PROINSULIN, PROAMYLIN AND THE BETA CELL ENDOPLASMIC RETICULUM: THE KEY FOR THE PATHOGENESIS OF DIFFERENT DIABETES PHENOTYPES

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Based on our clinical and epidemiological data, we have sustained for a long time the unitary character of the various phenotypes of the diabetic syndrome. In this paper, we add several arguments sustaining that the unitary character of diabetes is related to a common primary defect in the function of the beta cell endoplasmic reticulum, leading to an inadequate processing of the two main secretory molecules: pre-proinsulin and pre-proamylin. The post-translational changes of these molecules might explain the main proapoptotic and anti-regenerative pathogenic mechanisms leading to a progressive decrease in the β cell mass/function. In our view, the increased proinsulin levels encountered in various diabetes phenotypes could be not only a marker of beta cell dysfunction but also could indicate the main β cell defect, suggesting also its location.

Key words: Proinsulin; Proamylin; Beta cell endoplasmic reticulum.

INTRODUCTION – THE FOUNDATIONS FOR A NEW HYPOTHESIS REGARDING THE PATHOGENESIS OF DIABETES

One hundred years after the description of the beta cell by Lane[1], the main cell of pancreatic islets comes back in the center of diabetes pathogeny. After the big enthusiasm rised by the discovery of insulin in 1921[2], considered to be "molecule of century"[3], the discovery of the other beta cell secretory peptide, – proinsulin 1967[4], of amylin[5, 6] and of proamylin[7] – has been considered as second importance. Moreover, for three decades, the beta cell dysfunction has been shaded by the theoretical construction of *peripheral insulin resistance*[8] pushed in the first position as the main pathogenic mechanism at least in type 2 diabetes[9]. Not denying its contribution in modulating various clinical phenotypes of diabetes, it should be required a more precise definition, characterization and a better assessment. Otherwise, the risk for insulin-resistance to be taken "too seriously" (such as it is nowadays advocated by some) is high, since some diabetologists came to the brink of describing it as the "universal accelerator of diabetes", irrespective of the diabetes phenotype[10]. By associating obesity (a very concrete element) with *insulin-resistance* (a construction mostly theoretical and without precise boundaries) and the last with a supposed *hyperinsulinism* (frequently inexistent if from the routine determination of the plasma insulin we subtract the plasma proinsulin level), this theoretical construct can gain some supporters[11–13]. When the supporters come from the pharmaceutical industry, the danger is high, possibly leading to convulsions, both among physicians and patients. Anyway, the cautions of *Diabetologia*[14, 15] in the face of enthusiasm without a solid basis in "hard data" are more than welcome.

This material belongs too to the group of "theoretical constructs", but we think it can be supported by many concrete data. Our hypothesis is based on 4 decades of clinical activity, having at our disposal the epidemiological data provided by the Bucharest Registry of Diabetes, which from 1942 till present included almost 170 000 patients *of all ages*, representing *all the cases* of diabetes identified in the population from a geographical area. Thus, we could identify several epidemiological[16–19], clinical[20–22], genetic[23–31] and

biochemical[32–39] details which, put altogether, led us to a rather new interpretation of the pathogenesis of the various diabetes phenotypes, in the center of which is comfortably placed the pancreatic beta cell.

INCREASED PROINSULIN AS THE MAIN BETA CELL SECRETORY DEFECT

The first important finding that stayed behind our hypothesis was the finding of increased levels of plasma proinsulin in all diabetes phenotypes that we could investigate[35–39]. Using a literature search, both of older[40–48] and newer studies[35–39, 49–87] we found a confirmation of our hypothesis which places the proinsulin defect in the center of the pathogenic mechanisms which operates in the various phenotypes of diabetes. The more important as *pathogenetical significance* was the finding of increased proinsulin in *first degree relatives* or descendants from diabetic parents, both with *type 1*[48, 58, 86, 88–91] and *type 2*[70, 73, 82] diabetes. These were two confirmatory elements of our hypothesis: the proinsulin disorder in diabetes is not only *ubiquitous* but it is also *precocious*. For our hypothesis, the similarity between the beta cell secretion disturbances encountered early in the evolution of both major diabetes phenotypes (type 1 and type 2 diabetes) represents the strongest argument in favor of the *unitary character* of diabetes.

Since the source of increased plasma proinsulin proved to be the beta cell secretory vesicles[55], we focused our attention on these small "anatomic productions" of the pancreatic beta cell[93, 94]. The specific capacity of the beta cell to produce secretory vesicles, *i.e.* to distribute the astronomical number of insulin molecules included daily in equal quanta of ~200 000 molecules/vesicle, together with the other secretory (proinsulin, amylin, proamylin and C peptide) or non-secretory peptides (chaperones, enzymes, other small peptides of uncertain function), is explained by their main characteristic: to be *polarized* and *excitable* cells. The ~13 000 secretory vesicles/cell dominate the electronic microscopy image of a beta cell and highlight their major importance in the fulfillment of the beta cell function: to release insulin in a regulated manner according to the concentration of different nutrients in the blood.[93, 94]

In order to perform its function, the complex machinery of the beta cell has to exocytose „mature" secretory vesicles, *i.e.* vesicles in which proinsulin (the precursor of insulin) does not represent more than 1–2% of the initial translation of pre-proinsulin in the ribosomes. An immature secretory vesicle contains a higher percentage of proinsulin (perhaps of proamylin also) and, evidently, a lower percentage of mature insulin. Moreover, these "immature" vesicles, usually present only in the center of the beta cell, will be mingled with the mature vesicles in the different compartments of the beta cell dedicated to exocytosis. The exocytose of this immature vesicles will have two consequences: *the first consequence* is that the plasma levels of proinsulin will increase proportionally with the concentration of proinsulin inside the secretory vesicles while the proinsulin-to-insulin ratio (PI/I), (usually < 0,1) will increase, sometimes becoming supra-unitary; *the second consequence* is that immature vesicles will improperly react to the movement commands, such that the physiological insulin-secretion pattern (oscillatory secretion, biphasic response after a stimulus) will be attenuated or totally disturbed[95–102]. We don't know yet exactly the chronology of these disturbances (increased proinsulin *vs* disappearance of the first phase insulin secretion) but we know that they are both very precocious in the natural history of diabetes.

