

Acute-phase proteins among patients with type 1 diabetes

MB Gomes¹, LJ Piccirillo¹, VG Nogueira¹, HJ Matos²

SUMMARY

Objective: To determine whether young type 1 diabetic patients without clinical microvascular or macrovascular complications have altered levels of acute-phase proteins (AFP), α_1 -acid glycoprotein (AGP), C-reactive protein (CRP) and fibrinogen and whether their AFP levels are related to glycemic control.

Research Design and Methods: We studied cross-sectionally 48 type 1 diabetic outpatients (25 males) aged 19.9 ± 9.8 years with a duration of diabetes of 5 (1-21) years, without clinical chronic complications and 66 non-diabetic subjects (26 males) aged 23.1 ± 10.9 years. Inclusion criteria were normoalbuminuria, normal eye funduscopy, and no evidence of cardiovascular disease or neuropathy.

Results: High CRP [0.23 (0.01-2.90 l) vs (0.14 (0.01-2.41l) mg/dl $p = 0.01$] and AGP [53.5(40-78) vs 40.0(40-115) mg/dl $p = 0.0001$] levels were found in patients with type 1 diabetes compared to nondiabetic subjects. In the pooled group studied, AGP was correlated with CRP, HbA_{1c}, fasting plasma glucose (FBG) and AER and CRP was correlated with HbA_{1c} and AER. The correlation of AGP and CRP with AER persisted after controlling for HbA_{1c} and FBG. Stepwise multiple regression with AGP as the dependent variable showed that FBG and HbA_{1c} were the significant independent variables. No correlation between AFP and HbA_{1c} and FBG was observed in the diabetic group.

Conclusions: According to our results, AFP, a known marker of low-grade chronic inflammation, are increased in patients with type 1 diabetes probably independently of glycemic control and the presence of clinical microvascular or macrovascular disease. The influence of AFP on the development of chronic complications in patients with type 1 diabetes must be addressed in prospective studies.

Key-words: Acute-phase Proteins · Diabetes Type 1 · Chronic Complications.

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RÉSUMÉ

Protéines de phase aiguë chez des patients atteints de diabète de type 1

Objectif : Déterminer si des diabétiques de type 1 indemnes de complications microvasculaires ou macrovasculaires cliniques présentent des anomalies des concentrations plasmatiques des protéines de phase aiguë (AFP), d' α_1 - glycoprotéine acide (AGP), de C-réactive protéine (CRP) et de fibrinogène et s'il existe une relation entre les concentrations plasmatiques d'AFP et l'équilibre glycémique.

Méthodes : Nous avons étudié de façon transversale 48 diabétiques de type 1 ambulatoires (25 hommes) âgés de $19,9 \pm 9,8$ ans avec une durée de diabète de 5 (1-21) ans, sans complications chroniques cliniques et 66 sujets non diabétiques (26 hommes) âgés de $23,1 \pm 10,9$ ans. Les critères d'inclusion étaient une normoalbuminurie, la normalité du fond d'œil, et l'absence de maladie cardiovasculaire ou de neuropathie.

Résultats : On a observé chez les diabétiques de type 1 par rapport aux témoins des concentrations plasmatiques de CRP [0,23 (0,01-2,90 l) vs (0,14 (0,01-2,41l) mg/dl $P = 0,01$] et d'AGP [53,5 (40-78) vs 40,0(40-115) mg/dl $P = 0,0001$]. Dans l'ensemble du groupe étudié, l'AGP était en corrélation avec la CRP, l'HbA_{1c}, la glycémie à jeun et l'albuminurie (AER), et la CRP était en corrélation avec l'HbA_{1c} et l'albuminurie. La corrélation de AGP et CRP avec AER persistait après ajustement pour l'HbA_{1c} et la glycémie à jeun. La régression multiple pas à pas avec l'AGP comme variable dépendante a montré que la glycémie à jeun et l'HbA_{1c} étaient des variables indépendantes significatives. Aucune corrélation entre AFP et HbA_{1c} et glycémie à jeun n'a été observée dans le groupe diabétique.

Conclusions : Selon nos résultats, les AFP, marqueurs connus d'inflammation chronique de faible niveau, sont augmentées chez les diabétiques de type 1 probablement indépendamment du contrôle glycémique et de la présence de complications micro-ou macrovasculaires cliniques. L'influence des AFP sur le développement des complications chroniques chez le diabétique de type 1 doit être étudiée de manière prospective.

