Metabolism
Clinical and Experimental
VOL 38, NO 12 DECEMBER 1989

Correlation of Plasma Lipid Fractions With Colorimetrically Determined Glycated Hemoglobin in a Nondiabetic Population

Joan Prat, Reinald Pamplona, Albert Sorribas, Silvestre Martin, Merce Viñallonga, and Ramón Segura

To evaluate the incidence of protein glycation (measured as glycated hemoglobin) on plasma lipid levels in a free-living population, a colorimetric method has been used to detect 5-hydroxymethylforfural (HMF) released from glycated hemoglobin (GHb) and enzymatic methods have been used to evaluate total cholesterol, high-density lipoprotein (HDL) cholesterol, fasting glycemia, and total plasma triacylglycerides. These results have been used to calculate low-density lipoprotein-(LDL) cholesterol levels with the Friedewald formula. Results show that a positive correlation exists in men, but not in premenopausal women, between GHb and fasting glucemia ($P < 10^{-14}$), GHb and total plasma cholesterol ($P < .001$), GHb and LDL cholesterol ($P < .0001$), and GHb with the atherogenic index total/HDL cholesterol ($P < .0001$), whereas a negative correlation was shown between GHb and HDL cholesterol ($P < .05$). Fasting glycemia does not correlate with cholesterol lipoprotein fractions, but correlates well with total triacylglycerides. These correlations are significant when they are adjusted by age and body mass index. It has also been observed that the positive correlation between glycation of hemoglobin and total cholesterol occurs constantly at all glycohemoglobin levels, and affects all cholesterol fractions analyzed. This suggests an increased atherogenicity with increasing glycohemoglobin levels. Comparisons between groups with high and low GHb levels show variations in the order of 6.5% of total cholesterol levels and a major proportion in the different cholesterol fractions. The higher-range "glycated" populations show total cholesterol, LDL cholesterol and HDL cholesterol values significantly different from the lower-range "glycated" population. It is concluded, therefore, that GHb is a good indicator of cholesterol changes related to glucemic status, and that the glycation process may affect in an important way the plasma levels of cholesterol fractions, not only in diabetic levels, but also in nondiabetic levels.

NONENZYMATIC GLYCATION REACTION involves the covalent attachment of Glucose or other monosacharides to the amino groups of proteins in the absence of any type of enzyme. Consequently, the amount of modified protein only depends on the concentration of the reactants.

This reaction, known by nutritional scientists as a step of the Maillard transformation, was proposed by Bunn et al[1] in 1976 to be taking place in life tissues in his explanation of the chromatographic heterogenicity of the hemoglobin A found by Allen et al[2] in 1958. According to Bunn, blood hemoglobin is glycated by plasma glucose in a two-step reaction; in the first step, a labile aldoamine linkage between hemoglobin and glucose is formed; in the second step, this linkage is slowly replaced by a very stable ketoamine form via Amadori rearrangement of the modified glucose molecule.

Further demonstration of this nonenzymatic mechanism came after the finding by Trivelli et al[3] of elevated levels of this form of hemoglobin in diabetic patients. Later, several investigations (Koenig et al[4]) have reported the strong correlation between glycated hemoglobin and plasma glucose levels. All these findings have led to the conclusion that glycation levels of hemoglobin reflect the average glucemia levels during the erythocyte life span.

Recent studies have reported that the glycation process can affect almost all extracellular proteins. Sasaki and Cottam,[5] Witztum et al,[6] and Curtiss and Witztum,[7] for instance, reported that almost all plasma apolipoproteins can be glycated to some extent. Particularly, apolipoprotein (apo) B has been shown to have more than twelve glycation sites (Schleicher et al[8]).

Nonenzymatic glycation seems to have important functional consequences for some proteins. Thus, glycated low-

From the Facultat de Medicina, Departament de Ciències Mèdiques Bàsiques, Lleida, and the Facultat de Medicina, Departament de Fisiologia i Biologia Cellular, Bellaterra, Barcelona (Spain).

