table 3.21) may arise from the significantly greater levels of FC in LDL in DM₁, DM₂ and DM₃ groups reported in Table 3.5.

4.7.7.1 Fielding and Fielding (1995b) have further observed that uptake of cholesterol by receptor mediated endocytosis of LDL is 8 times stronger than the selective FC transfer from bound LDL. Similar binding of LDL and uptake of cholesterol appears to be taking place in the lymphocytes from our studies. Further studies are needed to investigate the structure of the coated pits, its uncoupling and intracellular recycling (observed in fibroblasts, smooth muscle cells and endothelial cells by several authors) in the lymphocytes, so that the same can be used as a model for lipid transfer and accumulation studies in human. HDL bound CE can be selectively taken up by adrenal cells (Gwynne and Hess, 1980) in gonadal cells (Reaven et al., 1995) and in hepatocytes (Pittman et al., 1987). Since LCAT activity is significantly elevated in NIDDM (Table 3.13) cholesterol in HDL gets esterified on standing, and may provide CE on the HDL for uptake into the cells.

4.7.8 While the influx of FC and CE occur through the initial binding of LDL to the "clathrin" coated pits (apo B receptor) of the cell membrane the dynamics of the cell provides for FC efflux through the "caveola" on the cell surface (Fielding and Fielding 1995a) the protein caveolin provides the FC, a raft to travel from its site of synthesis in the endoplasmic reticulum to move to the cell membrane (Smart et al., 1996).
4.7.8.1 A protease sensitive mechanism of FC efflux from cells have been observed (Reviewed Rothblat et al, 1992, Fielding and Fielding, 1995b and Oram and Yokoyama, 1996) FC rich rafts of "caveolin" reach the cell surface and are implanted on the exofacial leaflet of the plasma membrane to form the "caveolae", on which apo AI of HDL binds facilitating FC efflux. In NIDDM, VLDL is significantly elevated and the apoAI present may be competing with HDL to bind to the caveolae and inhibit cholesterol efflux from the cells.

4.7.9 Fielding and Fielding (1997) had observed that fibroblasts, vascular smooth muscle and endothelial cells are poor in coated pits, but rich in caveolae and in the number of high affinity scavenger receptors (also cited in Section 1.7.4). It is possible that with increased oxidant stress and oxidative modification of LDL and VLDL, the uptake of cholesterol through the scavenger receptors becomes greater, accelerating atherogenesis in NIDDM. More in depth studies on the regulation/modulation of caveolin synthesis in the cells and the FC efflux from cells in NIDDM may throw light on the dynamics of and efflux of cholesterol in the cells and factor affecting cholesterol accumulation and the process of atherogenesis.

4.7.9.1 The studies made on lymphocyte uptake of cholesterol from plasma (Table 3.21) is a preliminary study, but has revealed that in diabetes mellitus uncomplicated with ischaemic disease as in the DM1 group, metabolic and regulatory changes have already taken place making the cells more susceptible to cholesterol accumulation possibly through increased number of scavenger
receptors and/or oxidised LDL and/or competition of apolipoproteins of VLDL and HDL on the caveolae affecting cholesterol efflux.

4.7.10 Oxidation of VLDL, including modifications leading to lipid peroxidation of phospholipids, oxidation of cholesterol, carbamylation of the lysine residues of the different apoproteins, oxidation of SH groups and protein fragmentation is being studied utilising the models of chemical oxidation using \( \text{H}_2\text{O}_2 \) with \( \text{Fe}^{2+} \), \( \text{Cu}^{2+} \), \( \text{HOCl} \) by other workers in the laboratory. Comparison of the oxidative modification of VLDL in the experimented system and in diabetic plasma in structure, and uptake studies are also envisaged.