IN DEBATE: THE PRIMARY CAUSE OF TYPE 1 DIABETES

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Diabetes mellitus is a huge syndrome manifested by many phenotypes, each having some pathogenic particularities. Despite this large heterogeneity, the unitary character keeping them together is supported by the same “sine qua non” condition for all, which is the decompensation of blood glucose regulation. This is induced by a decrease in β-cell mass/function. According to the autoimmune or non-autoimmune mechanism in the β-cell loss, the age of onset and the pre-diagnostic period could be extremely different. In this state-of-art approach we have analyzed the early phases of the autoimmune diabetes, that often are very long, period in which it can be identified several particular steps. This approach was made with the conviction that prevention could be efficient only when it is applied before appearing the “no return” point for which we must find reliable markers.

Key words: Phenotypes of diabetes; Autoimmune diabetes; Markers of early phase of diabetes.

INTRODUCTION

Research from the last decade, that followed the results of the Human Genome Project1-3, and also the progresses registered in immunology4-7 and cellular biology8, require a reformulation of older hypotheses, not only in the field of diabetes, but also in autoimmune diseases and malign diseases, in order to find new therapeutic solutions for which the previous approaches were not proved to be viable.

In the field of type 1 diabetes (T1D), a challenging position recently published9, questioned the pathogenic pancreatic anti-β-cell autoimmune process, and was followed by the publication of such an unexpected point of view10,11. This strategy of Donath, Hess and Palmer9 has been probably adopted in order to create a debate for this topic that might pave the way towards the discovery of an efficient therapeutic solution12.

We have to mention from the beginning that the delay in the correct understanding of the pathogenesis of diabetes mellitus is owed, in the first instance, to the blockage that resulted from maintaining hyperglycemia as a single diagnostic criterion for diabetes13. In fact, for the alteration of blood glucose regulation to take place (hyperglycemia occurrence), it is obvious that the numerous compensatory mechanisms that are responsible for the normal control of energy metabolism (including the carbohydrate metabolism), must be overwhelming and severely deteriorated in their efficiency14.

In order to understand the difficulties encountered in deciphering such a vast syndrome as diabetes mellitus, we would like to comment shortly the two important causes that can explain the many failures registered in the discovery of the pathogenetic mechanisms operating in the various phenotypes of diabetes in humans, and, in consequence, in delaying the discovery of their optimal therapeutic solutions. The first one refers to excessive experimental data obtained in diabetic animal models that was transferred, without necessary precautions, in interpreting the pathogenesis and treatment of human diabetes.
second one refers to the phenomenon called heterogeneity, mentioned in numerous papers that were dedicated to clinical phenotypes of diabetes, to their genetic basis, pathogenic mechanisms or therapeutic response.\textsuperscript{15-20}

**Autoimmune mechanism in NOD mouse versus human diabetes**

Paying closer attention to literature, we could easily observe the high number of studies dedicated to NOD (Non-Obese Diabetes) mice or BB (Bio Breeding) rat- pancreas, both animal models of type 1 autoimmune diabetes, are in contrast with the scarcity of data obtained in humans. A first observation is that the prevalence of diabetes in these mice/rats models is very high, up to 100\%, whereas in humans the frequency of this disease is much lower\textsuperscript{21-26}. Moreover, the environmental conditions in animal experiences are well controlled, whereas in humans are totally uncontrolled. Sampling and killing mice at different ages give the opportunity to know very well what happens with different immune cells associated with changes in pancreatic islets. The experiments carried out in NOD mice were finally synthesized in a fair mice autoimmune prototype of diabetes, which however does not always overlap when the same investigations are performed in human patients with type 1 diabetes\textsuperscript{9}. Given the possibility of obtaining pancreas in multiple mice, but with progressively longer ages, it could be concluded that diabetes is almost always associated with the presence of peri-insular or intra-insular inflammatory processes, both with B lymphocytes (known as antibody producers, but also as antigen presenting cells), and also CD8\(^+\) T effector cytotoxic lymphocytes (Teff cells), dendritic cells and neutrophils. B cells were considered essential for inflammatory acceleration in NOD mice islets, but without knowing the mechanism and precise timing of their recruitment in the pancreas, T CD4\(^+\)Foxp3\(^+\) lymphocytes (that stimulate the expression of protective T lymphocytes clones called regulatory – Treg cells) play an important role, contributing to the attenuation of the inflammatory reaction mediated by cytotoxic T CD8\(^+\) lymphocytes.

