4. DISCUSSION
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4. DISCUSSION

4.1. PLASMA LIPOPROTEIN METHODOLOGY

4.1.1. About a decade ago, quantitative lipoprotein electrophoresis for phenotyping hyperlipidemic plasma samples was investigated with the classification of hyperlipoproteinemia by Fredrickson and Levy's group. The current resurgence of interest in lipoprotein analysis, is largely because of the inverse relationship between the HDL-cholesterol concentration and IHD risk, observed by Miller and Miller (1975) and confirmed by Miller et al. (1978). The diagnosis of remnant hyperlipoproteinemia (type III Broad-β disease) is also based on the quantitative analysis of the VLDL composition (Hazzard et al., 1972) and familial hyperalphalipoproteinemia has been recognized as a not so infrequent cause of mild hypercholesterolemia (Glueck, 1976) which needs no therapy.

4.1.2. Several methods are available of which the most widely used are precipitation procedures using heparin and manganese chloride, dextran-sulphate and calcium or magnesium chloride and sodium phospho tungstate and magnesium chloride.

4.1.3. Use of a high molecular weight (Srinivasan et al., 1978) or low molecular weight (Kostner, 1976) dextran sulphate for the differential precipitation of plasma lipoproteins has given satisfactory results. Another method employed is the phosphotungstate (Lopes Virella et al., 1977) precipitation of HDL. However, an increasing
proportion of laboratories including the Lipid Research Clinics Program of North America have adopted the heparin-manganese chloride method for this purpose.

4.1.4 Warnick and Albers (1978, 1979) have shown that a two-fold increase in the final manganese ion concentration to 0.92 M, improved precipitation of apolipoprotein B associated lipoproteins (LDL and VLDL), without excessive precipitation of HDL from the plasma. This increase in the magnesium ion level also provided improved sedimentation of apolipoprotein B containing lipoproteins from hypertriglyceridemic plasma. This procedure is suitable for EDTA-plasma in contrast with another procedure, which has been validated only for serum.

4.1.5 Current practice in many laboratories indicated that HDL-separation by precipitation method must be performed within one week, (Curb et al., 1980). Demacker et al. (1977) reported that specimens can be stored at 4°C for seven days without any effect on the reproducibility of HDL-cholesterol values obtained by heparin-manganese precipitation method.

In the present study, samples were analysed within three days of collection by the heparin and manganese precipitation procedure, and meanwhile the samples were stored at 4°C. Care was taken to see that EDTA was not more than 1 mg/ml of the blood collected.
4.1.6 The physical chemical basis for the aggregation of VLDL by the detergent sodium dodecyl sulphate (SDS) has been investigated by Bernstein and Scholnick (1972), who observed that VLDL aggregated at 0.6 to 0.75% SDS, while it did not aggregate at a concentration of 1.5 to 2%. The low concentration of SDS used increases the electrophoretic mobility of LDL and HDL and thus SDS appears to bind with these lipoproteins (Bernstein and Scholnick, 1970; 1972).

4.1.7 At higher concentrations (about 10 fold greater than that used for VLDL aggregation), purified LDL is dissociated into soluble lipid and apoprotein moieties.

Wilson and Spiger (1973) have shown that SDS precipitation can be used instead of preparative ultra centrifugation for the determination of LDL and HDL. Binding of SDS at low concentration did not interfere with measurement of LDL-cholesterol and HDL-cholesterol by this method.

4.1.8 Wilson and Lees (1972) and Wilson and Spiger (1973) compared the cholesterol content of LDL and HDL fractions after aggregation of VLDL by SDS as well as the VLDL-cholesterol content calculated by differences, with the preparative ultra centrifuge method. They observed excellent correlation between the two methods and recommended this procedure for screening the dyslipoproteinemias.

4.1.9 Ononogbu and Lewis (1976) have described a precipitation method, wherein VLDL is aggregated by SDS.
and dissolved in a solution of SDS (1%) in saline.
Cholesterol and triglyceride content is quantitated directly
in this VLDL solution. This method is comparable with that
of the ultracentrifugation method. This study of Ononogbu
and Lewis (1976, 1979) was used in population studies in
London and Nisakku (Nigeria).

4.1.10. Analytical ultracentrifugation has been the method
for research purpose, but is too expensive and time consuming
and cannot be used for routine clinical diagnosis.

Havel et al., (1955) developed a method for
fractionating lipoproteins by a multiple centrifugation
method in an angle-head rotor. It was Hatch and Lees (1968)
who pointed out the advantage of using a swinging bucket
rotor in which, convection disturbance and adherance of
large lipoprotein molecules to the walls of centrifuge tubes
can be eliminated.

4.1.11 The first step in the simplification of the ultra-
centrifugal method was the development of a one step
sedimentation on a discontinuous density gradient (sodium
chloride/potassium bromide) reported by Redgrave et al.,
(1975) and sodium chloride/sucrose gradient by Foreman
et al., (1977), which had reduced the spin time to 24 hours.

4.1.12 Further improvements in the procedure, were
made by cutting down the spinning time. The method employed
was that of Chung et al (1980, 1981) using a simple
continuous density gradient, in a Sorvall Vertical ultra-
centrifugal rotor in a single spin. A sodium chloride and potassium bromide density gradient was used.

4.1.13 Chung et al. (1980) have demonstrated that the properties of lipoproteins prepared by this technique are indistinguishable from the conventional methods (two stages of sedimentation) on the basis of apoprotein composition, electrophoretic mobility, and mean particle size, and equilibrium band density, from comparable lipoprotein fractions prepared by sequential flotation.

4.1.14 Further, Chung et al. (1980) have also observed that this method is highly reproducible and no measurable cross-contamination could be detected, except for albumin, which could be removed by sequential washing. Also, addition of potassium bromide to the plasma did not affect the analytical method, nor the stability of the lipoprotein.

Our observations included not only cholesterol analysis, but also the other lipid classes (Table 9 and figures 10 to 18) in the separation obtained by the dual precipitation method and ultracentrifugal method, on the normal and hyperlipidemic plasma samples.