Because increased plasma proinsulin results from the exocytosis of "immature" secretory vesicles, we had to answer to the question: what is the cause for the immaturity of the secretory vesicles? Since they are generated by the close cooperation between the *endoplasmic reticulum* (ER), that processes and sorts out the protein molecules transcribed in the nucleus and translated in the ribosomes, and *Golgi apparatus* (GA), that assembles the pieces of the vesicle cytoskeleton and includes inside the vesicle all the molecules mentioned above, the fundamental beta cell defect has to be placed somewhere between these two "sister" cell organelles. After the real explosion of information regarding the complex structure and function of the ER[103–110], we reached the conclusion that the site of the primary beta cell defect from the two major diabetes phenotypes (but also from gestational diabetes[111] and at least in some of the phenotypes classified as "other types of diabetes")[112] is represented by the *defective processing of the proinsulin and proamylin molecules inside the ER.* This faulty processing could have three causes: 1) a genetic defect of a functional protein from the structure of the *ER itself*, as it is the case for the Wolfram syndrome[113]. Such an alteration is extremely severe and cannot

be invoked as cause for the "common phenotypes" of diabetes that represent more than 95% of the total cases of diabetes; 2) a genetic defect in the transcription of proinsulin molecule, as it is the case in the Akita mouse diabetes model[114], leading to a blockage of the ER molecular flux by the *wrongly packaged proinsulin*[115]. Obviously this also cannot be the case for the common diabetes phenotypes; 3) a third defect, proposed by our hypothesis, is related not to a major alteration of a structural or functional molecule of the ER, but rather to a "degree of inflexibility" of the ER adaptability in the presence of an increased insulin demand, as for example in the presence of obesity. A structurally normal ER, but under-dimensioned for an increased or even normal secretory traffic, will have a lower capacity of pro-molecules processing. In the presence of a high molecular traffic through its secretory labirintic structure, this will allow the release towards the GA (the main site of secretory vesicles assemblage) of an increased percentage of un-split (or just partially split) proinsulin. In contrast with the ER, which has in the *Unfolding Protein Response* (UPR), a mechanism for a corrective reaction for the different types of "stress" generated inside this structure[104–107], the secretory vesicles have no mean for correcting the high level of proinsulin and maybe proamylin. The chaperone molecules inside the secretory vesicles will perform their function and try to limit "the local damage" but the generation of new chaperones with other corrective functions cannot be produced inside the vesicles anymore. This is why, a vesicular defect appeared in the stages of ER-GA vesicle generation will be finally reflected in the persistence inside the beta cell of a higher number of "immature vesicles" and finally in the increased levels of plasma proinsulin.

Once the *unitary* character of diabetes established on the basis of the *common beta cell proinsulin defect*, we have to explain the major differences that exist though between the two major diabetes phenotypes: the younger/older age at onset, presence/absence of weight excess, rapid/slow evolution, primary insulin-dependence/ insulin-independence. All these result, as correctly stated by Wilkin[10], from the different *speed* of *beta cell mass decrease*: rapid in the type 1 phenotype and slow in the type 2 phenotype. Unfortunately, we cannot agree with the statement (not sustained by proofs) that the main element which triggers the acceleration of this process is peripheral insulin resistance.