Address reprint requests to Joan Prat, MD, Departament de Ciències Mèdiques Bàsiques, Rovira Roure SN (Hospital Provincial) E-25006 Lleida, Spain.

0026-0495/89/3812-0001$3.00/0

density lipoprotein (LDL) shows a loss of affinity for the apo B/E receptor in human fibroblasts,[6] and in guinea pigs the catabolic rate of glycated LDL is clearly diminished (Steinbrecher et al[9]). Furthermore, glycated LDL shows a decreased ability to inhibit intracellular hydroxymethyl glutaryl (HMG) coenzyme A reductase activity,[6] and does not increase cholesteryl ester synthesis (Gonen[10]), two of the main effects of LDL endocytosis. Excessive glycation may then be a factor that contributes to the increase in plasma cholesterol levels observed in hyperglycemic diabetic subjects.

The effects of nonenzymatic glycation have been studied as a function of plasma glucose concentration on the different lipoprotein fractions and its consequences on the plasma cholesterol and triacylglycerol levels. A gradual but continuous increase has been observed in the levels of total, LDL, and high-density lipoprotein (HDL) cholesterol in proportion to the degree of hemoglobin glycation, going from the low to the high values found within the normal range.

MATERIALS AND METHODS

Study Population

From among the 475 workers of a small local enterprise, 340 agreed to participate in a screening for diabetes during the annual work force check-up. One of the men was excluded because he was known to be diabetic, as were six others on the grounds that they had a fasting plasma glucose level greater than 140 mg/dL. Eleven more of the participants were later excluded when it was learned that they had a diabetic among their immediate relatives. Finally, results of tests carried out on 20 others were not quantified because they were already under medication.

Because of the type of work force recruitment, the study group was composed mainly of adults (mean age 30 years, range 16 to 64 years), 219 (72.5%) of whom were males. Eighty-two of the 83 women participating were premenopausal.

All participants were interviewed personally to obtain data concerning age, diabetes and cardiovascular history, tobacco and alcohol consumption, and hormonal status (women). Moreover, some anthropometric measurements (weight, height, and arterial blood pressure) were taken.

Blood Handling

Blood obtained by vein puncture after overnight fasting was collected in tubes containing ethylenediaminetetraacetic acid as anticoagulant and immediately refrigerated at between 0° and 4°C. The cells were separated from the plasma by centrifugation at 3,000 rpm for 15 minutes.

Glycated Hemoglobin Quantification

For the purposes of this test, the chemical method described by Flückinger et al[11] and later modified by Nicol et al[12] was used. This method is based on the colorimetric detection of 5-HMF released from ketoamine-linked sugar by reaction with tiobarbituric acid.

All reagents used were made by Fluka AG (Buchs, Switzerland).

Hemoglobin Quantification

Hemoglobinometry was carried out according to the recommendations of the International Committee for Standardisation in Hematology.[13]

Packed erythrocytes (0.5 mL), carefully pipetted to avoid contamination with white blood cells, were washed three times with 9.5 mL isotonic saline and then lysed by the addition of 2.5 mL hemolyzing reagent (Triton X-100, diluted 1/1,000). The mixture was shaken vigorously for 15 minutes. The concentration of the resulting hemoglobin solution was adjusted to 10 mg/mL with the lysing reagent.

Quantification of Glycated Hemoglobin

The formation of 5-HMF from the sugar was accomplished by the addition of 1 mL oxalic acid reagent (63 g/L) to 1 mL hemoglobin (10 mg/mL) or to 1 mL fructose (0 to 80 μmol/L) solutions. The reaction test tubes were kept in a water-boiling autoclave at 124°C for 60 minutes. After 10 minutes of depressurization, the reaction tubes were placed in an ice bath for 10 minutes. Each lot contained between 80 and 100 glycohemoglobin tubes, 9 additional reaction tubes containing standards of from 0 to 80 μmol/L fructose solution, and 5 reconstituted-glycohemoglobin reference tubes.

