Genes associated with obesity in patients with atypical diabetes

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Abstract

Diabetes Mellitus has a high prevalence in Uruguay and around the world. It presents different and complex clinical characteristics and very frequently diagnosis is a challenge for treating physicians. Previous works described an atypical diabetic population showing genetic variations as HLA and non-HLA related to type 1 diabetes, presenting also type 2 diabetes clinical features.

Objectives: In the present work we studied variations in four genes (*IRS-1*, *PPAR-γ2, ENPP-1* and *UCP-2*) associated with obesity and diabetes.

Methods: 155 patients from two healthcare centers in Montevideo, Uruguay, were studied. They were divided into two populations as per type 2 diabetes criterion (84 patients) and atypical diabetes criterion (71 patients). Four SNPs were analyzed: rs1801278, rs1801282, rs1044498 and rs659366 in genes *IRS-1*, *PPAR-γ2*, *ENPP-1* and *UCP-2*, respectively.

Results: Significant differences in SNP, *IRS-1* and *UCP-2* were found. No significant polymorphism variations were found in *PPAR-γ2* and *ENPP-1*.

Conclusions: Our results would be in accordance with the evidence regarding the lack of correlation between genotype and phenotype in atypical diabetes patients, as there are differences in some of the susceptibility and protective variants for obesity and diabetes in genes involved in insulin resistance and faulty beta cells. On the other hand, other variants would be partaken by both populations, determining the phenotypic characteristics they have in common. Genetic studies could serve not only for the identification of this type of patients but also to optimize their treatment.

Key words: diabetes, SNP, obesity, atypical diabetes

1. INTRODUCTION

Diabetes Mellitus (DM) is a complex disease which drives the patient to an important decline in his quality of life and generates high costs in healthcare systems. There is a high prevalence worldwide, related with high morbid-mortality rates. Its frequency is constantly increasing due to the western countries' lifestyles, with a higher caloric intake and less physical activity (Inzucchi et al., 2012).

At present, diagnosing is an important challenge for clinical physicians as this disease shows heterogeneous and complex clinical traits presenting diverse and unique expressions in each patient (Royal College of General Practitioners, 2011). Similarities in clinical features could generate confusion between the different diseases' types and subtypes and furthermore, there is a group of patients with a very difficult disease classification (ADA, 2014; Stone et al., 2010). Type 2 diabetes (T2DM) is frequently associated with obesity and is developed when chronic overeating is combined with genetic susceptibility (damaging insulin-signaling pathways), together with a relative insulin deficiency of not-autoimmune etiology (Muoio & Newgard, 2008). Also, there is evidence showing these etiologies are combined with genes involved in insulin secretion and/or insulin signaling, modifiers of patients' phenotypic traits, datum not always taken into consideration (Di Paola et al., 2011; Liew et al., 2010; Ye, 2013).

Uruguay has the same problem as most countries have; there are patients sharing clinical, and presumably genetic, characteristics for both main diabetes types ("classical" types 1 and 2), mainly seen at the third level of medical attention (Steenkamp, Alexanian, & Sternthal, 2014). They are generally patients presenting classification difficulties, complex clinical approaches and also showing diverse responses to the different therapies. Some patients reach adequate disease control keeping up without drug treatments; other patients need antihyperglycemic drugs and a high percentage of patients behave as type 1 diabetic patients requiring high insulin dosage, reducing it up to a basal level during the night.

In previous works this diabetic population was characterized, defining the disease as "atypical diabetes" or "double diabetes". According to international guidelines (ADA, 2014), these patients were clinically diagnosed with type 2 diabetes, presenting phenotypes in accordance with this classification. Atypical diabetes presented a body mass index (BMI) ≥ 25 kg/m^2 and also showing HLA and non-HLA genes associated with type 1 diabetes (Fernández, Fabregat, Javiel, & Mimbacas, 2014; Mimbacas et al., 2009, 2012). Focusing on type 2 diabetes main traits (insulin resistance and beta-cell dysfunction) (Ashcroft & Rorsman, 2012; Ježek, Dlasková, & Plecitá-Hlavatá, 2012), in this research we have continued with the characterization of atypical diabetes, searching for possible genetic origins, which could explain why phenotype is not exactly in correspondence with genotype.