It can be seen that the dual precipitation method is comparable to the sedimentation method, in the fractionation of lipoproteins. Further it may be inferred that the precipitating agents do not produce any dissociation of the lipid components which go into the making of lipoproteins.
The values for the precipitation method are slightly higher than the centrifugal method, because the former accounts for the total plasma lipid components while the sedimentation method for the analysis of the individual fractions of the separated components. It may be noted that the statistical analysis of the comparative studies (Table 9) give very good correlation between the two methods.

4.1.15 The choice of the electroimmunoassay method for measurement of apolipoprotein B is supported by comparative studies made by earlier workers. Curry et al., (1978) examined three immunoassay techniques for measuring apolipoprotein B in the serum and lipoprotein fractions from normolipidemic and hyperlipidemic samples, by comparing values obtained by electroimmunoassay (EIA), radioimmunoassay (RIA) and radial immunodiffusion (RID), with those determined gravimetrically. He found that EIA is faster and simpler than RIA and equally precise, although Durrington et al., (1976) have found RIA practically suitable for the measurement of the low levels of apolipoprotein B.

Havelkes (1981) has pointed out that the LDL-preparation was a clear solution, when freshly prepared and became opalescent and/or contained a visible precipitate, when stored for a few weeks at 4°C.

4.1.16 In our experiments, standard LDL-apolipoprotein B was prepared and its concentration was measured by the
Lowry method for the estimation of protein. Further calibration of standard LDL apolipoprotein B was done with standard obtained from Behringwerke A.G., West Germany.

From section 2.5.5 it may also be noted that the LDL apolipoprotein B isolated, was found to be homogenous as tested by agarose electrophoresis and by immunodiffusion (plates I and II).

4.2. CHOLESTEROL LEVELS AND ITS DISTRIBUTION

4.2.1 The earliest attempt to investigate the biochemical nature of atherosclerotic disease incriminated cholesterol (Vogel, 1847; Windaz, 1910). The finding by Insull et al., (1966) supports this since cholesteryl ester is the principal lipid ingredient of the atherosclerotic lesions. The epidemiologic studies on the evolution of CVD were stimulated by the observation that the patients with CHD had higher serum cholesterol levels than those of control subjects [Davis et al., 1937; Steiner and Domanski, 1943].

4.2.2 Furthermore, the risk is strikingly related to the serum cholesterol level. But, at any level of cholesterol, the risk varies widely depending on a number of factors such as blood pressure and smoking. A heart attack could be predicted with a known degree of probability in clinically well people on the basis of serum cholesterol, blood pressure and smoking habits, which are classified as primary risk factors.
4.2.3 Methods for the prevention of CHD are based on the assumption, that there is a cause and effect relationship between the risk factors and the disease.

4.2.4 A partial explanation of these heterodox causes has been offered by the recognition of the fact, that the chemical composition of lipoproteins is more than a simple level of total cholesterol. Studies on a series of patients with CHD revealed that a combined elevation of VLDL and LDL cholesterol was the most frequent abnormality. This was also observed in the study by Lewis et al., (1974), Carlson et al., (1975b) and Avogaro et al., (1977).

4.2.5 In most of the studies, it was shown angiographically that serum cholesterol was higher among patients with abnormal coronary artery, while the reverse was true in the study carried by Tan (1980), Eisenberg (1976) and also from studies of Brunner et al., (1979).

4.2.6 Thus, lipid hypothesis of atherogenesis is weakened by cases which present one or more pathological pattern of the disease, while the lipid levels are still within normal limits.

4.2.7 Earlier, Gofman et al., (1954) have suggested that lipids or lipoproteins may be key factors in the atherogenic process. The hypothesis was revived in the seventies and the importance of distinguishing among
cholesterol bearing lipoproteins was realized because only LDL may be atherogenic while HDL (relatively rich in protein), carrying a small part of cholesterol, may actually counter some of the ill-effects of cholesterol in the lipoproteins of low density.

4.2.8 All these observations indicate that:
(a) There is no clinical level of serum cholesterol which is associated with angiographically determined CAD.
(b) Serum cholesterol by itself is not a good predictor of CAD.

Gordon et al (1977) have pointed out that the lipid profile (not cholesterol alone) consisting of the different lipoproteins may be used for a better prediction of CAD.

4.2.9 This study has been designed to understand the circumstances in humans, that favour elevation or depression of one or other of the lipoprotein classes and lipids, in the hope of devising a cut-off point to differentiate among CHD and healthy population in terms of our dietary and environmental factors.

From the study in healthy people (Table 19) it is to be inferred that there is a rise in total cholesterol with age \( (r = 0.175, \ p < 0.05) \), and this is mostly due to elevation in LDL-C and HDL-C \( (r = 0.909, \ r = 0.459, \ p < 0.01) \).
respectively versus total cholesterol) leading to an unfavourable increase in T-C/HDL-C ratio with rising age, well into the 5th decade (Table 18).

Table 43 shows the plasma cholesterol in different countries. It can be seen that the cholesterol values for the Indian subjects (healthy population) in the present study, are from urban, middle and high income groups. The cholesterol value for males of age 40 - 49 years is 241.5 mg/dl which is very high in comparison with data from other countries listed in table 43. It is almost ranking midway between English and Swedish populations. It could in a way be attributable to the dietary system of this upper class society and to reduced physical activity. As evident from table 8, only 1.02% of the population of healthy group was physically active, the remaining, approximately 99% never took part in habitual physical activity apart from their routine work.

4.2.10. This strength of association wanes progressively with advancing age and serum cholesterol can no longer be considered solely of predictive value for CHD risk below the age of 50 years. A similar observation has been reported by Gordon et al., (1977) for the age of 65 years in the Framingham study.

