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Serum High Density Lipoprotein in Diabetic Patients

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Summary. The purpose of the present investigation was the study of HDL lipoprotein changes in patients with diabetes mellitus. The comparison was made between 40 normal and 109 diabetic subjects and the following data was obtained: relative HDL concentration (polyacrylamide gel electrophoresis), HDL-cholesterol and apolipoprotein A concentrations. We found significant decreases in HDL (18–28%) and HDL-cholesterol (31–40 mg/100 ml) in most diabetics except in those with normalized serum levels of glucose and lipids (34% and 50 mg/100 ml respectively). There was a statistically significant difference in HDL and HDL-cholesterol concentrations between patients in the latter group and other diabetic patients. There was a negative correlation between HDL and HDL-cholesterol and serum glucose levels. No statistically significant difference was found when apolipoprotein A was compared in normal and diabetic subjects. Our results suggest that a deficient binding of cholesterol to apoprotein A might be present in diabetes.

Key words: High density lipoproteins (HDL), HDL-cholesterol, apolipoprotein A, serum glucose, diabetes mellitus, lipoproteins.

It is generally accepted that diabetics may have increased serum lipids and lipoproteins and a greater degree of atherosclerosis than normal individuals. Very few studies have been done on the phospholipid and cholesterol content of HDL in diabetic patients [1, 2]. These studies have been done in a small number of uncontrolled diabetic patients with marked hyperlipaemia and have shown a decrease in the HDL cholesterol and phospholipid levels, with an increase in those levels after control of the diabetes with insulin therapy.

Recently, there has been an upsurge of interest in the study of the role of alpha lipoprotein (HDL) in determining the risk of ischaemic heart disease. Glomset postulated a role of alpha lipoprotein in clearing cholesterol from tissues [3]. Miller and Miller [4] found a correlation between a low level of HDL and atherosclerotic disease. Rhoads et al. [5] described a lower concentration of HDL cholesterol levels in individuals with ischaemic heart disease. Recently Berg et al. [6] and Albers et al. [7] described a significantly lower concentration of apoprotein A-I in patients with coronary heart disease when compared with normal individuals. Special attention to diabetic patients was lacking in these studies.

The present study was undertaken with the purpose of determining whether similar changes could be found among diabetic patients, among whom the incidence of atherosclerotic heart disease is definitively increased [8].

Materials and Methods

Serum Samples

Serum was obtained from 109 male diabetics who had been admitted to the hospital or who were followed regularly in an outpatient clinic. The diagnosis of diabetes had been previously established in these patients by the repeated demonstration of fasting hyperglycaemia (> 125 mg/dl). In 3 of them, chemical diabetes was diagnosed using the criteria of Fajans and Conn [9]. If diabetes had its onset before age 20, a diagnosis of juvenile onset diabetes was

Table 1. Main clinical features of the patients studied

	Number of patients	Patients in each group			
		Group (2)	(3)	(4)	(5)[a]
Type of diabetes					
Adult onset	106	22	27	21	36
Juvenile onset	3	1	1	–	1
Duration of diabetes					
0–5 years	44	9	14	7	14
5–10 years	27	6	4	10	7
More than 10 years	38	8	10	4	16
Medication					
Insulin + diet	90	19	26	15	30
Oral agents + diet	9	1	2	2	4
Diet alone	10	3	–	4	3
Weight					
Degree of obesity					
Normal	87	18	26	15	28
Moderately obese[b]	14	5	1	5	3
Markedly obese[c]	8	–	1	1	6
Mean ± S. D. (lb)	169 ± 30	168 ± 24	156 ± 20	171 ± 34	180 ± 34
Percentage deviation from ideal body weight ($\bar{x}$±SE)	4.1 ± 1.1	3.6 ± 1.5	1.3 ± 1.9	4.6 ± 2.6	6.4 ± 2.6

[a] For definition of the groups refer to Table 2
[b] Between 15–25% above ideal body weight
[c] >25% above ideal body weight

made. If the onset was over age 20, a diagnosis of maturity onset diabetes was made. Ninety-four percent of the patients were on no other medication except the one prescribed for diabetes control. The remainder were also taking antihypertensive or antilipaemic medication.

