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Case Control Study

Association of *TCF7L2* mutation and atypical diabetes in a Uruguayan population

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Abstract

AIM

To investigate if mutations in *TCF7L2* are associated with "atypical diabetes" in the Uruguayan population.

METHODS

Healthy, nondiabetic controls ($n = 133$) and patients with type 2 diabetes ($n = 177$) were selected from among the presenting population at level-3 referral healthcare centers in Uruguay. Patients with type 2 diabetes were subgrouped according to "atypical diabetes" ($n = 92$) and "classical diabetes" ($n = 85$). Genotyping for the rs12255372 and rs7903146 single nucleotide polymorphisms (SNPs) in the *TCF7L2* gene was carried out with TaqMan® probes. Random samples were sequenced by MacroGen Ltd. (South Korea). Statistical analysis of the SNP data was carried out with the SNPStats online tool (<http://bioinfo.iconologia.net/SNPstats>). The best inheritance model was chosen according to the lowest values of Akaike's information criterion and Bayesian information criterion. Differences between groups were determined by unpaired t -tests after checking the normal distribution or were converted to normalize the data. The association of SNPs was tested for matched case-control samples by using χ^2 analysis and calculation of odds ratios (ORs) with 95% confidence intervals (CIs). All statistical tests were performed using SPSS v10.0 and EpiInfo7 statistical packages. Significant statistical differences were assumed in all cases showing adjusted $P < 0.05$.

RESULTS

We genotyped two *TCF7L2* SNPs (rs7903146 and rs12255372) in a population-based sample of 310 Uruguayan subjects, including 133 healthy control subjects and 177 clinical diagnosed with type 2 diabetes. For both SNPs analyzed, the best model was the dominant type: rs12255372 = G/G *vs* G/T+T/T, OR = 0.63, 95%CI: 0.40-0.98, $P < 0.05$ and rs7903146 = C/C *vs* C/T+T/T, OR = 0.79, 95%CI: 0.41-1.55, $P = 0.3$. The rs12255372 SNP showed high association with the type 2 diabetes cases (OR = 1.60, 95%CI: 1.20-2.51, $P < 0.05$). However, when the type 2 diabetes group was analyzed according to the atypical and classical subgroupings, the association with diabetes existed only for rs12255372 and the classical subgroup (*vs* controls: OR = 2.1, 95%CI: 1.21-3.75, $P < 0.05$); no significant differences were found for either SNP or atypical diabetes.

CONCLUSION

This is the first time SNPs_ *TCF7L2* were genotyped in a diabetic population stratified by genotype instead of phenotype. Classical and atypical patients showed statistical differences.

Key words: TCF7L2; Atypical diabetes; Type 2 diabetes; Latin America; TaqMan

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Core tip: This is the first time single nucleotide polymorphisms (SNPs) of the *TCF7L2* gene were genotyped and comparatively assessed in Uruguayan type 2 diabetes patients with "atypical" and "classical" cases. The results show that these two populations are genotypically different. The only statistical association found involved

one of the SNPs, rs12255372, and classical diabetes. No association was found to exist between either of the two SNPs examined (rs7903146 and rs12255372) and atypical diabetes. The findings in this study confirm the results of our previous investigations, which indicated that atypical and classical diabetes are two separate entities of the diabetes disease.

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INTRODUCTION

Diabetes mellitus is a global public health problem, and the Uruguayan population poses no exception. The prevalence of diabetes in Uruguay is 8%^[1], accounting for 3.3 million of the country's inhabitants^[2]. Worldwide, type 2 diabetes (T2D) is the most common form, with incidence and prevalence having reached epidemic proportions.

Since 2009, our group has published on patients that were difficult to classify from a clinical point of view because they did not present a correlation between phenotype and genotype^[3-5]. The current international guidelines for defining the type of diabetes present in an individual are still not sufficient to diagnose atypical diabetes. Patients with atypical diabetes do not fit exactly in any of the groups defined in the guidelines because they do not precisely follow the classical presentation and disease evolution and they show poor therapeutic response.

