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# THE FIRST DIABETOGENIC MOVES

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T1D is described classically as chronic autoimmune disease in which different environmental factors, acting on a genetic predisposition background, trigger the T cell mediated destruction of the pancreatic beta cells. Over time, autoimmunity will eventually lead to overt diabetes. During the last 4 decades numerous data were accumulated in support of this pathogenic paradigm. International genetic consortia efforts led to the identification of more than 50 susceptibility loci, the majority of which encode proteins involved in immune function. Of these, the HLA (Human Leukocyte Antigen) complex confers approximately 50–60% of the overall genetic risk. The main known autoantigens are insulin, the glutamic acid decarboxylase (GAD), the tyrosine phosphatase-like protein IA-2 and the zinc transporter ZnT8. Antibodies against insulin, GAD65, IA-2, ZnT8 and also the islet cell antibodies (ICA) are diagnostic for autoimmune T1D and help differential diagnosis with other phenotypes of "slim" diabetes. In first degree relatives of T1D patients, these antibodies can predict the occurrence of the disease. If T1D genes and antibodies are quite well defined, the environmental triggers of the disease are still elusive, despite data regarding the association with enterovirus infections, early contact of infants with different food antigens (cow milk or cereals), low levels of vitamin D, etc.
It seems that in humans, anti beta cell autoimmunity is promoted by a global defect of immune regulation. Thus the function of regulatory T cells is affected, with a concomitant resistance of the peripheral cytotoxic T cells to the suppressive signals from regulatory cells. In addition, in the pancreatic lymph nodes of T1D patients an imbalance between proinflammatory and regulatory T cells is reported. However, the studies of T lymphocytes have been largely limited to peripheral blood since few pancreata were available for research during the last decades. However, in the last couple of years, through the efforts of nPOD (Network for the Pancreatic Organ Donors with Diabetes), the study of insulitis in T1D patients gained momentum. The last years brought maybe more questions than answers. Thus, the relationship between the autoimmune response (evidenced by the presence of antibodies) and insulitis with beta cell destruction is still poorly understood. Insulitis in humans seems to be quite variable, with not all the islets being affected, and even in patients with clinical overt T1D, islets with normal beta cells can be found, challenging the paradigm that autoimmunity is the sole pathogenic mechanism leading to overt T1D. Our hypothesis is that the trigger of autoimmunity is represented in fact by the dysfunctional beta cells, the number of these cells and the magnitude of their secretory defect representing the key element differentiating the cases that will evolve rapidly towards complete beta cell loss and clinically overt diabetes, respectively those with "latent" and many times asymptomatic evolution even for decades.

*Keywords*: *Borreliosis*, Lyme disease, neuro-borreliosis, antibiotherapy.

## INTRODUCTION

Relatively soon after the publication of immunogenetic theory of "juvenile" diabetes by Nerup[1,2] and Bottazzo[3], George Eisenbarth[4] divided the evolution of type 1 diabetes (T1D) in four steps: (a) a *genetic predisposition*; (b) a *trigger of the autoimmune process*; (c) a progressive and, finally, almost complete *destruction of the pancreatic β-cells* by the autoimmune process and (d) *clinical onset* of diabetes. In order to offer an insight in this complex mechanism, even some mathematical models have been created[5]. Although the four stages mentioned above are generally accepted, the first two remain elusive, despite the efforts made for their detection and characterization.

For almost half a century, researchers that approached the pathogenesis of type 1 diabetes (T1D) have tried to identify the "trigger" of anti-β-cell autoimmunity, considered to be the mechanism responsible for the progressive destruction of the pancreatic β-cells. There are compelling arguments indicating that this destruction is mediated by the cytotoxic T lymphocytes[6–9]. However, an important question must be answered related to this statement: how manage these lymphocyte, produced in the peripancreatic lymph nodes, to enter through two biological fortresses: the glio-vascular capsule surrounding the Langerhans islets[10] and the peri/intercells matrix (a specific molecular network) surrounding each islet cell? In other words, how do the immune cells get close to β-cells, which normally are protected by the two mentioned barriers?[11].