In the studied population obesity is to be highlighted; it implies a low but chronic inflammation process involved in type 2 diabetes pathogenesis. Inflammation associated with obesity begins in the adiposite tissue and in the liver with macrophage infiltration and high expression of inflammatory pathways' signaling molecules (Ye, 2013). These pro-inflammatory cytoquines (as IL-6 and alpha TNF) are activated in the adipocyte tissue and in the liver and afterwards inhibit the gene expression required for the normal action of insulin, i.e. *IRS-1* (insulin receptor sustrate 1) and *PPAR-γ2* (Peroxisome Proliferator Activated Protein Gamma 2) (Peraldi, Hotamisllgil, Buurman, White, & Spiegelman, 1996). This inhibition process leads to a diminished insulin sensibility (Gao Z, He Qing, Peng Bailu, Chiao P, 2006; Wang et al., 2013). Other obesity- and insulin resistance-related genes participate directly in the inhibition of key genes as *IRS-1*. *ENPP-1* gene (ectonucleotide pyrophosphatase/ phosphodiestrase 1) encodes an antigen-differentiation membrane glycoprotein (PC-1)

which overexpression inhibits the tyrosine-kinase activity of insulin receptor (IRS-1) and the subsequent insulin downstream action (Marucci et al., 2013). The quantity of overexpressed PC-1 is directly correlated with the insulin resistance severity.

The expression and functionality of all these obesity-related and insulin-resistance genes depend upon the presence of some genetic polymorphisms which determine different activity levels and therefore greater or less predisposition for diabetes development.

IRS-1 gene is the prototype of the IRS protein family and is a fundamental mediator of the insulin-signaling pathway in several target tissues. Its prevaling polymorphism is Gly972Arg (rs1801278) substitution, presenting the Arg972 variant with a predominance in diabetic patients three-fold higher than in non-diabetic subjects (Almind et al., 1998; Marchetti et al., 2002; McGettrick, Feener, & Kahn, 2005; Porzio, Federici, & Hribal, 1999; Sesti et al., 2001). As IRS-1 is ubiquitously expressed in insulin-sensible tissues, including pancreas' alpha- and beta-cells, there is a possibility that this polymorphism in 972 codon could also affect differentiation, maturity or function of beta-cells islet, thus diminishing beta-cells response. It has been demonstrated that glucose-stimulated insulin secretion is modulated by autocrine activation of insulin-signaling pathways involving in this manner IRS-1 phosphorylation and PI-3 kinase activation (Yoshiuchi, 2013). Hence, defective interrelationships between these genes could contribute to insulin-resistance in peripheral tissues as well as to insulin-secretion deficiency.

Over the years, *PPAR-γ2* has been one of the most studied genes due to its relationship with insulin-resistance, T2DM and Metabolic Syndrome. It codifies for a ligand-inducible transcription factor belonging to the peroxisome-activated nuclear receptors family (PPARs). PPAR-*γ* protein is found mainly in adipocytes, kidneys, macrophages and gut mucosa, among other tissues. Its key role in adipocyte differentiation and to generating lipid accumulation in adipose tissue has also been proven. In addition, it plays an important role in maintaining adipose cells' viability and normal function. In macrophages, its link with bubble-cells formation, atherogenesis and with the suppression of inflammatory cytokines production has been demonstrated. Furthermore, it plays a fundamental role in lipid and glucide metabolism regulation in liver, skeletal muscle and adipose tissue, affecting in this way their plasmatic levels (Gurnell, Savage, Chatterjee, & O'Rahilly, 2003; He, 2009). Research has been undertaken regarding the role of several PPAR-*γ*2 variants in regard to insulin resistance; in epidemiological studies, Pro12Ala (rs1801282) polymorphism has been the most investigated. Pro12Ala (rs1801282) is a change of direction mutation in which substitution of C for G in codon 12 of B exon determines the change of a proline into an alanine in the protein. In different studies, Ala12 variant has been correlated with less adiposity and lower BMI, better response to Tiazoledinediones as well as less predisposition to developing insulin resistance, T2DM and Metabolic Syndrome (Buzzetti et al., 2004; Chistiakov et al., 2010; Frederiksen et al., 2002; Ghoussaini et al., 2005; González, Serrano, Fernández, Laakso, & Martínez, 2002; Ho et al., 2012).