These patients have been treated in level-3 health-care settings. The atypical cases could be bypassed inadvertently by healthcare providers if the appropriate genetic and immunological analyses are not carried out, primarily because overweight or obese status is a gross indicator of insulin-resistance. Indeed, these visibly assessed features are the primary disorder considered in classification of type 2 diabetes in the internationally used recommendations of the American Diabetes Association (ADA)^[6]. Atypical diabetes could be a confounder in latent autoimmune diabetes of adults (LADA) but the two are distinguishable according to several specific clues. LADA includes (1) patient onset at ≥ 30 years of age (we have found children and young people with atypical diabetes); (2) an absence of metabolic syndrome along with features of obesity, high blood pressure and high cholesterol levels (all of the atypical patients we have encountered have this condition); (3) uncontrolled hyperglycemia despite using oral agents; and (4) other autoimmune diseases (with clinical evidence for diagnosis) that are not necessarily present in atypical diabetes (*i.e.*, Graves' disease and anemia)^[7].

For the work presented herein, we analyzed the association of transcription factor 7-like 2 (*TCF7L2*), one

of the major genes related to T2D, continuing with the genetic characterization of atypical diabetes patients. Our choice of this gene was based upon the remarkable amount of research that has been carried out to date on the genetic factors of diabetes, and from which *TCF7L2* has emerged as one of the strongest T2D susceptibility genes^[8-10].

TCF7L2 is a Wnt signaling-associated transcription factor, expressed in the intestine, pancreas, others tissues and plays an important role in the β -cell proliferation and insulin secretion^[11]. Publications reviewing the possible mechanisms have led to several theories on the processes by which altered *TCF7L2* production or function may cause diabetes. Among these, reduced insulinotropic effect of incretin hormones, of GLP-1 signaling in β -cells especially, impaired insulin processing or release, and decreased β -cell mass seem to be the most probable etiological mechanisms^[12-15]. The fact that genes encoding Wnt signaling pathway factors are active in β -cells or the indication that they may be involved in insulin secretion supports the notion that β -cell dysfunction is a crucial final step on the path to diabetes^[16].

The *TCF7L2* gene is located on chromosome 10q25 and is composed of two major domains: a catenin-binding domain (exon 1) and a central DNA-binding HMG domain (exons 10 and 11). Variations in this gene have been consistently associated with T2D in studies of different populations, namely those of Caucasian, Asian and African origin, and specifically involving two intronic single nucleotide polymorphisms (SNPs): rs12255372 (G>T) and rs7903146 (C>T)^[17-25]. For rs12255372, homozygous carriers of the rare T allele produce 2.5-fold higher levels of *TCF7L2* transcript than wild-type carriers, while heterozygous carriers of both alleles produce 1.5-fold higher^[26]. This SNP in particular affects diabetes through deficiency in insulin secretion, more so than through insulin resistance^[14].

For rs7903146, it is more associated with capacity of insulin secretion than insulin resistance of the β -cell^[27]. The allele T carriers permit the decrease of insulin secretion in postprandial state. This is an important characteristic because it is possible measure the cell response to glucose, aminoacid and incretins^[28].

In this study, we investigated the association between the *TCF7L2* SNPs rs12255372 and rs7903146 in a control (nondiabetic) group and a case (diabetic) group consisting of patients with classical or atypical T2D in the Uruguayan population. This study represents the first time these SNPs have been investigated by stratifying the study population according to presence or absence of HLA and nonHLA susceptibility genes to T2D in patients with body mass index (BMI) ≥ 25 kg/m².

MATERIALS AND METHODS

We analyzed a total study population of 310 individuals, including T2D patients ($n = 177$) and controls ($n = 133$) that were enrolled in the study between 2004 and 2012.

Recruitment of patients was done by selecting from two referral diabetes healthcare centers in Montevideo, Uruguay, namely the Pasteur Hospital and CASMU-IAMPP.

The study was performed in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Ethics Committees of both participant institutions (Law 18331). All cases and controls signed an informed consent form for participation in this investigation.

T2D patients

Patients were selected according to ADA recommendations^[6]. We took into consideration another criterion, that being patients who had received a multidisciplinary care approach for their diabetes, showed good adherence to their treatment regimen (including a nutritional and physical activity plan according to their functional capabilities), and had received one or more oral antihyperglycemic drugs.

