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# Early and Late Onset Type 1 Diabetes: One and the Same or Two Distinct Genetic Entities?

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## 1. Introduction

Type 1 diabetes is a complex autoimmune disease in which genetic and environmental factors add up to induce an autoimmune destruction of the insulin-producing pancreatic  $\beta$  cells. Although type 1 diabetes is popularly associated to an onset in infancy or adolescence, it can begin at any age. The reasons behind this temporal difference in the onset of the disease are probably a mixture of genetic and environmental factors, just as the induction of the disease itself. Despite the great progress that the study of the genetics of type 1 diabetes has experienced in the last years, the genetic factors that could modify the age at diagnosis of type 1 diabetes have not been analyzed so deeply. This knowledge would be interesting to discover new routes to delay the disease onset and preserve the  $\beta$  cell mass as long as possible. In this chapter, we will review the characteristics of adult-onset type 1 diabetes patients and afterwards we will focus on the studies in type 1 diabetes genetics and the reported associations of genetics and age at onset of the disease. Finally, we will present an analysis of ten genetic associations in a group of Spanish patients with early and late onset of type 1 diabetes.

## 2. Diagnostic criteria for diabetes and further classification of the disease

Type 1 diabetes is the most prevalent chronic disease in childhood and it is also the most frequent form of diabetes in subjects diagnosed before age 19 (Duncan, 2006). Adults can also suffer from type 1 diabetes, given that the prevalence rate does not vary greatly with age, but the diagnosis of the disease in adult age is complicated by the higher prevalence of type 2 diabetes, which is the most frequent form of diabetes in adulthood (American Diabetes Association [ADA], 2010). Classical diabetes classifications used to categorize patients by their age at diagnosis or insulin requirement. Thus, type 1 diabetes was termed juvenile diabetes or insulin-dependent diabetes mellitus (IDDM) and type 2 diabetes could be diabetes of the adult or non-insulin dependent diabetes mellitus (NIDDM). However, type 2 diabetes can begin as an insulin-dependent condition or at an early age, type 1 diabetes can begin at any age, and a certain form of adult onset autoimmune diabetes termed latent autoimmune diabetes of the adult (LADA) is non-insulin-dependent by

definition at the time of diagnosis, nevertheless it is an autoimmune form of diabetes. Therefore, cataloging the different forms of diabetes is not so simple and classifications based in age at diagnosis or insulin requirement are no longer employed. Hence, we will review the diagnostic criteria for diabetes and define what can be considered adult-onset type 1 diabetes.

According to the most recent classification of the American Diabetes Association (ADA, 2010), the diagnostic criteria for diabetes are 1) levels of glycosilated haemoglobin over 6.5%, 2) fasting plasma glucose levels over 126 mg/dl, defining fasting state as no caloric intake for at least eight hours, 3) plasma glucose over 200 mg/dl at two hours during an oral glucose tolerance test (OGTT) or 4) random plasma glucose over 200 mg/dl in a patient with classic symptoms of hyperglycemia (poliuria, polidypsia or glucosuria). Patients with type 1, type 2 diabetes or LADA must meet these criteria. Then, further classification of the patient should be considered.

2.1 Type 1 diabetes

Type 1 diabetes accounts for 5-10% of the total cases of diabetes and it is the 90% of the cases of diabetes diagnosed in children (ADA, 2010). The disease is an autoimmune condition characterized by the destruction of the pancreatic  $\beta$  cells by autoreactive T lymphocytes. Hyperglycemia manifests when 60-90% of the  $\beta$  cell mass has been lost. As a result of the autoimmune insult, antibodies against pancreatic islets are synthesized and can be detectable in serum (see table 1). These antibodies precede in several years the clinical symptoms. They are not pathogenic (Wong et al, 2010), but its detection helps in the classification of the patient as type 1 diabetes, especially when the disease is diagnosed in adulthood. Antibodies against pancreatic antigens are positive at diagnosis in 90% of type 1 diabetes patients. Obesity is quite uncommon in these patients, but not incompatible with the disease. Patients are frequently insulin-dependent since diagnosis and insulin-replacement therapy is ultimately necessary for survival. Also, C-peptide levels (a measure of  $\beta$  cell activity) are usually low or undetectable. When untreated, type 1 diabetes leads to diabetic ketoacidosis, a life-threatening condition derived from the use of fat deposits (ADA, 2010).

Adult-onset type 1 diabetes patients tend to have a softer disease onset, with a lower frequency of diabetic ketoacidosis and a slower loss of insulin secretion capacity (Hosszufalusi et al, 2003; Leslie et al, 2006). These characteristics lead to think that a slower autoimmune reaction is taking place in the patient with adult onset.

	Autoantibody	Antigen expression
IAA	Anti-insulin antibodies	Pancreas
GADA	Anti-glutamate decarboxilase antibodies	Pancreas/nervous system
IA2-A	Anti-insulinoma associated 2 antibodies	Pancreas
ICA	Anti-islet cell antibodies (several antigens)	Pancreas
SCL38A	Antibodies against the zinc channel ZnT8	Pancreas

Table 1. Autoantibodies against pancreatic antigens in type 1 diabetes.

## 2.2 Type 2 diabetes

Type 2 diabetes includes 90-95% of the total cases of diabetes and accounts for 80-85% of the cases diagnosed in adulthood (ADA, 2010). The disease is a result of a combination of peripheral insulin resistance and relative insulin deficiency that develops into hyperglycemia. The hyperglycemia in type 2 diabetes appears gradually, usually with absence of the classic symptoms (polyuria, polydipsia) and can go undetected for long time before diagnosis. The etiologic factors of type 2 diabetes are unknown and probably this trait, more than type 1 diabetes, is composed of several different diseases with the common clinical manifestation of hyperglycemia. However, there is no proof of an implication of an autoimmune response, and thus autoantibodies against pancreatic antigens are always negative (ADA, 2010). Patients are usually obese and non-insulin dependent, although insulin can become a necessary therapy for a good control of hyperglycemia in some cases. However, insulin treatment in type 2 diabetes is not required for survival. C-peptide levels can be lower than in healthy controls, reflecting the relative insulin deficiency, but they are higher than in type 1 diabetes patients and decrease more gradually with time (Hosszufalusi et al, 2003). Diabetic ketoacidosis is quite rare and tends to develop due to underlying conditions, such as an infection (ADA, 2010).

## 2.3 Latent autoimmune diabetes of the adult (LADA)

Early after the discovery of antibodies against pancreatic antigens in the serum of type 1 diabetic subjects, it was noticed by clinicians that 10% of patients first diagnosed with type 2 diabetes tested positive for type 1 diabetes antibodies (mainly GADA and ICA) (Palmer et al, 2005). Those patients slowly but relentlessly progressed to insulin-dependency and showed signs of pancreatic islet dysfunction such as a progressive decrease of C-peptide levels. These characteristics defined a new category in the diabetes spectra called the latent autoimmune diabetes of the adult, and abbreviated LADA. Diagnostic criteria for these patients are age at diagnosis over 30 years, presence of antibodies against pancreatic islets, with a higher frequency of single positivity and GADA or ICA antibodies than type 1 diabetes patients, and no requirement of insulin for at least six months since diagnosis (Palmer et al, 2005; Leslie et al, 2006). There seems to exist a similar but milder genetic background to that of type 1 diabetic patients (Hosszufalusi et al, 2003; Palmer et al, 2005), and some researchers think of LADA as a slower and less aggressive form of type 1 diabetes, to the point that this condition is usually termed type 1.5 diabetes. Debate exists over LADA being an entity of its own or just a less aggressive form of type 1 diabetes at an older age (Hosszufalusi et al, 2003; Palmer et al, 2005; Leslie et al, 2006; Steck & Eisenbarth, 2008). Anyway, this group of patients poses a very interesting subset for testing  $\beta$  cell preserving therapies in an autoimmune form of diabetes due to its slow progression to insulin-dependency.