The mean age of the patient group was 50.1 years and the range was from 28–77 years. The mean weight of the group was 169 ± 30 lb and the range was from 120–245 lb.

Serum samples from 40 control males with verified normal values of serum lipids, lipoproteins and glucose were collected. Thirty of the controls had no known disease and were under no medication. The remaining ten were patients hospitalized in the services of Urology and Geriatrics with the following diseases: prostatic hypertrophy [4]; pulmonary tuberculosis [1], treated with Isoniazid; chronic bronchitis [2] treated with Tedral and aminophylline, femur and tibial fractures [2]; cataract [1]. The mean age of the control group was 39.0 and the range 20–82 years. The mean weight of the control group was 162 ± 22 and the range 110 to 210 lb. All blood samples were collected after an overnight fast.

Lipoprotein Electrophoresis and HDL Determination

Serum lipoproteins were separated by conventional lipoprotein polyacrylamide gel electrophoresis using Sudan Black B as the lipid stain [10, 11]. The separations were scanned with a Quick Scan Densitometer and the relative concentration of HDL determined from the integral values for each fraction provided by the scanner.

Separation of HDL

HDL was separated by precipitation of LDL and VLDL with sodium phosphotungstate in the presence of $MgCl_2$, as described by Burnstein [12, 13]. We added 100 µl of 4% sodium phosphotungstate solution and 25 µl of 2M $MgCl_2$ to 1 ml of serum. This resulted in an immediate, complete and selective precipitation of LDL and VLDL.

Sodium phosphotungstate was used as a precipitating agent instead of heparin to avoid the incomplete precipitation that occasionally occurs with some preparations of heparin [14].

The supernatant was used for determination of the cholesterol content of HDL. In highly lipaemic sera, if the initial precipitation was not complete (as judged by lipoprotein electrophoresis), the serum was diluted and the procedure repeated to ensure a complete precipitation.

In order to determine if the cholesterol determination by the method used in our laboratory was affected by the presence of sodium phosphotungstate or magnesium, supernatants of 10 sera with variable cholesterol, triglycerides and glucose concentrations

were precipitated with 4% sodium phosphotungstate as described above and with heparin (5000 U/ml) in the presence of 1M $MnCl_2$ [14]. We used 40 µl of heparin and 50 µl of 1 M $MnCl_2$ for 1 ml of serum.

Completeness of precipitation by both methods was assessed by electrophoresis. Equivalent values of cholesterol were found in the supernatants obtained after precipitation with both precipitating agents.

Quantitative Study of Serum Apolipoprotein A (Apo A)

Apolipoprotein A was determined in normal individuals and diabetic subjects by radial immunodiffusion [15]. Specific antisera to human alpha lipoprotein produced in rabbits was supplied by Behring Diagnostics (Lot 2792 C, titre 0.1 U/ml). This antiserum has been characterized by Lee and Alaupovic and found to contain antibodies to A apolipoprotein only [16].

A standard of HDL prepared in our laboratory following a method described by Burnstein [12] was used. The purity of HDL standard was demonstrated by immunoelectrophoresis [17].

Each radial immunodiffusion plate was prepared with 16 ml of 1% (w/v) agar-gel solution; the antiserum concentration used in each plate was 4%. The sera samples were diluted to 1:10 and 1:20 in saline and 5 µl of the sample applied. Each serum sample was tested at least in duplicate in each of the above dilutions. The immunological reaction was allowed to develop at room temperature for 24 to 48 h. In each plate we included 4 dilutions of the HDL standard as references, run in duplicate. The plates were washed with several changes of saline and finally rinsed overnight with distilled water and stained after drying with Coomassie brilliant blue R.