We sustained, with some convincing arguments[11], that in T1D pathogenesis, two distinct disturbances are involved: one present in the **pancreatic β-cell** (the chief regulator of the energy metabolism of the human body), and the other one in the **immune system** (the hidden branch of the defense system of the human body). Both are genetically determined and strongly influenced by environmental (epigenetic) factors. In order to trigger the β-cell destruction, these two systems must enter into collision, and this requires that both disturbances are found in the same (unknown) **time** and in the same known **place** (the pancreatic islets). In the last years, the already classic autoimmune mechanism based mainly on the experimental studies carried out in some animal models (the prototype being the Non Obese Diabetes (NOD) mice), has been challenged[12–14] asking for a reconsideration of the classical view of the human autoimmune diabetes. However, the implication of the immune system in pathogenesis of T1D is sustained by numerous arguments. We will mention only some of them.

## INSULITIS IN AUTOIMMUNE TYPE 1 DIABETES

The agglomeration of inflammatory cells around pancreatic islets of diabetic children deceased soon after the disease onset has been described several times in the last 112 years[15–21]. It took a long period of time before this process, known as insulitis, was explicitly recognized and properly defined in 2013[22] as an indicator of the presence of a β-cell destructive process with an autoimmune mechanism. The proposal for standardization of insulitis in the pancreas of diabetic patients, not only children but also adolescents or adults[22], was necessary because the term of insultitis was used before in cases where the number of leukocytes associated with an islet was sometimes below 5. This number of lymphocytes is often present in apparently healthy persons. As detailed in Table 1, the mentioned standardization was based not only on histological/immune-histochemical data obtained from pancreatic sections, but also on the analysis of the pancreatic islets isolated from organ donors, either diabetics or controls[23, 24].

*Table 1*

Consensus definition of insulitis released after the workshop of 5th annual meeting of Network for Pancreatic Organ Donors with Diabetes nPOD[22]

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|---|
| "*Patients with insulitis are defined by the presence of a predominantly lymphocytic infiltration specifically targeting the islets of Langerhans. The infiltrating cells may be found in the islet periphery (peri-insulitis), often showing a characteristic tight focal aggregation at one pole of the islet that is in direct contact with the peripheral islet cells. The infiltrate may also be diffuse and present throughout the islet parenchyma (intra-insulitis). The lesion mainly affects islets containing insulin-positive cells and is always accompanied by the presence of (pseudo) atrophic islets devoid of beta cells. The fraction of infiltrating islets is generally low (<10% of islet profiles). The lesion should be established in a minimum of three islets, with a threshold level of > 15 CD 45+ cells/islet before the diagnosis can be made. The pathology report should include the total number of islets analysed, the fraction of islets affected by insulitis, the fraction of (pseudo)atrophic islets, and a description of the spatial relationship of the infiltrate to the insulin-positive islet cells.*" |

We[11], like many other authors[14,24,25], have criticized excessive experimental studies carried out on NOD mice as proof for the autoimmune mechanism in humans. It is known that this mouse strain has been obtained through the successive selection of those mice developing autoimmune diabetes. It is questionable if NOD mice (or other similar animal models) can really reflect the pathogenic mechanism operating in human autoimmune diabetes[27]. The differences between mice and human are so big that the transfer of the data from one to another is at least imprudent. In contrast with rodent islets, human islets are more heterogeneous in structure, including a different cellularity (up to 70% beta-cells and other territorial distribution) and a more prominent intra-islet vascularisation[27].

After the consensus definition of insulitis, we felt that many questions remain to be answered, all related to the signification of this quite specific lesion.

## WHEN, HOW AND WHY DOES INSULITIS OCCUR?

The pancreas itself, especially the islets of Langerhans, can be considered as "**black boxes**" since attempts to evaluate imagistically their location, dimension and pathologic changes are not accurate enough to be clinically useful[29–31]. The above mentioned studies of insulitis were performed usually on patients deceased soon after the onset of clinical diabetes, *i.e.* after the full decompensation of blood glucose regulation. At this time about 80% of the β-cells are already irreversibly lost[32–34]. The attempts to preserve the remaining 20% of the β-cells that are still present in this late stage of disease using various combinations of immunomodulatory drugs, have failed[15,35]. It is obvious that we must look forward for a real primary prevention of this phenotype diabetes. To do that it is necessary to know, when and how the insulitis occur and when the first β-cells start to be destroyed[36–39]. This could be a fundamental objective of the future diabetes research.

