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Association of HDL & HDL subfraction levels with Microalbuminuria, in Type 1 Diabetes

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The prevalence of Type 1 Diabetes Mellitus is increasing worldwide.Diabetes mellitus has long term effects leading to damage, dysfunction and failure of various organs especially the eyes, kidneys, heart and blood vessels.In a patient of diabetes, the presence of persistantmicroalbuminuria is usually the first sign of diabetic nephropathy.The overall prevalence of microalbuminuria and macroalbuminuria is around 30-35 % in Type 1 Diabetes[1]. The diabetic sibling of a patient with Type 1 diabetes and nephropathy has a 72% cumulative risk of developing renal disease, whereas the diabetic sibling of a person with Type 1 Diabetes but without nephropathy has only a 25% risk [2]. This indicates that inherited factors play an important role in determining susceptibility to diabetic nephropathy. This view is consolidated by the observation of familial clustering of diabetic kidney disease[3,4].In general, the incidence of diabetic nephropathy follows a pattern of increase about 5 years after diagnosis, with highest incidence 5 to 15 years after diagnosis of type 1 diabetes[1].This changing pattern of risk indicates that the magnitude of exposure to diabetes is not sufficient to explain the development of diabetic nephropathy and suggests that only a subset of patients is susceptible to kidney complications.

Since nephropathy doesn't develop in all diabetic patients, other factors in addition to hyperglycemia must be operative in these patients at risk of development of diabetic nephropathy.

A large cross-sectional study was performed by Alicia.J.Jenkins et al, to assess the relationship between dyslipidemia and nephropathy in the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications cohort of subjects with type 1 diabetes[5]. They found no differences in plasma lipid profile among the 2 study groups i.e. those on conventional treatment and those on intensive treatment. Hence study subjects were classified based on albumin excretion rate into 3 groups (i.e. normoalbuminurics, microalbuminurics and albuminurics) and the level of triglycerides, total cholesterol and LDL cholesterol were compared between the groups. These parameters were found to be significantly associated with Albumin Excretion Ratio (AER). Among the NMR determined parameters, with increasing AER there was a shift in peak towards smaller LDL. Small HDL was found to be positively associated with AER. Analysis of the apolipoprotein a and Lp(a) levels showed no association with AER.

In the study by Molitch ME et al, LDL-C and triglyceride levels were found to be higher in those with albuminuria, but the increase was not statistically significant. However, HDL-C levels were significantly lower in those with albuminuria as compared to those without albuminuria [6].

In the study by Nish Chaturvedi et al (of the EURIDIAB PCS group), the differences in lipid and lipoprotein parameters with respect to microvascular and macrovascular complications in type 1 diabetes, were evaluated. It was found that abnormalities in lipid and lipoprotein levels were more closely related to albuminuria than to CVD in patients with type 1 diabetes. Total cholesterol, triglycerides, LDL-C and apoB levels were higher and LDL particle size was smaller in those with albuminuria. HDL-C and related parameters showed only trivial disturbances [7].

The present consensus is that all the above mentioned risk factors contribute to chronic low grade inflammation, which is already present in the diabetic state. This is said to be responsible for endothelial dysfunction.

Endothelial dysfunction(ED) is considered present when endothelial properties have changed in a way that is inappropriate with regard to the preservation of organ function, ex. Altered basement membrane synthesis, contributing to arterial stiffness and increased vascular permeability[8].Theoretically ED could cause albuminuria directly, by increasing glomerular pressure and glomerular basement membrane permeability and indirectly, by influencing mesangial cell and podocytefunction[9].

Though a pathogenic role of dyslipidemia characterized by increased triglycerides, LDL-C and low HDL-C has been suggested in a number of studies[5,7], their relation to progression of diabetic nephropathy is not yet proved. Therefore antihyperlipidemic agents are recommended only in those with abnormal lipid panels, as they are still useful in lowering mortality due to cardiovascular disease and other causes. Here we try to study the association of lipid profile specifically the HDL subclasses, with microalbuminuria in Indian diabetic patients.