To eliminate lipids and proteins, 1 mL trichloroacetic acid solution (400 g/L) and 200 μL carbon tetrachloride were added to each tube. After 15 minutes of vigorous shaking, the tubes were centrifuged at 10,000 rpm for 10 minutes. One milliliter from the resulting clear supernatant was transferred to test tubes containing 50 μL tiobarbituric reagent (7.21 g/L), and the rest to a tube not containing tiobarbituric acid (blank tube). Both types of tubes were incubated at 40°C for 45 minutes.

Quantification was performed by measuring the absorbance at 443 nm in a Zeiss spectralphotometer (Oberkochen, Federal Republic of Germany) equipped with a quartz automatic casting cell. The zero was adjusted with a tube treated in the same way as the rest of the lot but with 1 mL of distilled water in place of fructose or glycohemoglobin solution. The absorbance of the reaction solution (blank) was subtracted from the absorbance values shown by the tiobarbituric-containing tubes.

Method Precision

The correlation coefficient of the fructose calibration line was greater than 0.99, and linear regression equation variables were very similar in each lot. The intra-lot mean variation coefficient obtained from the five glycated hemoglobin references was 2.77%, and between lots was 3.89%.

Other Biochemical Quantifications

Glucose was determined using the GOD-PAP (Glucose oxidase) method (Boehringer, Mannheim, Federal Republic of Germany) in a Hitachi 737 autoanalyzer (Tokyo, Japan).

Total and HDL cholesterol were determined by a CHOD-PAP enzymatic method. A standard serum (Precilip, Boehringer) was used to assess the precision of the method. Mean relative error was 1.8%. The separation of HDL cholesterol was performed after precipitation of apo-B containing lipoproteins with heparin-$MnCl_2$, according to the method of Burnstein and Samaille[14] as modified by Wanick et al.[15] The variation coefficient of 20 samples of plasma pool was 3.8%.

Total triacylglycerides were determined by the GPO-PAP enzymatic method. The mean relative error of the method using a standard serum (Precilip) was 3.29%.

The LDL-cholesterol values were calculated according to Friedewald et al[16]: LDL cholesterol = Total cholesterol − (HDL cholesterol + triacylglycerides/5).

Statistical Procedures

The Pearson correlation coefficient and multivariate regression analysis were used to evaluate the statistical significance of the tests. The one-way ANOVA test was used to compare population groups. All tests were performed with the SPSS statistical program.

Table 1. Characteristic Parameters of the Study Population

	Total	Men	Women	Non-Alcohol Users	Alcohol Users	Nonsmokers	Smokers
Sample size	302	219	83	129	173	128	174
GHb (μmol/L)	50.7 (6.2)	50.8 (6.6)	50.2 (4.7)	50.7 (5.9)	50.7 (6.4)	51.1 (5.5)	50.4 (6.6)
Age (yr)	30.3 (10.6)	31.1 (11.1)	28.6 (9.2)	29.8 (11.2)	30.8 (10.3)	33.2 (11.6)	28.4 (9.5)
BMI (kg/m^2)	24.4 (5.7)	24.4 (3.4)	24.4 (9.2)	24.2 (3.9)	24.6 (3.5)	25.4 (3.9)	23.7 (3.3)
FPG (mg/dL)	86.7 (21.9)	88.5 (23.5)	81.9 (16.4)	86.0 (25.2)	87.2 (19.1)	85.8 (18.2)	85.8 (24.3)
CHO (mg/dL)	189.9 (35.6)	191.7 (38.1)	185.1 (27.4)	187.4 (35.1)	191.7 (36.0)	194.4 (36.9)	186.7 (34.4)
HDL cho (mg/dL)	53.0 (14.2)	51.7 (14.6)	56.4 (12.6)	54.2 (14.4)	52.1 (14.0)	56.1 (14.5)	50.7 (13.5)
TAG (mg/dL)	116.5 (66.5)	126.0 (77.3)	91.7 (37.9)	102.7 (51.6)	126.8 (72.2)	111.1 (59.4)	120.0 (71.4)
RAT	3.75 (1.25)	3.91 (1.31)	3.33 (0.95)	3.45 (0.89)	3.67 (1.02)	3.46 (1.17)	3.68 (1.11)

NOTE. Numbers in parentheses indicate SD.