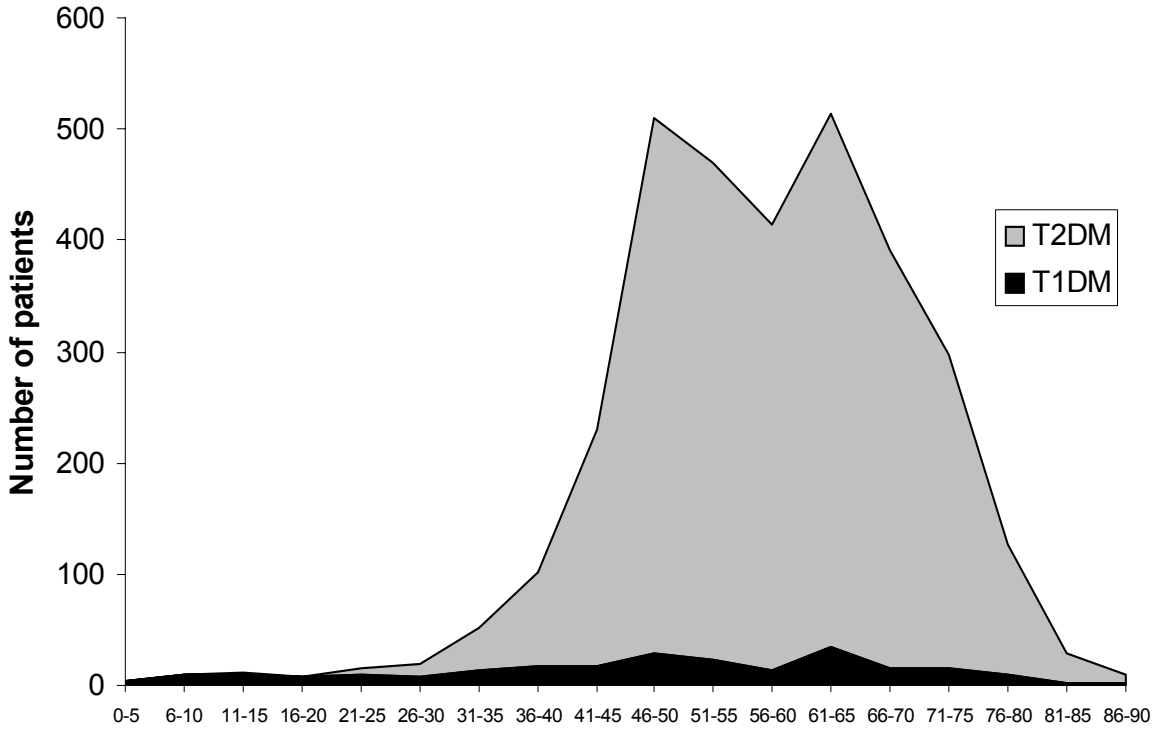

Fig. 1. The distribution of new discovered diabetic patients in 2001 by age and type of diabetes. The black area represents approximately 7% of T1DM new discovered patients.

If we take a close look to the distribution of the age at onset for the cases with primary insulin-dependence (highly overlapping with type 1 diabetes), we can notice that their distribution is almost constant during lifetime[17, 32, 37,116] (Fig.1). In one of our own studies[18], we showed that only 13.1% of all these cases have the onset before the age of 20 years, traditionally considered to be characteristic for T1DM. For this diabetes phenotype is operating a supplementary, intrinsic, genetically determined mechanism. We refer to the anti beta cell autoimmunity, consequence of a genetically inherited defect of the immune system, considered to be necessary[14, 117–121] but not sufficient[26, 120] for the genesis of T1DM. There are many carriers of this particular genetic defect that will never develop diabetes during their life. In other words, this "potential" genetic defect has to be activated by an external, supposedly environmental factor. However, all the attempts to clearly identify the exogenous environmental trigger for T1DM failed[14, 118, 122, 123]. The possibility that the exogenous trigger can be localized *inside the pancreatic beta cell* itself was not well explored. We consider that this trigger can be represented by the increased proinsulin in secretory vesicles, which has been proposed to have an important auto-antigenic potential[124–126]. We don't think we can talk about the existence of a "double diabetes"[127] but we can talk about a double genetic defect. The different age at onset for T1DM in subjects that inherit the same „autoimmunity genes" could be explained by the different interval required for the proinsulin level inside the beta cell to reach a "critical threshold" for the initiation of autoimmunity. In parallel with the aging process, we can expect also a decrease of the intensity of the "autoimmune reaction", as illustrated by the lower number and titer of anti beta cell antibodies in LADA and T1DM in adults[128–130].

If we look to the age distribution of T2DM cases, a major difficulty is represented by the attempt to explain the peak of T2DM incidence that appears after the age of 40–50 years, reaching a maximum between the ages of 55–65 years. We think that in these cases, to the beta cell proinsulin secretory defect is added a second pro-apoptotic defect, represented by the *conformational changes* of the proamylin/amylin molecules. It can be speculated that between the proinsulin/insulin secretory defect and that of proamylin/amylin can exist some pathogenic relationships. Such a relationship could explain the amyloid transformation of amylin[131–133]. The generation of amylin toxic oligomers[110, 133–137] will transform the slow beta cell apoptosis in a more rapid apoptosis, in the same time involving also a higher number of beta cells.

The supplementary *pro-apoptotic amyloidogenic mechanism* is the third important element of our hypothesis. We owe to Westermark and his team not only the discovery of amylin[5] and its gene cloning[6], but also the demonstration of the correlation between the hormonal peptide known as amylin and the amyloid deposits[6, 131, 133], described for more than a century to be associated with T2DM[138].

We recognize that beside the strong elements of our hypothesis: 1) increased proinsulin; 2) defect of proinsulin processing inside the ER; 3) immature secretory vesicles; 4) increased intra beta cell proinsulin as a trigger for beta cell autoimmunity; 5) the relationship between amylin / toxic amylin oligomers / amyloid deposits / beta cell apoptosis, there are still some unanswered questions regarding the chain of events that leads from the beta cell proinsulin defect in the ER to the decrease of the beta cell mass. At the speed of the confirmatory data published in 2007[110, 139–141], we are convinced that these questions will be soon answered.

THE HETEROGENEITY OF THE DIABETES SYNDROME

The profound cause of many uncertainties regarding the pathogenesis of diabetes is related to the amazing **heterogeneity** of the disorders present in every diabetes phenotype that was analyzed. This derives maybe form the great structural and functional heterogeneity of the beta cell mass, a concept launched by the school of Pipeleers[142–145] and later developed by other researchers also[146–149]. Clinical, pathogenetical and epidemiological heterogeneity is without doubt related to the genetic heterogeneity not only in T1DM but also in T2DM. We repeatedly showed that differences in the prevalence of high risk HLA alleles in the general population across Europe can explain at least in part the T1DM epidemiological heterogeneity in this region[26, 120]. Thus, there are some differences within Europe in the distribution of the HLA risk alleles among patients affected by type 1 diabetes. The proportion of DQB1*02 – positive subjects is higher among patients from

southern Europe, whereas the DQB1*0302 haplotype is more common in northern Europe.