I. Research Design & Methods:

The study composed of a total number of 144 subjects, of which the apparently normal subjects, who formed the control group, were 48, out of which males were 14 and females were 34 in number. The control group comprised of volunteers from among the staff and students of Madras Medical College.The remaining subjects were those with Type 1 DM, enrolled into the study as cases. These individuals were from among the Type 1 DM individuals attending the Diabetology outpatient clinic in Madras Medical College & Government General Hospital, Chennai.

Inclusion criteria:

- Patients with Type 1 DM of duration >5 years.

Exclusion criteria:

- Presence of UTI.
- Presence of other complicating conditions such as infections, diabetic ketoacidosis, coronary artery disease, etc.

Among the cases, 48 were Type 1 DM individuals without microalbuminuria and remaining 48 were Type 1 DM individuals with microalbuminuria. A urine albumin:creatinine ratio(UACR) of > 30 mg/g creatinine was used as a cut off for diagnosis of microalbuminuria& assigning of cases to the 2 study groups.

Estimation of Urine Albumin CreatinineRatio (UACR).

Early morning midstream urine sample was collected for UACR estimation. Urine Albumin was estimated by Quantitative Immunoturbidimetric Method using kit manufactured by Randox Laboratories Ltd. Urine Creatinine concentration was estimated by Jaffe's Method based kit, manufactured by Bayer diagnostics. The UACR was calculated using the following formula:

$$\text{UACR(mg/gcreatinine)} = \left(\frac{\text{Urine Albumin conc. mg/dl}}{\text{Urine Creatinine conc. mg/dl}}\right) * 1000$$

Blood sample was collected for estimation of Fasting Plasma Glucose (FPG), Urea, Creatinine, HbA_{1C} and Lipid profile. Samples were collected in the morning, after a minimum of 12 hour fast period. The blood samples were analyzed on the same day within 4 hours of collection. All the parameters except HDL and its subfractions were estimated on the same day of collection. For estimation of HDL and its subfractions, 1 ml of serum was stored in eppendorf tubes at –20°C and analysed within 1 week.

Glucose, Urea, Creatinine, Total cholesterol & Triglycerides were estimated using kits manufactured by Autopak Limited. Estimation of $HbA_{1C}$was done using the Glycated Hemoglobin (GHb) kit manufactured by Crest Biomedicals Ltd. It is based on Ion-exchange resin chromatography. The value of HbA_{1C} is obtained from GHb using the conversion table provided in the kit.

II. Estimation of HDL & its Subfractions.

Methodology

Dual precipitation method adapted from the original procedure proposed by Gidez et al[10].

Principle

Polyanionic reagents form lipoprotein polyanion complexes with the serum lipoproteins.The lipoproteins with lower protein to lipid ratio are the first to be precipitated and get precipitated at lower concentrations of these polyanionic reagents.

Lipoproteins of density <1.063 g/dl and apo B associated lipoproteins of density >1.063 g/dl (i.e. all lipoprotein fractions except HDL) are precipitated by Heparin-$MnCl_2$ precipitation procedure. This was originally described by Lipid Research Clinics, later modified by Warnick and Albers.

In case of HDL subfractions, HDL_2 which has a lower protein to lipid ratio than HDL_3, can be precipitated under conditions which will not precipitate HDL_3. Dextran sulphate is used at a concentration of 0.13g/dl in the final solution to precipitate only HDL_2. Centrifugation is done to sediment the precipitate and HDL_3cholesterol is estimated in the supernatant.

Reagents :

1. Heparin-$MnCl_2$ solution: 0.06 vol of heparin sodium (40,000USP units/ml) and 1.0 vol of 1.06 M $MnCl_2$.
2. Dextran sulphate solution: 1.43 g/dl in 0.15 M NaCl.
3. Cholesterol estimation kit from Bayer diagnostics.