## VIRAL HYPOTHESIS: AN UNFINISHED CONTROVERSY

Insulitis is an inflammatory process, so that the hypothesis that a viral or microbial infection might contribute as a "trigger" of the autoimmune process in young ages was the first hypothesis at hand. Isolation of Coxackie B virus from a pancreas of a child dead soon after the onset of diabetes and induced diabetes in mice by injecting the isolated virus, give a strong impetus to the "infectious" origin of T1D[40–45]. However, in the majority of young patients developing autoimmune diabetes, no such acute infection was reported. In a *pro* and *con* debate published in 2008 in Diabetologia[42], no compelling conclusion has been reached. Recently, Schneider and von Herrath[46] analyzed the plethora of results linked to the topic. They mention 22 papers in a synopsis table, but finally don't reach a clear conclusion. Despite controversies, viral infections, not only with Coxackie B, but also with rotaviruses, echoviruses, rinoviruses, mumps or congenital rubella have been discussed as possible triggers of autoimmunity[46,47]. The changes in the intestinal microbiome have been also associated with higher incidence of T1D in adult population.

In children younger than 5 years, both early (< 4 months of age) and late (> 4 months of age), the first exposure to solid foods has been associated with an increased risk for the development of T1D[48,49]. These or other environmental factors (excessive sanitation, chemical additives, lifestyle changes) or perinatal factors (birth weight, infant growth, maternal age) have been considered to be responsible for the accelerated increase in the incidence of T1D in many countries[50–55]. An increased mucosal intestinal permeability (*via* tight junction modulators) has been also proposed as increasing exposure to the diabetogenic antigens, of viral or non-viral bio-pathogens[56].

In 2009, using sections from paraffin-embedded pancreatic autopsy samples, Richardson *et al.*[57] found also a focal staining for enteroviral capside marker VP1 in 44 of 72 of young recent-onset T1D patients, but also in 10 of 25 cases with T2D tested for VP1, and also in 10% of non-diabetic controls of all ages. Considering that enteroviral infection is the most common infection worldwide[58], the presence of VP1 could be not a specific marker for T1D[59–60].

It has been recently shown that the intestinal microbiota promotes enteric virus replication[61] and that Coxackie virus B3 can infect the bone marrow[59], influencing its capacity to produce new and specific clones of lymphocytes. If a such pathological phenomenon is involved also in T1D it is not known.

In our view, the various enteral pathogenic agents (viral antigens or other biopathogens) could occasionally trigger an autoimmune mechanism against B-cells. They might migrate from the complex gut microbiome via abundantly omento-mesenteric lymph vessels and lymph nodes which in their way toward the Cisterna Cyli pass through the numerous peri-pancreatic lymph nodes[59,60] and then to the pancreatic tissue.

Despite the excellent biologic material, and the state-of-art technology, a heavy drawback can be clearly observed: the results, otherwise outstanding, presented in the latest studies[57] could not give us any image about what happened during the long "prehyperglycemic" stage of diabetes, lasting several years, if not decades. It will be interesting to compare the islet architecture data obtained in these studies of pancreases of living diabetic donors with that obtained by us through a careful histological

analysis of a normal human pancreas from an organ donor[62].

As a conclusion (similar with other authors' opinion) a viral infection as a trigger of autoimmunity in T1D cannot be confirmed nor denied. It is quite possible that after the onset of autoimmunity (triggered by other factors) a viral or other type of infection could amplify and maintain the inflammatory process in islet cells.

## AN UNPREDICTABLE IMMUNE GENETIC CONSTELLATION

A careful genetic analysis[63], including results obtained using *the candidate gene method*[64], and also those provided by the *genetic wide scans*[65] described a high number of genes associated with the T1D phenotype (over 60 at present). The majority of these genes expressed molecules that were present in some cells belonging to the immune system. Extremely important is the fact that still a part of them codify other molecules that do not have a known relationship with the immune system. Among these, the most important is the insulin gene (*INS*), but also the *ERBB3* gene, and many others that do not have a known function yet.

However, the involvement of HLA alleles (DR3/DR4; DQ2/DQ8 especially, and also some other variants that are strongly diabetogenic) suggests the primary role of the Major Histocompatibility Complex (MHC) in the pathogenesis of this phenotype. The non-HLA genes, *CTLA4, PTPN22*, *IL2-RA* or *IFIH1* endorse the presence of a genetic predisposition for autoimmunity, considering the frequent association with other autoimmune diseases[63,64].

We have to mention that the presence of predisposing gene variants is important, but not mandatory. Their effect can be neutralized if their carrier has in the genome one or more protecting alleles[63,64]. In other terms, a single protecting gene can have a *veto* power, in the same way that this right is used in politics when the interest of the powerful states come into play. We should accept that the immune system can be seen as a "*great biologic power*", being a constant and active component of the human body defense system.