In some conditions, B lymphocytes could play an active part in antigen presentation, mediating, not only antibody production, but also stimulating T CD8\(^+\) cytotoxic lymphocyte clone formation. Altogether, B cells can play an important role in NOD mice in maintaining a sufficient number of cytotoxic lymphocytes (Teff) that explain the large percentage mice (of \(\sim\) 80\%) which, in NOD mice, develop autoimmune diabetes. This large percentage is in flagrant contrast to the small prevalence of autoimmune type 1 diabetes in humans (under 1/1000 persons in the general population). It is one of the reasons why the extrapolation of data obtained from NOD mouse in interpreting human diabetes can lead to deceiving conclusions, especially concerning immune-modulatory treatment, efficient in stopping the insulitis process in NOD mice, but inefficient in humans.\textsuperscript{27-38} This is the main reason why Donath, Hess and Palmer\textsuperscript{9} questioned the implication of immune process in human T1D, an obvious exaggeration, intentionally released in order to start a debate on this theme. As we will see, the autoimmune mechanism in human diabetes is based on numerous robust objective data, even though the traditional interpretation of them requires some adjustments, at least regarding the duration of the autoimmune process before the clinical onset of diabetes, which is short in NOD mice and long or very long in human T1D. A first conclusion is that experimental data obtained in animals can be useful to a certain extent, but they cannot be transferred in humans before checking if they are superposable. As we will see, the data resulted from long duration prospective clinical studies of newborn FDRs (First Degree Relatives) regarding human T1D, are by far preferred to those from easier experimental studies on animals.\textsuperscript{39,40}

**The anatomic and functional heterogeneity of endocrine pancreas**

The second important difficulty in understanding the primary cause of diabetes is related to the great heterogeneity of pancreatic structure and the pathology of this complex organ\textsuperscript{2,26,40-42} whose embryological steps are given in Figure 1.
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It has to be added that from the embryologic point of view, the pancreas is heterogeneous because of its origin derives from two distinct buds: a dorsal and a ventral one. The last is originally common with the biliary ducts development. Later it expands and migrates, bypassing the duodenum in order to attach to the major pancreatic fragment (the dorsal one). The last forms the superior part of the head, body and tail of the pancreas (Fig. 2).

The pancreas appearance from two embryonic buds could partially explain the heterogeneous structure of pancreas, the more so as the pancreatic head is molded and intimately connected to duodenal flexure, probably being the most important supporting point of this unique organ by its extraperitoneal location. Interesting to note is that in the head of pancreas the pancreatic polypeptide secretory cells (named γ or PP – pancreatic polypeptide- cells) are more frequent than the other cell types. Their presence in the head of the pancreas, the place where the cells originated in the two embryonic buds, might create cell instability which, in our view, can explain why the pancreas cancer is mainly found in the head of this organ.

The presence in the same organ of two glands, one with external and the other with internal secretion, is more an exception than a rule. While the exocrine pancreas has a regulated lobular structure, the endocrine pancreas proves to have a heterogeneous organization, whether we refer to the total number of islets (between 500.000 and 2.5 million, possibly many more), their size (the smaller and the larger islets have a ratio > 1:20 between them) (Fig. 3 – Acta Endocrinologica iulie – sept 2014, pag 323) as well as the cellular composition of islets, making the accurate calculation of their total number more difficult.

Yet, it is estimated that the number of pancreatic β cells (insulin secreting, but also amylin and C peptide), in a normal human pancreas is of approx 3 billion.

They represent approx 70% of the islet cells, followed by α glucagon secreting cells (approx 15%), the rest being divided between δ cells (somatostatin secreting), γ cells (pancreatic polypeptide secreting cells also called PP), and ε (ghrelin secreting) (Fig. 3).
Figure 3. The ratio between a small and a big islet is $> 20$ after reference Ionescu-Tîrgoviște C., Acta Endocrinol., Buc, 2014.

Unfortunately, it is obvious that this cellular ensemble (possible to be histologically studied only in vitro) develops complex anatomical and functional communications hard to observe in their physiological dynamic relationships as they act in vivo and in situ.

We have to mention that pancreatic islets are better vascularized and innervated than the acinar tissue, the only separation of them being a poorly known glio-endothelial capsule\textsuperscript{43,44}.

At the rich arterio-venous vasculature of the pancreas (Fig. 4) we must add a rich network of lymphatic vessels that drains the lymph in a specific manner, heading to the numerous afferent peripancreatic lymph nodes (LN), divided by the Japanese Pancreas Society (JPS) in 18 distinct groups\textsuperscript{45}.

Finally, the lymph flux from the pancreas is heading in four directions that could be well represented by the four cardinal points (Fig. 5).

We will return to the role played by the lymphatic tissue in the autoimmune diabetes pathogeny, only adding here the fact that the pancreatic structure remains only partially understood, and the “abdominal drama” induced by acute pancreatitis, as it is called by surgeons\textsuperscript{45,46}, has become a subject of great interest because of the abundant sensitive innervation of the pancreatic “capsule”, and, possibly, of the abdominal retroperitoneal wall which is in direct contact with its posterior surface. In the 70’s, Gilorteau named this structure “the pancreatic reflexogenic area”\textsuperscript{47}.
The collaboration between diabetologists and surgeons became a priority after the North-European group nPOD performed 6 tail pancreatic laparoscopic resections in 6 young type 1 diabetes patients (aged between 20 and 30 years), soon after the onset of diabetes (less than a month), in order to carefully analyze the intrapancreatic insulitic process. All these interventions had obviously an informed consent from the patients. Surprisingly, 3 of 6 cases had some incidents (one had a spleen hemorrhage and two had a peritoneal reaction because of the acinar liquid leakage through the suture blunt). These were finally resolved by a second surgical procedure or medical treatment. The program of the nPOD group stopped (we hope only temporarily) this interesting and potential useful approach.