Mots-clés : Protéines de phase aiguë · Diabète de type 1 · Complications chroniques.

Address correspondence and reprint requests to:
MB Gomes, Estrada Barra, 1006 bl. 3/502, Rio de Janeiro,
Brazil CEP 22648-900.
mariliab@uerj.br

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¹ Department of Medicine, Diabetes Unit, State University Hospital of Rio de Janeiro, Brazil

² Medical Informatics Department, State University Hospital of Rio de Janeiro, Brazil.

Today diabetes mellitus is considered as an independent risk factor for cardiovascular disease. Although most studies have been conducted on patients with type 2 diabetes, a high mortality and morbidity from cardiovascular disease in patients with type 1 diabetes was also observed [1]. The underlying mechanisms that cause accelerated atherosclerosis in patients with type 1 diabetes are poorly understood. In these patients the major cardiovascular risk factors like arterial hypertension and lipid abnormalities are related to the development of microalbuminuria and could partly account for the increased mortality observed [2]. Many recent observations have related atherosclerosis to chronic low-degree inflammation characterized by an increase in circulating acute-phase proteins produced by the liver such as α_1 -acid glycoprotein (AGP), C-reactive protein (CRP) and fibrinogen [3-6]. Based on the above facts, it is possible that high levels of acute-phase proteins in patients with type 1 diabetes may be one of the multiple factors involved in the network of endothelial dysfunction in these patients. All of these acute phase proteins, AGP, CRP and fibrinogen, have been analyzed in patients with type 2 diabetes and most of the studies have shown their effects on cardiovascular risk [3-6] and on insulin resistance [7-8] in non-diabetic subjects as well in diabetic patients [9]. However, few studies have been conducted on young patients with type 1 diabetes without clinical cardiovascular disease and microvascular complications or on young subjects without diabetes to assess whether increased levels of acute-phase proteins could exist independently of the presence of diabetes and its related chronic complications [10-15].

The objectives of the present study were to determine whether levels of acute-phase proteins are increased in patients with type 1 diabetes without evidence of clinical chronic complications and whether the levels of these acute-phase proteins are related to glycemic control.

Research design and methods

The study was performed on a group of 48 type 1 diabetic outpatients (25 males and 23 females) consecutively attended at the diabetes clinic of the State University of Rio de Janeiro, aged 19.9 ± 9.8 years and with a duration of diabetes of 5(1-21) years, and on 66 non-diabetic subjects (staff members, hospital employees, and medical students with FBG < 110 mg/dl), 26 males and 40 females aged 23.1 ± 10.9 years. Pa-

tients and non-diabetic subjects were matched for age, gender, smoking habit and Tanner stage [16]. According to duration of diabetes there were 25 (52.1%) patients with less than 5 years, 15 (31.3%) ranging from 5 to 10 years and 8 (16.7%) with more than 10 years. The inclusion criteria were patients with diabetes diagnosed before 30 years of age using insulin since the diagnosis without symptoms of diabetes decompensation or acute infection and with satisfactory clinical control. The exclusion criteria were evidence of clinical cardiovascular disease, and self-reported underlying diseases that could be associated with an increase of acute-phase proteins. All patients with microalbuminuria, hypertension, retinopathy and clinical neuropathy and peripheral arterial disease were also excluded. The subjects were asked to provide three accurately timed overnight urine samples over three months. The insulin dose was 0.8 ± 0.4 U/kg/day. As mentioned earlier, subjects with a self-reported past or present history of myocardial infarction, angina pectoris, stroke and peripheral vascular disease and in response to the World Health organization cardiovascular questionnaire [17] were excluded. All subjects were submitted to a 12-lead resting ECG and when alterations were detected according to the Minnesota coding [18] they were also excluded from the study. Normal serum creatinine and urinary sediment were used to exclude primary renal disease. All subjects received written instructions and gave informed consent to participate in the study. The experimental design was approved by the local Ethics Committee.