Protein Determination

Protein determination of the HDL standard was done by the biuret reaction.

Cholesterol and Triglyceride Determinations

Total cholesterol was determined by an automated colorimetric method described by Block et al after extraction with isopropanol [18]. The cholesterol content of HDL was assayed in a 1:10 isopropanol extract instead of the usual 1:20 extraction done for total sera.

Triglycerides were determined by an automated fluorimetric method [19].

Glucose Determination

Glucose levels were assayed using an automated method [20].

Lipoprotein Ultracentrifugal Separation

Venous blood samples were collected in EDTA (1 mg per ml of blood) from 5 diabetic patients after a 12–14 h fast. The plasma was separated by centrifugation at 2500 rpm for 20 min at 4° C.

Lipoprotein separation was initiated in the same day of collection, by sequential ultracentrifugation.

Ten ml of plasma were pipetted and overlaid with 0.16 M NaCl, pH 9, density 1.006 g/ml. These samples were ultracentrifuged at 40000 rpm for 24 h at 16° C in a type 40 rotor in a Beckman Model L5-75 ultracentrifuge.

The tubes were sliced at the middle of the clear zone separating the supra and infranatant. The bottom fraction was stirred and aspirated to a 10 ml volumetric flask. The walls of the tube were carefully washed with saline and the sample adjusted to a final volume of 10 ml.

Five ml of that fraction containing LDL and HDL lipoproteins were adjusted to a density of 1.063 g/ml with solid NaCl and run at 40000 rpm during 24 h at 16° C.

The tubes were sliced again in the middle of the clear zone separating the supra and infranatant and the bottom fraction was transferred to a volumetric flask of 5 ml.

Total plasma, HDL+LDL fraction and HDL fraction were used for cholesterol and apolipoprotein A assay as described.

Statistical Methods

Mean differences between groups were tested using the two tailed t test.

The correlation coefficient (r) was determined by linear regression analysis. Statistical significance of the correlation coefficient was determined by the method of Fisher and Yates.

Results

We included 149 individuals in the present studies, divided into one group of normal subjects and four groups of diabetic patients. Table 1 shows a summary of clinical data. In Table 2 our laboratory studies are summarized.

Diabetic patients have low levels of HDL and HDL-cholesterol. These findings are seen mainly in

Table 2. Serum lipids, glucose, HDL, HDL-cholesterol and apolipoprotein A levels in normal and diabetic subjects

	No. of Subjects	Serum Total Cholesterol (mg/100 ml)	Serum Triglycerides (mg/100 ml)	Serum Glucose (mg/100 ml)	HDL Levels (Polyacrylamide Electrophoresis) Percentage Values	Cholesterol Content of HDL (mg/100 ml)	Apolipoprotein A Levels (mg/100 ml)
Group 1	40	188±29[a] (134–250)[b]	86±32 (35–160)	88±13 (55–108)	38±7.0 (25–57)	53±10 (40.5–74)	201±33 (121–270)
Group 2	23	194±40 (136–247)	94±30 (30–135)	101±14 (75–117)	34±8[c] (23–55)	50±10[c] (34–70)	203±34[c] (161–265)
Group 3	28	197±31 (160–249)	100±26 (55–147)	188±57 (126–362)	28±6[d] (16–42)	40±11[d] (24–66)	187±30[c] (121–239)
Group 4	21	225±50 (132–300)	410±461 (104–1168)	110±11 (97–123)	21±7[d] (9–33)	40±16[d] (14–74)	193±41[c] (120–251)
Group 5	37	238±45 (157–329)	376±378 (114–1885)	197±65 (126–368)	18±7[d] (7–35)	31±9[d] (13–47)	187±38[c] (100–270)

Group 1 – Normal individuals
Group 2 – Diabetic subjects with normal serum lipid and glucose levels <125 mg/100 ml
Group 3 – Diabetic subjects with normal serum lipid and increased glucose levels
Group 4 – Diabetic subjects with increased serum lipid (cholesterol and/or triglycerides) and normal glucose levels
Group 5 – Diabetic subjects with increased serum lipid (cholesterol and/or triglycerides) and glucose levels