According to some surgeons that frequently perform pancreatic surgery and that we have consulted, the view that the structure and consistency of this “peripancreatic capsule” is variable (meaning heterogeneous as consistence) from patient to patient was confirmed to us. In some cases it is so loose (inconsistent) that it cannot permit the blunt re-suturation. We have even suggested that if the immuno-histologic information received from such pancreatic approach (that we still expect from the nPOD group) are sufficiently important in understanding the autoimmune diabetes pathogeny, then this procedure should be continued with a
supplementary element of precaution naming the careful laparoscopic assessment of the pancreatic capsule consistency. The procedure should be continued only if it is robust enough to permit the post-resection suturing.

Clinical heterogeneity of diabetes

From the beginning, we have to mention that the diabetic syndrome includes different phenotypes, that can appear from the first day of life (neonatal diabetes) to the last one of long living individuals (“senile diabetes” - see Fig. 6)\(^4\).

Besides monogenic forms of diabetes, the number of which exceeds 20, but whom global frequency is small enough (less than 2% of the diabetic patients), there are the main polygenic phenotypes that can manifest in different manners. From practical reasons, in the actual classification they were divided in two major categories depending on the presence or absence of autoimmune mechanism: type 1 and type 2 diabetes. On its turn, type 1 diabetes was classified in type 1A (autoimmune) - that appears in children, adolescents and young adults (18-35 years) or sometimes in more advanced ages (a phenotype known as Late Autoimmune Diabetes of the Adult - LADA), and type 1B non-autoimmune. Type 2 diabetes is frequently associated (~80% cases) with overweight/obesity, the rest being of normal weight, and is frequently encountered at older ages\(^5\).

Within each of these two major traditional phenotypes (T1D and T2D), there are numerous sub-phenotypes, explainable not only through clinical, but also genetically, anatomical and pathogenic heterogeneity, that finally leads in each patient to a unique, unrepeatable form of diabetes. Because the autoimmune diabetes is in the last year under a critical scrutiny we will try to shed a new light on the early phases of this dramatic phenotype, especially when it appears in young ages.

![Figure 6. Distribution of diabetes in Bucharest (1994).](image)
**Autoimmune diabetes and a new genetic approach**

We must start by saying that autoimmune diabetes can be encountered from childhood to adulthood (Fig. 6). Our data prove that between phenotype 1 (autoimmune and non-autoimmune) and phenotype 2 of diabetes stand a large group of patients, approximately 10-15%, that show clinical, biochemical and immune characteristics, which are sometimes closer to type 1 diabetes and some other times to type 2 diabetes\(^{50}\). For this phenotype we proposed the term of Intermediary Diabetes Mellitus (IDM), that seems more appropriate than LADA since this last name doesn’t accurately express the characteristics of this phenotype\(^{50,51}\). It is worthy of note that the large majority of the genetic studies using the new approach of the genome wide scanning (GWAs) were carried out separately selecting from T1D analyses mainly “pediatric” cohorts of diabetics\(^ {52} \), whereas, for T2D cohorts were selected the typical patients with onset over 40 years and never insulin treated. The post adolescent and young adults with diabetes were excluded without a clear explanation\(^ {1,52,53} \).

Between 2007 and 2014 many such genetic studies and meta-analyses have been published, ending to the conclusion that each of the two classical phenotypes (T1D and T2D) are associated with more than 60 genes each\(^ {55} \).

In support to the existence of an intermediary type of diabetes, we could bring a supplementary genetic argument\(^ {56-60} \). In the first analysis of gene promoters associated with the two major phenotypes of diabetes (T1D and T2D), we have observed that some genes traditionally associated with both phenotypes form a distinct group (Fig. 7). Its emplacement as a pattern of promotors lies between type 1 and type 2 diabetes, respectively (Fig. 8), a fact that objectively supports the intermediate character (IDM) of this phenotype.

Frequently, these patients are initially treated with oral antidiabetics, although they require an insulin treatment over the course of the disease. The marked decrease of β-cell mass/ function in these patients is proved by the progressive decrease of plasma C peptide level.

The pattern of promotors through which the diabetes phenotypes are characterized was based on our original method\(^ {56-60} \) that we consider taking part of what was called post-genomic approach of nucleotide sequence analyses. This original method uses the relationship between two specific parameters: the content of the nucleotide segment analyzed (in our study the promotors of genes associated with type 1 and respectively with type 2 diabetes) cytosine and guanine (CpG) and a new parameter, Kappa index of coincidence (KIC) (Fig. 9).

This index is derived from a cryptographic method used in the two World Wars for decrypting messages sent between different armies. Considering that the genome represents an encrypted message, apparently as a disordered succession of four nucleotides (adenine, guanine, cytosine and thymine), we used the two parameters obtained with a sliding scale window that goes through the analyzed sequence, nucleotide by nucleotide. From each step of the sliding window a point was plotted generating a pattern as shown in

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**Figure 7.** The distribution of promotors of genes associated with type 1 and 2 diabetes.
Figure 8. The distribution of promoters of genes associated with type 1 diabetes, type 2 diabetes and IDM.

Figure 9. DNA pattern analysis method.
Figure 9ABC. Essentially, this method analyzes information complexity that is held in the ~500 nucleotides segment from which the gene promoters that precede its starting point are formed. This particular genomic segment is important because in precise regions over their length, the transcription factors of the respective gene are coupled. Some of these up-regulate (stimulate) gene transcription, while others down-regulate (inhibit) gene expression\textsuperscript{57,58}.