From the pathogenetic point of view, an important question for which we don't have yet an answer based on objective data is the following: Is the beta cell defect of protein processing inside the ER present in all the pancreatic beta cells or just in part of them? According to the theory regarding the structural and functional heterogeneity of pancreatic islets/beta cells[142–145] it can be stated that this defect is rather scattered than generalized. Since immunohistochemically the pattern of distribution of proinsulin between the different islets and, inside an islet, between the different beta cells[151] as well as the pattern of distribution of amyloid deposits[131, 133, 147, 149] are scattered, it is presumable that the dysfunctional beta cells are also scattered, having an unpredictable number and distribution. This finding is not at all surprising taking into account that the structural and functional heterogeneity involves even the secretory vesicles, in a way that inside the same beta cell some vesicles can be normal (mature) while others, coexisting inside the same cell, could be immature[151]. The normal vesicles could be generated during the periods of lower secretory load (associated evidently with a lower molecular traffic inside the ER) while the abnormal vesicles could be formed in the periods of more intense beta cell secretory demand. In the conditions of a more rapid molecular traffic inside the ER, the risk for an incomplete processing of proinsulin will be higher, the defect being transferred to the vesicles produced during this interval, with the consequence of higher proinsulin content.

The pathogenic heterogeneity of T1DM (expressed in pathological terms) results well from the histological (necroptical) features of the pancreas of some young patients deceased shortly after the clinical onset of the disease, showing that the "insulitis" process was found in some islets but absent in others[152–155]. In the case of an exclusive defect of the immune system, it is expected that autoimmunity should have targeted equally all the Langerhans islets inside the pancreas. The finding that, at least initially, only part of the islets is involved can be explained by the anatomical and functional heterogeneity not only of the islets, but also of the beta cells inside an islet[142–145, 149]. Finally, *the pathogenic heterogeneity* will be reflected in the clinical – biochemical phenotypes heterogeneity and their incidence along the life-span. From our published[17–20, 37] and unpublished epidemiological data, two observations are important for our discussion: a) the preferential distribution of T1DM at young ages and of T2DM in old ages is only apparent. Moreover, at least in Romanian population the highest incidence of T1DM is encountered not below 20 years, but between 50 and 60 years; b) on the contrary, T2DM is more heterogeneous, with two particularities: in the first decade, its incidence is very low, while after 20 years the increase in incidence is marked by three increasing steps: one between 25–30 years; the second between 30–40 years and the third between 40–65 years (Fig. 1). In our view, these three steps of incidence increase correspond to the intervention of three additional pathogenic mechanisms on top of the initial proinsulin defect. These are: the "physiologic" *decrease in the regenerative capacity* of the beta cells; the slow *increase in beta cell apoptosis*; and the *amyloidogenic mechanism* which quantitatively represents the main pro-apoptotic mechanism operating after 50 years, corresponding to the upper third of the "incidence bell" of diabetes (Fig. 1). The arguments for this interpretation will be given later. In fact, all pathogenic factors operating more or less in the pancreatic islets and beta cells will lead to a decrease of the beta cell mass. The speed of this decrease will influence not only the age at onset but also the clinical character at the onset of the disease. The high clinical heterogeneity reflects well the complexity of the pathogenic mechanisms involved in the various phenotypes of diabetes.

As for the pathogenesis of T2DM, beta cell heterogeneity should be taken into account for both the beta cell secretory components, since in this phenotype appears, apart the obvious heterogenic proinsulin/insulin secretory dysfunction[151], a proamylin/amylin defect having also a heterogeneous distribution inside the pancreas (only some lobules involved), inside the islets (only some beta cells affected) and also inside the beta cell itself (only some secretory vesicles involved)[6, 131, 132, 147, 149]. It becomes evident that the primary beta cell defect in the processing of proinsulin and proamylin (molecules co-secreted and co-exocytised by the secretory vesicles) can be seen as evolving on two distinct but parallel pathways: the *proinsulin/insulin* defect can explain the increased levels of plasma proinsulin (associated automatically with decreased plasma insulin levels as well as with the mentioned defects of the insulin-secretory pattern); and the *proamylin/amylin* defect that could be secondary to

the first, but which pathogenetically could be placed in the front seat of diabetogenic mechanisms operating after the age of 50 years[131, 132, 133, 156].

ENDOPLASMIC RETICULUM: A REFINED BUT ALSO VULNERABLE STRUCTURE

By its anatomic position (the first post-nucleus organelle) and by its function (processing of translated protein molecules), ER appears to be not only an essential cell structure but also the most vulnerable regarding the secretory dysfunction of the beta cell. Its complex function in protein processing make it vulnerable to any type of defect, either genetical or acquired as the consequence of an increased insulin-secretion demand[79–81, 157, 158] or of some pathogenic biochemical stimuli, such as some unsaturated fatty acids[74, 78, 159–161]. Before presenting the more recent data regarding the function of ER and the mechanisms of which alteration can lead to diabetes, we shall present some models of diabetes in animals or humans that could have suggested ER as the possible site of the beta cell defect.

In the Akita mouse diabetes model[114, 162], the genetic defect in the processing of proinsulin (characteristic for this animal model) was identified in a single mutation leading to an amino-acid change in the proinsulin molecule (Cys96Tyr). This mutation affects one of the disulfide bonds inside the insulin molecule, preventing its proper processing and packaging. The first (and most important) beta cell morphological changes were noticed in the ER, suggesting for the first time that this cell structure, blocked by the agglutinated un-processed proinsulin molecules, can lead to a precocious and extended beta cell apoptosis[114]. Diabetes in the Akita mouse model is a severe form, characterized by a progressive and irreversible decrease of the beta cell mass. The secretory vesicles occasionally generated by the GA contain high amounts of proinsulin but no insulin. The level of plasma insulin is high.

Hyperproinsulinemia in obese/fat mice is also associated with a point mutation in the carboxy-peptidase E[163], a disorder rarely encountered in humans[83, 85, 164]. The fact that PC2 transgenic mice[165] have marked hyperproinsulinemia while hyper-expression of PC2/PC3 in MIN cells has the opposite effect[166] explains the interest for the study of the beta cell convertases as a possible explanation for the proinsulin processing defect.[83, 167–169].