[a] mean and standard deviation
[b] range
[c] N. S. (as compared to Group 1)
[d] $p<0.001$ (as compared to Group 1)

Table 3. Cholesterol and Apo A levels in total plasma and ultracentrifugal lipoprotein fractions

Patient	Glucose mg/100 ml	Trigl. mg/100 ml	Total Chol. mg/100 ml	HDL Chol. mg/100 ml	VLDL Chol. mg/100 ml	LDL Chol. mg/100 ml	Total Apo A mg/100 ml	HDL Apo A mg/100 ml	LDL + HDL Apo A mg/100 ml
Group 2									
H. L.	89	114	188	53	5	130	180	187	185
B. V.	124	80	221	46	17	158	190	192	188
Group 3									
G. B.	140	140	179	44	29	106	200	210	195
L. B.	254	108	171	39	28	104	220	210	220
Group 5									
H. M.	239	580	198	30	74	94	225	213	215

Table 4. Age-related variation of HDL and HDL cholesterol in controls

Age	Number of subjects	HDL Cholesterol mg/100 ml	HDL percentage (% of total lipid)
20–30 years	16	56±12	36±8
30–40 years	8	52±10[a]	39±7[a]
40–50 years	5	53± 6[a]	40±5[a]
50–60 years	6	51± 8[a]	38±8[a]
60–82 years	5	51± 9[a]	39±3[a]

[a] N. S. (as compared with the group with ages between 20–30 years)

the group of hyperglycaemic and hyperlipidaemic patients (Groups 3–5). The levels of apolipoprotein A do not show significant differences among the several groups of individuals included in the study.

The differences in the proportion of HDL lipoproteins and in the concentration of HDL-cholesterol among the different groups were statistically analyzed. There were significant differences among both determinations when normals (Group 1) were compared with diabetics with elevated serum glucose and/or lipid values (Groups 3, 4, and 5) while there was no difference between normals and diabetics with normal serum glucose and lipid levels (Group 2).

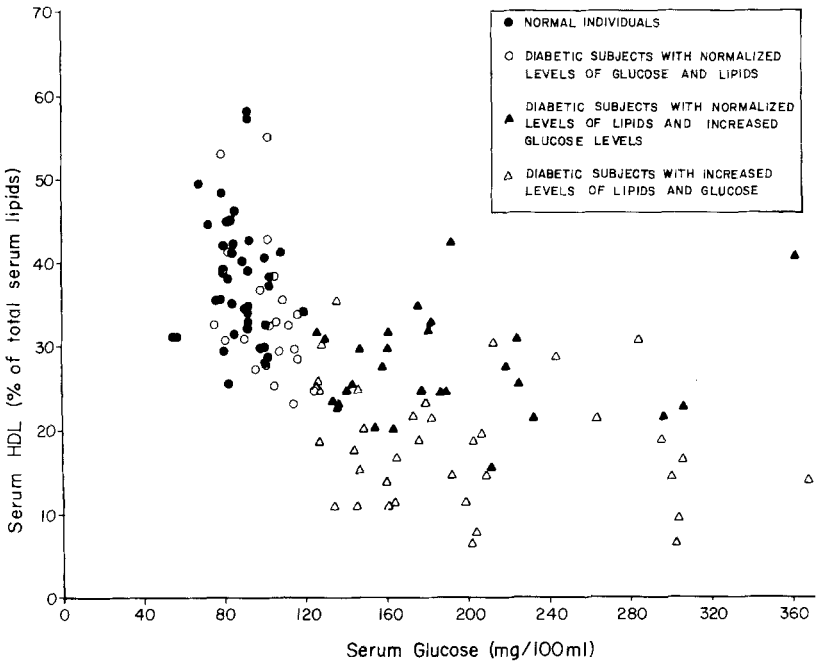

Fig. 1. Relation between glucose concentrations and the percentage value of HDL among total lipoproteins. The patients with normalized glucose and hyperlipoproteinaemia (Group 4) were excluded ($n = 128$, $r = -0.50$, $p < 0.01$)