All subjects passed urine immediately before 8 p.m., discarded this sample and recorded the time. All the urine passed until 6 a.m. was collected into containers without a preservative to determine albumin excretion rate (AER). The urine volume was recorded and aliquots were stored in glass tubes at -70° Celsius until analysis. Fasting blood samples were also obtained. Urinary albumin concentration was estimated by double antibody radioimmunoassay (Diagnostic, Los Angeles, CA, sensitivity of $0.3 \mu\text{g/ml}$) with an intra-assay and interassay coefficient of variation of 2.7% and 3.5%, respectively. Based on AER, only subjects with normoalbuminuria (AER < $20 \mu\text{g/min}$ in two out of three overnight urine specimens) were included. Each urine specimen was tested for bacteriuria and when the latter was present ($> 10^5/\text{mm}^3$) the urine was discarded. Blood pressure was measured by the same observer 3 times after a 5-min rest in the supine position using a standard mercury sphygmomanometer. Diastolic blood pressure (DBP) was recorded at the disappearance of Korotkoff sounds (phase 5). The mean of the measurements of systolic and diastolic blood pressure was used. Hypertension was defined in adults as systolic blood pressure (sBP) > 140 mmHg and/or DBP > 90 mm Hg or any value in patients under antihypertensive treatment and in children and adolescents according to described in a consensus [19]. Retinopathy was assessed by fundoscopy through a dilated pupil by ophthalmoscopy performed by

Abbreviations

AER :	albumin excretion rate
AGP :	α_1 -acid glycoprotein
BMI :	body mass index
CRP :	C-reactive protein
FBG :	fasting blood glucose
HbA _{1c} :	glycated hemoglobin
dBp :	diastolic blood pressure
sBP :	systolic blood pressure

the same ophthalmologist and only patients with normal examination in both eyes were included. Blood samples were drawn in the morning between 7:30 and 8:30 am after the last urine collection and an overnight fast. After centrifugation at 2500 g for 15 min at room temperature (19° Celsius) aliquots of plasma and sera were stored at -70° Celsius until analysis. Serum α_1 -acid glycoprotein (AGP) and plasma fibrinogen (4.5 ml of blood collected without stasis in vacuum tubes containing 0.5 mL 3.2% buffered sodium citrate) were determined by immunoturbidimetry assay (Behring Turbimer, Germany) and serum C-reactive protein (CRP) was measured using a highly sensitive immunonephelometry assay (APTEC-Selectra Merck, Germany). Detection limits and intra-assay CV% were: AGP 40 mg/dl (4.1%), fibrinogen 180 mg/dL (5.2%), and CRP 0.01 mg/dL (2.9%). HbA_{1c} was determined by high performance liquid chromatography (L-9100 Merck Hitachi-Frankfurt, Germany) (reference range: 4.5-6.2%). FBG, triglyceride, HDL cholesterol and total cholesterol levels were measured by enzymatic techniques using an auto-analyzer (Cobas-Mira Roche). LDL cholesterol was calculated by the method of Friedwald [20]. Weight and height were measured to the nearest 0.1 kg and 0.1 cm, respectively. Body mass index (BMI) (kg/m²) was calculated from these measurements. Body fat composition was assessed by bioimpedance in the fasting state.

Statistical analysis

The Kruskal-Wallis and Mann-Whitney U tests were used for comparisons between groups of variables not normally distributed and the Student t-test and ANOVA were used for the other comparisons. The Chi-square test with Yates correction and Fisher's exact test were used for comparison of categorical variables. For Pearson correlation and stepwise multiple regression analysis the variables not nor-

mally distributed were log transformed. Stepwise multiple regression analysis was fitted to log AGP and CRP as the dependent variable and to all the variables with $p < 0.1$ in Pearson correlation as independent variables. Variables of interest were also added to the model (age, gender, BMI, fat mass, smoking habit, diabetes duration and Tanner stage). In the final fitted model only variables with $p \leq 0.05$ were considered. Partial correlation using Pearson's correlation coefficient was also performed. Sensitivity, specificity, and positive Likelihood Ratio were calculated for different cut-offs of AGP as the diagnosis of diabetes type I was concerned. When indicated 95%CL was presented. These analyses were performed using the statistical package for the social sciences (SPSS, version 10.0), EPI INFO (version 6.0), and Splus 2000. Normally distributed values were expressed as mean (SD) and values not normally distributed as median (minimum/maximum). A two-sided P value of less than 0.05 was considered to be significant.