Disorders in the processing of proinsulin of lesser magnitude were noticed also in other diabetes animal models, such as the sand rat *Psammomys obesus*[170], in which has been discovered a defect in the transcription factor PDX1 (IPF1) suggesting that the dysfunction could be related with the proinsulin processing inside ER.

These data focused the attention to the ER as a beta cell structure extremely sensible to the defects in protein processing. The high interest for the study of ER function registered during the last years[103, 104–1110, 171, 172] led us to believe that the most probable beta cell defect encountered in the common forms of human diabetes is a defect in the processing of pro-hormones (proinsulin and pro-amylin) inside the ER. Differently than the Akita mouse model, in human diabetes it seems that neither proinsulin nor the processing enzymes are structurally altered[84]. It is possible that the defect in the folding and packaging of proinsulin can be too discrete, so that the un-splitted precursor molecules can pass further into the GA and be included in the insulin secretory vesicles. Why the protein convertases (included also in the insulin secretory vesicles where they remain until the final exocytosis) cannot perform a complete cleavage of proinsulin and proamylin is not known yet. It is possible that the conformational changes of these two molecules, even if they are very discrete, can render more difficult the access of convertases to the splitting sites of the respective pro-hormones.

The role of the ER in the pathogenesis of diabetes, suggested by the findings in the Akita mouse model already mentioned above, could be sustained also by the alterations present in the human Wolfram syndrome, known also as the DIDMOAD syndrome (Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy and Deafness). Evidently, in this case we refer to a rare point genetic defect affecting the ER from several tissues[113]. Its rarity suggests that the protection of the ER against such genetic defects is high, indirectly highlighting its particular importance for any type of cell. Wolfram syndrome is a rare autosomal-recessive disorder, characterized by a juvenile onset insulin dependent diabetes and optic atrophy[113]. The gene involved is *WFS1* on chromosome 4[173, 174] which encodes an ER functional membrane protein[175, 176], expressed at high level in the brain, heart and pancreatic islets[173]. Association of this gene both with type 1[177] and

type 2[178] diabetes has been reported. This gene might be important in the regulation of intracellular calcium level[113, 176] and the function of ER. *WFS1* beta cell KO mice ($\beta Wfs^{-/-}$) is associated with beta cell ER stress, enhanced apoptosis, reduction of the beta cell mass and diabetes[113].

THE ENDOPLASMIC RETICULUM AS THE PRIMARY SITE OF THE BETA CELL DEFECT

In the last decades (and with a higher intensity during the last two years) the endoplasmic reticulum became the subject of intense studies[103-110] that can shed a new light on the pathogenesis of diabetes. Similar to the few hundreds of structural or functional protein molecules specific for the beta cell that are translated in the ribosomes attached to the walls of ER, the two secretory molecules (proinsulin and proamylin) arrive together with the rest in the labyrinth of ER membranes. The function of the last is one of the most complex: the processing (folding and packaging) of globular protein molecules and the control of their quality, with the aim of sorting the normal from the defect molecules. The defect molecules can be eliminated either by their proteolysis inside the ER or by their redirectioning towards the lysosomes or the constitutive (un-regulated) secretory pathway[84, 94, 179]. A second type of sorting refers to the selection of the protein molecules that will be included in the secretory vesicles from the various protein molecules (receptors, ion channels, cytoplasmic enzymes, etc.) that have other cell destinations than the secretory vesicles. We mention that all these processes take place with great speed since the number of proteic molecules that pass through the ER is in the range of several thousands per second[55, 94, 180] in the high demanding courtions.

Since the secretory vesicles represent the final "anatomic" product of the beta cell (they may reach ~50% of the molecules translated in the ribosomes and transferred to the ER), the correct fulfillment of this function has to be prioritary. This is confirmed by the high number of secretory vesicles present inside a beta cell (~13 000 in humans), vesicles that dominate the histological image in electronic microscopy. The high number of secretory vesicles implies an extremely high traffic of molecules inside the ER, especially during periods of unusual high insulin need. This traffic is regulated by numerous types of sensors (for pressure, pH, and spatial conformation) capable to "sense" any kind of modification occurring at this level. Since the main function of the beta cell is that of secretion, any defect in the processing of proinsulin and/or proamylin can trigger a prompt and efficient corrective reaction. This kind of reaction, known as *Unfolding Protein Response* (UPR), is an universal mechanism of response for various sorts of dysfunctions inside ER[103, 104, 106–110].

The main components of UPR mechanism are the following[108]: a) *Translational attenuation* in ribosomes (reduced synthesis of new proteins in order to prevent further accumulation of unfolded proteins). This attenuation includes not only the pre-proinsulin and pre-proamylin translation, but also the synthesis of other structural or functional proteins with various destinations in beta cells; b) Up-reglation of the genes encoding *ER chaperons* in order to increase the process of folding and packaging the various proteins, which in β cells are mainly proinsulin and proamylin molecules. Intervention of these chaperons will decrease the „crowding tension" inside ER[181]; c) The increase in *proteosomal degradation* of miss-folded proinsulin molecules or their sorting for the „constitutive" (non-regulated) pathway of release. (However, in type 2 diabetes, a part of these miss-folded proinsulin molecules will be transfered to the GA, where the secretory vesicles are generated. We mention that the access of the specific convertases – PC3 and PC2 – to the dibasic amino-acids, where the split of the proinsulin and proamylin molecules takes place, is highly dependent on a correct folding and packaging of these molecule. This is why the inclusion of these "marginally wrongly folded and packaged insulin molecules" in the secretory vesicles will lead finally the high plasma proinsulin concentrations encountered in the various phenotypes of diabetes); d) If the UPR fails to restore normal ER function, the process of apoptosis will be triggered[106–108, 182, 183]. Several proteins involved in the ER stress – induced apoptosis have been identified, including CHOP transcription factor, the bcl-2 family members (Bak/Bax), Caspase 12 and C-Jun Nh2 terminal kinase (JNK)[106, 107, 184]. This type of apoptosis may play also a role in the progressive decrease of the β cell mass encountered in type 2 diabetes, but in this case the apoptosis appear in the cells in which the proinsulin level reach a critical threshold. The deciphering of this pro-apoptotic mechanism offered an explanation for

the anatomo-pathological observations of Butler *et al.*[147], that in type 2 diabetes, according to the evolution stage of the disease, the beta cell mass presents important decreases which, in the moment of diagnosis, can overpass 50% of the initial value[147, 185, 186].