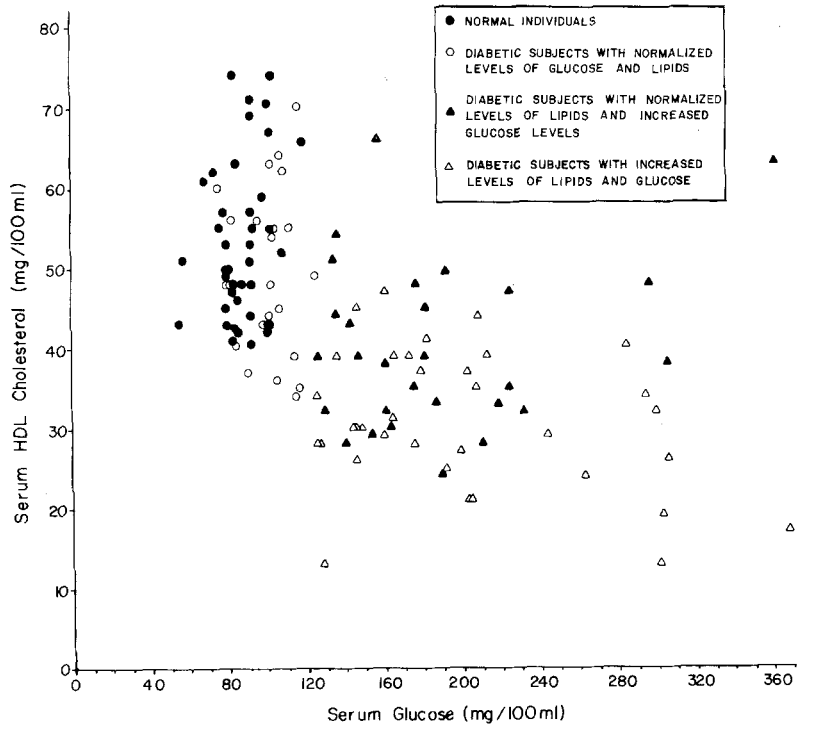

Fig. 2. Relation between glucose concentrations and HDL cholesterol levels. The patients with normalized glucose and hyperlipoproteinaemia (Group 4) were excluded ($n = 128$, $r = -0.51$, $p < 0.01$)

Statistically significant differences were also found when the group of well controlled diabetics (Group 2) was compared with the other groups of diabetics (3, 4, and 5). This was particularly striking in the comparison of Groups 2 and 5.

Statistical analysis confirmed the apparent lack of a significant difference between the apoprotein A concentrations in the several groups of patients and normal individuals.

In view of the normal levels of Apo A in diabetics with low levels of HDL cholesterol, we performed experiments to determine if, in these patients, there was a shift of Apo A from HDL to another lipoprotein fraction mainly VLDL.

In Table 3 our results are summarized. It is apparent that there is no alteration in the total amount of Apo A in each one of the fractions separated by ultracentrifugation. As in normals most of the Apo

A was in the HDL fraction. The small differences that we found are due to the normal variation of the method used for Apo A assay.

The correlations between the several variables studied were further explored by linear regression analysis comparing glucose concentrations with a) percentage value of HDL among total lipoproteins, b) HDL-cholesterol, and c) apolipoprotein A concentrations. Some of these studies are graphically illustrated in Figures 1 and 2. There were negative correlations between HDL and HDL-cholesterol and glucose levels, but no correlation was apparent when apolipoprotein A and glucose concentrations were compared.