4.2.11 Male population had higher total cholesterol levels than their female counterparts in all age groups
<table>
<thead>
<tr>
<th>Population</th>
<th>Age (years)</th>
<th>Cholesterol (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>HDL</td>
<td>LDL</td>
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<tr>
<td>North America</td>
<td>45-49</td>
<td>212.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>North America</td>
<td>40-49</td>
<td>213.4</td>
<td>45.5</td>
<td>143.4</td>
</tr>
<tr>
<td>Canada</td>
<td>40-44</td>
<td>202.8</td>
<td>45.4</td>
<td>131.6</td>
</tr>
<tr>
<td><strong>Europe</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>London</td>
<td>30-39</td>
<td>231.4</td>
<td>54.8</td>
<td>154.1</td>
</tr>
<tr>
<td>Naples</td>
<td>30-39</td>
<td>191.2</td>
<td>52.2</td>
<td>115.9</td>
</tr>
<tr>
<td>Uppsala</td>
<td>30-39</td>
<td>251.1</td>
<td>52.2</td>
<td>168.0</td>
</tr>
<tr>
<td>Geneva</td>
<td>30-39</td>
<td>225.9</td>
<td>52.9</td>
<td>137.9</td>
</tr>
<tr>
<td>New Zealand</td>
<td>41-50</td>
<td>176.0</td>
<td>49.0</td>
<td>115.0</td>
</tr>
<tr>
<td>US Vegetarian</td>
<td>41-50</td>
<td>126.0</td>
<td>43.0</td>
<td>73.0</td>
</tr>
<tr>
<td>Tarahumara</td>
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<td></td>
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</tr>
<tr>
<td>adults</td>
<td>41-50</td>
<td>118.0</td>
<td>23.0</td>
<td>75.0</td>
</tr>
<tr>
<td>Ethiopia</td>
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<td>32.0</td>
<td>48.0</td>
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<td>Africa (Nigeria)</td>
<td>40-59</td>
<td>202.5</td>
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<tr>
<td>Israel</td>
<td>35-44</td>
<td>221.3</td>
<td>47.1</td>
<td></td>
</tr>
<tr>
<td>Japan</td>
<td>40-49</td>
<td>202.0</td>
<td>55.0</td>
<td></td>
</tr>
<tr>
<td>Argentina</td>
<td>41-59</td>
<td>250.0</td>
<td>52.6</td>
<td></td>
</tr>
<tr>
<td>India (lower</td>
<td>41-50</td>
<td>218.8</td>
<td>61.1</td>
<td>122.6</td>
</tr>
<tr>
<td>socio-economic</td>
<td></td>
<td></td>
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<tr>
<td>group)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>India (present</td>
<td>41-50</td>
<td>242.0</td>
<td>70.0</td>
<td>140.0</td>
</tr>
<tr>
<td>study)</td>
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</table>
studied except in the fifth decade, where a cross-over is seen and females have higher total cholesterol (Table 10 and 11).

4.2.12 Dietary protein has been considered to have little effect on the serum cholesterol level [Anderson et al., 1971; Connor, 1979] but epidemiological data has shown a positive correlation between animal protein in diet and mortality from cardiovascular disease [Yudkin et al., 1957, Connor, 1979].

Huff and Carroll (1980 a and b) found that cholesterol turnover was more rapid in plasma of animals fed with soy protein compared to those fed casein. The rate of excretion of fecal steroids and bile acids were also higher in animals fed soy protein [Carrol, 1981]. It seems possible that a greater loss of sterol from the body pool as a result of decreased reabsorption from the intestine and increased fecal excretion may be a reason for the lower level of plasma cholesterol in rabbits fed soy protein compared to those fed casein.

Experiments with $^{125}$I labelled lipoproteins have indicated that the turnover of the protein components of plasma intermediate density lipoprotein (IDL) was faster in rabbits fed soy protein compared to those fed casein [Carrol et al., 1979]. These observations raise the possibility that the effect of dietary protein on the plasma lipid levels may be secondary to their effect on
the production and/or metabolism of the apoprotein of plasma lipoprotein.

Indians (Vellore, South India) and Ugandians consuming vegetarian diets [Hill and Aris, 1971] have low fecal bile acid output. Since the vegetarian diet (mainly rice - which has low fiber content) and the diet of the primitive population are particularly low in saturated fats, simple sugars and to some extent also in calories, the difference in cholesterol metabolism may not be solely caused by the dietary fiber.

4.3. HIGH DENSITY LIPOPROTEINS

4.3.1 Cholesterol in the body is conceived as behaving as two pools, one which equilibrates rapidly with the plasma cholesterol (pool A) and another which equilibrates slowly (pool B). Pool A comprises most of the cholesterol within the erythrocytes, liver, spleen and ileum, while cholesterol on the adipose tissue, muscle, xanthomata and arterial wall belongs predominantly to pool B.

4.3.2 The cholesterol pool size remains unrelated to the mean plasma concentration of total cholesterol, triglyceride, VLDL and LDL. Miller et al., (1975) have observed a negative correlation between pool A and B and plasma HDL cholesterol.
4.3.3 Cholesterol must therefore be transported from those tissues, to the liver during cholesterol turnover, through HDL. Cholesterol exists in the body in esterified and unesterified forms, but only the latter exchanges readily between the plasma lipoproteins and tissues [Glomset, 1968]. The lecithin cholesterol acyl transferase enzyme has been shown in vitro to promote the transfer of cholesterol from the erythrocytes to the plasma by maintaining the free cholesterol equilibrium between the plasma and the cell-membrane [Murphy, 1962]. This process was shown to be accelerated by the addition of HDL to the incubation mixture [Glomset, 1970]. This evidence for a role of HDL in tissue cholesterol clearance is now strengthened by the demonstration of an inverse relationship between the HDL-concentration and the pool size of the body cholesterol by Miller and Miller (1975), suggesting that the concentration of HDL may be rate limiting in the removal of cholesterol.

4.3.4 Brunner et al., (1979) suggested that it is preferable to express HDL-C as a % of serum cholesterol rather than in absolute units. In doing so, the HDL cholesterol becomes independent of the serum cholesterol level and misleading interpretation based on the absolute units can be avoided. For it is seen, that 50% (higher proportion) of serum cholesterol is carried by HDL in infants and this is comparable to that found in animals such as the herbivores (cows, rabbits etc.) HDL is a major lipoprotein class although there is a certain classical exception, like
guinea pig. The carnivorous species like rat, which are resistant to spontaneous atherosclerosis exhibit elevated levels of HDL, which usually dominates their lipoprotein pattern [Chapman, 1980].