For the stratification of T2D patients into the classical and atypical diabetes groupings, we used the same classification standards as in our previous studies^[3-5]. For atypical diabetes, 92 of the patients met the following inclusion criteria: (1) BMI ≥ 25 kg/m² and categorization following the World Health Organization overweight and obesity guidelines (25-29.9 kg/m² and ≥ 30 kg/m² respectively); (2) having reached the education and nutrition plans' objectives as per international guidelines; (3) having presented doubts about their disease classification and/or not having reached a good therapeutic response (*i.e.*, no decrease of 1.5% in HbA1c levels shown in two consecutive measurements after 3 mo^[29]); and (4) presence of autoimmune-diabetes-susceptibility HLA alleles (the HLA DQB1* 0201-0302 and DR 3-4 susceptibility alleles were considered for the Uruguayan population^[30]). For classical diabetes, 85 patients fulfilled the (1) and (2) inclusion criteria listed above but did not present doubts about their diagnosis and did not show presence of autoimmune-susceptible genes.

Individuals who fit the inclusion criteria but had other metabolic disorders or were undergoing tumor processes were excluded from this study.

Control (healthy, nondiabetic) subjects

One hundred and thirty-three healthy, nondiabetic subjects were recruited from among blood donors at the Hemotherapy Department of Pasteur Hospital between 2014 and 2015. The blood donors presented for this service on a volunteer basis. Prior to the sample extraction, the attending doctors carried out an exhaustive questionnaire survey of each donor, which included information on chronic or infectious diseases and taking of any medication(s). Blood pressure was taken and weight and height were recorded in order to calculate the BMI (kg/m²). Laboratory tests were carried out and individuals with infectious diseases such as human immunodeficiency virus and hepatitis A/B/C were excluded from enrollment. At the time of sample extraction, none of the donors

Table 1 Anthropometric characteristics of the study population

	Healthy controls	Type 2 diabetes cases
<i>n</i>	133	177
Male/Female	76/57	87/90
Age (yr)	37.49 ± 13.04	63.05 ± 11.66
BMI (kg/m ²)	25.17 ± 5.35	31.73 ± 5.65

BMI: Body mass index; SD: Standard deviation.

had diabetes, but this did not rule out the possibility that they could have developed it in the future since it is a multifactorial disease. The majority of the population that donates blood has an average age of approximately 38 years.

Of the 300 individuals that were initially included as the control population, we selected those who had a BMI similar to that of the sample of patients with atypical diabetes in order to avoid a confounding variable. In addition, we had already shown in a previous study that this variable does not have a statistically significant difference between classic and atypical diabetes cases^[5].

Genetic typification

DNA was obtained from peripheral blood using the standard phenol/chloroform technique. The rs12255372 (G>T) and rs7903146 (C>T) SNPs in the *TCF7L2* gene were genotyped using *TaqMan*[®] Probes for real-time PCR in the Rotor-Gene[™] 6000 PCR machine (Corbett Research, Sydney, Australia). The primers and probes for SNP rs12255372 were designed with the Primer3Plus software (Boston, MA, United States) and AlleleID[®] software (Palo Alto, CA, United States), respectively. The primer sequences were as follows: forward, TCTGGCTTGGAAAGTGTA; reverse, GAGGCCTGAGTAATTATCAGAA. The probe sequence was as follows: FAM/HEX-CCAGGAATATCCAGGCAAGAAT[T/G]ACCA-BHQ1. The rs7903146 primers and probe were obtained from the catalog of genotyping experiments in *TaqMan*[®] SNP Genotyping Assays (Life Technologies[™], Carlsbad, CA, United States). The primer sequences were as follows: forward, GCCTCAAAACCTAGCACAGC; reverse, GTGAAGTGCCCCAAGCTTCTC. The probe sequence was as follows: VIC/HEX TAGAGAGCTAAGCACTTTTATAGATA[C/T]TATATAATTTAATTGCCGTATGAGG. In both cases, a commercial genotyping kit (Platinum[®] Quantitative PCR SuperMix-UDG); Invitrogen[™] by Life Technologies[™]) was used for the genotyping procedure.

Melting curve analyses were performed using the Rotor-Gene[™] 6000 software v.1.7 (build 75) and the accompanying algorithm. Random samples were sequenced by MacroGen Ltd. (Seoul, South Korea) and were aligned using MEGA4 (Molecular Evolutionary Genetics Analysis software, Tempe, AZ, United States).