## 2.4 The diagnosis of an adult-onset diabetic patient

When recruiting patients for genetic studies, a careful evaluation of adult-onset patients must be carried out to avoid misclassification. In most cases, the three more common forms of diabetes in adults can be distinguished with a test for antibodies against pancreatic antigens at diagnosis and the requirement for insulin therapy (see table 2).

	Adult-onset type 1 diabetes	Type 2 diabetes	LADA
Insulin requirement	Ultimately needed for survival	Useful for improved glycemic control in some cases	Not for the first 6 months after diagnosis. Patients eventually evolve to insulin-dependency
Corporal phenotype	Usually lean	Usually obese	Variable
Antibodies	2 or more positive	Negative	At least 1 positive High frequency of GADA+ and/or ICA+
C-peptide levels	Low or absent	Normal or slightly decreased	Low
Diabetic ketoacidosis	Present	Rare	Rare
T-cell response against pancreatic islet antigens	Positive	Negative	Positive
Type 1 diabetes HLA susceptibility	Present	Absent	Present
% of total adult diabetes	5-10%	80-85%	5-10%

Table 2. Summary of the characteristics of the three more common forms of diabetes in adulthood: adult-onset type 1 diabetes, type 2 diabetes and LADA (Leslie et al, 2006; ADA, 2010).

The subgroup of adult-onset type 1 diabetic patients is relatively easy to separate from the other two clinical manifestations of diabetes in adulthood: patients should be insulin-dependent since diagnosis, positive for at least two type 1 diabetes autoantibodies and usually of lean body type. However, many genetic studies in the last years have excluded the adult subset of type 1 diabetic patients as a precaution to avoid contamination with type 2 diabetic patients. This conservative measure has excluded a group of patients that could give us important information about the genetics of the disease: the existence of genes that modify the progression of the disease, together with unknown environmental factors, which would be the causatives of the fast immune  $\beta$  cell destruction in a child and the slower destruction in an adult patient.

From now on, we will focus on the genetics of type 1 diabetes and the study of the influence of genetics in age at disease onset.

3. Genetic studies in type 1 diabetes

The existence of a genetic basis that influences the development of type 1 diabetes is known since the first studies associated alleles and haplotypes in the Human Leukocyte Antigen (HLA) complex with type 1 diabetes risk in the late 70s (Nerup et al, 1974). Familial studies have been able to quantify the genetic basis of the disease in a range between 30-70% of the total contributing factors, being the remainder due to

environmental factors (Redondo et al, 2001a; Pociot et al, 2010). Half of this genetic contribution is due to alleles and haplotypic combinations in the HLA region (Erlich et al, 2008), which is the strongest genetic modifier of type 1 diabetes risk. The rest of the genetic load is composed of genes with smaller effects, some of which have been unveiled in the last thirty years through different approaches developed as the technology for DNA study evolved. These studies ranged from the association studies of candidate genes to the complex hypothesis-free genome-wide association studies. In this section, we will briefly review the methodology employed in these studies and its importance in the unraveling of the type 1 diabetes genetic component.

3.1 Association studies of candidate genes

The association studies are suited for the detection of variants with moderate or low effects on the disease, as long as the studied variants are relatively frequent in the population of study (minor allele frequency over 5%) (Pociot et al, 2010; Steck & Rewers, 2011). Association can be measured in a case-control design (study of the differences between a set of unrelated patients and healthy controls) or a family design (analysis of deviations in the theoretical 50% transmission of the variant from healthy parents to patients). Previous to the massive knowledge that the Human Genome Project provided to genetic studies in humans, association studies had to focus on the selection of candidate genes, which limited these studies to genes with known function and biased the selection by what was known (or believed) about the pathogenesis of the disease at the moment. Nevertheless, this approach discovered the five classical regions associated with type 1 diabetes and detailed in table 3.

First reported	Gene	Function
1970-1980 (Nerup et al, 1974)	HLA class II	Antigen presentation in antigen-presenting cells.
1984 (Bell et al, 1984)	<i>INS</i>	Expression levels in the thymus regulate the presence of insulin-reactive T cells.
1996 (Nistico et al, 1996)	<i>CTLA4</i>	Modulator of inactivation of the immune response. Constitutively expressed in regulatory T cells, a lymphocyte subset specialized in the suppression of autoimmunity.
2004 (Bottini et al, 2004)	<i>PTPN22</i>	Suppressor of signals through the TCR. Susceptibility variant is believed to favor the survival of auto-reactive T cells in the thymus.
2005 (Vella et al, 2005)	<i>IL2RA</i>	Alpha subunit of the high affinity IL2 receptor. Constitutively expressed in regulatory T cells, it is essential for the maintenance of this cell subset.

Table 3. Classical type 1 diabetes associated genes discovered through association studies of candidate genes. TCR: T cell receptor.



### 3.2 The genome wide association studies

The first genome-wide association study was published in 2007 (The Wellcome Trust Case-Control Consortium [WTCCC], 2007). Samples from seven diseases (among them type 1 and type 2 diabetes) were collected, recruiting 2000 cases of each disease and a common subset of 3000 healthy controls. This single study described four new regions strongly associated with type 1 diabetes, almost the same number of regions that the previous association studies had taken three decades to discover.

Genome-wide association studies were possible thanks to the great development in the knowledge of human genetics and in the techniques to study DNA, derived from initiatives such as the Human Genome Project. The design of a genome-wide study is based on the analysis of over 500.000 single nucleotide polymorphisms (SNPs) throughout the genome in each subject recruited. The study design is usually a case-control approach, but both case-control and family studies are commonly used to replicate the stronger associations in the recent genome-wide studies (Hakonarson et al, 2007; Barrett et al, 2009). Since their object of analysis is the whole genome, these kind of studies are hypothesis-free and are able to detect associations in regions and genes that a candidate gene study would have never considered, such as regions with genes of unknown function and genes in routes not classically considered to take part in the pathogenesis of the disease. Thus, from the four regions associated with type 1 diabetes and described in the WTCCC study (WTCCC, 2007), one (16p13) covered a gene of unknown function, and two (12q13 and 12q24) pointed to regions with several candidate genes.

Despite the advantage that poses the hypothesis-free design, the genome-wide association studies have a great disadvantage in return: the high number of polymorphisms studied implies an elevated number of statistical comparisons and an increased probability of obtaining false-positive associations. Therefore, these studies are subject to a strong statistical correction (WTCCC, 2007), and, depending on the number of markers analyzed, p values should be as low as  $10^{-7}$  to be considered statistically significant (Todd et al, 2007). Moreover, the results obtained (especially those that are borderline significant) should be replicated in independent populations to assure that the result is not a false positive and it is not influenced by population variability (McCarthy et al, 2008). At this stage is where follow-up studies take place. Follow-up studies select associations from genome-wide studies for replication purposes, being the more interesting those that are borderline significant.