As it can be seen in Figure 10, the promoters of genes associated with the two main phenotypes of diabetes contain different DNA patterns. T1D promoters exhibit image-based patterns which show that they are part of a special class of promoters called “AT-based”\textsuperscript{57,58}. The promoters of genes associated with T2D exhibit patterns that show they are part of a special class of promoters called “CG-based”\textsuperscript{57,58}. This separation of classes shows that genes associated with these two phenotypes rarely share transcription factors, therefore these genes cannot be co-expressed. The third type of pattern is presented by a number of genes such as CD55, C1QTNF6, INS, ERBB3, HMGAA2, CTS\textsubscript{H}, SLC30A8, CDKN2AIP, PROX1, PPARG, TCF7L2 which suggest a new phenotype, an Intermediary Type of Diabetes (IDM), (Fig. 10). The shape of these patterns indicate that genes associated with IDM can use transcription factors from both phenotypes, further indicating that IDM may contain the driver genes for triggering T1D and T2D. It is worthy of note that the gene SLC30A8, which encodes the isoform 8 of the Zinc transporters specific for \textbeta{}-cells, although it is nominally associated with T2D, in fact it encodes a \textbeta{}-cell antigenic molecule inducing Zn-T8 antibodies, encountered in autoimmune type 1 diabetes. This supports the view that the genetic background of various phenotypes of diabetes is more complex than previously thought.

As it is observed in Figure 8, in which we reproduce the position of the gene promoters associated with T1D, T2D, but also IDM and obesity, it becomes obvious that each phenotype of diabetes results from a gene network mobilization, specific for every phenotype of diabetes. Since the number of genes associated with a certain phenotype varies from patient to patient, there are important variations even within a phenotype that was clinically defined by us that make from each case an unrepeatable (unique) phenotype of diabetes.

### Diabetes heterogeneity and the lymphatic system

It is the right time to bring into discussion the second great heterogeneity of diabetes syndrome, the one that depends on the immune system. If the fundamental and intrinsic cause of diabetes is hidden in the secretory dysfunction of pancreatic \textbeta{} cells, we can accept that sometimes the acinar ducts inflammatory processes may intervene sometimes in the pancreatic inflammation, including its both endocrine and exocrine components\textsuperscript{20,61-64}. This factor could soon become “viewable” due to the progress in imagistic methods that are addressed to pancreatic \textbeta{}-cells identification\textsuperscript{65-67}, or to the local inflammatory process called “insulitis” characteristic of type 1 diabetes, but also to a possible inflammation of the acinar ducts\textsuperscript{68,69}.

Autoimmune diabetes results from a conflict that appears between pancreatic \textbeta{}-cell (which represents the center of command and control of energy metabolism) and body’s immune system (which is responsible for anatomic and functional integrity of the human body that sometimes operates in a hostile medium). Usually, its activity is not sensed by the patient, although the structures involved in the body’s immune defense are vast and only partially known (despite its great importance in the maintenance of human body integrity).

The lymphatic system starts and increase its size from the four body extremities, gathering from more than 2/3 of the supero-inferior body (the inferior half and a great part of the superior left side) in the “cisterna chyli” situated in the abdominal cavity in the supra-umbilical region. From here starts the lymphatic duct that drains in the left sub-clavicular vein (Fig. 11). A second great lymphatic vessel (right lymphatic duct) gathers the lymph from the less than 1/3 of the right superior part of the body and drains in the right sub-clavicular vein.

A staining of this system would allow the reproduction of a mold of the human body with its numerous extensions that exist in the skin (where the network of lymphatic capillaries originate blindly in the extracellular spaces of all tissues (except the brain, eye and internal ear) and also around all internal organs (where the lymphatic originates in the same way).
Figure 10. DNA patterns generated from promoters of genes associated with type 1 and type 2 diabetes.
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Figure 11. Lymphatic system from Clinically Oriented Anatomy by Moore L. Keith, A.F. Dalley and Anne M. Agur.

In the lymphatic vessels already collected in small trunks there are abundant valves that guide the lymphatic flux centripetally towards the cisterna chyli, and then in larger vessels that drain in the venous systemic circulation. If we take into consideration the hundreds of lymphatic nodes (~ 600 in humans) located along the course of lymphatic vessels filtering the flow of lymph originating in various regions of the body’s segments, and especially around important organs, to whom we add the bone marrow, spleen, thymus and Payer plaques in the intestine, we have to accept that the importance of this system is very special. We mention also that immunocytes (the cells involved in the body’s immune system) circulate not only through the lymphatic system or circulatory system, but they are also capable to insinuate in the different regions of the human body, and so we can understand why the function of this system is so little known. This incomplete knowledge is explained by the great dynamism of these cells and especially through their infinite capacity of reacting to all the existent antigens on Terra, but also to those that existed in the past and probably to those that will appear in the future.