Statistical analysis

The statistical analyses for the polymorphisms were done

with the online tool for SNP analysis, SNPStats (<https://www.snpstats.net/start.htm?http://bioinfo.iconcolgia.net/SNPstats>). The best inheritance model was chosen according to the lowest values of Akaike's information criterion (AIC) and Bayesian information criterion (BIC). Continuous variables were expressed as means and standard deviations. Differences between groups were determined by unpaired *t*-tests after verification of normal distribution, or converted to normalize the data.

The association of SNPs in matched case-control samples was tested using χ^2 analysis and calculation of odds ratios (ORs) with 95% confidence intervals (CIs). All tests were performed using the SPSS statistics package version 22 (IBM Corp., Armonk, NY, United States) and Epi Info[™] statistics package version 7 (Atlanta, GA, United States). Significant statistical differences were assumed in all cases having adjusted *P* < 0.05.

RESULTS

We genotyped two *TCF7L2* SNPs-rs7903146 and rs12255372-in a population-based sample of 310 Uruguayan subjects, including 133 control subjects and 177 patients clinically diagnosed with T2D. The most relevant anthropometric values for the T2D and control groups are presented in Table 1. The T2D atypical and classical subgroups are presented in Table 2, showing the main clinical characteristics of each.

The best inheritance model was the dominant model for both SNPs analyzed: rs12255372= G/G vs G/T+T/T, AIC = 423.4, BIC = 430.8; and rs7903146 = C/C vs C/T+T/T, AIC = 426.4, BIC = 433.9. The rs12255372 SNP was the only variation that showed high association with the disease (Table 3). Comparative statistical analysis of the atypical and classical diabetes subgroups showed association only between the classical diabetes *versus* controls for rs12255372 (Table 4).

DISCUSSION

Diabetes mellitus is a complex disease, in which genetic and environmental factors are interweaved. After the discovery of *TCF7L2* as a key player in T2D etiology, several works in multiethnic populations identified two main SNPs in this gene, rs12255372 and rs7903146, and characterized them as the most relevantly associated to T2D^[9,10,17,21]. In our previous works^[3-5], we reclassified patients who are clinically diagnosed as T2D into two subgroups, representing the classical and atypical cases, as described in the Materials and Methods section. Intriguingly, these previous studies consistently found that the two case categories were different at the genetic level, involving several genes. In the current study, we amplified the genetic characterization of atypical diabetes in Uruguayans to include the analysis of two SNPs strongly related with T2D according to other populations studied and reported.

Analyzing the T2D study population in comparison to

Table 2 Clinical characteristics of the atypical and classical diabetes cases

	Atypical diabetes, <i>n</i> = 92	Classical diabetes, <i>n</i> = 85	<i>P</i>
Age (yr)	61.29 ± 13.08	65.74 ± 9.93	0.011 ^b
Age (yr)	43.36 ± 12.62	45.93 ± 14.67	0.304
BMI (kg/m ²)	32.22 ± 5.48	31.32 ± 5.96	0.288
HbA1c, %	8.27 ± 1.80	8.27 ± 1.77	0.993
Total cholesterol (mmol/L)	5.18 ± 1.14	5.53 ± 1.16	0.044 ^a
HDL (mmol/L)	1.24 ± 0.29	1.32 ± 0.33	0.080
LDL (mmol/L)	2.84 ± 1.02	3.26 ± 1.11	0.010 ^b
Triglycerides (mmol/L)	2.24 ± 1.40	2.24 ± 1.4	0.991
TG/HDL	4.47 ± 3.41	4.43 ± 4.73	0.941

^a*P* < 0.05, ^b*P* < 0.01, for all parameters. BMI: Body mass index; HbA1c: Glycated hemoglobin; HDL: High-density lipoprotein-cholesterol; LDL: Low-density lipoprotein-cholesterol; TG/HDL: Insulin resistance index.