Genome-wide association studies and their follow-up have been successful in uncovering associations of high to moderate effect (odds ratio over 1.15) in variants with a minor allele frequency over 5%. Now, the remainder of the genetic component in type 1 diabetes is proposed to reside in rare variants with high effect and common variants with low effect on the disease (Pociot et al, 2010). Due to the stringent statistical correction required, genome-wide studies are not suitable for detecting these associations and new approaches will be necessary. Despite their limitations, in just five years genome-wide studies have revealed ten times more genetic regions than the older approaches did in thirty years, providing fifty genetic regions associated with type 1 diabetes. A brief summary of these regions can be consulted in table 4.

### 3.3 The problem with age at diagnosis

Although type 1 diabetes has a similar prevalence in all ages, the restriction to pediatric patients has been a popular criterion for recruitment of patients in studies on the genetics of the disease, as it is shown in table 5. Its purpose is to avoid the inclusion of misdiagnosed

Chromosome	Candidate gene	OR	Reference study	Published age-at-onset analysis
1p13.2	<i>PTPN22</i>	2.05	(Smyth et al. 2008)	Yes
1q31.2	<i>RGS1</i>	0.89	(Smyth et al. 2008)	
1q32.1	<i>IL20-IL10-IL19</i>	0.84	(Barrett et al. 2009)	
2q11.2	Several	--	(Barrett et al. 2009)	
2q24.2	<i>IFIH1</i>	0.86	(Smyth et al. 2008)	
2q32.2	<i>STAT4</i>	1.10	(Fung et al. 2009)	Yes
2q33.2	<i>CTLA4</i>	0.82	(Smyth et al. 2008)	
3p21.31	<i>CCR5</i>	0.85	(Smyth et al. 2008)	
4p15.2	<i>AC111003.1</i>	1.09	(Barrett et al. 2009)	
4q27	<i>IL2-IL21</i>	1.13	(Barrett et al. 2009)	
6p21	<i>HLA</i>	0.02-49.2	(Ounissi-Benkalha and Polychronakos 2008)	Yes
6q15	<i>BACH2</i>	1.13	(Cooper et al. 2008)	
6q22.32	<i>CENPW</i>	1.17	(Barrett et al. 2009)	
6q23.3	<i>TNFAIP3</i>	0.90	(Fung et al. 2009)	
6q25.3	<i>TAGAP</i>	0.92	(Smyth et al. 2008)	
7p15.2	Several	0.88	(Barrett et al. 2009)	
7p12.1	<i>COBL</i>	0.77	(Barrett et al. 2009)	
9p24.2	<i>GLIS3</i>	0.88	(Barrett et al. 2009)	
10p15.1	<i>IL2RA</i>	0.62	(Smyth et al. 2008)	Yes
10p15.1	<i>PRKCQ</i>	0.69	(Lowe et al. 2007)	
10q22.3	<i>ZMIZ1</i>	--	(Barrett et al. 2009)	
10q23.31	<i>RNLS</i>	0.75	(Barrett et al. 2009)	
11p15.5	<i>INS</i>	0.42	(Smyth et al. 2008)	Yes
12p13.31	<i>CLEC2D-CD69</i>	1.09	(Barrett et al. 2009)	
12q13.3	<i>CYP27B1</i>	1.22	(Bailey et al. 2007)	
12q13.2	<i>ERBB3</i>	1.31	(Barrett et al. 2009)	Yes
12q24.12	<i>SH2B3</i>	1.28	(Smyth et al. 2008)	Yes
13.32.3	<i>GRP183</i>	1.15	(Heinig et al. 2010)	
14q24.1	Several	0.86	(Barrett et al. 2009)	
14q32.2	Several	1.09	(Barrett et al. 2009)	
14q32.2	Several	0.90	(Wallace et al. 2009)	
15q14	<i>RASGRP1</i>	1.21	(Qu et al. 2009)	
15q25.1	<i>CTSH</i>	0.86	(Smyth et al. 2008)	
16p13.13	<i>CLEC16A</i>	0.81	(Smyth et al. 2008)	Yes
16p11.2	<i>IL27</i>	0.86	(Barrett et al. 2009)	
16q23.1	Several	1.28	(Barrett et al. 2009)	
17q12	Several	0.87	(Barrett et al. 2009)	
17q21.2	<i>SMARCE1</i>	0.95	(Barrett et al. 2009)	

Table 4. Summary of the 50 chromosomal regions currently associated with type 1 diabetes (continues in next page). The odds ratio in the table has been extracted from the reference study. Data come from the on-line database [www.t1dbase.org](http://www.t1dbase.org), belonging to the Type 1 Diabetes Genetics Consortium (T1DGC). The reference study does not correspond with the published age-at-onset study. Genes *CLEC16A* and *SH2B3* were analyzed in Todd et al (2007) and where not associated with age at onset. The rest of the age-at-onset associations are reviewed in section 4.



Chromosome	Candidate gene	OR	Reference study	Published age-at-onset analysis
18p11.21	<i>PTPN2</i>	1.28	(Smyth et al. 2008)	Yes
18q22.2	<i>CD226</i>	1.16	(Smyth et al. 2008)	
19p13.2	<i>TYK2</i>	0.86	(Wallace et al. 2009)	
19q13.32	Several	0.86	(Barrett et al. 2009)	
19q13.4	<i>FUT2</i>	--	(Barrett et al. 2009)	
20p13	Several	0.90	(Barrett et al. 2009)	
21q22.3	<i>UBASH3A</i>	1.13	(Smyth et al. 2008)	
22q12.2	Several	1.10	(Barrett et al. 2009)	
22q12.3	<i>IL2RB</i>	--	(Barrett et al. 2009)	
22q13.1	<i>C1QTNF6</i>	1.11	(Cooper et al. 2008)	
Xp22.2	<i>TLR8</i>	0.84	(Barrett et al. 2009)	
Xq28	Several	1.16	(Barrett et al. 2009)	

Table 4 (continuation). Summary of the 50 chromosome regions currently associated with type 1 diabetes.

patients (type 2 diabetics or LADA patients) within adult-onset diabetic patients. However, enough criteria exist to discriminate type 1 diabetic patients from the remainder of diabetic adults, and the exclusion of adult type 1 diabetes patients limits the knowledge of the genetics of the disease only to its early onset. Adult-onset patients show signs of a slower immune reaction to  $\beta$  cells. The factors that cause a rapid destruction of  $\beta$  cells in a child but a slower degeneration in an adult-onset patient are unknown, nevertheless they are probably a mixture of genetic and environmental factors. Some hypotheses may explain the different speed in clinical manifestations: the genetic load of adult-onset diabetes could be composed of a lower number of associated genes than in the early-onset patients, or could be the same genes but with less effect in the adult disease, or maybe the adult-onset population has genes associated that are exclusive of adult-onset. Besides, the simple replication in adult-onset patients of associations found in pediatric type 1 diabetes is interesting to prove that, from a genetic perspective, adult-onset patients are as much type 1 diabetes as the pediatric-onset ones.

Four genome-wide studies and a series of follow-up have been published in type 1 diabetes in the last five years (table 5). Two included systematically some adult-onset patients in their populations; however most of these studies lacked an analysis of the influence of genetics in the age of diagnosis. The characteristics of the four genome-wide and some selected follow-up and major genetic studies, and the populations included in them, can be consulted in table 5.