If we refer to diabetes, we should note that the high number of lymph nodes surrounding the pancreas - that the Japanese Pancreas Society divided in 18 groups (Fig. 5), reflect the maximum protection that the pancreas needs since it is situated in the proximity of the digestive system, and confronts numerous aggressions that it is obliged to resolve rapidly and as completely as possible. There are three situations in which the pancreatic damage generates a pathology that is difficult to control. The first is acute pancreatitis (defined by Dieulafoy in XIX century as an “abdominal drama”), that sometimes appears suddenly, without any premonitory symptoms, even though its frequency is greater in diabetic patients, obese subjects, subjects with gallbladder stones and in dyslipidemia45,46.

The second situation is pancreatic cancer, one of the most aggressive forms of cancer, without an optimal therapeutic solution. Because the main location of pancreatic cancer is the head of pancreas, we wonder if this location is not related to a possible “cell instability” in this region in which the tissue results from intermingled cells, originating from the two separated, dorsal and ventral embryonic buds (Fig. 2).

Finally, the third situation is represented by diabetes mellitus, irrespective of its phenotype, but particularly autoimmune diabetes, that through its preferential appearance at small ages, represents one of the most dramatic conditions, when in a family a toddler or a schoolchild is diagnosed with this disease.

Is diabetes heterogeneity compatible with a unitary mechanism?

Our point of view is that the element that makes the heterogeneous diabetic syndrome unitary is just its sine qua non condition, represented by the β-cell mass/dysfunction which is present in every phenotype. The heterogeneity of the diabetic syndrome originates from the different mechanisms through which the β-cell damage is
produced. This is due to the genetic heterogeneity of classic phenotypes\textsuperscript{52,55}, and also to epigenetic factors intervention that are assigned to the influence of the surrounding environment, including infectious, alimentary, chemical or some other factors\textsuperscript{50-74}. These factors can directly influence the β-cell function, or can intervene by modifying the immune response of the organism, that can be vicious in the case in which the immunity genes (associated with type 1 autoimmune diabetes) are present in a higher or a lower number, some of them involving the innate immunity, and others adaptive immunity\textsuperscript{52,55,7}

Irrespective of the diabetes phenotype, for the glycemic alteration to take place, it is necessary that the β-cell mass/function decreases below a certain threshold. It is estimated that this level is approximately 50-60\% in type 2 diabetes phenotype, 50-70\% in IDM (CIT 2013), and 80-90\% in type 1 autoimmune diabetes\textsuperscript{75-79}

It was noticed that the decrease of the β-cell mass before the clinical onset of the disease is different. It is higher in T1D, smaller in IDM, and even smaller in T2D. Despite this age related heterogeneity, in an apparently paradoxical manner, the unitary character of diabetes is confirmed, considering that the speed of β-cell mass loss is less important, than the fact that the common consequence for all diabetic phenotypes is ultimately a progressive loss in the β-cell mass/function that leads to the blood glucose decompensation.

Heterogeneity of clinical phenotypes is registered also within the autoimmune “juvenile” type of diabetes that can appear any time after birth and up to beyond 16 years. The multiple seroconversions (the occurrence of 2, 3 or 4 anti β-cell antibodies) predicts the onset of clinical diabetes at a younger age\textsuperscript{32,33}, especially in first degree relatives (FDR) of T1D patients\textsuperscript{39} associated with strong diabetogenic haplotypes (HLA-DR/DQ) and in the absence of HLA protecting genes\textsuperscript{55}

**When does the real onset of diabetes take place?**

It is known at present that the clinical onset of diabetes is preceded by years or decades of a slower or less slow decrease in β-cell mass. It is also known that this process is progressive and leads in time to the blood glucose decompensation. Why do we continue to ignore this prehyperglycemic period of diabetes, whose evolution could be easier to influence through specific methods, as it was demonstrated for T2D\textsuperscript{79,80}?

We are aware of the fact that by diagnosing diabetes in its prehyperglycemic period, the number of T2D patients would double or even triple in a first phase. However, the secondary positive effect could be that of sparking the interest for deciphering the pathogenic mechanism and diagnosing this progressive prehyperglycemic stage by not expensive, but clear predictive markers. This will stimulate also the development of a real prevention method used at a right time, i.e. for T1D before the “point of no return” of the autoimmune process.

Immunology, biochemistry and genetic scientists will have to solve the problem of conceiving a diabetogenic risk score with a good predictability, capable of indicating the persons that must be included in structured prevention programs. Accepting the Hippocrate’s principle “primum non nocere”, such a program should include administering of bioactive molecules that come from plants. It is known that these contain molecules that are compatible with human body, some of them being eatable (blueberries, cranberries, mulberries, sea buckthorn- Hippophae rhamnoides, Morus alba, morus nigra). Some of them might contain insulin-like molecules or antioxidants or other compounds\textsuperscript{82-84} whose therapeutic efficiency has been recently experimentally proven\textsuperscript{85-88}.

The comparative genetics (plants/animals/humans) should evolve in the direction of honestly testing the therapeutic qualities of some natural products that have already been used for centuries in treating most of the entities framed in metabolic pathology.

We should not forget to mention that the most efficient and safe class of antidiabetic agents – the biguanides -, was identified in Galega officinalis. This says a lot. In some academic circles there is still a detrimental mentality arguing that this approach comes as a second hand method, at least from the scientific point of view. It is the time to reevaluate this therapeutic resource, taking precautions for avoiding quackery and misleading information.