Results

According to Tanner stage, the total diabetic population studied consisted of six pre-pubertal and 15 pubertal individuals and 27 adults, and the non-diabetic population consisted of eight pre-pubertal and 18 pubertal individuals and 40 adults. BMI and % body fat were higher in non-diabetic subjects than in patients with type 1 diabetes (22.1 ± 3.1 vs 20.4 ± 2.9 kg/m², $p = 0.01$, and 26.4 ± 6.5 vs $21.6 \pm 7.7\%$, $p = 0.0008$, respectively). No difference was noted between patients and non-diabetic subjects with respect to the other clinical and/or demographic variables analyzed. Concerning the acute-phase proteins, higher serum levels of AGP [53.5(40-78) vs 40.0(40-115) mg/dl $p = 0.00001$] (Fig 1) and CRP [0.23 (0.01-2.90) vs (0.14 (0.01-2.41)) mg/dl $p = 0.01$] (Fig 2) were found in patients with type 1 diabetes compared

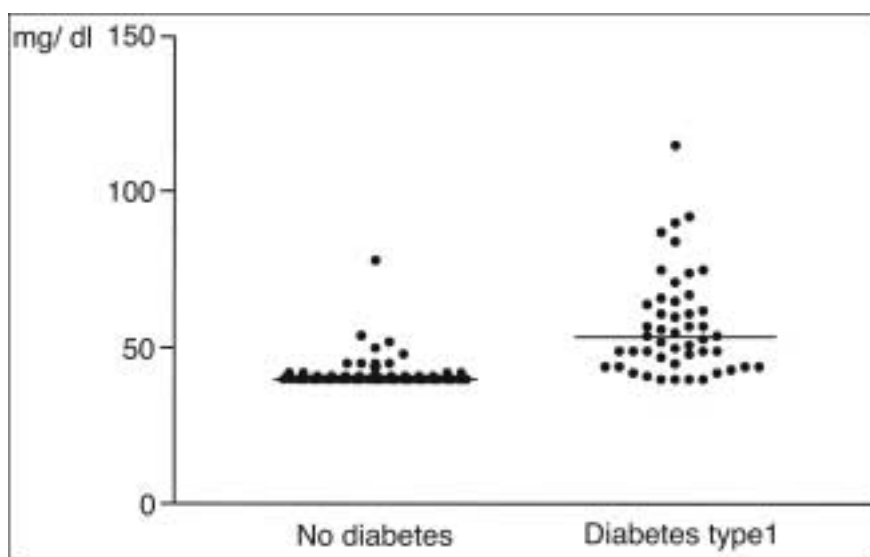


Figure 1
Alpha-1 Acid Glycoprotein distribution in patients with diabetes type 1 and non-diabetic subjects.

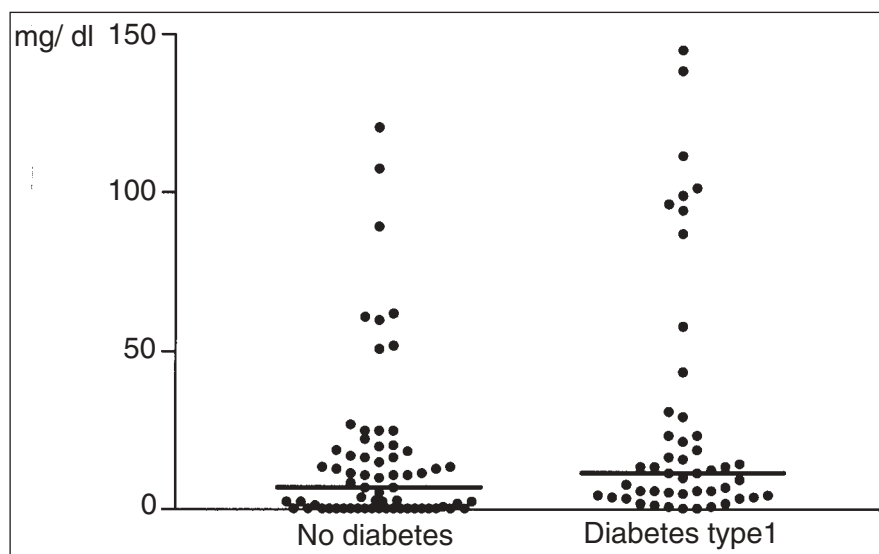


Figure 2
C-reactive protein distribution in patients with diabetes type 1 and non-diabetic subjects.

to non-diabetic subjects. These results persisted after adjustment for smoking habit. These data are shown in *Table 1*.