Moreover, new types of methods have been designed in recent years for a direct analysis of the DNA sequences, which may present new correlations between genes associated with diabetes[66–70]. The DNA structure is fundamental in understanding local interactions with different molecules. For instance, when gene promoters are analyzed using DNA patterns, it appears that diabetes pathogenesis is divided into at least three phenotypes[39].

## BETWEEN THE PREDISPOSITION TO DIABETES AND ACTIVATION OF AUTOIMMUNITY

In 2009, we have published in this Journal a wealth of data showing that an increased proinsulin level in peripheral circulation could reflect the main defect of the β-cell: that of producing immature secretory vesicles – SVs[71]. This defect can result from the hundreds of molecules involved in the maturation of SVs, but also in the maintenance of the surrounding extracellular matrix. That is a well organized molecular network through which the β-cells are interconnected with other β-cells or non-β-cells from the Langerhans islets[72]. A normal extracellular matrix results from the close cooperation between the β-cells and endothelial cells, including also the pericytes[72–77]. It is known that the islets receive 5–15% of the whole pancreatic blood supply, even if they represent less than 3% of the pancreatic mass according to our recent data[62]. Such a dense capillary network is essential for the proper islet function which includes the building up of two strong barriers: one that surrounds the islet itself, and the second surrounding each islet cell, forming a specific inter-cellular matrix between β-cells – endothelial cells, β-cells -alpha cells or other secretory cells (gamma, delta and epsilon). This is a powerful protective barrier against any pathogens or inflammatory infiltrates.

In a recent study, Pathiraja has identified 53 distinct cell clones of CD4+ T lymphocytes from the pancreas of a T1D patient[78]. A significant proportion (25%) of these clones responded to proinsulin epitopes restricted to HLA-DQ8 and DQ8 transdimers that form DQ8/DQ2 heterozygous subjects. These data confirm our data[66] sustaining the main role of proinsulin as an intiating pathogenic process, stimulating the specific T cell clones against beta cells.

It is important to understand that the β-cell dysfunction may affect not only the capacity to produce the mature SVs, but also to maintain the integrity of the inter-cellular matrix. In such circumstances, the functional relationship between

the β-cell and other islet cells could be broken. In Fig. 1 is given the image of the "broken matrix" between a β-cell and an α-cell in the pancreas of a patient suffering a surgical intervention for an insulinoma. An intrusion of secretory vesicles from the β-cells in the α-cells can be seen[79].

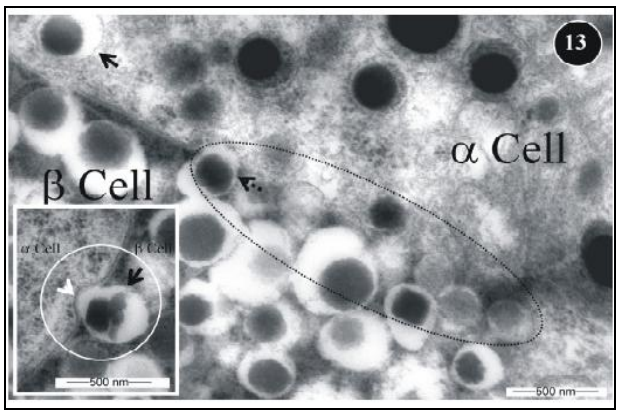


Figure 1. An argument for the major importance of intercellular matrix disorganization is demonstrated by the penetration of the secretory vesicles of a β-cell into an α-cell. Adapted from Mirancea GV *et al.*[79].

This image is in line with the concept of the high plasticity of the islet cells. In a mice model with the ablation of the transcription factor FoxO1 (inducing diabetes and the loss of the pancreatic beta cells), a de-differentiation of the beta cells in alpha cells has been observed[80].

If in an islet there are many dysfunctional β-cells, such a condition can release a "danger" signal[81–83] which is received by a hyperactive immune system (probably dendritic cells) that will trigger a chain of pathogenic events that can be summarized like that: the circulating dendritic cells (DCs) can migrate towards those islets containing, apart normal β-cells, some dysfunctional β-cells, in order to "test" their functionality and, if it is necessary, to detect and probe potential antigens using their multiple prolongations, by which hundreds or thousands of β-cells might be assessed. In an ingenious animal model (the cinematographic recording of the normal islets introduced in the anterior chamber of the eye of another mice during the development of diabetes), it could be observed the normal islets were infiltrated with DCs that, after several days, leave these islets, presumably migrating to the closest lymph nodes. From an *in vivo* pancreas, this migration might take place towards one of the several regional groups of peripancreatic lymph nodes. Here, a specific clone of cytotoxic T cell will be generated with a specific destination: the islets containing the tested dysfunctional cells. Initially, a peri-insulitic process is induced and, if any counter-reaction appears, the infiltration of the entire islet with the occurrence of a full-blown insulitis process will develop. The destruction of β-cells can be rapid if the diabetogenic constellation is enough powerful. In this case, the rapid spreading of the autoimmune reaction against β-cells could lead soon (in the first years of life) to the clinical onset of diabetes.