Beyond the mentioned mechanisms, cellular energetic metabolism is another important factor to be considered in regard to obesity and insulin resistance, as it determines cell health and longevity. Therefore, mitochondria plays a role controlling circulating glucose and lipid levels at the central and peripheral metabolism regulation mechanisms. Recent advances in the knowledge about lipid and glucose cellular metabolism have identified *UCP-2* gene as a critical regulator in the use of cellular "fuel" (Diano & Horvath, 2012; Ye, 2013). As this gene plays a role in glucose and lipid physiological and pathological metabolism, there is a link between the above mentioned genes and mitochondrial genes.

UCP-2 gene (uncoupling protein 2) codifies for a protein which forms part of the internal mitochondrial membrane; this protein uncouples glucose oxidative metabolism from ATP production (Bell, Walley, & Froguel, 2005; Das & Elbein, 2006). 866G/A polymorphism in *UCP-2* promoter (rs659366) has been associated with obesity, insulin secretion and diabetes (Bell et al., 2005; Freeman & Cox, 2006). The *UCP-2* promoter region contains response elements to *PPAR*, which is activated by free fatty acids. Experiments carried out in animals treated with PPAR antagonists have shown an increase in *UCP-2*'s ARNm, supporting the hypothesis that free fatty acids directly induce *UCP-2* via *PPARs* (Chan, Saleh, Koshkin, & Wheeler, 2004; Li Li-xin, Skorpen, Egeberg, Jørgensen, & Grill, 2002). A recent meta-analysis has confirmed that allele -866G is related with a greater risk to develop obesity (Andersen et al., 2013).

In the present study we evaluated the prevalence of the main polymorphisms of *PPAR-γ2*, *IRS-1*, *ENPP-1* and *UCP-2* genes in "atypical diabetes" patients in order to compare them with classical type 2 diabetes patients, hence trying to explain the non-correlation between phenotype and genotype. Detecting genetic differences between these two populations could serve not only to have a better understanding of the disease pathogenesis, but also to start more individualized and adequate treatments.

2. MATERIAL AND METHODS

For this study 155 patients were selected from two referral healthcare centers in Montevideo, Uruguay: Pasteur Hospital and CASMU. In order to be enrolled in the research protocol, the following considerations were taken into account:

- Patients with a multidisciplinary approach for their diabetes, who showed a good disposition regarding the treatment (including a nutritional and physical activity plan according to their functional capabilities and as per ALAD/ADA recommendations) and who had received one or more oral antihyperglycemic drugs. An informed consent was obtained from each patient, which had previously been approved by the Ethics Committees of both participant institutions.

- "Atypical diabetes" sample patients: 84 patients fulfilling the following criteria were included: a) Patients showing a good response to treatment; b) Body mass index (BMI) ≥ 25 kg/m2 (calculated as per the weight/height2 formula) and categorized following the OMS overweight and obesity guidelines (25-29,9 kg/m2, ≥ 30 kg/m2 respectively); c) Patients who had reached the education and nutrition plans' objectives as per international guidelines; d) Patients who presented doubts about their disease classification and/or not having reached a good therapeutic response (no decrease of 1.5% in HbA1c levels shown in two consecutive measurements after three months (Nathan *et al*., 2009); e) Patients with autoimmune-diabetes-susceptibility HLA alleles (HLA DQB1* 0201-0302 and DR 3-4 as susceptibility alleles were considered for Uruguayan population) (Mimbacas *et al*., 2004).

- "Classical diabetes" sample patients: 71 patients fulfilling the same a), b) and c) inclusion criteria but who did not present diagnosis doubts, showed a good response to treatment and did not have autoimmune-susceptible genes. Individuals who accomplished inclusion criteria but had other metabolic disorders or were undergoing tumor processes were excluded from this study.

Interventions in all patients were done by their treating physician following a standardized protocol.