In the total population studied (patients with type 1 diabetes and non-diabetic subjects), AGP was correlated with CRP ($r = 0.28$ $P = 0.002$), HbA_{1c} ($r = 0.53$ $p = 0.000$), fasting plasma glucose ($r = 0.54$ $p = 0.000$) and AER ($r = 0.52$ $p = 0.000$). Further analysis showed that the correlation with AER persisted after controlling for HbA_{1c} ($r = 0.23$ $p = 0.01$) and fasting plasma glucose levels ($r = 0.29$ $p = 0.002$). After adjustment for age, gender, smoking habit, BMI and fat mass, stepwise multiple regression applied to the data with HbA_{1c} , CRP, fasting plasma glucose, and fibrinogen as independent variables and AGP as dependent variable showed that fasting plasma glucose ($r = 0.53$ $r^2 = 0.29$ [95% CL (6.5 E-05 to 8,5 E-04), B 3.7 E-04 $p = 0.000$]) and HbA_{1c} ($r = 0.57$ $r^2 = 0.32$ [95% CL (0.002 - 0.02), B 0.01 $p = 0.000$]) were the significant independent variables. In the total population studied, CRP was correlated with HbA_{1c} ($r = 0.18$ $p = 0.048$) and AER ($r = 0.26$ $p = 0.006$) and tended to be correlated with fasting plasma glucose ($r = 0.16$ $p = 0.07$). Further analysis showed that the correlation with AER persisted after controlling for HbA_{1c} ($r = 0.19$ $p = 0.04$) and fasting plasma glucose levels ($r = 0.20$ $p = 0.03$). In the same model of stepwise multiple regression above described with CRP as dependent variable no significant independent variable was observed.

In the diabetic group no correlation was found between acute-phase proteins and all the demographic, clinical and laboratory variables analyzed. In non-diabetic subjects a correlation was found between fibrinogen and BMI ($r = 0.25$ $p = 0.04$) and HbA_{1c} ($r = 0.24$ $p = 0.044$). Further analysis did not show any other correlation in the groups studied.

To evaluate the importance of AGP concentration with respect to diabetes type I, a ROC curve was constructed for different AGP concentrations, ranging from 45 to 50 mg/dL, showing that the best cutoff was 47mg/dL. Sensitivity and

Specificity for 47 mg/dL were 72.9% and 92.4%, respectively, with a positive likelihood ratio of 9.59, area under the curve of 0.827, 95% CL (0.743-0.911), and SEM of 0.0429 (*Fig 3*).

Conclusions

Our cross-sectional study confirmed elevated serum levels of acute-phase proteins in patients with type 1 diabetes without clinical microvascular or macrovascular complications compared to subjects without diabetes. A high association was observed with CRP and AGP. AGP is a glycoprotein with a 42% content of carbohydrate whose specific immune regulatory function is related to its carbohydrate moiety, mainly in terms of the increase of fucosylation and degree of branching [22-24]. With respect to the role of AGP as a marker of disease, it has been discussed that high AGP levels may be associated with acute myocardial infarction [25], cancer of the lung [25] and diabetes type 2 [21]. Recently it was described that urinary excretion of AGP was a predictor of all cause and cardiovascular mortality in patients with type 2 diabetes [26] and that high levels of serum AGP may predict the development of diabetes type 2 [27]. In the present study the concentrations of AGP in patients with diabetes type I were shown to be over 47 mg/dL according to ROC data. Whether AGP could also be a predictor of diabetes type I as shown in diabetes type 2 [27] remains to be demonstrated. So far few studies have been conducted on patients with type 1 diabetes [12, 28], with different results. One study reported high levels of AGP in patients with diabetes without distinguishing the type of diabetes [28] and another did not find differences between patients with type 1 diabetes and matched controls [12]. This latter study examined some details of the structure of AGP in type 1 diabetes and found increase fucosylation of AGP with a similar degree of branching (diantennary glycans) in comparison to matched controls [12]. In the total population studied here

Table 1

Demographic, clinical and laboratory data of the patients with type 1 diabetes and matched subjects without diabetes.