Abbreviations: GHb, glycated hemoglobin; BMI, body mass index; FPG, fasting plasma glucose; CHO, total plasma cholesterol; HDL cho, HDL cholesterol; TAG, total plasma triacylglycerides; RAT, ratio of HDL cholesterol to total plasma cholesterol.

RESULTS

In the total population, glycohemoglobin values are compatible with a normal distribution at a 95% confidence level, with a mean value of 50.73 mol/L fructose and a SD of 6.20 (Table 1), and a range of between 35 and 86 μmol/L. To study the different aspects that can affect these results, this population has been divided into different subgroups according to important criteria such as sex, smoking habits, and alcohol consumption. Table 1 shows the characteristic values of these different population subgroups.

A comparison of these different groups shows that women have slightly lower values of glucose, glycated hemoglobin, cholesterol, and triacyglycerides, and higher mean levels of HDL cholesterol. However, all these differences are stastically insignificant. In the same way, alcohol users exhibit similar values to those of non–alcohol users, with the exception of triacylglyceride values, which are clearly higher in alcohol users ($P < .01$). No important disparities were found between the different levels of ethanol consumption.

Significant increase with age was found in almost all parameters measured (body mass index, cholesterol, HDL cholesterol, triacylglycerides, glucose, and glycated hemoglobin) in the different population groups. Moreover, age correlates with the other parameters in all population groups. These results show the important role played by age on these values.

As expected, glycated hemoglobin levels show a significant correlation with fasting plasma glucose levels ($r = .5649$, $P < 10^{-14}$). Furthermore, six subjects with biochemically diagnostic diabetes (fasting plasma glucose > 140 mg/dL), who were excluded from the study, exhibited very high glycated hemoglobin levels (all above the 95% population percentile). More variable results have been obtained for broad borderline fasting plasma glucose values (100 to 140 mg/dL).

The correlations between glycohemoglobin and plasma lipids in the different population groups are shown in Table 2 (owing to the lack of significant differences, the results obtained in the smoking and nonsmoking populations have been omitted). As can be seen, glycated hemoglobin levels correlate in a high degree of statistical significance with total cholesterol, LDL cholesterol, and total-cholesterol/HDL-cholesterol ratio, but not with triacylglyceride concentration in the different population groups, with the exception of women. However, a minor correlation can be observed in alcohol users. Correlation with ages appears significant in all population groups. In contrast (Table 3), FPG correlates only with triacylglycerides, HDL-cholesterol levels and the total-cholesterol/HDL-cholesterol ratio. Additional studies, using multiple regression analysis, have shown that age and glycated hemoglobin are the only determinants of the plasma cholesterol values in this study. Moreover, body mass index, which correlates well with lipid values, does not covariate with glycated hemoglobin in any population group. Accord-

Table 2. Correlation Coefficients and Statistical Significance of the Lipid Parameters of Glycated Hemoglobin in the Different Population Groups

	Total Population		Men		Women		Non-Alcohol Users		Alcohol Users	
		Adjusted		Adjusted		Adjusted		Adjusted		Adjusted
Age	0.248*		0.279*		0.205†		0.372*		0.160†	
BMI	0.073		0.101		0.094		0.084		0.065	
CHO	0.168*	0.088	0.178*	0.069	0.094	0.101	0.323*	0.235‡	0.066	0.023
HDL cho	0.148‡	0.173*	0.178*	0.190‡	0.102	0.112	0.088	0.152	0.192‡	0.158
LDL cho	0.207*	0.157*	0.223*	0.161‡	0.117	0.144	0.323*	0.279*	0.127	0.134
TAG	0.090	0.026	0.088	0.004	0.050	0.026	0.173†	0.090	0.055	0.048
RAT	0.256*	0.185*	0.272*	0.215*	0.131	0.046	0.347*	0.201‡	0.206†	0.154

NOTE. Adjusted columns indicate age- and BMI-adjusted correlations.

Abbreviations: See Table 1.