Procedure :

Step 1: Precipitation of lipoproteins with d <1.063 g/dl and apo B lipoproteins of d >1.063 g/dl. 0.1vol (100 µl) of heparin-$MnCl_2$ solution was added to 1.0 vol (1.0ml) of serum. After 20 min at R.T., the samples were centrifuged for 1 hr at 3700 rpm (1500*g) at 4°C, using a microcentrifuge. HDL-C was estimated in the supernatant using the cholesterol estimation kit.

Step 2: Precipitation of HDL_2 and estimation of subfractions.

0.1vol (50 µl) of dextran sulphate solution was added to 1 vol (500 µl) of heparin-$MnCl_2$ supernatant from step1. After thorough mixing, the sample was left at R.T. for 20 min. Centrifugation was carried out at 4°C at 3700 rpm (1500*g) for 1/2hr.HDL_3-C was estimated in the supernatant using the cholesterol estimation kit. HDL2-Cwas calculated by subtracting HDL_3-C levels from that of total HDL-C.

LDL-C levels were calculated by Friedwald's equation.

$$\mathrm{LDL-C} = \mathrm{TC} - (\mathrm{HDL-C} + \mathrm{VLDL}/5)$$

III. Results:

Table 1 shows an overview of the demographics & blood biochemical parameters of the different groups in the study.

Table1: Overview of demographic & biochemical parameters in the study groups.

Sr. no.	Parameter	Controls		Type 1 diabetics without microalbuminuria		Type 1 Diabetics with microalbuminuria	
		Males Mean(SD)	Females Mean(SD)	Males Mean(SD)	Females Mean(SD)	Males Mean(SD)	Females Mean(SD)
1.	Age(in yrs)	23.14(6.9)	22.73(7.3)	28.59(9.13)	22.57(7.81)	29.57(7.28)	24.95(9.23)
2.	Duration of diabetes(in yrs)	NA	NA	10.24(4.95)	8.05(2.63)	11.86(7.29)	10.4(6.5)
3.	FBS(mg/dl)	73.3(6.2)	74.47(5.9)	141.8(59.7)	140.31(58.24)	157.03(61.05)	175.96(124.59)
4.	HbA1C	4.4(0.5)	4.3(0.5)	8.66(0.99))	8.46(1.2)	8.31(1.2)	8.33(1.11)
5.	TAG(mg/dl)	80.7(8.7)	74.2(6.3)	85.86(37.34)	76.81(32.6)	87.31 (50.23)	105.65 (84.73)
6.	TC(mg/dl)	137.05(15.6)	130.4(12.3)	147.58 (39.08)	150.12 (28.36)	155.8(29.1)	158.1 (26.03)
7.	LDL-C(mg/dl)	72.9(16.2)	65.1(11.4)	83.9(30)	82.19 (20.35)	93.5(27.9)	94.46(24.1)
8.	HDL-C(mg/dl)	47.9(3.5)	50.46(3.6)	46.16(10.12)	50.85(9.7)	43.83(6.91)	42.9(7.15)
9.	HDL_3-C(mg/dl)	31.36(3.2)	32.17(3.6)	29.25(6.61)	32.11(4.9)	32.13(5.9)	30.26(7.1)
10.	HDL_2-C(mg/dl)	16.57(3.7)	18.29(3.9)	16.6(4.8)	18.74(7.32)	11.7(3.1)	12.59(3.17)
11.	HDL_2: HDL_3 ratio	0.54(0.15)	0.58(0.16)	0.58(0.2)	0.58(0.13)	0.37(0.1)	0.42(0.11)

The biochemical parameters of the controls were analyzed & the data was compared between males & females to ensure that the control group enrolled in the study was representative of the general healthy population. Similar comparisons were made between males & females in the 2 diabetic case groups. Refer table 2 for results of these comparisons.

In all the comparison tables, the statistical significance of the difference in level of the biochemical parameters among the study groups was arrived at from the p-value which is obtained from the student's 't' Test. Mann Whitney test was performed for those parameters with a non-normal distribution.