In total opposition could be the cases in which the dysfunctional β-cells are detected only in a small number and only in a few islets. Even if during the collision of immune cells with the dysfunctional β-cells, the activation of B lymphocytes will initially produce insulin autoantibodies (IAAs), the autoimmune process can be canceled if the capacity of the immune system will stimulate the production of regulatory T cells (Tregs), inducing a complete remission of the autoimmune process.

Between these two extremes, there are many intermediate models, explaining why the age at onset of clinical T1D can vary between a couple of months and many decades. Such an extreme heterogeneity can be explained by two main factors: a) genetic architecture is unique for each patient and, apart the genes with diabetogenic effect, there are several genes with a protective effect; b) the transcription of human genes is under the endogenous control, but also under that of environmental (epigenetic) factors, some with an up-regulation or down-regulation effect upon each of these genes, either with protective influence or with pathogenic influence. The above mentioned increase (or decrease) in the incidence of T1D has been explained by the influence of many environmental factors: nutritional, chemical, infectious, socio-economic or behavioral[48].

## SIMPLE AND MULTIPLE SEROCONVERSION

The frequent appearance in the serum of T1D predisposed subjects (either First Degree Relatives-FDR or High Risk Subjects- HRS carrying strong T1D-associated genotypes) of the antibodies against some specific β-cell antigens represents an important moment for the diagnosis of the *pre-hyperglycemic* evolution of autoimmune diabetes. The most studied antibodies are anti-insulin/proinsulin (IAA), anti-GAD (glutamic acid decarboxylase)), anti-IA-2 (insulinoma associated

protein 2/tyrosine phosphatase) and anti-ZnT8 (zinc transporter isoform 8)[52, 84–86]. In 2014 some other anti-islet antibodies were described, with unknown clinical significance and not yet reproduced in other studies[87]. In the prospective cohorts that were actively monitored for 10, 15 or even 25 years, it has been demonstrated that the evolution towards overt diabetes is proportional with the number of these antibodies and with their titer (Fig. 2). The presence of 3 antibodies predicts the onset of diabetes in approximately 80% of the cases. The prediction is practically 100% if, in the presence of multiple antibodies, the strongly diabetogenic genotypes are also present[89,89].

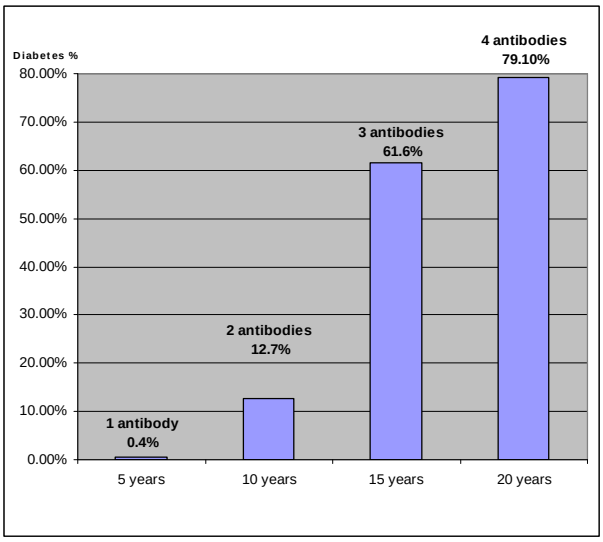


Figure 2. The progressive increase of T1D prevalence according to the number of positive antibodies. Adapted after[52].

There are some exceptions for this pro-autoimmune argument, namely the rare cases in which the onset of clinically overt diabetes has not been preceded or followed by the identification of any anti-islet antibodies[52,85]. However, it is very likely that these antibodies could have been present between the annual evaluations and was absent at the moment when blood sampling took place[52].