Three important studies published in 2007[139–141] investigated the genetics of some functional proteins from the ER including those involved in proinsulin processing. Thus, Chu *et al.*[141] studied the *ATF6* (Activating Factor 6) gene localized on chromosome 1q21-23, which serves as a key proximal sensor in ER. From 64 SNPs evaluated on a dataset of 191 cases/188 control Caucasian subjects, six SNPs showed nominal association with type 2 diabetes, but only one (rs1159627) remained significant on permutation testing. This is a field of high interest for the study of some candidate diabetes loci that encode proteins from the most important functional structure present in the beta cell.

The two other important papers[139, 140] refers to the TCF7L2 gene (10q25.2). It is known that a strong association between common variants of the *TCF7L2* gene and type 2 diabetes has been first reported by Grant *et al.*[187] at the beginning of 2006 and then, repeatedly confirmed by many authors in various populations[188–191]. *TCF7L2* is a nuclear receptor gene localized on chromosome 10q which encodes an entero-endocrine transcription factor that has a role in the WNT signaling pathway which is fundamental for growth and development. This pathway is also fundamental for embryogenesis, growth and cell proliferation and operates through several tens or hundred of genes. From the study of Loos *et al.*[139] carried out on a large number of normoglycemic individuals (1697 Europid men and women) results a *strong association* between four *TCF7L2* SNPs (including rs7903146) and *proinsulin level*. Thus the authors showed that T (minor) allele of this SNP was strongly and positively associated with fasting proinsulin and 32,33 split proinsulin relative to total insulin levels, but not to insulin-to-glucose ratio (IGR) at 30–min oral glucose tolerance test. The authors made the supposition that this beta cell defect could be related with both the major genes involved in proinsulin processing (PC3, PC2) because these contain TCF binding sites in their promoters. The same association of TCF7L2 with plasma proinsulin levels has been reported by Dalhgren *et al.*[140], for both polymorphisms (rs7903146 and rs12255372) the T allele was associated with increased plasma proinsulin. These findings strongly suggest that *TCF7L2* risk allele may predispose to type 2 diabetes by affecting beta cell proinsulin processing. Because the proinsulin processing defect has been noted long time ago in offspring of type 2 diabetic patients[70, 73, 192] this proves that the defect appears early in the natural history of type 2 diabetes. Unfortunately, the authors didn't study in parallel the proamylin levels whose splitting is mediated by the same enzymes, PC3 and PC2. Anyhow, the hypothesis that the *TCF7L2* gene is involved in the processing of proinsulin (and maybe proamylin) inside the beta cell is in line with our view that the main genetically determined secretory defect can be found somewhere between the ER/GA and the beta cell secretory vesicles.

In order to illustrate how different studies from different directions can complement each other over time, we should mention that in 1991 Dornhorst *et al.*[111] showed that gestational diabetes is associated with increased levels of plasma proinsulin. In the first half of 2007, Shaat *et al.*[193] reported that a common variant of the same *TCF7L2* gene is associated with an increased risk for gestational diabetes while in the second half of 2007 Loos *et al.*[139] establish the correlation between a SNP of this gene and the plasma proinsulin levels. In order to get a clearer picture of this correlation, we should identify the functional protein inside the ER or secretory vesicles that, expressed under the control of *TCF7L2*, determines the defect of proinsulin processing.

PROINSULIN IN THE COMMON PHENOTYPES OF DIABETES

Soon after the discovery of proinsulin by Steiner and Oyer[4], an increase of its concentration in the blood was repeatedly reported in different diabetes phenotypes[41–45, 47, 194–196]. After the discovery of amylin – the second beta cell secretory product[5, 6, 197] – Porte and Kahn[195] launched the hypothesis that proinsulin and amylin could be the two key molecules for the understanding of diabetes pathogenesis. However, until the end of the last century, the interest for proinsulin and pro-amylin remained at a low level. In the following years, the increase of plasma proinsulin continued to be reported from time to time[49, 54, 56, 57, 64, 66, 71].

The much lower interest for the increase in proinsulin levels in comparison with that for the

decrease in insulin secretion seemed to be justified for several reasons. The first was that the "antidiabetic" beta cell product was insulin, the effect of proinsulin on the peripheral glucose uptake being much lower: only 7% in the muscle cells and 12% in the liver[198, 199]; the second reason was that the laboratory methods for the assessment of intact proinsulin (and later of partially split des-31,32 or des-64,65 proinsulin) were very expensive and, consequently, rarely utilized[82, 200]; finally, the third reason was that no-one concentrated on the pathogenic significance of the increased plasma proinsulin as indicating the potential mechanism and location of the beta cell defect.