Barr (1953) recognised that a form of the longevity syndrome is hyperalphalipoproteinemia [Glueck, 1976]. Elderly black Africans in Western Transval do not suffer from CHD. 45% of their serum cholesterol is present in the HDL.

4.3.5 In the population under study, HDL-cholesterol was lowered in the case of patients (table 28) HDL-cholesterol was higher in women than men of the healthy group (table 28) but not to the same degree as reported in the Framingham study by Castelli et al (1977). No significant increase of HDL cholesterol with age was observed in either sex.

4.3.5.1. The T-C/HDL-C increased in patients and in healthy persons with rising cholesterol levels as seen in tables 21 and 19, \( r = 0.357, p < 0.01 \) and \( r = 0.723, p < 0.01 \) respectively. The higher the serum cholesterol, the smaller is the percentage bound to HDL.

The ratio of T-C/HDL-C represents the most discriminative factor in this survey, since it showed a higher significance \( p < 0.001 \) sexwise and also among healthy and CHD patients (table 28). This lends further support to the conclusion reached in the Framingham study.
that T-C/HDL-C ratio is one of the best predictors for CHD risk [Kannel et al, 1979, Wilson et al, 1979]. The ratio T-C/HDL-C was found to be highest in the fifth decade for the healthy population while it was the fourth decade in the patient population.

4.3.6 On examining table 43 it is seen that HDL cholesterol was higher than in most of the countries listed except that of the African countries. This high level of HDL-cholesterol correlates with the low incidence of CHD. This could be attributed to the dietary pattern in India. As seen from table 8, 70% of the males were non-vegetarian but it should be borne in mind that the Indian diet is richer in fiber and starch content in proportion to fats, than any other country and the bulk of the dietary calories and proteins arise from plant food even in the non-vegetarians. Table 5 illustrates the net food supplies in the different countries.

4.3.7 The energy giving dietary constituent is predominantly carbohydrate in the Indian population. Further, India being one of the developing countries, the physical activity among the Indians in their day to day life is comparatively higher than that of their American or European counterparts. The poor response in the questionnaire for physical exercise, cannot be directly translated into energy expenditure in comparison with Western countries.
Muscular work is done by most Indians in going to the place of work and returning home on bicycles and by the public transport systems such as buses and trains. On an average, the distance covered by foot for going to work is about a mile per day. In the absence of washing machines, dish washers and vacuum cleaners, the physical work done by Indian women (mostly housewives) should be considered while interpreting their response to the questionnaire on physical exercise.

4.3.8 In table 43, the risk factors \((\text{total cholesterol/ HDL cholesterol})\) in the Indians is indicated as 3.43 [Suresh, 1981] and in the present study the ratio is 3.45, which is very close to the above value although the former was a study in the lower socio-economic groups which showed lower total cholesterol levels \((211 \pm 44 \text{ mg/dL})\).

4.4. LOW DENSITY LIPOPROTEINS

4.4.1 Havel and Carlson (1962) suggested that the hyperlipoproteinemia associated with CHD could be due to elevated VLDL triglyceride and/or LDL-cholesterol and/or levels of triglyceride and cholesterol in VLDL and LDL respectively.

Several workers have observed that among the survivors of myocardial infarction, elevated levels of LDL-cholesterol are common and the magnitude of the change in LDL cholesterol is smaller than the changes observed in VLDL-cholesterol and HDL cholesterol [Lewis et al., 1974, Carlson et al., 1975].
A critical ratio of LDL-C/HDL-C is postulated to be essential for the normal clearance of cholesterol from the arterial wall [Glueck et al, 1976]. Persons with hypo-β lipoproteinemia or hyper-α-lipoproteinemia live longer and do not as a rule have cardiovascular problems. They have low LDL-C/HDL-C ratio. Patients at an increased risk of developing atherosclerosis have high LDL-C/HDL-C ratio [Bagdade et al, 1977].

In the population under study, the LDL cholesterol and LDL-C/HDL-C was higher in the case of patients than in the healthy groups (table 28; p < .001). This is similar to the observation made by earlier workers in different populations.

There is no significant difference in their ratio between the two sexes in the healthy population analysed.

In table 43, it was seen that like total cholesterol, LDL-cholesterol was higher in Indians, which ranked midway between the English and Swedish, in the countries listed. In the case of Americans, their LDL cholesterol was higher and VLDL cholesterol was lower in comparison with the present study.

The ratio of LDL-C/HDL-C for Indian and American people was 2.0 and 3.15 respectively, which explains the higher incidence of CHD in Americans in spite of the higher level of total cholesterol in Indians. Thus a lower
ratio in Indian population could be due to dietary, environmental or physical factors peculiar to the Indian population.

4.5 VERY LOW DENSITY LIPOPROTEINS

4.5.1 Epidemiological studies have demonstrated that LDL levels were positively associated with risk, while HDL levels were negatively associated with the risk of developing clinical sequelae of atherosclerosis. VLDL levels are not usually accepted as an independent risk factor for CHD. It is considered as an additional risk to the elevated LDL levels and probably acts independently at extremely high levels as in Diabetes Mellitus [Kannel, et al, 1979].

4.5.2 Age-wise variation of VLDL cholesterol in the healthy patient population is not uniform (Tables 18, 20). The highest level is observed in the fifth decade in the healthy population. Males are found to have higher values of cholesterol (p < .001) than females (Table 28).

It can also be seen that VLDL cholesterol formed about 12% of the total cholesterol in the healthy people while it accounted for more than 20% in the patient population investigated. This has been viewed along with the elevated total and LDL cholesterol levels. Further, it has been noted that the % of cholesterol in the LDL fraction is about 59% both in patients and normals while the distribution of cholesterol in HDL is lowered by about 10% in CHD patients (p < .001).
4.5.3 The ratio of VLDL-C/HDL-C may also depict the picture of metabolic clearance. It was observed in patients that this ratio is nearly double that of the healthy population \((p < .001)\). Hence this may also be considered as a risk factor for CHD. It may be inferred that there is a shift in the cholesterol distribution which may be due to the decreased catabolism of VLDL and the property of this complex to retain cholesterol for longer periods.