The two populations that recruit late-onset patients deserve a more detailed commentary. The GoKinD (Genetics of kidneys in diabetes) population, included in Cooper et al in 2008, belongs to a United States project that aims at studying the genetics of kidney diseases in type 1 diabetes. Selection criteria for type 1 diabetes were age at diagnosis before 31 years, insulin therapy needed within the first year of diagnosis and not interrupted for any reason ever since. Patients had a minimum disease duration of 10 years. Analysis of the influence of genetics in age at diagnosis was not carried out in this genome-wide study.

Study	Year of publication	Population	Age limit for selection of participants	Study of genetics and age at diagnosis
(1) WTCCC	2007	2000 cases, British	Diagnosis before 17 years	No
(2) Todd et al	2007 Follow-up from WTCCC	4000 cases and 2997 British families	Diagnosis before 17 years	Yes
(3) Hakonarson et al	2007	563 cases and 1422 families from Britain, the US and Australia	Most diagnosed before 18 years	No
(4) Cooper et al	2008	GoKinD population 1785 US cases	Diagnosis before 31 years	No
(5) Smyth et al	2008 Major genetic study	8064 cases and 3064 families from US, Finland, Ireland, Norway and Romania	Diagnosis before 17 years	No
(6) Barrett et al	2009	T1DGC population 3983 cases and 2319 families from Britain, the US and Australia	Diagnosis before 35 years	No

Table 5. Genome-wide and major genetic studies performed in type 1 diabetes. Studies 2, 4 and 6 performed metanalysis with the WTCCC data. Also, the last genome-wide carried out by Barrett et al (study 6) performed a meta-analysis of the three larger genome-wide studies (1, 4 and 6) performed in type 1 diabetes.

The T1DGC (Type 1 Diabetes Genetics Consortium) is an initiative constituted in 2002 with the aim of providing resources to the research in type 1 diabetes. Since 2007, the consortium has published several studies on type 1 diabetes genetics (Erlich et al, 2008; Hakonarson et al, 2008; Howson et al, 2009; Qu et al, 2009) and, although the genome-wide study in which the T1DGC population was genotyped did not include an age-at-onset analysis, this group is lately including age-at-onset analyses in their publications and some of their studies have provided the first evidences of influence of genetics in age at onset of the post-genome wide era (Hakonarson et al, 2008). The selection criteria for adult patients are year at diagnosis under 35 years and uninterrupted insulin treatment for at least 6 months (Hakonarson et al, 2007). However, despite the wider limit in age at onset in this population, the majority of the patients included are pediatric, as reflected in the mean age at onset (around ten years) found in the published studies (Hakonarson et al, 2008; Howson et al, 2009).

4. Reported genetic associations of genes and age-at-diagnosis

The influence of genetics in age at onset of type 1 diabetes has been analyzed in some studies. However, initiatives to replicate these first studies or to establish a protocol to analyze the genetics or early and late onset are lacking, and therefore there is disparity in the definition and selection of late-onset type 1 diabetes patients, and in the statistical methods employed to analyze the associations. In this section, we will review some selected studies on the influence of genetics in age at diagnosis of type 1 diabetes.

- Familial studies: analysis in monozygotic twins with one member affected with type 1 diabetes have shown that the probability of developing the disease in the non-affected twin is considerably higher (38%) when the affected twin developed type 1 diabetes at an early age (under 24 years) than when the affected twin developed the disease after 25 years of age (6% risk for the non-affected twin) (Redondo et al, 2001b). This observation might suggest that early-onset type 1 diabetes has a stronger genetic component (responsible of the higher concordance rate) than the same disease with a late onset.
- HLA associations to age at onset: several studies (Redondo et al, 2001a; Leslie et al, 2006; Klinker et al, 2010) have pointed out to the higher risk and earlier onset of type 1 diabetes in patients that are heterozygote for the HLA class II risk haplotypes *DRB1\*03* and *DRB1\*04-DQB1\*03:02*. On the other hand, the influence of HLA class I alleles in age at diagnosis has been thoroughly studied, and alleles *B\*39* and *A\*24* have been consistently associated to an earlier onset of the disease (Valdes et al, 2005; Nejentsev et al, 2007). The *B\*39* allele, for example, precipitates the age of diagnosis in four years (Valdes et al, 2005).
- *IL12B*: the gene *IL12B* codes for the p40 subunit of the interleukin 12, also shared with interleukin 23. A 2004 study (Windsor et al, 2004) carried out in an Australian cohort including early and late onset patients found association of a polymorphism in position +1188 of the gene with late-onset of the disease (over 25 years). Neither associations on the *IL12B* gene with type 1 diabetes nor the described age-at-onset association have been replicated in recent studies.
- *CAPSL-IL7R*: this region was first associated with type 1 diabetes in a study of non-synonymous polymorphisms, finding a marker in the *CAPSL* gene that was highly associated with the disease (Smyth et al, 2006). Another polymorphism in the *IL7R* gene has been associated to type 1 diabetes and multiple sclerosis (Hafler et al, 2007; Todd et al, 2007). Our group undertook a replication study on both polymorphisms briefly after the discovery of the first signal (Santiago et al, 2008). We found association with type 1 diabetes in both polymorphisms and also described that both markers were associated with an earlier onset, an effect more noticeable in the *IL7R* polymorphism.
- Region 12q13 (*ERBB3* gene): in a replication study of borderline significant signals from a previous genome-wide (Hakonarson et al, 2007), the T1DGC found evidence of the association of three 12q13 polymorphisms with age at diagnosis (Hakonarson et al, 2008). The influence of this region on age at onset of type 1 diabetes has been subsequently analyzed in two independent studies (Awata et al, 2009; Wang et al, 2010) that, with different statistical methodology, did not replicate the effect seen in the first study. Finally, we have studied several signals in this region and found an age-at-diagnosis effect stronger than the previously described, with homozygotes for the susceptibility allele having an age at onset five years earlier than carriers of protective alleles (Espino-Paisan et al, 2011b).
- Region 2q32 (*STAT4* gene): a study in 2008 described an association of polymorphisms in the *STAT4* gene with type 1 diabetes patients with an onset earlier than 8 years (Lee et al, 2008). Among the polymorphisms studied was rs7574865, which we will include in our study on age at onset in section 5. This study was carried out in a pediatric Korean population, therefore population differences have to be taken in consideration, given that the genetics of Asian and Caucasian type 1 diabetes patients present some important differences (Ikegami et al, 2007).