2.1 Genetic typification: DNA was obtained from peripheral blood using the standard phenol/chloroform technique. SNPs: rs1801278 *(IRS-1)*, rs1801282 *(PPAR-γ2),* rs1044498 *(ENPP-1)* and rs659366 *(UCP-2)* were genotypified using PCR-RFLP and PCR real time techniques and confirmed by sequentiation.

Oligonucleotides were designed using Primer 3 plus software. Rotor Gene 6000 (Qiagen) with HRM (High Resolution Melting) analysis capacity and a commercial kit (Bioline) were used for real time PCR technique. The analyzed sequences were sent to Macrogen Ltd. (Korea) and were aligned using MEGA 4 software.

2.2 Statistical studies: Data was expressed as median and their corresponding standard deviations. All tests were performed using SPSS 10.0 and EpiInfo7 statistical packages. Significant statistical differences were assumed in all cases with adjusted $p < 0.05$ and 95% of confidence interval.

3. RESULTS

Clinical characteristics of both populations are shown in Table 1. Body mass index (BMI) similar values and differences in lipid profile between both populations are highlighted. Although Type 2 diabetes population shows increased LDL and total cholesterol values, the atypical population shows increased triglycerides values and decreased HDL values.

For the purposes of the genetic analysis, allelic and genotypic frequencies were calculated (Table 2 and 3, respectively). Among the analyzed patients, it is important to highlight that there were no homozygous individuals for genes *PPAR-γ2* and *UCP-2 "*protective" alleles. For a better comprehension, firstly the analysis of each gene will be described separately and afterwards the interactions between them will be analyzed.

PPAR-γ2 (rs1801282): No significant differences were observed in this polymorphism between patients with atypical diabetes and patients with classical type 2 diabetes. Alanine allele was found in only 15% of samples and no homozygous individuals were found.

IRS-1 (rs1801278): The obesity and diabetes risk variance (Arg972 allele) was more frequent in atypical rather than in classical patients (Odds: 2.21, CI: 1.07-4,5, p= 0.03), as well as in patients with GA heterozygous genotype (Table 3). No A allele homozygous individuals were found in either population.

UCP-2 (rs659366): A allele was more frequent in atypical diabetes population, with an odds of 2.15 (1.24-3.74; adjusted p = 0.005). No protective A allele homozygous individuals were found in either of both populations.

ENPP-1 (rs1044498): In both populations, a very low frequency of Q risk allele homozygous individuals (7% and 3%) was found. There were no significant differences for this allele in either of both populations, as only the dominant genetic model could be tested comparing carriers (KQ + QQ vs KK) due to the small number of QQ homozygous individuals (Table 3). It is to be highlighted that 43% and 45% of samples showed the risk allele in their genotype.

Sex influence was analyzed in every sample, not finding any significant difference (data not shown).

When analyzing genes as a whole, it was observed that all patients presented homo- or heterozygous obesity or T2DM risk variants in at least two genes. Figure 1 shows the combinations found in both groups of patients. It is interesting to highlight the differences between *PPAR-γ + UCP-2* combination: in this case, 54.76% of classic diabetes patients and 24.29% of atypical diabetes patients showed both homozygous risk variants. The remaining patients (38.09 vs. 68.57%) were heterozygous in one of the variants (CC + AG, CG + GG). It is also to be noted that the risk allele proportion is higher in the atypical population than in the classical one (34%), as it adds the *IRS-1* gene effect to other genes effects.

4. DISCUSSION

At present, clinical physicians are increasingly recognizing the existence of a diabetic subpopulation that shows complex characteristics,

in clinic as well as in paraclinical examinations, and in the response to the established routine treatments (Steenkamp et al., 2014). However, there is a paucity of information about this topic and even less studies on metabolic genes in this group of atypical diabetic patients. The results of the present study would be supporting the current evidence regarding the lack of correlation between genotype and phenotype in this particular population (Mimbacas et al., 2009, 2012). These patients would show differences in the variants for susceptibility and obesity/diabetes protection in genes involved in insulin resistance and faulty beta-cells. The presence of genetic variabilities shared by both populations could be determining a similar phenotypic expression in both groups. This fact makes an accurate classification difficult, while on the other hand polymorphisms showing significant differences between both populations could play an important role in clinical behavior and in the different responses to treatment observed in the atypical population.