*$P < .001$.

†$P < .05$.

‡$P < .01$.

Table 3. Comparison of the Age- and BMI-Adjusted and Nonadjusted Correlations of Glycated Hemoglobin and Glucose With Plasma Lipid Fractions

	Not Adjusted		Adjusted	
	GHb	Glucose	GHb	Glucose
Age	0.2488*	0.1561†		
BMI	0.0736	0.0372		
CHO	0.1682*	0.1047	0.0888	0.0714
HDL	0.1486†	0.1216	0.1734†	0.1500†
LDL	0.2076*	0.0986	0.1577†	0.0700
RAT	0.2562*	0.1865*	0.1857*	0.1624†
TAG	0.1606†	0.1925*	0.0269	0.1797†

*$P < .001$.
†$P < .05$.

ingly, age-adjusted and body mass index–adjusted correlations of glycated hemoglobin with plasma cholesterol fractions remain significant for all values in all population groups with the exception of total cholesterol.

To evaluate the size of the effect of glycated hemoglobin on plasma lipid age-adjusted values for all glycated hemoglobin levels, the study population has been divided into quintiles. As can be seen in Table 4, a gradual increase in total cholesterol as well as in LDL cholesterol and triacylglycerides takes place from lower to higher quintiles, parallel to the increase in glycated hemoglobin. Likewise, mean values of HDL cholesterol decrease with glycohemoglobin levels. Figure 1 shows the changes in total cholesterol and its fractions in the different quintiles in this male population group. Not only the sharp increase in total cholesterol levels but also its distribution in the different fractions may be easily appreciated.

Quintiles I and V represent the extreme groups for glycated hemoglobin levels in the healthy population. In these groups, the observed differences in cholesterol fractions concentration are statistically significant, founding an increase in total and LDL cholesterol, the atherogenic index of total-cholesterol/HDL-cholesterol, and a reduction in HDL cholesterol. However, important differences in triacylglyceride levels are not significant, probably because of the important variance of this value.

In conclusion, in our study population lipoproteic fractions of plasma cholesterol are elevated in individuals with high glycated hemoglobin levels compared with those subjects who have low glycated hemoglobin levels. Such differences are observed in a higher-level atherogenic distribution of plasma cholesterol in the population that has high glycated hemoglobin levels because of a slow increase of mean levels. Despite the covariation found with age, adjustment of the data with age, and body mass index does not have a substantial effect on the significance of the correlations.

DISCUSSION

Main cohort studies have shown that clinical diabetes increases twofold the chances of dying of coronary and cerebrovascular diseases. Likewise, studies based on mortality records show that cardiovascular disease accounts for 60% to 70% of total causes of death in diabetics. This increased cardiovascular mortality rate is usually attributed to the high incidence of atherosclerotic disease found in diabetics.[17]

These observations have stimulated the analysis of cardiovascular risk factors shown by diabetics, especially plasma lipid alterations. There is evidence that plasma triacylglycerides are elevated in diabetic patients, mainly in those who are not well controlled. At the same time, a decrease in the levels of HDL cholesterol have been reported by several, but not all, authors.[18] However, total- and LDL-cholesterol levels have not always been found to be elevated in diabetic patients.[19]

Table 4. Mean, Standard Deviation, and Range Values of the Different Parameters Measured in the Study Population Divided Into Quintiles of Glycated Hemoglobin Distribution