- **Insulin gene:** the T1DGC group analyzed several classical type 1 diabetes genes (Howson et al, 2009) and found association of the susceptibility variant in the insulin gene to an onset of type 1 diabetes two years earlier than the protective allele. However, this effect was not replicated in one of the cohorts included in the study. We will analyze the same polymorphism in our study in section 5.
- ***IL2RA*:** a Finnish study analyzed several classical type 1 diabetes genes in a group with late-onset of the disease (Klinker et al, 2010). They found associations of the insulin, *PTPN22*, *IFIH1* and *CTLA4* genes with late-onset patients, and replicated the age-at-onset effect of the *DRB1\*03-DRB1\*04-DQB1\*03:02* heterozygote. They also found that *IL2RA* was associated with an earlier disease onset. However, the T1DGC studied the *IL2RA* gene and did not find any effect in age at onset, although they did not include the stronger association in the gene that the Finnish study did analyze (Howson et al, 2009). Our group conducted a replication study in polymorphisms in the *IL2RA* gene and we found them associated to both early and late disease onset (Espino-Paisan et al, 2011c).
- ***PTPN22*:** a German group studied the C1858T polymorphism in the *PTPN22* gene and found that the susceptibility polymorphism was associated to an earlier onset of the disease in a group of pediatric-onset patients (Kordonouri et al, 2010). Patients with the susceptibility polymorphism had an onset of the disease two years earlier than homozygotes for the protective allele. However, this observation was not replicated in the T1DGC study (Howson et al, 2009). We will study this polymorphism in our group of pediatric and adult patients in section 5.
- ***PTPN2*:** our group studied the influence in age at disease onset of two polymorphisms in the *PTPN2* gene that had been previously associated with type 1 diabetes (Todd et al, 2007; WTCCC, 2007). We found that one of the studied polymorphisms was associated with an earlier disease onset, with carriers of the susceptibility allele having a disease onset almost three years earlier than homozygotes for the protective allele (Espino-Paisan et al, 2011a).

## 5. A practical study: Genetic analysis of a population with early and late-onset type 1 diabetes patients

We have selected ten chromosome regions (five classical genes and five genome-wide discoveries) previously studied in type 1 diabetes to test their association with pediatric and late-onset patients. We will briefly review their role in the pathogenesis of the disease and compare our results with the previous reported associations.

### 5.1 Population of study and methods

A total of 444 type 1 diabetes patients (47% female) were included in this study. All patients were recruited from the Madrid area (Spain), all where Caucasoid and diagnosed according to the criteria of the American Diabetes Association (ADA, 2010). Age at diagnosis was available for 415 patients and ranged from 1 to 65 years. Mean age at onset of the population was  $18.6 \pm 11.1$  years and median age at onset was 16 years. All patients were insulin-dependent since diagnosis and had been on uninterrupted insulin treatment for at least 6 months. Adult patients diagnosed over 35 years were included on the basis of positivity to autoantibodies, lean body type and insulin-dependency status. Also, a maximum of 888 ethnically matched controls (53.7% female) with no history of type 1 diabetes in first degree relatives were recruited.

Genes in the HLA complex were genotyped by two SSOP (Sequence Specific Oligonucleotide Probe) procedures: dot-blot hybridization and Luminex technology. The remaining genes were studied through genotyping of single nucleotide polymorphisms by TaqMan Assays in a 7900HT fast real-time PCR system (Applied Biosystems Foster City, CA, USA). The call rate for each SNP was 95%. A summary of these studied polymorphisms can be consulted in table 6.

Chromosome	Candidate gene	SNP	Assay reference	Control MAF
1p13	<i>PTPN22</i>	rs2476601	By design	T (0.06)
2q24	<i>IFIH1</i>	rs1990760	C__2780299_30	G (0.46)
2q32.3	<i>STAT4</i>	rs7574865	C__29882391_10	T (0.21)
2q33.2	<i>CTLA-4</i>	rs231775	C__2415786_20	T (0.29)
		rs3087243	C__3296043_10	G (0.48)
6q23.3	<i>TNFAIP3</i>	rs10499194	C__1575581_10	T (0.32)
9q33.2	<i>TRAF1</i>	rs2269059	C__15875924_10	A (0.07)
11p15.5	<i>INS</i>	rs689	C__1223317_10	A (0.28)
12p13.31	<i>CLEC2D</i>	rs11052552	C__32169467_10	G (0.49)
16p13.13	<i>CLEC16A</i>	rs2903692	C__15941578_10	A (0.42)

Table 6. Summary of the genotyped SNPs in each gene. MAF: minor allele frequency.

No statistically significant deviations from Hardy-Weinberg equilibrium were found in the control subset for each polymorphism. A case-control analysis was performed to asses association of the selected variants with type 1 diabetes. Differences were calculated through Chi-square and Fisher’s exact tests when necessary. Analysis of age at onset was performed through a stratified and a continuous approach. For the stratified analysis, cases were classified in early-onset (age at diagnosis under 17 years) or late-onset (age at diagnosis over 16 years) and compared with Chi-square test or Fisher’s exact test. Associations were estimated by the odds ratio (OR) with 95% confidence interval. All Chi-square and Fisher’s exact test comparisons were calculated with Epi Info v.5 (CDC, Atlanta, USA). For the continuous analysis approach, ages at onset associated to each allele were compared with the non-parametric U Mann-Whitney test implemented in SPSS v.15.0 (Chicago, Illinois, USA).



## 5.2 Selected genes and results

### 5.2.1 Classical type 1 diabetes associations

**Class II HLA alleles (chromosome 6p21):** class II HLA binds extra-cellular antigens processed by antigen presenting cells and presents them to CD4+ helper T cells. The proposed mechanism in the pathogenesis of type 1 diabetes takes place at the negative selection process during lymphocyte thymic maturation (Redondo et al, 2001a; Ounissi-Benkalha and Polychronakos, 2008). Negative selection occurs when a T cell with an autoreactive T cell receptor (TCR) binds a HLA molecule loaded with an autoantigen. This union sends a strong activation signal through the TCR that is deleterious to the autoreactive T cell. Theoretically, susceptibility HLA alleles bind pancreatic antigens less efficiently, lowering the activation signal to non-deleterious levels and allowing the autoreactive T cell to escape from thymic selection.

The HLA associations detected in our group in the case-control analysis and the study on age at onset can be consulted in table 7. Due to the high number of alleles and haplotypic combinations in this region, and the low frequency of some of them, a stratified analysis would imply a marked loss of statistical power, so we choose to perform only the continuous analysis. We did not find evidence of an influence in age at diagnosis in any of the haplotypic combinations included, which means that our adult-onset patients have the same HLA contribution to type 1 diabetes than our pediatric patients. Of notice, there are two haplotypes with a marked difference in the mean age at diagnosis: the *DRB1\*04-DQB1\*03:02* homozygote (carriers have an onset three years earlier) and carriers of the protective haplotype *DRB1\*15:01-DQB1\*06:02* (carriers have an onset ten years later than non carriers). None of these comparisons are statistically significant, but it could be a problem of low statistical power since both genotypes are quite infrequent. A larger sample would be needed to elucidate the possible associations.

Many studies describe the *DRB1\*03-DRB1\*04* heterozygote as associated with earlier age at diagnosis of type 1 diabetes, and also as the haplotypic combination that confers a higher risk to the disease. In our population we do not see an age effect ( $p=0.9$ ). Moreover, the *DRB1\*03-DRB1\*04* heterozygote does not confer the higher risk, but the *DRB1\*04* homozygote, the *DRB1\*03* or *DRB1\*04* carrier, and the *DRB1\*03* homozygote.