## OUR VIEW REGARDING THE EVOLUTION AUTOIMMUNITY

Between 1974 (the year when the immuno-genetic theory of "juvenile diabetes" has been published)[1–3] and 2014, various immunotherapic approaches have been tested with positive results obtained in NOD mice and with some "encouraging" results in human diabetes[45, 90–91]. Over the years, various β-cell antigenes (proinsulin, insulin, GAD and others) have been assessed as agents whom administration in small quantities will help to train the adaptive immune system (essentially T or B cells) to eliminate "insulitis" and to produce long-lived immunological memory. Despite some "encouraging" results, it was not possible to obtain a balance between a low dosage without positive effects, and a high effective dosage but with unacceptable side-effects[90–92].

The activation of cytotoxic T cells has been considered to be under the control of the complex formed by an antigen (either foreign or autoantigen) and the MHC (Major Histocompatibility Complex) molecules. However, this is not enough for the activation of T-cell clones with a specific target, in our case, β-cells. A co-stimulatory signal is necessary, which in the case of autoimmune diabetes is provided by the dendritic cells (DCs)[60]. These cells seem to enter first in the islet cell in order to test and to sample antigenes from non-healthy β-cells. When a certain number of such β-cells are dying, the DCs phagocytes the specific antigenes and migrate to the most appropriate lymph nodes close to the islets found to contain unhealthy or not useful β-cells. This message plus peptide/MHC complexes will trigger the activation of cytotoxic T cells having as target the pancreatic islet or islets containing dead β-cells. These "cadavers" must be eliminated with the help of several inflammatory cells (including innate immune cells) that appear around or inside islets and produce the first "insulitic" process. After the phagocytosis of the dead β-cells during a longer or shorter period of time, the insulitic process disappears leaving behind the islets emptied of β-cells[22].

Recently, an analysis has been published[93] regarding an immune "check-point" considered to be the product of CTLA4 gene, acting as an activator of oncogenesis. As a consequence, a blockage with anti-CTLA4 antibodies (ipilimumab) has been proposed as an anti-tumorigenic agent. If this immune "check-point" is a real partner in the fight against such hopeless malignant conditions, what future could it have for the treatment of T1D, a similar immune-mediated disease?

There are some data which suggest that the immune defect that could precipitate the start of the anti-β-cell attack could not be related with the excessive number of cytotoxic T lymphocytes clones (Teff), but with the small number of

regulatory T lymphocytes (T regs), making the equilibrium between these two opposing forces to be in favor of the first.

Let's suppose that all the pro-immunity arguments listed before are fulfilled. For the anti-β cell immune process to be produced, other factors must be present. The defense capacity of the pancreatic islet must be sufficiently weakened in order to allow the immune system to lead to the destruction, not of the whole pancreatic islet with all its cells, but in particular, only of β-cells. This view assumes that the β-cell is an important player in the autoimmune defense system.

## A DIFFICULT DECISION

For many years we have sustained the need for a radical change in the *criteria for defining* diabetes and, as a consequence, a radical *change of the classification* of diabetes.

It is obvious that what we call today diabetes is the result of the progressive decline in the β-cell function/mass[94]. This decline starts when the first dead β-cell cannot be replaced by a new one. This decline is very rapid in the cases of diabetes with onset in the first years of life, and attenuated for those with onset in young ages (1–18 years), slow or very slow in the young adults (18–30 years) and even slower in adults (> 30 years). A non-autoimmune loss of β-cells can result from an inherited or specific set of diabetogenic genes, usually in subjects older than 40 years at diabetes onset, absence of immune markers, and often with overweight or obesity. This is the characteristic phenotype of type 2 diabetes (T2D).

If such an interpretation is agreed, then we are obliged to ask: what is more important for defining diabetes, the true pathogenic *phenomenon*, which is the decline of the β-cell mass/function, or its *epiphenomenon* which is the decompensation of blood glucose regulation? All of us will accept that the phenomenon must be the main criterion since the epiphenomenon appears very late, sometimes when several vascular complications are already irreversibly established. This is the case for T2D. For T1D, immune markers in association with a high genetic risk score and the presence of some proinflammatory cytokines could indicate with a high probability that ongoing β-cell destruction is on its irreversible slope/way. For a real and true prevention approach, this would be too late if the autoimmune process is beyond the point of "no return".

The actual return to the careful study of human pancreatic islets in normal subjects and then in the different stages of the various phenotypes of diabetes could be the small light seen at the end of a long tunnel.

**Conflict of interest**: We declare no conflict of interests.

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