4.5.4 It has been noted that VLDL cholesterol levels in the healthy population studied are higher than the values observed in most of the American and European people as presented in table 43. The dietary differences (in addition to probable genetic variation) in the populations studied may be responsible for the high VLDL-C in our plasma samples.

4.5.5 Higher VLDL Cholesterol and lower LDL cholesterol in our population compared to the Americans (table 43) could also be attributed to the high carbohydrate diet consumed by Indians. Schoifeld and Pfleger (1971) have observed that high carbohydrate diets increase the secretion of VLDL by the rat liver, and Ruderman et al, (1971) have shown that these particles are larger than in the normals. The VLDL particles in high carbohydrate fed men are richer in triglyceride also [Schoifeld, 1970], while there are apoprotein variations in VLDL of diabetic subjects fed high carbohydrate diets [Witztum and Schonfeld, 1978].
VLDL triglyceride levels in our normals are higher than those reported by Falko et al. (1979). Our levels are much higher at $68 \pm 5.3 \text{ mg/dL}$ than their $(36 \pm 12 \text{ mg/dL})$ normals but close to their high carbohydrate administered subjects $(77 \pm 35 \text{ mg/dL})$.

The same trend was observed in the low density lipoprotein fractions. But, VLDL cholesterol/VLDL triglyceride ratio is higher in our population compared to those of the carbohydrate treated subjects studied by Falko (1979). Indeed, this may be primarily due to the higher level of cholesterol in VLDL.

4.5.6 The VLDL triglyceride, VLDL cholesterol and VLDL protein rose by a factor 2.4, 1.67 and 1.88 respectively. [Schonfeld et al., 1976]. When a carbohydrate rich diet was given, VLDL became enriched in triglyceride, LDL cholesterol fell by a factor 0.78 while LDL triglyceride and LDL apoB remained constant.

HDL triglyceride rose by 1.42 and HDL cholesterol fell by 0.74 i.e. HDL became enriched in triglyceride. Plasma apo A-I fell by a factor 0.84. Thus there were alterations in both the levels and composition of all lipoproteins.

4.5.6.1 Witztum and Schonfeld (1978) observed that apo C are increased relative to apo B and apo E and the relative proportions of various apo C's were also changed. Thus the liver responds to this particular dietary stimulus to
secrete more VLDL not only by raising the number of particles it produced but also by altering their compositions.

The changes in VLDL apoprotein composition are of interest because apo B and apo E serve as recognition markers for cellular LDL receptors, through which some lipoproteins are taken up by the cells [Brown and Goldstein, 1976] and apo C modulates the activity of lipoprotein lipase [Havel et al, 1973].

Smaller VLDL particles are more easily taken up by the cells due to the difference in the conformation of apo B on the VLDL particle [Schonfeld, 1979] but larger VLDL particles are readily catabolised in plasma than the smaller VLDL [Stroja, 1977].

In our laboratory other apoprotein levels are being investigated, which may throw some light on the possible differences existing between the lipoprotein composition in the American and European populations.

4.5.7 In the gut, cholesterol is incorporated into chylomicron, VLDL and HDL [Green et al, 1978], while the liver secretes LDL that contains cholesterol. The studies by Balmer and Zilversmit (1974) and Chang et al, (1976) suggest that plant fiber intake enhances cholesterol removal from plasma. Conceivably, plant fiber could influence the size of the chylomicron released from the gut and this would influence subsequent metabolism. It may alter the proportion of cholesterol incorporated into chylomicron,
VLDL and HDL or influence the size of the VLDL particle released from the gut or liver. This may be done by altering the ratio of cholesterol ester to cholesterol, triglyceride or phospholipid fraction to apoprotein or one apoprotein to another, suggesting that altering the composition of lipoproteins could influence the subsequent metabolism of the particles.

4.6. TRIGLYCERIDE LEVELS AND ITS DISTRIBUTION

4.6.1 Besides cholesterol, triglyceride is regarded as a major risk factor for the development of atherosclerosis. Many studies have been carried out to understand the mechanism leading to hypertriglyceridemia.

Circulating fatty acids are long chain acids, which serve as a source of calories for peripheral tissues, during periods of fasting. They are transported from adipose tissue to the peripheral tissue by plasma albumin. A larger part of free fatty acids (FFA) are removed by the liver, where they may be oxidised for energy purposes or incorporated into triglycerides of VLDL. This fraction is dependent on diet and is higher after a carbohydrate diet than during a period of fasting [Sailer, 1979].

4.6.2 In table 19, the correlation data of the healthy population shows a positive significance of triglyceride and HDL-triglyceride with age ($r = 0.197$, $p < .05$ and $r = .297$, $p < .01$ respectively). It is seen from the
table 29 that VLDL triglyceride, TG/HDL-TG, and VLDL-C/VLDL-TG were lower for the female population (p < .05, p < .01 and p < .01 respectively;) which implicate the finding that VLDL-C/VLDL-TG may be a powerful indicator for risk rather than VLDL triglyceride alone [Knopp et al., 1981], but more useful indication could be predicted in [Tatami et al., 1981] in combination with VLDL cholesterol (i.e. cholesterol rich VLDL = \(\frac{VLDL-C}{VLDL-TG}\) x VLDL-C).

4.6.3 The relative proportion of cholesterol/triglyceride in the plasma and the lipoprotein fractions in normals (Table 26) and patients (Table 27) indicate wide differences in the two groups studied.

While the cholesterol/triglyceride ratio for the plasma remains between 1.7 and 2.1 in the normal healthy adults in the age group studied, it is comparatively lower (1.6 to 1.8) in the patients. This change is because the triglyceride levels are elevated in the patients. More significant differences are observed in the cholesterol/triglyceride ratio in the HDL. In the healthy adults, this ratio is progressively lowered from 4.2 to 2.5 in this group with the most vulnerable age remaining around 35. In the patients the ratio is around 2.5 irrespective of age and is significantly lower than the normal level. Similar trends are observed with LDL also.
4.6.4 Serum triglyceride and its distribution in the lipoproteins reflects the dynamic state of lipoprotein metabolism. Carlson and Ballantyne (1976) showed that in the VLDL complexes with higher TG/protein ratio, there is a significant change in the relative proportion of apo-protein CII and CIII. In the case of hypertriglyceridemic VLDL (i.e. type V), almost absence of CII is found whereas CII is normally higher than CIII. This confirms the hypothetical role of CII in activating lipoprotein lipase suggested by Havel et al. (1973).