It is possible that in atypical patients beta-cells functional reduction plus its association with insulin resistance could be more clearly seen. In these patients, beta-cells are dysfunctional, perhaps due to the presence of HLA and no-HLA alleles associated with autoimmune problems (Fabregat, Fernandez, Javiel, Vitarella, & Mimbacas, 2015; Mimbacas et al., 2004) or due to changes in insulin-signaling pathways, as mutations in the analyzed genes show.

There are no important differences between both populations in gene *PPAR-γ2* Pro12Ala polymorphism. Pro12 is the most usual variation in both groups and it has been correlated with a higher BMI in diabetes patients. It is to be highlighted the high frequency of Pro/Pro homozygous in both populations. This result is consistent with phenotypic findings, shown in similar BMI and insulin resistance indexes, hindering the detection of an atypical population as per routine clinical criteria.

In relation to *IRS-1* (rs1801278), the risk allele was frequently found in atypical patients compared with classic patients, as well as the heterozygous GA genotype. The greater presence of A allele has been related to beta-cell apoptosis (Niessen, 2006). The existence of this allele shows less insulin levels during fasting and C peptide decrease, expressing a diminished beta-cells secreting capacity. It has also been proven that in human islets this allele confers a significant increase of beta-cells apoptosis with respect to its wild counterpart. In A allele carriers insulin requirements start earlier (Luo & Luo, 2006; Sesti et al., 2001, 2003). In fact, during patients' follow-up it was also observed an early decrease of C peptide. This decrease should be further analyzed using a different epidemiologic model.

Perhaps one of the most radical changes to be taken into account is the recognition that beta-cells function reduction at early stages is a key problem in type 2 diabetes and not only an insulin resistance associated disease. This change of paradigm is already being validated as a result of human islets studies demonstrating a glucose-dependent reduction of insulin secretion in type 2 diabetes, even when the insulin decrease is taken into consideration.

Another argument supporting this change of paradigm comes from ligand studies and genome-wide association studies (GWAS) (Bonnefond et al., 2010). As our results suggest, this early effect over beta-cell function could be greater in atypical patients. In this sense, Steenkamp et al., (2014) mention the benefits of an early short-term insulin therapy in this group of patients.

There are several controversies and contradictions regarding these genes' alleles association with risk factors (Gable, Stephens, Cooper, Miller, & Humphries, 2006; Lyssenko et al., 2005; Qin, Wen, Qu, & Huang, 2013; Sasahara et al., 2004) The reasons for these contradictions could be: a) the diverse ethnic mixtures (and therefore different genetic variations which could lead to diverse functional combinations) or b) frequently, during the selection process of patients for this

kind of studies, these atypical cases having genes related to both classic diabetes, are not taken into consideration.

The analysis of several genes taken all together enables us to achieve a better understanding of the behavior and interrelationships between different polymorphisms. For example, interrelationships between *UCP-2* and *IRS-1* with *PPAR-γ2* are well known (Vergotine, Yako, Kengne, Erasmus, & Matsha, 2014; Villarroya, Iglesias, & Giralt, 2007). Our results show a higher proportion of 866A allele in atypical patients. Yang and col. works proved an association of *UCP-2* 866A variant with less therapeutic efficacy of Rosiglitazone multiple dosage (Yang et al., 2009). Therefore, this research could be a point of reference for new treatment guidelines. For future investigations it would be convenient to analyze a higher number of variants and genes, plus a higher number of samples, in order to obtain more general conclusions.

As DM is a polygenic and multifactorial disease, diabetes researchers and physicians have difficulties elucidating its sometimes complicated picture, frequently generating conflicts between both groups. These difficulties may restrain basic investigation as well as genetic findings' translations (Bonnefond & Froguel, 2015). In conclusion, the present investigation contributes to elucidating diabetes genetics showing the existence of differences in patients' traits so as to promote translational and personalized medicine.

Table 1. Clinical characteristics of the studied populations.