**Insulin gene (chromosome 11p15):** the insulin gene is also proposed to participate in the generation of autoreactive T cells in the thymus. The polymorphism associated with type 1 diabetes is a VNTR (variable number of tandem repeats) that locates upstream of the *INS* gene and modifies its expression in the thymus (Pugliese et al, 1997; Vafiadis et al, 1997). Alleles in this VNTR range from 26 to 210 repetitions of a consensus sequence and are usually classified in three groups: short class I alleles (26 to 64 repetitions), intermediate class II alleles (64 to 139 repetitions, infrequent in Caucasian and Asian populations) and large class III alleles (140 to 210 repetitions). Large alleles are associated with protection from type 1 diabetes and are related to a higher expression of insulin in the thymus (Vafiadis et al, 1997). This is thought to favor the negative selection of insulin-reactive T cells, a theory that would be consistent with the lower levels of insulin antibodies detected in patients that carry the large class III alleles (Hermann et al, 2005). We have selected a polymorphism (rs689) in linkage disequilibrium with the two main classes of alleles that is usually employed in genetic studies (Hermann et al, 2005; Todd et al, 2007; Smyth et al, 2008) as a proxy to the VNTR genotyping.



Genotype	Case-control analysis			
	Genotype frequency		p	OR
	T1D	Controls		
DR4-DQ8 homozygote	0.052	0.002	6.0x10 <sup>-8</sup>	33.59 (5.40-1386)
DR3-DQ2 carrier or DR4-DQ8 carrier	0.894	0.384	2.1x10 <sup>-61</sup>	13.24 (9.27-18.96)
DR3-DQ2 carrier	0.143	0.015	3.1x10 <sup>-16</sup>	11.19 (5.31-24.41)
DR3-DQ2 DR4-DQ8	0.249	0.031	1.6x10 <sup>-26</sup>	10.36 (6.11-17.75)
DR4-DQ8 - X (not DR3)	0.256	0.123	3.7x10 <sup>-8</sup>	2.43 (1.74-3.39)
DR3-DQ2 - X (not DR4)	0.369	0.221	2.0x10 <sup>-7</sup>	2.03 (1.53-2.69)
DR2-DQ6 - X	0.011	0.186	4.5x10 <sup>-19</sup>	0.05 (0.02-0.12)

Table 7. Case-control analysis of selected HLA haplotypes. Comparisons were calculated with Chi-square and Fisher’s exact test when necessary. HLA haplotypes have been abbreviated: DR4-DQ8 (*DRB1\*04-DQA1\*03:01-DQB1\*03:02*), DR3-DQ2 (*DRB1\*03-DQA1\*05:01-DQB1\*02:01*), DR2-DQ6 (*DRB1\*15:01-DQA1\*01:02-DQB1\*06:02*). T1D: type 1 diabetes.

Genotype	Continuous analysis		
	Mean age at onset		P
	Carrier	Non carrier	
DR4-DQ8 homozygote	15.9 (11.5)	18.6 (11.1)	0.2
DR3-DQ2 carrier Or DR4-DQ8 carrier	18.3 (11.1)	19.4 (10.2)	0.4
DR3-DQ2 carrier	17.5 (11.2)	18.6 (11.0)	0.2
DR3-DQ2 DR4-DQ8	18.0 (10.7)	18.6 (11.2)	0.9
DR4-DQ8 - X (not DR3)	18.0 (10.5)	18.9 (11.5)	0.7
DR3-DQ2 - X (not DR4)	18.8 (11.9)	18.4 (11.5)	0.9
DR2-DQ6 - X	28.0 (13.7)	18.5 (11.0)	0.2

Table 7 (continuation). Analysis of age at onset in selected HLA haplotypes. Comparisons were calculated with the U Mann-Whitney test. HLA haplotypes: DR4-DQ8 (*DRB1\*04-DQA1\*03:01-DQB1\*03:02*), DR3-DQ2 (*DRB1\*03-DQA1\*05:01-DQB1\*02:01*), DR2-DQ6 (*DRB1\*15:01-DQA1\*01:02-DQB1\*06:02*). T1D: type 1 diabetes.

In our study, we replicate the association previously described in the *INS* gene and we do not find effects on age at onset in the stratified and continuous analysis. Results are provided in table 8.

Gene	CASE-CONTROL AND AGE-STRATIFIED ANALYSES				CONTINUOUS ANALYSIS		
	MAF		p	OR	Mean age at onset		P
					Major allele	Minor allele	
<i>INS</i>							
T1D-control	0.175	0.282	8.3x10 <sup>-9</sup>	0.54 (0.43-0.67)	19.3 (11.8)	18.0 (10.7)	0.4
Pediatric-adult T1D	0.162	0.177	0.6				
<i>CTLA4 rs231775</i>							
T1D-control	0.328	0.299	0.1	1.14 (0.96-1.37)	18.4 (10.9)	18.9 (11.4)	0.6
Pediatric-adult T1D	0.323	0.339	0.6				
<i>CTLA4 rs3087243</i>							
T1D-control	0.477	0.518	0.05	0.85 (0.72-1.00)	18.9 (11.0)	17.8 (10.8)	0.2
Pediatric-adult T1D	0.493	0.451	0.2				
<i>PTPN22</i>							
T1D-control	0.115	0.064	7x10 <sup>-6</sup>	1.91 (1.42-2.56)	18.4 (11.2)	19.0 (10.6)	0.4
Pediatric-adult T1D	0.113	0.127	0.5				
<i>IFIH1</i>							
T1D-control	0.373	0.409	0.1	0.86 (0.70-1.06)	17.2 (10.3)	17.2 (10.6)	0.9
Pediatric-adult T1D	0.373	0.394	0.6				

Table 8. Analysis of classical gene associations in type 1 diabetes. Minor allele frequency (MAF) is provided in case-control and age-stratified analyses, and comparisons were calculated with Chi-square and Fisher’s exact test when necessary. In the continuous analysis, mean ages at onset associated to each allele and the p value from the U Mann-Whitney test are presented. *INS*: insulin gene. T1D: type 1 diabetes.

**CTLA4 (chromosome 2q33):** this gene encodes a negative regulator of lymphocytic activation. Its expression is induced in activated lymphocytes, but it is also constitutively expressed in regulatory T cells, a lymphocyte subpopulation specialized in the suppression of autoimmunity. Also, a soluble form of CTLA4 is secreted in the serum, and it is believed that this form contributes to the downregulation of activation in the immune system (Ueda et al, 2003). Several polymorphisms have been identified and associated with type 1 diabetes (Ueda et al, 2003; Qu et al, 2009). We have selected two functional polymorphisms: one aminoacidic change related to lower membrane expression of the protein (rs231775) and a polymorphism in the 3’ end that is related to higher expression of soluble CTLA4 (rs3087243), which also is one of the strongest associations with type 1 diabetes in the gene (Ueda et al, 2003; Qu et al, 2009).

We replicate the association described in *CTLA4* rs3087243. Differences in *CTLA4* rs231775 do not reach statistical significance, but this could be due to low statistical power to detect the previously described association (Ueda et al, 2003). We do not find effects of any of the polymorphisms on age at onset. Results can be consulted in table 8.

**PTPN22 (chromosome 1p13):** this gene encodes a lymphoid-specific phosphatase called LYP, which is an important downregulator of T cell activation through the TCR. We selected the classical non-synonymous polymorphism C1858T that causes a substitution from arginine to tryptophan in the aminoacid 620 of the encoded protein (Bottini et al, 2004). The mutant form shows a higher phosphatase activity, and therefore it suppresses T cell activation more efficiently. Its role in type 1 diabetes is believed to be at the thymic selection process, where the mutant PTPN22 would lower the activation signal sent to the autoreactive T cell through its TCR, thus contributing to its survival (Bottini et al, 2006). It also has been proposed that the increased suppression of activation associated to the mutant form could affect negatively the activation of regulatory T cells (Bottini et al, 2006).