Table 3 Genotype frequencies of rs12255372 and rs7903146 in controls and cases

SNPs	Healthy controls, %	Type 2 diabetes cases, %	OR (95%CI)	<i>P</i>
rs12255372 G > T				
G/T + T/T	48.9	60.5	1.6 (1.02-2.51)	0.04
G/G	51.1	39.5		
T allele	30	37	1.37 (0.97-1.92)	NS
G allele	70	63		
rs7903146 C > T				
C/T + T/T	46.6	52.5	1.27 (0.81-1.99)	NS
C/C	53.4	47.5		
T allele	29	34	1.22 (0.87-1.72)	NS
C allele	71	66		

CI: Confidence interval; NS: Non-significant; OR: Odds ratio; SNP: Single nucleotide polymorphism.

Table 4 Genotype frequencies of rs12255372 and rs7903146 in controls, atypical diabetes and classical diabetes patients

SNPs	Controls, %	Atypical diabetes, %	Classical diabetes, %	OR (95%CI)	<i>P</i>
rs12255372 G > T					
G/T+T/T	65	-	57	2.1 (1.21-3.75)	0.008
G/G	68	-	28		
G/T+T/T	65	50	-	1.2 (0.73-2.12)	NS
G/G	68	42	-		
rs7903146 C > T					
C/T + T/T	62	-	47	1.4 (0.82-2.45)	NS
C/C	71	-	38		
C/T+T/T	62	46	-	1.2 (0.67-1.95)	NS
C/C	71	46	-		

SNP: Single nucleotide polymorphism; CI: Confidence interval; NS: Non-significant; OR: Odds ratio; SNP: Single nucleotide polymorphism.

the control population showed us a significant association of the rs12255372 SNP. The same results have been found in other studies of different populations, and the presence of the T allele was also found to be associated to major proneness to T2D^[21,31,32]. Subsequent analysis of our results from the T2D patients upon subgrouping according to atypical and classical cases, and in comparison with controls, showed an increase in OR when we removed the atypical patients from the analysis for rs12255372. This finding could indicate that the atypical subpopulation of T2D patients could serve as a confounding factor in general analyses of T2D patients, highlighting the potential of the overall T2D population being a mixture of case subgroups. This subgroup profile may help to explain

previous results observed in different studies that used the T2D pooled population as a unique group.

The ideas expressed above are in accordance with the notion that atypical patients could be framed as a separated group from patients with classical T2D^[3]. Another perspective sets atypical diabetes in a mid-course status between type 1 diabetes and T2D, as described by Pozzilli *et al.*^[33]; as such, the atypical diabetes case would be located in the middle of the T2D disease spectrum. This concept goes along with the so-called "accelerator hypothesis", which states that β -cell loss could be variably accelerated by the conjunction and different weight of three different processes: insulin resistance, autoimmunity and constitution^[34,35]. The β -cells of those

individuals carrying the *TCF7L2* gene mutations are more susceptible than others to the metabolic demands of insulin resistance^[36], but not as susceptible as those carrying the HLA DR3/DR4 haplotype, as in the case of the atypical diabetes population, providing a combination of unfavorable genetic background, impairment in β -cell secretion and a diminished survival upon challenge with hyperglycemic stress, as well as establishing an auto-immune cell environment^[14,26,37].

Interestingly, the rs7903146 SNP did not show significant association with T2D in our analyses. The Uruguayan population has a three-hybrid admixed origin-European, African and Amerindian; the Caucasian component represents a major proportion, but there is a significant mixture degree and a noteworthy Amerindian component of maternal origin^[38]. Studies performed in different Asian populations^[39-42] have found no significant association between this SNP of the *TCF7L2* gene and T2D. Thus, one could ponder the idea that the Uruguayan population may be more related to Asian populations than expected; this idea might also be supported by the theory that the American continent was populated by Asian ancestors^[38].

Beyond the ethnic influence that has also been found in other studies of rs12255372 and rs7903146^[17-25], this study showed that the association of rs12255372 with diabetes was increased when the population was reclassified as subgroups of case types and only the classical T2D patients were compared with controls. This finding suggests the importance of taking into consideration the existence of an atypical group, which could serve to obscure the real association of SNPs in the *TCF7L2* gene. On the other hand, it is important to note that studies using populations of patients with LADA have found differences in the polymorphisms of the *TCF7L2* gene as well as with T2D^[43,44]. This finding reinforces the theory that LADA and atypical diabetes are distinct entities.