	I		II		III		IV		V	
Quintile	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
N	60		60		61		60		61	
Age	27.5	9.2	28.3	11.3	28.2	8.5	31.3	11.7	34.7	13.0
Range	16-55		17-58		18-53		18-56		17-64	
BMI	23.8	3.7	24.1	3.5	24.4	5.2	24.8	3.8	24.4	3.1
Range	19-42		20-33		18-42		18-37		18-32	
GHB	43.7	2.6	47.8	0.8	50.3	0.6	52.7	0.8	58.9	7.3
Range	35-46		47-49		50-51		52-54		55-86	
GLU	85.4	20	82.4	16	82.1	16	87.9	18	95.5	31
Range	52-134		50-130		50-127		54-123		62-129	
CHO	183.1	27	187.0	30	191.9	36	192.5	39	195.3	41
Range	137-252		132-260		141-305		142-333		151-339	
HDL	55.2	14	54.9	12	54.3	12	50.3	14	50.2	16
Range	25-89		33-92		24-82		26-89		26-108	
LDL	103.6	29	113.9	26	114.4	31	116.9	35	119.3	40
Range	37-179		61-164		57-219		65-205		61-167	
RAT	3.55	1.1	3.36	0.9	3.69	1.0	3.96	1.5	4.18	1.4
Range	2.2-7.2		2.3-6.2		2.0-7.4		2.4-8.4		1.9-8.5	
TAG	121.4	65	90.1	37	115.5	68	126.3	66	129.1	81
Range	46-382		42-200		48-432		42-357		42-503	

Abbreviations: See Table 1.

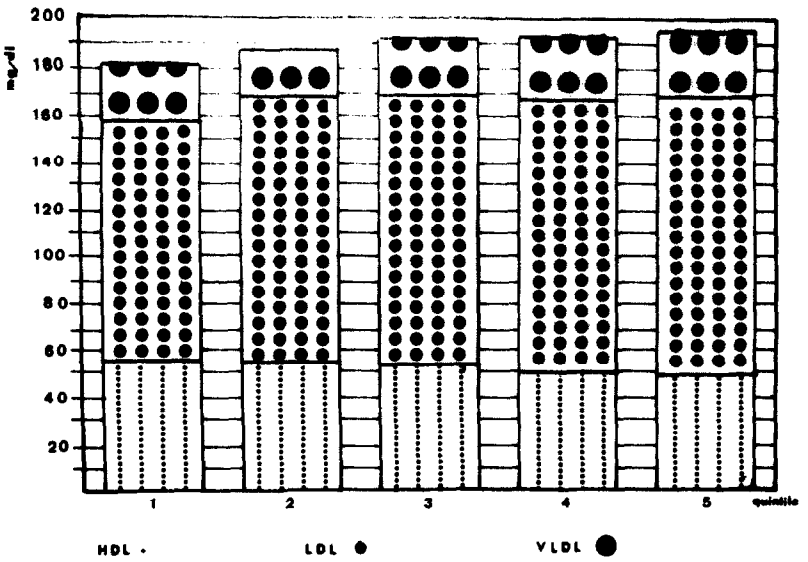

Fig 1. Total cholesterol levels and lipoprotein distribution in quintiles 1 to 5. (Very-low-density lipoprotein [VLDL] is calculated according to Friedewald; triacylglycerides/5.)

Thus, these changes cannot explain the excessive cardiovascular mortality rate seen in diabetics.[20]

Clinical diabetes is only the end stage of a progressive impairment of insulin action. It is known to be preceded by a long period of time (more than a decade) of asymptomatic loss of insulin activity, a period which may be crucial for the initiation and establishment of vascular lesions and clinical complications.[21]

Some prospective studies have shown that lipid metabolism is altered in the prediabetic state.[22] However, data obtained by classical methods used to assess diabetic status (fasting plasma glucemia and postload plasma glucemia) do not show a clear and constant relationship with the cardiovascular mortality risk and plasma lipid alterations in this type of population.[23,24] It is important to note that the only etiological link between diabetes and dislipemia, prior to the protein glycation hypothesis that we propose, was based on the metabolic effect of insulin on plasma triacylglyceride concentrations.

In contrast to the classical parameters used until now, the degree of protein glycation provides not only a tool for an accurate evaluation of the averaged glucemia, independent of short-term variations, but also a pathophysiological mechanism to explain the cardiovascular complications perceived in this kind of patient.