We replicate the association previously described in the *PTPN22* gene and we do not find effects on age at onset in the stratified and continuous analysis. Results can be consulted in table 8.

**IFIH1/MDA5 (chromosome 2q24):** certain viral infections such as that caused by Enterovirus are more prevalent in type 1 diabetes patients than in the healthy population, and it has been proposed that they could participate in the development or acceleration of the immune response against the  $\beta$  cell (Hober & Sauter 2010). The helicase IFIH1 recognizes viral double stranded RNA (dsRNA) and it is expressed in the cytoplasm of several cells, including  $\beta$  cells. In the presence of a viral infection, IFIH1 binds the dsRNA and induces the synthesis of pro-inflammatory cytokines. Functional experiments show that protection from type 1 diabetes is achieved through a lower performance of the sentinel role of IFIH1 that would end up in lower activation of the immune system in response to the viral infection (Colli et al, 2010).

We detect a lower frequency of the minor allele of *IFIH1* in type 1 diabetes patients respect to controls; however this difference is not statistically significant, probably due to low statistical power of our study. We do not find effects on age at diagnosis of type 1 diabetes in the stratified and continuous analysis. Results are provided in table 8.

### 5.2.2 Genome-wide associations

**Region 2q32 (STAT4):** the gene *STAT4* is an interesting candidate for type 1 diabetes. Member of a family of transcription factors, *STAT4* activates the transcription of several genes including IFN- $\gamma$  in response to interleukin-12 signaling. The pathway IL12-*STAT4*-IFN $\gamma$  polarizes the immune response to a Th1 type, the kind of response that is thought to be responsible of the type 1 diabetes autoimmune reaction (Raz et al, 2005). We have selected a polymorphism that was first discovered associated with rheumatoid arthritis (Remmers et al, 2007). Our group studied this polymorphism in several autoimmune diseases and described its association with type 1 diabetes (Martinez et al, 2008), as it can be seen in table 9. Influence of this polymorphism in age at onset has been previously studied in a pediatric-onset Korean population, as we described in section 4. We performed a stratified and continuous analysis of age at onset and we did not find evidences of the influence of this polymorphism in age at onset of the disease. Results can be consulted in table 9.

Gene	CASE-CONTROL AND AGE-STRATIFIED ANALYSES				CONTINUOUS ANALYSIS		
	MAF		p	OR	Mean age at onset		P
					Major allele	Minor allele	
2q32 (STAT4)							
T1D-control	0.240	0.192	0.01	1.33 (1.05-1.68)	17.6 (10.6)	16.7 (9.4)	0.6
Pediatric-adult T1D	0.237	0.250	0.7	--			
6q23 (TNFAIP3)							
T1D-control	0.282	0.321	0.05	0.83 (0.69-1.01)	18.7 (11.1)	17.8 (10.6)	0.3
Pediatric-adult T1D	0.297	0.271	0.4	--			
9q33 (TRAF1)							
T1D-control	0.085	0.071	0.2	--	18.4 (11.1)	21.2 (10.5)	0.02
Pediatric-adult T1D	0.063	0.104	0.04	--			
12p13 (CLEC2D)							
T1D-control	0.462	0.504	0.06	0.85 (0.71-1.01)	17.6 (10.7)	18.5 (11.0)	0.4
Pediatric-adult T1D	0.471	0.460	0.8	--			
16p13 (CLEC16A)							
T1D-control	0.368	0.416	0.05	0.82 (0.66-1.01)	16.9 (9.7)	18.0 (11.5)	0.5
Pediatric-adult T1D	0.359	0.394	0.4	--			

Table 9. Analysis of selected gene associations from genome-wide studies in type 1 diabetes. Minor allele frequency (MAF) is provided in case-control and pediatric vs adult onset analyses, and comparisons are calculated with Chi-square and Fisher’s exact test when necessary. In the continuous analysis, mean ages at onset associated to each allele and the p value from the U Mann-Whitney test are presented. T1D: type 1 diabetes.

**Region 6q23 (TNFAIP3):** two polymorphisms located in an intergenic space adjacent to the *TNFAIP3* gene have been associated to several autoimmune diseases, among them type 1 diabetes (Fung et al, 2009). We have selected the polymorphism that shows a stronger association with the disease. The gene *TNFAIP3* is expressed in  $\beta$  cells and serves as an anti-inflammatory mechanism by its downregulation of the NF- $\kappa$ B activation (Liuwantara et al, 2006); therefore, it poses an interesting candidate gene in the pathogenesis of type 1 diabetes. To our knowledge, this is the first time that this region is studied in relation to its influence in age at onset.

In our study, we replicate the association previously seen in type 1 diabetes and we do not find differences in age at onset either in the stratified or continuous analyses. Results are provided in table 9.

**Region 9q33 (TRAF1):** this region was first associated to rheumatoid arthritis (Kurreeman et al, 2007). Our group took part in a collaborative study that analyzed this region in several autoimmune diseases and found association with type 1 diabetes, among others

(Kurreeman et al, 2010). As an extension of the cited study, we selected and studied new polymorphisms in the *TRAF1* gene and analyzed their influence in age at onset of the disease. We present in table 9 the results obtained in one polymorphism that has not been previously studied in type 1 diabetes. Like *TNFAIP3*, the gene *TRAF1* is expressed in  $\beta$  cells and protects them against cytokine-mediated apoptosis in an inflammatory environment (Sarkar et al, 2009).

The polymorphism in *TRAF1* shows interesting data (table 9). We do not see statistical differences in the case-control analysis, but the age-stratified analysis shows an elevation of the minor allele frequency only in the adult-onset patients, that is statistically significant when compared to pediatric patients ( $p=0.04$ ) and to controls ( $OR=1.52$  [ $1.00-2.31$ ];  $p=0.04$ ). On the other hand, the pediatric patients are similar to controls ( $p=0.6$ ). The continuous analysis confirms the difference observed in the stratified analysis, showing an age at onset associated to the minor allele (mean 21.2) that is almost three years higher than the age at onset associated to the major allele (mean 18.4). Therefore, there seems to exist an association in this gene that is exclusive of our adult-onset type 1 diabetes patients.

**Region 12p13 (CLEC2D):** first associated with type 1 diabetes in the WTCCC study (WTCCC, 2007), it includes several genes. Polymorphisms associated with the disease (WTCCC, 2007; Barrett et al, 2009) have been identified in the surroundings of two genes with immunological function: *CLEC2D* and *CD69*. We will focus on the polymorphism near *CLEC2D* (coding the NK receptor *LLT1*), which was the strongest signal reported by the WTCCC (WTCCC, 2007) in this region. To date, the influence of region 12p13 on age at onset has not been studied.

In our study, we detect a trend towards association with type 1 diabetes of the studied polymorphism. We do not find differences in age at onset in the stratified and continuous analyses. Results are provided in table 9.

**Region 16p13 (CLEC16A):** this region was one of the most strongly associated with type 1 diabetes in the WTCCC (WTCCC, 2007) and was also discovered independently in a parallel study (Hakonarson et al, 2007). It covers a gene of unknown function termed *CLEC16A*, which is expressed in antigen presenting cells and NK cells, but little else is known about this gene or its possible role in the pathogenesis of the disease. Our group replicated the association detected in the WTCCC study (Martinez et al, 2010). Here we will analyze its influence in age at onset.