Variables	No Diabetes	Type 1 Diabetes	Significance level (p)
N (number)	66	48	
Gender (M/F)	26/40	25/23	
Age (years)	23.1 ± 10.9	19.9 ± 9.8	0.11
Age at diagnosis (years)		13.7 ± 9.3	
Duration of diabetes (years)		5 (1.0-21)	
BMI (kg/m ²)	22.1 ± 3.7	20.4 ± 2.9	0.01
Fat mass (%)	26.4 ± 6.5	21.6 ± 7.7	0.0008
sBP (mmHg)	106.3 (90.0-130.7)	107.6 (96.0-133.0)	0.29
dBP (mmHg)	68.9 ± 7.2	70.7 ± 7.2	0.2
FBG (mg/dl)	78.7 (64.0-97.0)	176.0 (59.0-489.0)	
HbA _{1c} (%)	4.28 ± 0.45	8.25 ± 2.14	
Cholesterol (mg/dl)	165.5 ± 32.8	169.5 ± 40.6	0.7
HDL- Cholesterol (mg/dl)	45.7 ± 10.8	50.2 ± 15.8	0.8
LDL- Cholesterol (mg/dl)	105.2 ± 30.2	105.5 ± 33.8	0.2
Triglycerides (mg/dl)	73.5 ± 31.4	68.2 ± 32.3	0.2
Smoking (yes/no)	3/63	4/44	0.4
Fibrinogen (mg/dl)	210.0 (180.0-319.0)	210.0 (180.0-299.0)	0.9
CRP (mg/dl)	0.14 (0.01-2.41)	0.23 (0.01-2.90)	0.01
AGP (mg/dl)	40.0 (40.0-78.0)	53.5 (40.0-115.0)	0.0001
AER (µg/min)	1.6 (0.3-7.0)	8.8 (2.8-17.7)	0.0001

Values are mean ± SD or median (minimum/maximum); BMI, body mass index; FBG, fasting blood glucose; HbA_{1c}, glycated hemoglobin; sBP, systolic blood pressure; dBP, diastolic blood pressure; AGP, α₁-acid glycoprotein; CRP, C-reactive protein.

(patients with type 1 diabetes and non-diabetic subjects), despite the weakness of the correlation between serum AGP and AER after adjustment for FBG and HbA_{1c}, our data cannot exclude that the relationship between the two proteins is partly independent of measurements of glycemic control. Recently an association between high levels of AER and degree of AGP fucosylation was described in patients with diabetes type 1, suggesting a relationship between low-grade inflammation and endothelial dysfunction [29]. In the group of patients with diabetes type 1 no correlation was noted between HbA_{1c}, FBG and AGP, in agreement with other studies [12, 26].

Concerning the other acute-phase proteins, CRP and fibrinogen, most studies have shown their effects on cardiovascular risk [3-6] and on insulin resistance [7, 8] in non-diabetic subjects as well in diabetic patients [9]. Few studies have investigated the relationship of fibrinogen and/or CRP

in patients with type 1 diabetes [10, 12-15]. We did not observe a relationship between fibrinogen, type 1 diabetes, BMI and HbA_{1c}, in agreement with other studies [12]. High levels of fibrinogen have been described in patients with type 1 diabetes but with microalbuminuria [13] and macroalbuminuria [14, 15]. The EURODIAB IDDM study has also shown a probable influence of smoking habits on the association between fibrinogen and albumin excretion rate, neuropathy and lipid abnormalities [15]. Despite the clustering of risk factors associated with high levels of fibrinogen in this latter study, no association was found between fibrinogen and cardiovascular disease. Probably high levels of fibrinogen in patients with type 1 diabetes are related to the presence of chronic clinical complications and could be one of the abnormalities that indicate a hypercoagulable and hypofibrinolytic state in these patients, both of them known risk factors for cardiovascular disease [9].