Based on data accumulated during the last 4 decades[40-87, 201–203] and our data[34–39] we can state that, irrespective of the type of diabetes, the primary and probably the main beta cell change could be identified in the increase of the *proinsulin levels* and of proinsulin-to-insulin ratio or proinsulin/C peptide (PI/C) ratio. Simplifying, the percentage of proinsulin inside the secretory vesicles (and subsequently in the peripheral circulation) increases, while the percentage of insulin decreases. It is possible that a similar change may occur for the proamylin-to-amylin ratio which refers to the second main secretory peptide of the beta cell[204–206], whose intervention in the diabetogenic process, for an unknown reason seems to be more prominent in the old ages. We don't have concrete data in order to exclude as the first beta cell secretory change the proamylin/ amylin secretory alteration, but this is less probable for two reasons: quantitatively the proinsulin/ insulin secretory line is 100 times higher; by the most complex structure of proinsulin, which requires multiple conformational changes, proinsulin is more susceptible to such alterations.

According to our hypothesis, the proinsulin secretory alteration, initially not accompanied by any clinical symptom, is common for both T2DM and T1DM phenotypes. Despite the great clinical and biochemical differences between these two phenotypes, their common root could have a logic and apparently simple explanation.

Proinsulin in type 1 diabetes

Increased plasma proinsulin levels in type 1 diabetes was reported as early as the 1970' and confirmed several times after that[35–40, 58, 86, 207, 208], without receiving a proper explanation until now. In some prospective studies, the random PI/C ratio has been proposed as a dynamic parameter who's increase overtime may indicate a progressive evolution from prediabetes to diabetes[48, 58, 60, 86, 88, 91]. The increase in proinsulin level in this phenotype could have the same significance with that of increased proinsulin in the early stages of type 2 diabetes: a defect in the ER processing of proinsulin, defect that is transmitted to the secretory vesicles in the form of an increased proinsulin percentage. In support to this interpretation come the extremely important data showing that proinsulin is increased also in the *siblings or offspring* of T1DM patients[48, 58, 60, 86, 88, 91].

Our hypothesis is supported by the capacity of proinsulin to function as a strong beta cell autoantigen[124–126]. This capacity can be amplified in the presence of increased proinsulin concentration inside the secretory vesicles of the beta cells that carry this dysfunction. It could solve the mystery of the trigger for beta cell autoimmunity which is still under debate. In our view, the great importance of intra beta cell proinsulin increase in T1DM is related to its increased antigenicity. This molecule can become more easily the target for an anti beta cell autoimmune reaction even in not yet diabetic descendants of T1DM subjects[48, 58, 86], in the condition of the coexistence of a supplementary defect in the immune system, an issue at which we shall come back to.

It is well known that some doubts regarding the solidity of the immunogenetic theory of diabetes started to appear when it was found out that the "diabetogenic" HLA types can explain only a part of the genetic predisposition for the autoimmune diabetes phenotype. In the years that followed, the number of genes (or chromosomal loci) thought to have a role in the pathogenesis of type 1 diabetes increased quickly[209, 210], but finally only a part of these were confirmed repeatedly in various ethnic groups: IDDM1 – Class II HLA alleles[119]; IDDM2 – the insulin gene VNTR[29]; IDDM12 – the CTLA4 (cytotoxic T lymphocite associated protein 4) gene [28]; the PTPN22 (lymphoid tyrosine phosphatase) gene[191, 211] and the IL2RA/CD25 gene[212].

The uncertainties regarding the relationship between the "diabetogenic genes" and the disease itself increased when it was found out that a high proportion of the subjects carrying genetic markers for T1DM did never develop the disease. For instance, in one of our own studies we showed that up to 50% of the non-diabetic first degree relatives of T1DM patients carry both HLA and INS

diabetogenic alleles[120, 121]. The situation was the same even for some of the subjects carrying both genetic and immune (plasma autoantibodies) markers of the disease. For instance, in the Finnish Type I Diabetes Prediction and Prevention Project (DIPP), from 84 children with high risk HLA genotypes + IA-2 antibodies, 68 (*i.e.* almost 81%) had remained non-diabetic for > 7 years. Similar data were reported in other studies[117, 119]. Instead, sometimes, subjects carrying no genetic and no autoimmune markers could evolve towards a clinical overt form of T1DM. Thus, between 30% and 70% of subjects from prospectively followed-up cohorts who become diabetic have low HLA genetic risk and antibody titers[117]. It became clear that the immunogenetic theory could not be applied to all the cases of type 1 diabetes. This led to the introduction in the classification of T1DM of the non-autoimmune (1b) type 1 diabetes subtype.

The same explanatory difficulty appears when we try to answer the question: why the diabetogenic anti beta cell autoimmune response is not triggered in subjects carrying all the currently known diabetogenic alleles. Between the genetic defect of the immune system and the autoimmune disease itself (attested by the presence of islet antibodies) there was a missing link. It was hypothesized as trigger of T1DM in genetically predisposed individuals the intervention of some *environmental factors*. From these, the most studied were the viral factor and the alimentary/chemical factor[122, 213, 214]. Thousands of studies were dedicated to the investigation of the environmental factors but with any conclusive result[123, 130, 213].

Our hypothesis of a **double genetic defect**, one of the **beta cells** and one of the **immune system**, started from two observations: *the first* was the presence of increased plasma proinsulin in the descendents of type 1 diabetic patients[48, 58, 86, 88, 91]. Truyen *et al.*[86] considered that the increased plasma proinsulin levels can be an additional marker for the prediction of type 1 diabetes. Moreover, increased plasma proinsulin was reported also both at the clinical onset of T1DM[35–39, 58], and later during the evolution of the disease[35–39]; *the second observation* referred to the contribution to the pathogenesis of T1DM of *IDDM2* (the pre-proinsulin gene), a gene that apparently does not belong to the classical autoimmunity genes as the other 4 genes confirmed to be associated with T1DM (class II HLA's, CTLA4, PTPN22 and CD25/IL2RA). Indeed, the insulin gene is not directly involved in the function of the cytotoxic lymphocytes (CTLs) considered to be the major contributor to β cell destruction[215].