Table 2: Statistical comparison of data between the males & females in each study group

Parameter	Control group		Type 1 diabetics without microalbuminuria		Type 1 diabetics with microalbuminuria	
	P value	Significance	P value	Significance	P value	Significance
Age(in yrs)	0.86	NS	0.02	S	0.07	NS
Diabetes Duration (in yrs)	NA	NA	0.05	NS	0.47	NS
FBS(mg/dl)	0.55	NS	0.93	NS	0.54	NS
HbA1C	0.62	NS	0.56	NS	0.95	NS
TAG(mg/dl)	0.02	S	0.38	NS	0.39	NS
TC(mg/dl)	0.17	NS	0.80	NS	0.77	NS
LDL-C(mg/dl)	0.12	NS	0.81	NS	0.90	NS
HDL-C(mg/dl)	0.03	S	0.12	NS	0.66	NS
HDL_3-C(mg/dl)	0.45	NS	0.09	NS	0.27	NS
HDL_2-C(mg/dl)	0.17	NS	0.32	NS	0.34	NS

HDL_2: HDL_3 ratio	0.39	NS	0.89	NS	0.15	NS

Females in the control group have significantly lower Serum triglycerides and significantly higher HDL-C than males. This observation is consistent with the fact that estrogen in females stimulates LDL receptor mediated clearance of remnant particles. This significantly higher HDL-C levels are absent in diabetic females.

Comparison of biochemical parameters between the males in each group is depicted in Table 3.There is no significant difference in lipid profile between the controls & Type 1 diabetes normoalbuminurics, similarly there is no significant difference between Type1 Diabetes normoalbuminurics&microalbuminurics. However Type 1 diabetic microalbuminurics have significant difference in routine lipid profile when compared to controls. On examining the mean of individual lipid profile parameters in each study group (Table 1), it is obvious that there is a gradual increase from the control population to the microalbuminuria group.

Table 3: Statistical comparison of parameters between males in the 3 study groups.

Parameter	Controls Vs Type 1 diabetic normoalbuminurics		Controls Vs Type 1 diabetic microalbuminurics		Type 1 Diabetic NormoalbuminuricsVsMicroalbuminurics	
	P value	Significance	P value	Significance	P value	Significance
Age(in yrs)	0.04	S	0.01	S	0.7	NS
Diabetes Duration (in yrs)	NA	NA	NA	NA	0.3	NS
FBS(mg/dl)	1.1E-06	S	7.9E-08	S	0.3	NS
HbA1C	3.5E-21	S	1.1E-17	S	0.2	NS
TAG(mg/dl)	0.98	NS	0.5	NS	0.9	NS
TC(mg/dl)	0.21	NS	0.01	S	0.4	NS
LDL-C(mg/dl)	0.13	NS	0.005	S	0.2	NS
HDL-C(mg/dl)	0.40	NS	0.01	S	0.3	NS
HDL_3-C(mg/dl)	0.17	NS	0.59	NS	0.1	NS
HDL_2-C(mg/dl)	0.85	NS	0.0003	S	1.75E-05	S
HDL_2: HDL_3 ratio	0.39	NS	0.002	S	1.3E-07	S

HDL_3-C shows no significant difference between the different study group males. However HDL_2-C&HDL_2:HDL_3 ratio is significantly higher in controls & Type 1 diabetes normoalbuminurics when compared to Type1diabetes microalbuminurics.

Comparison of biochemical parameters between the females of each group is depicted in Table 4. There is significant difference in TC & LDL-C levels between the controls & Type 1 diabetes normoalbuminurics as well as between controls & Type 1 diabetes microalbuminurics. However there is no difference in the above parameters, between Type 1 diabetic normoalbuminurics&microalbuminurics. So they can be concluded to have no association with microalbuminuria. Total HDL-C is found to be significantly decreased only in type 1 diabetic microalbuminurics when compared to control females.