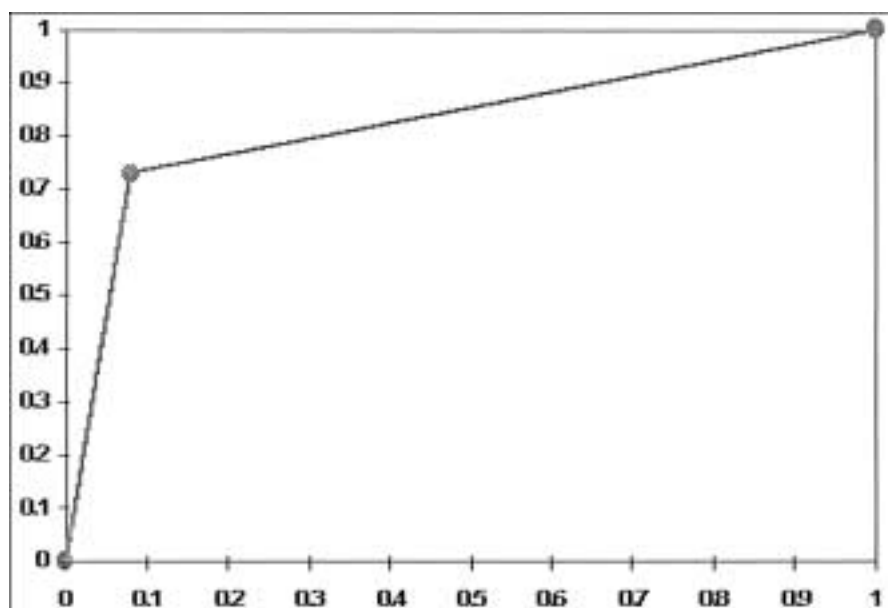


Figure 3
ROC curve showing cut-off of 47 mg/Dl of Alpha-1 Acid Glycoprotein as an optimal cut-off for distinguishing diabetic patients from non-diabetic subjects.

As described in other reports, high CRP levels were observed in the group of patients with type 1 diabetes compared to matched controls [10-12]. Nowadays, CRP is considered to be one of the strongest predictors of cardiovascular events in the general population [30] and in patients with diabetes type 2 [31]. Nevertheless a cut-off level of CRP for clinical use has not yet been defined [30]. Considering that in our patients clinical cardiovascular disease was excluded using the WHO questionnaire and Minnesota coding of 12 - lead resting ECGs, we cannot rule out the possibility that patients with preclinical atherosclerosis may have been included. Recently different studies on young patients with type 1 diabetes demonstrated that high levels of CRP were correlated with intima-media thickness of the carotid artery, a subclinical index of early carotid atherosclerosis, [10] and were associated with coronary artery calcification, an index of coronary atherosclerosis, in women [11]. Nevertheless, both studies showed that high levels of CRP were a risk factor regardless of the presence of type 1 diabetes. It is important to emphasize that no difference was found between our patients and matched controls concerning other cardiovascular risk factors like blood pressure, smoking habit and lipid profile. So we considered the hypothesis that the increased levels of CRP and AGP could be some of the other operational risk factors accelerating atherosclerosis disease in patients with diabetes type 1. As our patients had a lower BMI and percent of fat mass than non-diabetic subjects, the increased levels of AGP and CRP must be related to hyperglycemia and so to the diabetic condition itself. Hyperglycemia induces an increase in oxidative stress and in glycation products which activate macrophages [32]. As a consequence of this process there is up-regulation of cytokines resulting in an overproduction of acute-phase proteins by the liver [32]. This fact is quite different from what is

observed in patients with diabetes type 2 and obesity for whom high levels of acute-phase proteins may also be secondary to the increased levels of interleukin-6 and tumor necrosis factor α derived from adipose tissue [21].

Some limitations of our study concerning the criteria to define retinopathy and/or neuropathy and peripheral disease must be discussed. Probably our measurement of retinopathy has less sensitivity in detecting the initial steps of non proliferative retinopathy than fundus photographs and/or fluorescein angiography. Concerning neuropathy and peripheral disease both were excluded by clinical examination. Although funduscopy and neurological clinical examination cannot exclude subclinical microvascular diseases, these complications are very unusual in the range of duration of diabetes of the majority of our patients (less than 5 years).

Another point to be mentioned is the heterogeneity of duration of diabetes in our patients with diabetes type 1 which could have influenced the lack of correlation between acute phase protein and measurements of glycemic control in this group.

In conclusion, this study demonstrated elevated levels of AGP and CRP in patients with type 1 diabetes without clinical cardiovascular disease or microvascular complications. This relationship could be probably independent of glycemic control. Finally, the causal and temporal relationship of low-grade inflammation in the development of chronic complications in patients with type 1 diabetes must be addressed in prospective studies.

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