We replicate the association previously seen in type 1 diabetes. We do not find differences in age at onset in the stratified and continuous analyses. Results are provided in table 9.

### 5.3 Discussion

In this study we analyzed ten genetic regions, five of them classical type 1 diabetes genes and another five extracted from recent genome-wide studies. The analysis of age at onset (either age-stratified or considering age as a continuum) did not provide statistical differences in nine of the ten regions studied, therefore we propose that our adult-onset patients have the same genetic background in the studied genes that our pediatric-onset patients. Hence there is no reason to exclude these late-onset patients from genetic studies on type 1 diabetes. The results we observe in the classical type 1 diabetes susceptibility genes (table 8) are concordant with a recent Finnish study (Klinker et al, 2010) that analyzes these genes in late-onset patients and finds the same associations previously described in



pediatric-onset type 1 diabetes. Also, a recent study from the T1DGC (Howson et al, 2009) that replicated 19 genes, including *PTPN22*, *IFIH1* and *CTLA4*, studied age at onset in each one and did not find statistical differences, supporting the idea that the classical genetic associations to type 1 diabetes are shared between the early and late onset patients.

Interestingly, in our population we do not find differences in the HLA associations with early and late onset. It has been described before that the *DRB1\*03-DRB1\*04* heterozygote is the combination that confers a higher risk and it is associated with an earlier age at onset of type 1 diabetes (Redondo et al, 2001a; Leslie et al, 2006; Klinker et al, 2010). However, in our population the heterozygote is the fourth combination in risk conferred to the disease after the *DRB1\*04-DQB1\*03:02* homozygote, the *DRB1\*03* or *DRB1\*04-DQB1\*03:02* carrier and the *DRB1\*03* homozygote, and we do not see an effect on age at diagnosis of the heterozygote. This could be due to populational differences, quite important in the HLA complex. It is well known that not all the *DRB1\*03* haplotypes confer the same susceptibility to the disease. An extended conserved haplotype marked by *B\*18-DRB1\*03-DQB1\*02:01* is described to confer higher susceptibility among the *DRB1\*03*-carrying haplotypes (Johansson et al, 2003; Urcelay et al, 2005). This haplotype is more frequent in the Mediterranean area and its frequency descends in Northern Europe. Therefore, it could be possible that the higher frequency of this high risk haplotype enhances the risk conferred by being a *DRB1\*03* homozygote in a Mediterranean population such as the Spanish.

A recent study with the T1DGC family cohort (Howson et al, 2009) found a mild effect of the insulin gene in age at diagnosis of the disease, with the susceptibility allele conferring an onset two years earlier than the protective allele. We do not see an effect on age at diagnosis (continuous analysis,  $p=0.4$ ). Moreover, in the aforementioned Finnish report, the authors also studied the *INS* gene and found association in late onset patients (Klinker et al, 2010). It is possible that our study lacks statistical power to detect a difference of two years in the age at diagnosis. However, the authors of the T1DGC study tried to replicate their findings in a case series of 900 patients and did not find the effect they saw in the family cohort, opening the door to the possibility that the described effect is a false positive.

Finally, we find that *TRAF1* is only associated to late-onset type 1 diabetes. Although our study is limited by low statistical power and would require replication in an independent late onset type 1 diabetes cohort, this is an interesting finding that would justify the study of the late-onset patients as a distinct set among the type 1 diabetes patients.

From the study of these ten genetic regions in our group composed of early and late onset patients, we propose that there are no major genetic differences between patients with an early and a late onset of type 1 diabetes.

## 6. Conclusions

Although the knowledge on the genetics of type 1 diabetes has experienced a great development in the last years, it has not provided many hints on the basis of the genetic components that could modify the age at onset of the disease. Several studies have approached the subject, but few of the reported associations have been properly replicated in independent populations. Also, the heterogeneity in the methodology of the published studies should be discussed: some studies select only paediatric patients. If the influence of a genetic region reaches a peak in the paediatric age and then decreases with



time, a study with paediatric and adult patients would better estimate the difference than a study with only paediatric patients in which the difference may be seen, but also may be smaller than it really is. The majority of the published studies analyze the age at diagnosis of type 1 diabetes as a continuous variable, but some studies adopt a stratified analysis that implies the fragmentation of the patients in two or more groups and the individual analysis of each subgroup. This strategy defines artificial groups with limits that do not have a biological justification, and makes it more likely to produce false results in underpowered studies. We recommend the age-stratified analysis as a screening method or as a confirmation analysis, but we consider the analysis of age as a continuous variable a more accurate method to detect differences, given that it only takes into consideration the genotype studied and the ages at onset of all the patients carrying this genotype. Therefore, we propose that age at diagnosis of type 1 diabetes should be studied in groups that include paediatric and adult onset patients, and that the statistical analysis should include at least one method that considers age at diagnosis as a continuum.

Despite the improvements that can be incorporated to age-at-onset analysis, what is known to date about the influence of the genetics on the early and late onset of type 1 diabetes allows us to formulate a tentative answer to the question that gives title to this chapter: are early and late onset type 1 diabetes the same or two distinct genetic entities? Our data, presented in section 5, and the previous studies reviewed in section 4 suggest that there are no major differences in the genetic component of paediatric and adult patients. Both share the risk conferred by the main type 1 diabetes risk modifiers such as the HLA, the insulin gene or *PTPN22*. However, two points have to be taken into consideration: first, minor differences can be found between the adult and the paediatric patient, such as the elevated prevalence of the higher risk HLA alleles in subjects with early onset. Second, the genetics of adult-onset type 1 diabetes patients has frequently been studied only in relation to genes that already showed association in paediatric patients. This approach precludes the possibility of discovering genes that could be associated exclusively to late-onset type 1 diabetes. The study of these two points is of great interest given that it could point to metabolic routes that take part in the acceleration of the disease and, therefore, genes in these routes would be excellent candidates for therapeutic strategies focused on the delay of the autoimmune  $\beta$  cell destruction.

In conclusion, we propose that type 1 diabetes, whether in its early or late-onset, is an autoimmune disease defined by a number of primary risk genes and a constellation of minor genetic modifiers that, together with environmental factors, define the pace of the autoimmune reaction that will determine the age at onset.

## 7. References

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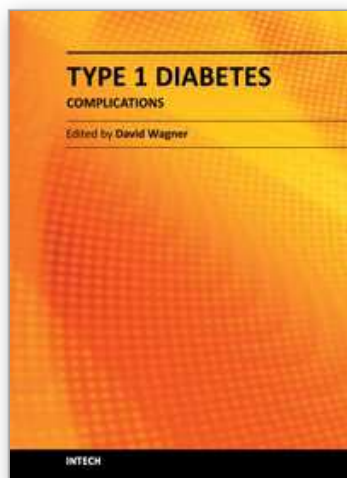
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This book is a compilation of reviews about the complication of Type 1 Diabetes. T1D is a classic autoimmune disease. Genetic factors are clearly determinant but cannot explain the rapid, even overwhelming expanse of this disease. Understanding etiology and pathogenesis of this disease is essential. The complications associated with T1D cover a range of clinical obstacles. A number of experts in the field have covered a range of topics for consideration that are applicable to researcher and clinician alike. This book provides apt descriptions of cutting edge technologies and applications in the ever going search for treatments and cure for diabetes.

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