Variable	Diabetes type	
	Classical	**Atypical**
Age	68.11 ± 9.02	65.63± 13.72
Age at diagnosis	49.08± 9.03	45.90±13.69
Age onset	19.08±8.21	20.12±8.79
BMI	31.40±5.71	31.41±4.51
Cholesterol	210.41±41.04	200.23±44.34
HDL	51.77±12.93	47.75±11.00
LDL	129.78±69.55	112.49±39.41
Triglycerids	186.98±98.18	191.84±95.78
Tg/HDL	3.98±2.8	4.3±2.5

Table 2. Allelic frequency of each SNP in both populations.

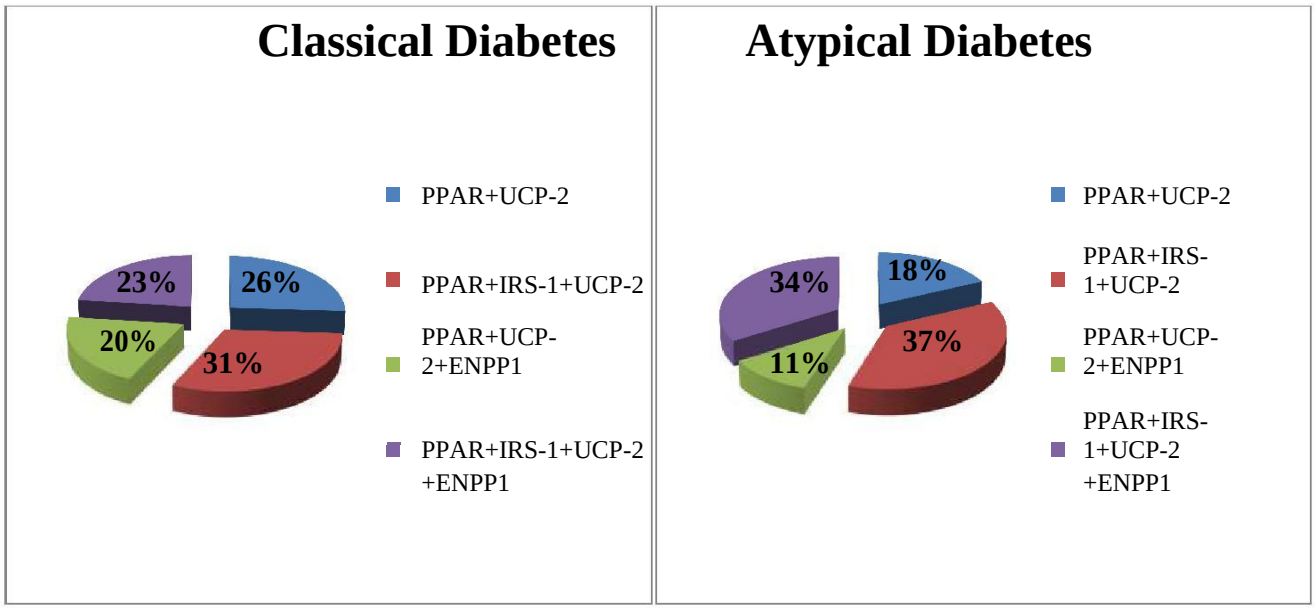
Gene	Allele	Classical diabetes	Atypical diabetes
PPAR-γ2 **(rs1801282)**	C	0.93	0.92
	G	0.07	0.08
IRS-1 **(rs1801278)**	G	0.73	0.65
	A	0.27	0.35
ENPP-1 **(rs1044498)**	K	0.75	0.76
	Q	0.25	0.24
UCP-2 **(rs659366)**	G	0.81	0.66
	A	0.19	0.34

Table 3. Genotypic frequencies in both groups.

Gene	Classical diabetes (%)		Atypical diabetes (%)		Odds (CI 95%)
PPAR-γ2	CC (%)	CG (%)	CC (%)	CG (%)	NS
(rs1801282)	86	14	84	16	
IRS-1	GA	GG	GA	GG	**2.06**
(rs1801278)	54	46	70	30	**(1.06-4.02)**
ENPP-1	KK	KQ + QQ	KK	KK + KQ	NS
(rs1044498)	57	43	55	45	
UCP-2	GA	GG	GA	GG	**3.32**
(rs659366)	38	62	67	33	**(1.71-6.46)**

NS: not significant

Figure 1. Genotypic combinations found in patients taking into account the existence of at least one risk allele in whichever of the analyzed genes.



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