Fourteen of the patients were moderately obese. They were distributed in groups 2, 3, 4 and 5 and their values for HDL cholesterol and HDL percentage were not significantly different from the mean values for the non-obese members of the same group. Among the eight patients with marked obesity, six of them were in Group 5. There was no significant difference between the mean values for HDL and HDL-cholesterol for these individuals (15% and 29 mg/100 ml) and the non-obese members of Group 5 (18% and 31 mg/100 ml).

The other two markedly obese individuals were in Groups 3 and 4. In both cases, the values for HDL (24.8% and 18.7%) and HDL-cholesterol (28 and 22.5 mg/100 ml) were markedly below the mean values for the non-obese members of the group.

Discussion

This study demonstrates that male diabetic patients have abnormalities in their HDL-lipoprotein system, consisting of a decrease of the percentage value of HDL among total lipoproteins and in a decrease of HDL-cholesterol. On the contrary, apolipoprotein A concentrations were not significantly changed. An interesting correlation between other indices of metabolic dysfunction and HDL abnormalities exists. Well controlled diabetics with normal glucose and normal serum lipid levels have normal HDL values, while those patients with normal serum lipids, but with abnormal glucose concentrations, show abnormal HDL values. Furthermore, linear regression analysis demonstrated a correlation between glucose concentration, HDL percentage and HDL-cholesterol.

This finding can be significant considering the evidence that has been recently accumulated suggesting that low alpha cholesterol values are associated with atherosclerotic coronary disease [4, 5]. The increased incidence of atherosclerotic disease in diabetics could then be related to the postulated clearing action of alpha lipoprotein on tissue cholesterol.

Recent studies have also shown a correlation between HDL cholesterol levels and apolipoprotein A-I values [7]. This was not the case in our study, but we used an antiserum that would recognize both polypeptides of apoprotein A (apo A-I and A-II). It is known that the ratio of Apo I to Apo II is very important in determining the binding capacity of these polypeptides to HDL lipids [21].

It is possible that our lack of correlation between Apo A concentrations and HDL cholesterol levels results from an imbalance of Apo I and Apo II concentrations that somehow would result in a decreased binding capacity for cholesterol. This is a point that deserves further investigation. Alternatively, a possible consequence of the metabolic changes occurring in diabetic patients is that the availability of cholesterol to apoprotein A decreases.

One possible criticism of our study is the age difference between the group of normal individuals and the patient group. However, Berg et al. [6] have reported that HDL concentrations, although sex-dependent, do not vary significantly with age. HDL-cholesterol has also been found not to vary with age [6] or alternatively, to increase with increasing age [14]. In our studies, we saw no age-related changes of HDL or HDL-cholesterol in the control group (Table 4). Further, in spite of the fact that the ages did not differ significantly in the 4 diabetic groups (Group 2: 48.8±11.1; Group 3: 52.1±12.6; Group 4: 48.9±8.5; and Group 5: 50.1±10.3), we saw significant differences in HDL and HDL-cholesterol when Group 2 was compared with the others. There does not appear to be a major influence of obesity on HDL or HDL-cholesterol levels in our studies. Occasionally, however, the presence of marked obesity was associated with unusually low levels of HDL and HDL cholesterol. This observation requires further study.

We conclude that HDL and HDL-cholesterol levels may be a very critical index of metabolic normalization in diabetics. They provide a better evaluation than total lipid determinations, since we have found that hyperglycaemic and normolipaemic patients have significantly lower values for both HDL and HDL-cholesterol.

Finally, the implications of these findings regarding precise control of blood glucose and its possible influence on vascular disease in diabetes deserves comment. In view of the fact that HDL-cholesterol and percentage is only normalized at plasma glucose levels of about 125 mg/100 ml or below (Figs. 1 and

2), a strong argument for diabetic management to normoglycaemia can be made. If the postulate of Miller et al that lowered alpha lipoprotein relates to cholesterol clearing from tissues is correct, then normalization of this value in diabetics, a group highly susceptible to atherosclerosis, could be critical in the prevention of atherosclerosis.

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