The HDL_3-Csubfraction again has no significant difference in levels between the 3 female study groups. HDL_2-C&HDL_2:HDL_3 ratio is significantly higher in controls & Type 1 diabetes normoalbuminurics when compared to Type1diabetes microalbuminurics.

Table 4: Statistical comparison of parameters between females in the 3 study groups.

Parameter	Controls Vs Type 1 diabetic normoalbuminurics		Controls Vs Type 1 diabetic microalbuminurics		Type 1 Diabetic NormoalbuminuricsVsMicroalbuminurics	
	P value	Significance	P value	Significance	P value	Significance
Age(in yrs)	0.94	NS	0.37	NS	0.39	NS
Diabetes Duration (in yrs)	NA	NA	NA	NA	0.15	NS
FBS(mg/dl)	0.0001	S	0.002	S	0.26	NS
HbA1C	2E-12	S	7.13E-14	S	0.74	NS
TAG(mg/dl)	0.14	NS	0.31	NS	0.17	NS
TC(mg/dl)	0.009	S	0.0002	S	0.36	NS
LDL-C(mg/dl)	0.002	S	3.04E-05	S	0.09	NS
HDL-C(mg/dl)	0.87	NS	0.0002	S	0.01	S
HDL_3-C(mg/dl)	0.96	NS	0.18	NS	0.27	NS
HDL_2-C(mg/dl)	0.81	NS	5.55E-07	S	0.002	S
HDL_2: HDL_3 ratio	0.92	NS	9.44E-07	S	0.005	S

From the results of the above preliminary statistical comparison between the study groups, an association between HDL_2-C,HDL_2:HDL_3 ratio &microalbuminuria seems to exist.

Univariate logistic regression was used to examine the contribution of the different individual variables in predicting risk for microalbuminuria in Type 1 Diabetic individuals. Urine albumin excretion was considered as the dependent variable and the individual demographic & biochemical parameters were considered as independent variables. The results are depicted in Table 5

Table 5: Analysis of individual variables' ability to predict microalbuminuria development in Type 1 diabetics. (Univariate logistic regression)

Sr.no.	Independent Variable	Odds ratio (O.R.)	95% CI	p-value
1.	Age	1.02	0.97,1.07	0.42
2.	Sex	1.09(with males as reference)	0.48,2.46	0.84
3.	Duration of DM	1.06	0.99,1.14	0.12
4.	FBS	1.00	0.99,1.01	0.15
5.	HbA1c	0.81	0.67,1.12	0.26
6.	TAG	1.01	0.99,1.01	0.26
7.	TC	1.01	0.99,1.02	0.21
8.	LDL-C	1.02	1.00,1.03	0.05
9.	HDL-C	0.94	0.89,0.99	0.02
10.	HDL_2-C	0.74	0.65,0.85	0.00
11.	HDL_2: HDL_3ratio	0.39	0.26,0.58	0.00

The above results show a 6% & 26% protection from development of microalbuminuria for every unit increase in HDL-C&HDL_2-Clevels respectively. The regression analysis with HDL_2:HDL_3 ratio as independent variable indicates a 61% risk protection from development of microalbuminuria for every 0.1 unit increase in the ratio.

Multivariate logistic regression analysis was performed to examine the contribution of the above parameters in predicting risk for microalbuminuria in type 1 diabetics, when adjusted for age & sex; and when adjusted for duration of diabetes, FBS & HbA1c. The results are depicted in Table 6.

Table 6: Analysis of HDL-C, HDL_2-C&HDL_2:HDL_3 ratio ability to predict microalbuminuria development in Type 1 diabetics when adjusted for other co-variates. (Multivariate logistic regression)

Parameters	Regression analysis adjusted for age & sex			Regression analysis adjusted for FBS, duration of DM, HbA1c		
	O.R.	95% CI	p-value	O.R.	95% CI	p-value
HDL-C	0.94	0.89,0.99	0.02	0.94	0.89,0.99	0.02
HDL_2-C	0.74	0.64,0.85	0.00	0.73	0.63,0.85	0.00
HDL_2: HDL_3 ratio	0.38	0.26,0.57	0.00	0.38	0.25,0.58	0.00

The risk protection ratio remains almost the same even in multivariate regression when adjusted for age, sex, duration of diabetes, FBS & HbA1c.