To continue the characterization of the atypical diabetes subpopulation it will be important to obtain measurements of C-peptide from the patients, so as to study if there is any difference for this marker between the subpopulations classified. The C-peptide is a precursor of insulin whose measurement shows the reserve of secretion of the same by the pancreatic β -cell. It is very useful in those patients with poor response to antihyperglycemic medication, as is the case of our atypical patients. Therefore, this technique would represent another resource to help in the classification and a more appropriate therapeutic in this population of complex patients.

Today, in our country, the investigation of C-peptide is not routinely performed and is only carried out in some specialized centers. Therefore, it becomes of paramount importance to implement the C-peptide measurement in the Healthcare Centers and Hospitals in our country. In addition, it will be important to continue characterizing the atypical diabetes subpopulation through the study of genetic markers. Identification and characterization of disease-specific genetic markers will help doctors to more

readily and more accurately classify these cases, according to an etiopathogenic base. Such could also lead us to designing and implementing a therapy that will avoid or minimize trial and error time.

At the same time, therapeutic inertia would facilitate advancement of the chronic complications of this pathology. In the group of patients investigated in this study, we took into account the presence of clinical biomarkers. Although we cannot speak from a statistical point of view (due to the short time elapsed during the study period), we have managed to individualize the therapy (data not shown). In turn, this has led our patients with atypical presentation to have greater confidence in the treatment used, such as the acceptance of a timely insulinization.

Ultimately, this study showed that the application of a translational medicine research approach provides knowledge of basic science that can be applied directly in the clinic towards the resolution of complex clinical cases.

We have studied two of the most relevant SNP variants related to T2D, in the *TCF7L2* gene, in a Uruguayan diabetic population stratified by genotype differences. The present and previous works support the idea that the combined effect of several predisposition variants would turn the atypical subpopulation into a new classification and serve as therapeutic targets^[45].

Currently, there are different classifications that encompass atypical patients, placing them within different categories. Steenkamp *et al.*^[46] refer to a group of patients who would meet some of the criteria described herein as diabetic (ketosis-prone diabetes), while other authors locate these patients within a subset of the LADA patients^[47]. Overall, this reaffirms the necessity to continue the genetic analysis of this particular population to achieve a more adequate classification and treatment of these patients.

ARTICLE HIGHLIGHTS

Research background

In a high percentage of patients, clinical presentation alone does not define the type of diabetes. This is very important, since it hinders implementation of an individualized and safe treatment. The current classification system of diabetes is useful and easy for typical patients. However, there are many situations in which it is difficult to determine what type of diabetes is presenting due to the great heterogeneity in the pathogenesis. The current classification of diabetes is not satisfactory and its revision has been under consideration for many years. Previous studies carried out in the Uruguayan population have demonstrated the existence of patients for who it is not possible to classify into any of the categories provided in the international guidelines. We continue to investigate this type of patient because it is very important to assist them appropriately and improve their quality of life. In this way, it is possible to abolish the trial stage and error that patients suffer from when not being correctly diagnosed. At this time, different researchers have proposed that the classifications of diabetes should be revised, and this is the principal objective of our work. We have emphatically proposed the inclusion of genetics determination for HLA to elucidate atypical diabetes patients. Such an approach and related data will permit correct classification and treatment for these kinds of patients.

Research motivation

To date, we have investigated genes related to type 1 diabetes in patients with atypical diabetes. In this study, we sought to analyze the major gene related to

type 2 diabetes, the *TCF7L2* gene, in the atypical diabetes patients.

Research objectives

To analyze the association of the two most important single nucleotide polymorphisms (SNPs) of the *TCF7L2* gene-rs12255372 and rs7903146-with atypical diabetes.

Research methods

This case-control study was conducted in atypical and classical cases of type 2 diabetes using genotyping with *TaqMan* probes for the rs12255372 and rs7903146SNPs of the *TCF7L2* gene.

Research results

The SNPs of the *TCF7L2* gene that were analyzed in this work showed no association with atypical diabetes; nevertheless, the rs12255372 SNP was associated with classical diabetes.

Research conclusions

As has been shown in previous studies, the genetics of atypical diabetes are different from those of classical diabetes, despite a shared phenotype.

Research perspectives

To continue the characterization of the atypical diabetes subpopulation it will be important to obtain measurements of C-peptide in these patients and to study if there is any difference for this marker between the populations classified.

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