4.6.5 Schonfeld et al. (1976) has shown an increase in CII and decrease in CIII-2 in both chylomicron and VLDL after the intake of dietary carbohydrates. During the same period HDL became enriched with triglyceride and its apo A-I levels were lowered. Thus, it is believed that apoprotein changes may also be considered as the metabolic indices for lipid clearance. It is possible that in patients with hypertriglyceridemia, there is increased synthesis of VLDL-particles poorer in lipoprotein lipase activators.

4.6.6 Vessby and Lithell (1976) studied patients with hypertriglyceridemia (type IV) treated with carbohydrate restriction which were divided into two groups:

a) those showing an increase of LDL cholesterol

b) those showing a decrease, the cut-off point between the two being LDL-cholesterol level of approximately 150 mg/dl.
Analysis of the lipoprotein composition of the two groups of patients showed different cholesterol/triglyceride ratios in LDL and HDL.

In particular, the cholesterol/triglyceride ratio, was lowered in patients responding to therapy with an elevation of LDL cholesterol [Vessby and Lithell, 1976]. These patients had significantly low HDL. After carbohydrate restriction, patients responding with increased LDL cholesterol had increased cholesterol/triglyceride ratio in LDL, whereas no significant changes were noted in the HDL-composition.

4.6.7 The inverse relationship between tissue lipoprotein lipase (LPL) activity and circulating VLDL level is also seen with exercise. Long distance male runners showed low triglyceride levels especially so in VLDL and an increased HDL-cholesterol level [Wood et al., 1976; Nikkila et al., 1978]; this triglyceride lowering is found even when caloric intake is increased to maintain body weight [Gyntelberg et al., 1977]. In addition, increased lipoprotein lipase (LPL) activity is found in the adipose tissue and skeletal muscle of these male athletes [Nikkila et al., 1978] and total calculated enzyme in these tissues is increased by a factor of 2.7 and 1.7 respectively over the normal sedentary levels. With respect to the mechanism of increased LPL in exercise, serum insulin levels were reduced in runners, but Nikkila et al. (1978) has pointed out the increased
tissue sensitivity to insulin with exercise may also be a contributing factor. It has also been suggested [Tall and Small, 1978] that the increased HDL cholesterol levels seen with exercise may reflect the increased lipolysis of VLDL-triglyceride.

4.6.7.1 Baggio et al. (1980) have shown that in the post-prandial phase, the HDL₂ and HDL₃ subfractions show variation with greater fluctuation in HDL₂. An increase in phospholipid and triglyceride as well as a slight reduction in cholesterol was evident in HDL after 4-5 hours. At the same time, both lipid and protein were decreased in HDL₃ and increased in HDL₂. This phenomenon is more evident in females who showed a significantly higher basal HDL₂ level.

However, since HDL₂ formation appears to be related to chylomicron metabolism [Havel et al., 1973; Tall and Small, 1978 and Eisenberg et al., 1978] it may be assumed that the higher increase in HDL₂ in the fertile female is due to their more pronounced capacity to remove triglyceride. It may not be excluded that transformation of HDL₃ into HDL₂ contributes to the increase in the latter. In vitro studies seem to favour this possibility [Patsch et al., 1978]. More detailed investigations into the apoprotein and the HDL subfraction are warranted.

4.6.8 High fiber diet intake is usually accompanied by a reduction in the total lipid and triglyceride content of liver in experimental animals [Riccardi and Fahrenbach,
1967, Chang and Johnson, 1976]. Anderson and Chen (1979) have observed that when high plant fiber diet is coupled with a high carbohydrate diet, a reduction in triglyceride occurs, especially in the (hypertriglyceridemia) treated cases, while the reverse was true when they were subjected to only high carbohydrate diet with low fiber content.

It is interesting to note that while the dietary pattern in the population investigated (Madras) is high in carbohydrate but low in fiber, in the healthy group, the triglyceride levels are higher (100-150 mg/dl) than in the North Indians, where high fiber and a high carbohydrate diet is consumed. A study by Kumar (1975) shows that the triglyceride levels are lower at 80-100 mg/dl in healthy and CHD group.

The triglyceride levels for age adjusted males in India, in the present study, were more or less the same as that of Japan but lower than that of US, Canada and Argentina. This could be due to nutritional factors and physical activity. Ho and Chan (1974) observed in the Chinese population that the amount of rice consumed per day by their subjects correlated significantly (r = 0.91) with their serum triglyceride level. Rice is the staple food in Southern India and this may correlate to the high triglyceride levels.
PHOSPHOLIPID LEVELS AND ITS DISTRIBUTION

4.7.1 From correlation matrix table 19, it is observed that total phospholipid, HDL phospholipid, LDL phospholipid show positive correlation significance \( (r = .554, r = .469, r = .285, p < .01) \) with age, while VLDL-PL showed a weak positive significance \( (r = .209, p < .05) \).

On going through the table 30, it was observed that females had higher HDL phospholipid \( (p < .001) \) while lower T-PL/HDL-PL and LDL-PL/HDL-PL ratios \( (p < .05) \) than males.

It is interesting to note that T-C/T-PL was lower in women in the healthy group, while HDL\(_{PL}/T-C\) was higher in women of the same group \( (p < .01 \) and \( p < .001 \) respectively). It must be noted that the cholesterol/phospholipid ratios are not significantly altered in the patients with age.

4.7.2 Studies carried out by Naito et al (1980) have shown that patients with coronary artery disease, HDL-cholesterol by itself is not as effective a predictor of CAD as HDL-PL/T-C. This relation suggests that HDL-PL/T-C ratio can be considered as a risk factor for CAD, but is not very dependable for clinical diagnosis.