**Reviewing a fine, but an obsolete theoretical concept**

One of the most evoked staged evolution of T1D was that proposed by Eisenbarth \textit{et al.}\textsuperscript{89}, over
25 years ago. It was inspired by the immune-genetic theory of “juvenile diabetes” – established by Nerup et al. and confirmed in the same year by demonstrating the pancreatic tropism of anti-islets antibodies from diabetic blood. Eisenbarth et al. placed the well-known genetic predisposition for T1D as the first step (stage I), followed by a presumed step named “autoimmunity trigger” (stage II), and then by a progressive and almost complete destruction of β-cell mass by the specific autoimmune process (stage III), progressing through the prediabetes stage IV and ending with stage V, the clinical onset of diabetes.

Ordering the stages of a certain pathology, especially in the absence of some known pathogenic mechanisms, is always broadly accepted, because something it’s better than nothing. However, the questions remained unanswered after the classification of Eisenbarth was released: when does the anti-β-cell autoimmunity activation takes place? is the β-cell destructive process linear as the image of Eisenbarth suggests? does this destructive process lead to the disappearance of all β-cells?

Although this stepwise evolution of T1D initially had a positive effect, further it acted as a break in understanding the complex mechanisms that stood behind a “beautiful” simplification. In the following pages, we shall try to detail some of the complex processes that take place in the “black box” of the endocrine pancreas, as we could characterize the events that take place in the ensemble of Langherhans islets.

**Genetic predisposition – a new interpretation**

As a term, genetic predisposition suggests only a potentiality which can or cannot progress to a specific disease. Indeed, the genetic predisposition for T1D is, no doubt, present not only in future diabetic patients, but also in a number of newborns with a genetic risk score (the presence of the main genes that are associated with T1D) that could be similar with that of other individuals that will develop diabetes. Yet, only part of them would finally develop a clinical disease.

Considering the actual knowledge, we shall try to imagine the stages that could precede the appearance of first anti-insulin antibodies, merging in a single step the genetic predisposition and the autoimmune trigger, probably misunderstood as being two distinct stages.

One of the important observations we made while participating in the EURODIAB epidemiological study of T1D in children (0–14 years) was the progressive increase in the incidence of T1D in the age group 0–14 years. If in 1988, the incidence of diabetes in Romania in this age group was 3.5/100.000/year, at present it reached approximately 10/100.000/year, meaning an increase of more than 100% per decade. It is obvious that in this period of time, the genes remained the same, so that the only logical explanation for this increase was the change of environmental factors. As such, the high incidence increase in T1D in East European countries might be explained by the fast modification of lifestyle (the outbreak of fast food diet and sedentary lifestyle imposed first of all by computers, mobile communication technology and TV, but also the rise in inter-individual contacts through globalization process), without finding a clear causal agent.

The predisposition should be associated with the existence of an already variable β-cell secretory defect. If the β-cell secretory defect is important, the immune destructive process will be rapidly set in motion, leading to the clinical onset of diabetes at a young age, even in the first months or years after birth. Depending on the various ratio between β-cell dysfunction and autoimmune dysfunction, the process will continue up to puberty, and then beyond the age of 20, up to the age of 40 (19). Even within the “juvenile” phenotype of diabetes, an important clinical and pathogenetic heterogeneity could be observed. The explanation could be a variable inheritance in both β-cell secretory and immune defects. These early manifestations are difficult to identify because the β-cell defect and the immune system increased auto-reactivity could be only intermittent and without any clinical expression.

In our view, the initiation of the first autoimmune “compensatory” reaction results from the “danger signals” released by the dysfunctional β-cells, probably located only in some islets and in those islets just in a few β-cells. The main marker of β-cell dysfunction could be the higher proinsulin plasma levels or an increased proinsulin-to-insulin ratio. It is difficult to understand how researchers had overlooked such an important marker of the β-cell dysfunction that can give in the same time a pathogenic information.

We argued many times based on data obtained from the descendants of parents with T1D or FDRs of persons with T1D, that the presence in
their serum of an elevated level of plasma proinsulin\textsuperscript{12,106,109,110} indicate a clear β-cell dysfunction. Because this elevated proinsulin comes from pancreatic β-cells, the primary defect results from the cell inability to produce mature secretory vesicles (SV)\textsuperscript{109}. This is why we have designed the β-cell not to be a simple and truly "insulin factory" \textsuperscript{112} but a "mature secretory vesicles factory" \textsuperscript{109}. Since in SVs maturation participate a great number of β-cell structures/molecules, the defect could be situated anywhere in the long chain of events starting with intranuclear transcription of many genes (preproinsulin included), up to their natural and physiological processing and assembly mechanisms, the only one capable to generate mature SVs. The immature β-cell vesicles are unable to respond promptly and efficiently to various requests, day by day, year by year, and decade by decade. That is why we gave such a high importance to the β-cell dysfunction as a starting point in the diabetes pathogenic process.

What can be invoked at present time as an explanation, is the fact that the environmental factors, whatever they are, would be capable to recruit a higher number of associated genes with T1D that have a smaller diabetogenic power, but who, together with the main diabetogenic genes, (HLA DR/DQ, INS, PTPN22, CTLA-4, IL-2RA), will lead to diabetes onset (see [55]) in a greater number of persons that carry these genes.