Several studies designed to establish the correlation between glycated hemoglobin and plasma cholesterol metabolism indicators in both diabetic[25-29] and nondiabetic populations[30] show a strong correlation between glycated hemoglobin and total plasma cholesterol, LDL cholesterol, and total/HDL or LDL/HDL-Cholesterol ratios. Nevertheless, the negative correlation between glycated hemoglobin and the HDL-cholesterol fraction seems to be weaker, being found insignificant by Kennedy et al[25] and Barret-Connor et al,[30] and slightly significant in this study and others.[26-29] In addition, some studies reported a strong correlation in respect to triacylglyceride levels.

The results obtained in this study show an acceptable concordance with the data obtained by Barret-Connor et al,[30] despite the differences in the expression of the glycated hemoglobin values. The distribution of glycated hemoglobin values in both studies is normal. Fasting plasma glucose, cholesterol and triacylglycerides values are lower in our study population, while HDL-cholesterol levels are higher. This can be explained by the lower age of our population (mean 30 *v* 65 years) and the predominance of blue-collar employees in our group, together with other factors which also have cultural and dietary implications. Correlation coefficients established in this study are slightly elevated in respect to cholesterol, LDL-cholesterol, and HDL-cholesterol levels, but are much higher in respect to the LDL/HDL-cholesterol ratio ($r = .251$ to 0.12) and of fasting plasma glucose ($r = .56$ to 0.20). The high correlation coefficient found between FPG and glycated hemoglobin may be attributed in this case to the accuracy and precision of the TBA-colorimetric method used in this study.

An important discrepancy between these papers appears in the correlation of the glycated hemoglobin values with age. In this population, age correlates significantly with almost all parameters measured, including glycated hemoglobin levels, in all categories, whereas in the Barret-Connor study, the correlation coefficient of age with this value is very low and, therefore, insignificant. In fact, other populations studied with a more elevated mean age showed a lower correlation between these two parameters.

A second important discrepancy is found in the fact that, whereas Barrett-Connor et al mention correlations that are similar in both the female and the male group, in our female study group correlations between glycated hemoglobin and lipids are lower than in males and are insignificant. In our opinion, these differences can be attributed to the different hormonal status of both populations. Thus, in spite of the lack of information on this question, it may be the case that the female group in Barret-Connor's study, with a mean age of 65 years, was composed mainly of postmenopausal women, while in our population (mean age 28 years) only one subject declared herself to be postmenopausal. The importance of the hormonal status in females has been discussed at length and it appears to be a crucial factor in the determination of the cholesterol metabolism in women.

In our opinion, the results obtained by using glycated hemoglobin for the characterization of diabetic status are much more homogeneous than those obtained with punctual observations of carbohydrate metabolism state, despite the lack of a definite reference method and reference levels for protein glycation analysis. Thus, the determination of glycohemoglobin levels suggests that the continuous distribution of average glucemia levels found in a normal population has a corresponding continuous distribution of plasma lipoprotein cholesterol indicators. This variation is not due to an increase of the number of hypercholesterolemic subjects, but rather to an increase of the average in the normally ranged population, an interpretation which appears to be in accordance with the hypothesis of a direct effect of glycation of plasma apolipoproteins on plasma lipid levels. Furthermore, the increase in plasma cholesterol levels should not be underestimated; by applying risk coefficient for plasma cholesterol found in the Multiple Risk-Factor Intervention Trial,[31] people found in the higher-level quintile are already at double the risk for cardiovascular disease in comparison with those found in the lower-level quintile.

Microcolumn cation-exchange chromatography has been used in all epidemiological studies performed until now because it is the usual routine method in clinical laboratories. However, microcolumns are difficult to use when the daily flux of samples is intense, as occurs in large epidemiological studies. In contrast, a colorimetric method, which is tedious and may be cumbersome to use with a small number of samples, is appropriate for processing large quantities of samples because all incubation steps can be accomplished simultaneously. Other advantages of the method included the alternative use of capillary[32] or dried[33] samples and the possibility of keeping the samples prior to the analysis for a long time. Correlations with other methods found in our (unpublished results) and other laboratories have been very satisfactory.

ACKNOWLEDGMENT

The authors acknowledge the help of Montse Iglesias and Jaume Prat in the preparation of this manuscript, and thank Dr Brian J. Worsfold for correction and improvement of the English text.

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