In another study in Japan by Tsuji (1980) among normal and IHD patients, it was observed that HDL-C/HDL-PL ratio of females was higher than that of males. No such observation was made in our study. But they have found
HDL-C/HDL-PL was lowered in the case of IHD significantly and HDL-C/HDL-PL was influenced by ageing.

A similar observation was made in our population in that influence of age on HDL-C/HDL-PL showed a negative significant correlation ($r=-0.359, p < .01$) Table 19.

4.7.3 Gofman and Tandy (1967) have provided evidence that the total mass of HDL₂ is frequently reduced in CHD patients to an extent exceeding that which could be produced even by a total loss of cholesterol ester. Barret al. (1951) demonstrated that HDL-phospholipid concentration was also reduced in CHD patients. In the Livermore study [Gofman, 1966] of CHD patients, reduction in HDL₂ and HDL₃ amounted to 32% and 8% respectively. We have not been able to fractionate the HDL in the population investigated.

4.7.4 It is known that HDL serves as an acceptor for the polar components, which are generated from the enzymatic analysis of triglyceride rich particles. Edlestein (1981) has noted the important differences in HDL subfractions HDL₂ and HDL₃, although in both cases saturation of phospholipid uptake was reached causing HDL to shift to the lighter density. Apolipoprotein AII of HDL had a lesser capacity to accept phospholipid, the excess of which was found to organise into a particle binding in the low density range of the gradient profile (peak density 1.04 g/ml) and containing both apolipoprotein A-I and B apoproteins.
Thus, the relative proportion of AI and AII may be a factor in modulating the acceptor capacity of HDL for the surface constituents of VLDL.

4.7.5 The availability of dietary fats, their quantity and quality influences the composition and fatty acid content of the circulating phospholipids. Hence, cellular metabolism may be selective in handling these essential phospholipid.

Linoleic and linolenic acid are required for the glycerophosphatides (phospholipids) of cellular membranes, the transport and oxidation of cholesterol and the formation of prostaglandins [Sinclair, 1980].

4.7.6 The most important function of essential fatty acids (EFA) is that they are a part of the glycerophosphatides of all animal cellular membranes and most of the signs of deficiency of EFA's arise from the structural needs of the cells. A low ratio of EFA to non-essential fatty acids (NEFA) in the region where cellular membranes are being formed causes NEFA to be incorporated into the membrane in place of EFA thus altering the fluidity and shape of the membranes [Sinclair, 1980]. But one of the most marked effect of deficiency of EFA was increased permeability of capillaries, epidermis, plasma membrane of cells, etc.) and so the local deficiency would facilitate LDL entry into the intima, particularly if the plasma concentration was high [Smith, 1974].
Platelet plasma membranes like all other mammalian plasma membranes are mainly composed of a phospholipid bilayer, free cholesterol and protein. [Singer and Nicolson, 1972]. A major determinant of the membrane fluidity [Shattil and Cooper, 1976] is the molar ratio between the free cholesterol and phospholipid content (C/P ratio) of the membrane. Following the increase of cholesterol/phospholipid ratio in vitro (causing an enhanced membrane fluidity), the aggregation tendency of platelets increases [Shattil et al., 1975] as does the thromboxane production in response to thrombin [Stuart et al., 1980]. Indeed, platelets of patients suffering from type IIa hyperlipoproteinemia show an elevated cholesterol/phospholipid ratio [Shattil et al., 1977]. Investigations on the platelet aggregation, and phospholipid analysis of the lipoproteins are envisaged as an extension of the studies reported.

4.8. CLASSIFICATION OF PATIENTS STUDIED INTO FREDRICKSON'S HYPERLIPOPROTEINEMIC GROUPS

4.8.1 It can be seen from table 42 that the patients with symptomatic coronary artery disease investigated fall into 43 normals, 47 type IIa, 36 type IIb and 39 cases of type IV. 25% showed normal cholesterol and triglyceride levels but suffered from coronary artery disease. Thus the importance of lipoprotein studies is apparent.

4.8.1.1 The studies on apolipoprotein B in these cases (included in figure 33 and tables 33 to 36) have greater predictive value since the apo B levels in the CHD group
4.8.1.2 Unlike the Lipid Research Clinics program which includes patients other than those suffering from cardiovascular disease, we have not come across types I, III and V, possibly due to the limitations in our sampling.

4.9 CUMULATIVE RISK SCORE

4.9.1 Williams et al. (1979) have observed an inverse relationship between HDL-C/T-C (% ratio) against cumulative risk factor score.

4.9.2 Likewise LDL-C/LDL-TG, T-C/LDL-C and T-C/T-PL ratios showed a rise while T-C/TG showed a slight fall with the risk rating score (figures 38, 39).

4.9.3 When smoking, blood pressure, weight, cholesterol and triglyceride levels were taken into consideration, they showed a relation to the incidence of CHD. It has been already well documented in a number of studies, that these primary risk factors correlate well with incidence of CHD, which in turn reflects the plasma lipid or lipoprotein levels.

4.9.4 LDL-cholesterol, phospholipid and triglyceride were found to increase with the risk score. HDL-cholesterol was more or less constant except for score 5 in which there were only 2 cases.
4.10 APOLIPOPROTEIN B - A BETTER DISCRIMINATOR OF AHEROGENESIS

4.10.1 Quantitation of plasma apolipoprotein B reported in the present study represents an attempt to establish the risk associated with the protein moiety of the lipoprotein recognised as the main carrier of cholesterol.

It is observed (from tables 33 to 36) that apo B levels are significantly higher (about 50%) and thus run parallel to LDL levels, and the higher levels of cholesterol and triglyceride respectively, and also with VLDL-TG.

The clear demarkation between healthy and CHD levels of apolipoprotein B is illustrated in figure 33 which gives the % frequency distribution.

4.10.2 From the correlation table 19 apolipoprotein B appears to rise with age \( (r = 0.392, p < .01) \) in the healthy group.