**Seroconversion and its significance**

For a long time, seroconversion was considered to be the first stage of type 1 diabetes mellitus, reflecting the open conflict between pancreatic β-cell and the immune system. Since the presence of a single antibody was associated with only a minor increase in the progression to clinically manifest diabetes \textsuperscript{18-20,39,111-120}, the problem of their real significance was raised \textsuperscript{113,114}. Often, the anti-insulin/proinsulin antibodies have a fluctuating titer and sometimes can regress without the person involved developing a clinical form of diabetes during lifetime. That is an important observation mirroring the long term indecision regarding the progress or regression of the insulitis process.

Besides this favorable evolution of the autoimmune process, a percentage of the cases that develop a first anti-islet antibody can evolve in time (sometimes after months and other times after years or decades) to multiple seroconversion. These anti-β-cell antibodies were only four by 2008: anti-insulin antibodies (IAA), anti-GAD antibodies (anti-glutamic acid dehydrogenase), and anti-tyrosine phosphatase (IA-2A) and ICA (Islet Cell Antibodies). In 2008, soon after the discovery of SLC30A8 gene\textsuperscript{53} that encodes the isoform 8 of the zinc transporter (Zn-T8) \textsuperscript{121,122}, along the four antibodies mentioned before, was added this fifth specific anti-β-cell antibody. Recently, in 2014\textsuperscript{123}, to the five major anti-islet antibodies (ICA, IAA, GADA, IA-2A, Zn-T8), were added at least two more: EEF1A1 (Elongation Factor 1α1) and UBE213 (Ubiquitin-conjugated Enzyme 213). The prevalence of anti-EEF1A1 and anti-UBE213 antibodies in T1D was of 29.5%, and 35.8% respectively. Interestingly enough, these two new islet antibodies were also detected in fulminant T1D, but not in IDM (Intermediary Diabetes Mellitus) known also as LADA\textsuperscript{124}. The fact that > 40% of GAD-negative patients were positive for one or both of these newly identified antibodies is of great importance, increasing the positivity in T1D patients with evidence of autoimmunity from 76.3% to 86%. Overall the prevalence of these new antibodies was about 30% in T1D patients sera vs. only 5% in normal glucose tolerance subjects, a figure similar with the presence of ZnT8 antibodies in T1D patients, especially in those with adult onset of T1D\textsuperscript{123}.

The number of anti β-cell antibodies could increase in future, since Koo \textit{et al.}\textsuperscript{123} claim that according to their data, other 66 potential antibodies might be discovered, opening a new perspective for a better understanding of the complicated conflict between β-cells and the immune system. Such hypothesis is plausible considering the high number of antigenic molecules present in the pancreatic β-cell, or which became antigenic after their posttranslational changes\textsuperscript{72,125}.

The relationship between sero-conversion and T1D was well studied in the past years\textsuperscript{39,126,127}. They will remain an important milestone for the objectivation of the gradual paths of different diabetogenesis stages and confirm the existence of a long or a very long prehyperglycemic period, sometimes exceeding three decades\textsuperscript{115}. However, the refine changes which precede this more visible pathogenic step, remains largely unknown.
How could the anti β-cell autoimmunity be initiated?

The β-cell secretory defect, regardless of the cell zone where it comes from, can be explained through the stress from its endoplasmic reticulum. This damage can have a good experimental basis \(^{108,110,128-131}\). The same defect that impedes SVs maturation can also be responsible for the vicious formation of the extracellular matrix (ECM) inside the Langerhans islet. Such disorganized matrix would negatively influence the adaptation of the β-cell to the oxidative stress \(^{6,132,133}\), and in the experiments done on mice it negatively influenced the β-cell survival and expansion \(^{6,134-137}\).

A stressed pancreatic β-cell (but also many other cells, as endothelial cells or immune cells) is capable to release around it or directly in the systemic circulation, various chemical messengers through exosomes (EXOs). Exosomes are nanosized (30-100 nm) membrane vesicles that contain some powerful imuno-stimulatory chemokines. They are secreted as micro vesicles by many types of cells. Their biogenesis is unclear. Several small exosomes can fuse with each other to form multivesicular bodies. By the fusion with plasma membrane this organelles release their content in the extracellular space or directly into circulation. In these vesicles are also found specific RNAs which have recently \(^{138}\) been proposed as markers of the place (cells) where they originated from \(^{139-143}\). Exosomes can be found also in various body fluids (saliva, breast milk, urine, broncho-alveolar lavage) and can be isolated by ultracentrifugation or density gradient centrifugation \(^{138}\).

It is worthy of note that the mesenchimal stem cells (MSCs) have been found also inside Langerhans islets as stromal cells with a broad potential to become either islet cells or immune cells. In some unknown conditions (ER stress in β-cells for instance), these cells could release also exosomes containing various molecules acting as chemokines or cytokines probably from only a small number of islets and in these islets only from the dysfunctional β-cells. Such a scenario has been recently reported in NOD mice \(^{138}\). The mesenchimal like cells cultured ex-vivo proved to release highly imuno-stimulatory exosomes capable to activate autoreactive T and B cells via the release of IFNγ, initiating a vicious circle ending with the β-cell death. A possibility is that from the immune system the main player could be the dendritic cells \(^1\), which are able to send the information collected from dysfunctional β-cells to the peri-pancreatic lymph nodes, where specific T cells cytotoxic clones are generated against one or more antigens detected in the β-cell \(^{144}\).