IV. Discussion

Insulin deficiency results in decreased LDL fractional clearance [11], which is the cause for increase in cholesterol as well as LDL levels, observed in Diabetic individuals. This effect is observed in both sexes & in normoalbuminuric as well as microalbuminuric individuals.

Insulin deficiency results in slow catabolism of VLDL by LPL, which could lead to low HDL formation [12]. The point to be noted here is that the association of microalbuminuria is more significant with HDL_2-C levels &HDL_2:HDL_3ratio.Thismay be due to the protective role of HDL – C, specifically HDL_2 in prevention of diabetic nephropathy. HDL, by virtue of its role in reverse cholesterol transport, may have a protective effect on vascular endothelium by decreasing cholesterol accumulation in macrophages, and thus preventing endothelial dysfunction[13], which is responsible for pathogenesis of glomerular damage and microalbuminuria. A decrease in HDL_2-C&HDL_2:HDL_3 ratio indicates decrease in reverse cholesterol transport. Factors influencing reverse cholesterol transport would result in decreased HDL_2 and increased predisposition to endothelial dysfunction and diabetic nephropathy.

HDL_2 is formed from HDL_3 by the activity of LCAT and ABCA1[14]. So a decrease in activity of LCAT or ABCA1 may be the reason behind decreased reverse cholesterol transport leading to low HDL_2-C&HDL_2:HDL_3 ratio. Increase in CETP activity resulting in increased cholesterol ester transfer to other apo B containing lipoproteins such as LDL, lead to increased conversion of HDL_2 to HDL_3[15]and may also be the reason for decrease in HDL_2-C levels &HDL_2:HDL_3 ratio in some individuals.

Decrease in activity of ABCA 1 [16] and increase in CETP activity [17]has been shown to produce a similar profile of HDL subfractions in other studies. The above mentioned alteration in level of activity of these

proteins could be due to polymorphisms of genes encoding the above proteins[18].These polymorphisms have already been found to be linked to coronary artery disease [19,20]. This would explain the difference in predisposition of different Type1 individuals to development of microalbuminuria. It also explains the familial clustering of microalbuminuria cases.

Insulin deficiency and glycemic control may also be hypothesized to influence the activity of these proteins and enzymes but in this study, we observe that the difference in HDL and its subfraction levels between the Type1 diabetic normoalbuminurics&microalbuminurics persist even after adjustment for duration of diabetes, FBS & HbA1c levels. All this strongly favour the presence of genetic reasons such as polymorphisms, in the genes encoding the above mentioned proteins which result in decreased HDL_2-C&HDL_2:HDL_3 ratio & predispose to development of microalbuminuria.

V. Conclusion

Individuals with Type 1 Diabetes having low serum HDL levels, associated with low HDL_2 subfraction and HDL_2:HDL_3ratios are more likely to have microalbuminuria. The association between HDL and microalbuminuria is independent of the individuals' age, gender, duration of diabetes and glycemic control.

Comparing the facts that there is familial clustering of diabetic nephropathy and that the individuals with low HDL_2-C are at increased risk of nephropathy, there is a possibility of polymorphisms of proteins involved in HDL metabolism, that is CETP and ABCA 1 may be the reason for the familial clustering and the differential susceptibility of individuals to develop nephropathy.

Contrary to the previous belief that dyslipidemia is associated only with macrovascular complications of diabetes namely cardiovascular disease, the dyslipidemia documented in Type 1 diabetics in our study and in others, is associated with nephropathy. Therefore Type 1 Diabetic individuals should have regular monitoring of their lipid profile for early detection of any abnormalities. Dyslipidemia if present should be treated aggressively to prevent complications.

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