In a prospective study of the Framingham and Livermore Populations, Gofmann et al, 1966, have demonstrated that LDL is a powerful risk factor by CAD in men younger than 50-55 years of age. However, above 55 years of age, the predictive power of LDL and HDL deteriorates with increasing age. Similarly, Whayne (1981) observed that under 50 years of age apo B and LDL cholesterol were most significant, whereas from patients above 50 years or older, VLDL-cholesterol was the most reliable variable.
4.10.3 When viewed according to the concept of lipoprotein families [Alandovic et al., 1971, 1972 and Osborne et al., 1977] the presence of atherosclerotic lesions earlier in life seem to be associated primarily with the free form of LP-B and later in life with triglyceride rich complexes of lipoprotein B, C and E. In fact, both forms of apo B containing lipoproteins (LDL and VLDL), may contribute equally to the presence of coronary atherosclerosis. They also observed that segregation between these two lipoprotein forms might be caused by diminishing the lipolytic activity, which in turn increases the concentration of incompletely hydrolysed triglyceride rich lipoproteins.

4.10.4 Onitri et al. (1976) have shown a positive correlation between the concentrations of apo B and C in the arterial intima with the levels of these apolipoproteins in sera of patients undergoing coronary surgery. In patients with elevated VLDL levels (of triglyceride rich complexes of lipoproteins B, C and E) arterial concentrations of apolipoproteins were increased two fold, while in patients with elevated LDL (free form of LP-B) arterial concentration of apo B was increased five fold.

Sniderman et al. (1980) have shown that there is a certain group of patients with normal levels of LDL-cholesterol and plasma cholesterol but with elevated apoB levels.
Possible mechanism for these differences are now becoming apparent. Hamond and Fischer (1971) suggested that there are subfractions of LDL and that a lowered ratio of LDL cholesterol to apo B might result in enrichment of certain types of LDL. Alternatively, the changed composition might indicate an abnormal LDL that has increased atherogenic potential.

Sniderman et al. (1978) experimented on the uptake of cholesteryl ester from LDL across the human splanchnic bed with corresponding output in VLDL and LDL. The transfer of cholesteryl ester between intact lipoproteins has been demonstrated by Chajak & Fieldraja (1978). The cholesterol and in particular the cholesteryl ester portion of LDL may be malleable, if so, apo B protein, a structural component of LDL, may reflect more accurately the amount of LDL in plasma than the LDL cholesterol. This would result in a better differentiation between normo cholesterololemic coronary patients of type II hyperlipoproteinemia.

Both have not only coronary disease but also elevated LDL protein levels. This concept is further confirmed by the finding of apo B lipoprotein levels reported in figure 42 in our study.

The lipid hypothesis may need a reexamination as a lipoprotein hypothesis, that is, the risk being more proportional to the number of LDL particles and not the level of LDL cholesterol alone. For, it may be the LDL
particle and not LDL-cholesterol which enters the arterial intima and damages it, thus initiating the atherogenic process [Ross and Glomset, 1973].

Further studies on the apoprotein are envisaged.
5. SUMMARY
5. SUMMARY

5.1 The distribution of plasma lipids and lipoproteins in a group of healthy South Indian subjects (Madras, Tamilnadu) and patients with cardiovascular disease (CVD) were investigated.

5.2 The fractionation of plasma lipoprotein lipids was carried out by a dual precipitation technique involving heparin manganese chloride precipitation of LDL and VLDL and aggregation of VLDL alone by sodium dodecyl sulphate.

5.3 A comparison made between the dual precipitation and ultracentrifugation procedures in twenty-three samples, showed a very good correlation between the two methods in arriving at the cholesterol, triglyceride and phospholipid distribution in HDL, LDL and VLDL.

5.4 Apolipoprotein B was determined in 48 healthy subjects and 36 patients with CVD by the electroimmunoassay method.

5.5 The analyses of plasma lipids and lipoprotein concentration in 156 healthy male and female subjects (higher and middle socio economic groups) provided the following salient findings:

5.5.1 Plasma cholesterol, triglyceride and phospholipid were found to correlate positively with age.
5.5.2 Men had higher total cholesterol, LDL-cholesterol & VLDL-cholesterol than women.

5.5.3 Higher values of HDL-C were found in women (70.3 mg/dl) than in men (67.5 mg/dl). The ratios T-C/HDL-C, LDL-C/HDL-C and VLDL-C/HDL-C, showed significant differences with age and sex.

5.5.4 Total and VLDL-triglyceride as well as their ratios total/HDL, VLDL/HDL were higher in males.

5.5.5 Total phospholipid and its distribution in the lipoprotein classes were higher in women, but their ratios total/HDL, LDL/HDL, VLDL/HDL were lower than males.

5.6 Analysis of plasma lipid and lipoprotein concentration in 165 patients revealed the following:

5.6.1 The cholesterol, triglyceride and phospholipid fractions of HDL, LDL and VLDL as well as their ratios Total/HDL, LDL/HDL, VLDL/HDL were increased and were significantly higher in the case of patients.

5.6.2 In the HDL fractions cholesterol and phospholipid were found to be lowered significantly in the case of patients, while HDL-TG remained constant when compared to healthy subjects.

5.6.3 HDL-C/HDL-PL, HDL-PL/T-C, HDL-C/HDL-TG and T-C/TG were found to be significantly lower in the patients.
5.6.4 There is further support for the view that HDL-C is a negative risk factor for clinical CHD independent of the VLDL cholesterol and LDL cholesterol concentrations.

5.6.5 Comparison of the frequency distribution of apolipoprotein B values in normals and patients showed a better demarcation between healthy and CVD groups than any of the lipid components investigated suggesting its usefulness in the evaluation of the atherogenic index.

5.6.6 Correlation of HDL cholesterol with triglyceride was negative in the Framingham study. No such observation was made in our study. But HDL cholesterol showed a negative correlation with total cholesterol/total triglyceride in healthy and a positive correlation in the case of CHD subjects.

5.7 Indian adults investigated have higher levels of VLDL-cholesterol and triglyceride than the populations studied elsewhere. The possible role of dietary carbohydrate and fiber are discussed in the light of earlier observations.

5.8 The need for a thorough investigation on the apoprotein groups in the investigation of atherosclerosis has been discussed.