Recently, Bogdani et al. \(^{144}\), analyzing the human islets obtained from normal donors and diabetic patients, found that the hyaluronan (HA) molecule, a long chain polysaccharide, involved in generation of islet inflammation, in cooperation with hyaladherins (HA-binding proteins), were dramatically increased, both inside and outside the islets surrounding immune cells in areas of insulitis. Such changes were only observed in tissues of younger pancreas donors with disease duration of < 10 yrs \(^{144}\). Interestingly enough, HA and their binding proteins were also clustered in follicular germinal centers in T-cell areas in lymph nodes and spleen. Such changes were not found in the islets of non-diabetes controls.

The signification of the high number of lymph nodes surrounding the pancreas Figure 15 can now be put in an another interesting perspective. Indeed, the lymph nodes are the place where B and T cell activation takes place in T1D. Moreover, HA production was reported to induce dendritic cells phenotype maturation \(^{146}\). These cells might be the main cells responsible for the initiation of inflammation in those islets in which a large number of β-cells carry the defect in the maturation of their secretory vesicles. An increased hyaluronan can also stimulate antigen presentation and T cell production \(^{147}\).

A question to be answered is the following: during the long-term evolution of diabetes, when do such changes take place? Are they a cause or a consequence of the β-cell defect?

In our hypothesis, a defect in the extracellular matrix structure could be secondary to the β-cell secretory defect, thereby creating a supplementary vulnerability of the pancreatic β-cell in front of an excessively reactive immune system. The cytotoxic immune cells (especially Teff cytotoxic lymphocytes) could not reach the proximity of pancreatic β-cells if the extracellular matrix texture, that has a protective role, would not be deteriorated by the β-cell defect itself, and possibly the endothelial cells associated with them. The crosstalk between β-cells and their endothelial cells is strong and continuous.

These new data make us to take into consideration the hypothesis of the β-cells inability to create the fibrilar and molecular intercellular
network with a protective role, as a concomitant important change in the early phases of a diabeticogenic process.

**Biochemical modifications that precede seroconversion**

In the last years, an increased interest has been shown for identifying the biochemical markers that are present and characteristic to the early hyperglycemic phases of diabetes. The recent metabolomic data managed to identify numerous small molecules present in the systemic circulation in the early stages of both T1D and T2D. In T1D, the numerous researches were more intended to the identification of the diabeticogenic process manifested before sero-conversion, meaning the occurrence of the first anti-β-cell antibodies, followed by other types (multiple sero-conversions).

In a more extensive study, Dutta et al. evaluated through mass spectroscopy a large number of chemical compounds and found modifications in the plasma level of 330 metabolites belonging to 133 metabolic pathways, in response to a 8 hour deprivation of insulin in type 1 diabetic patients. As it was expected, the main disorders were found in the glucose, amino-acid and fatty acid metabolic pathways. According with the author’s statements, the possibility of identification “of the chemical fingerprints of cellular events” exists. Such changes have been described already in 1912 and 1921 by Paulescu, when he defined diabetes to be a dysfunction in the normal utilization of all the three fuels (carbohydrates, proteins and lipids) in peripheral cells.

At present, numerous metabolomic studies were published by various authors for the identification of the early biochemical modifications in patients predisposed to develop T1D and also for those predisposed for T2D. This is an interesting and promising approach, because for every major diabetic phenotype there could be identified a number of specific chemical compounds.

In the close future, such selected markers could be determined at a larger scale through the development of diagnostic laboratory kits dedicated to the evaluation of these markers for the populational studies.

**In conclusion**, at present, we cross a challenging period in which we must clarify when, where and how the first diabeticogenic “movements” take place. We hypothesize that in the majority of cases, this occurs many decades before the onset of clinical TID.

The increase in intracellular proinsulin, and subsequently in the systemic circulation must be seriously taken into account as a first detectable molecular change, which can become also an unvaluable early marker of the disease. Such defect can easily allow the hyperactive immune cells to enter in the pancreatic β-cell proximity, because of the already disorganized extracellular matrix.

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**REFERENCES**

10. Faustman D. Why were we wrong for so long? The pancreas type 1 diabetic patients commonly function for decades. Diabetologia 57:1-3, 2014.


59. Guja C, Gagniuc P, Ionescu-Tirgoviste C. The promoters of genes may be the closest link between type 1 diabetes and other autoimmune diseases (Abstract) EASD, Vienia 2014.


64. Atkinson MA. Losing a grip on the notion of β-cell specificity for immune responses in type 1 diabetes: can we handle the truth? Diabetes 63:3572-3574, 2014.


68. Gialleonardo VD, EFJ de Vries, MD Grolano et al., Imaging of β cell mass and insulin in insulin dependent (type 1) diabetes mellitus. Endocrin Rev 33:892-919, 2012.


The Primary Cause of Type 1 Diabetes