It is currently known that the first auto-antibodies that appear in the circulation of the subjects that will later develop type 1 diabetes are the insulin auto-antibodies (IAA)[126, 216–218]. They precede with weeks or even months the appearance of the other types of anti-beta cell autoantibodies (GADA or IA2A). The main issue that arises is represented by the true nature of the so called insulin autoantibodies that could be in fact anti-proinsulin antibodies. It is known that insulin has some antigenic epitopes, identified as the amino-acid sequences 9–23 or 15–23 of the beta chain[125, 161, 219-222], fragments included also in the molecule of proinsulin. On the other hand, proinsulin itself has some specific epitopes, localized also on the beta chain between amino-acids 24–33 or 24–36[223].

Recently, Wagner *et al.*[224] demonstrated that post-translational protein modifications can potentially create new antigenic epitopes, which may trigger the autoimmune reaction induced by T lymphocytes hyper reactivity. The incomplete processing and packaging of proinsulin inside ER can be one of the post-translational protein changes that could explain the initiation of the anti beta cell autoimmune response. For instance, it was shown that a post-translational change in the conformation of the A chain of insulin is sufficient in order for it to expose a new epitope that is recognized by the T cells[225].

In some animal models (NOD mice, for instance) proinsulin/insulin is a key autoantigen for diabetes development and immune response against proinsulin epitopes has been found to be strongly correlated with diabetes[219, 223]. Anti proinsulin autoimmunity was more recently studied in relation with its decreased expression in the thymus, a finding that could explain its increased antigenicity[124–1266, 219]. It is known that *IDDM2* (chromosome 11p15) is represented by the VNTR region located in the promoter region of the pre-proinsulin gene, at ~ 600 bp 5' of its start site [29]. It has been showed that an allelic variation in the VNTR-*IDDM2* locus correlates with the level of insulin mRNA expression in thymus[124, 219]. A reduction of thymic insulin expression may lead to the suppression of the process of negative selection of insulin specific autoreactive T cells, or to the impaired selection of regulatory T cells, and thus facilitate the development of autoimmune T1DM in humans[226].

Ten years ago, Vafidis *et al.*[124] showed that proinsulin is the only type 1 diabetes autoantigen that is exclusively expressed by the β cells and the only one that maps to a confirmed susceptibility locus. In the NOD mice model of spontaneous autoimmune diabetes that resembles human T1DM, proinsulin/insulin is a key autoantigen for diabetes development and immune responses against proinsulin epitopes has been found to be strongly correlated with diabetes[219, 223, 226].

It is worthy of note that NOD mice knock-out both for the proinsulin 1 and proinsulin 2 genes do not develop autoimmune diabetes[125]. In a CD4-TCR transgenic mice, the target molecule is a natural autoantigen – the insulin B:9–23 peptide – and the modification of that specific target influences disease pathogenesis[125, 227].

An important argument favoring the hypothesis regarding the antigenicity of proinsulin as the main inductor of anti beta cell autoimmunity[228] is the fact that plasma proinsulin was found to be increased in descendents of T1DM parents that subsequently developed the same form of disease[48, 58, 86]. Moreover, the risk haplotypes of T1DM (DR3/4 class II HLA's) predispose type 2 diabetic relatives not only to an antibody positive status, but also to impaired insulin secretion, irrespectively of antibodies status[48].

Recently, Hermann *et al.*[229] showed that the DR4-DQ8 haplotype of *IDDM1* (HLA class II) is associated with the appearance of IAA (as well as the IA2 antibodies – IA2A), while GAD antibodies appear more frequently in subjects carrying the DR3-DQ2 haplotype. So, it can be concluded that proinsulin has a central role in the emergence of IAA, and that the subsequent appearance of multiple autoantibodies is linked to IAA. Hermann *et al.*[229] considered that, at least in children who develop IAA, proinsulin/insulin autoimmunity may represent the primary event and is controlled, at least in part, by the *IDDM2* locus. In a more recent study, the same group[230] showed that the T1DM new gene PTPN22 (Lymphoid tyrosine phosphatase; 1p13) is associated with the emergence of IAA, also accelerating the insulin specific autoimmunity. In this process, the interplay between polymorphisms in the insulin gene (VNTR-*IDDM2*) and PTPN22 gene (C1858T) seems to have an additional effect on the initiation of anti β cell autoimmunity[229, 230].

Why proinsulin becomes autoantigenic in some individuals is still a matter for debate. However, we are confident that an increase of the level of proinsulin inside the β cells increases the chances for this event to occur. The increased percentage of proinsulin in different compartments of the beta cell could leads to its increased exposure as an autoantigen, against which carriers of the defect in the immune system (hyper-reactivity of the T lymphocytes) will react by secreting IAA and generating clones of cytotoxic T cells reactive against the proinsulin epitopes. This could explain why the anti-insulin/proinsulin antibodies are the first to be detected during the long pre-hyperglycemic period of T1DM natural evolution. After a first autoimmune attack, the beta cell will become more and more vulnerable, exposing more and more other beta cell autoantigens (GAD, IA2) which explains the successive waves of autoimmunity[216–218] that lead finally to the quasi-total destruction of the beta cell mass.

If the increased levels of plasma proinsulin at the onset of type 1 diabetes can be quite easily explained[48, 58, 86], the increased proinsulin described in some patients with long standing T1DM was really surprising[35–39], moreover as the C peptide negative diabetic patients were considered to have a quasi total destruction of the pancreatic beta cells. A possible explanation for this finding could be found in the more recent data[147, 231] that evidenced (using immunohistochemical methods) the presence in the pancreas of long standing T1DM patients of some markers for beta cell regeneration and also positive markers for proinsulin/insulin. Since these subjects were however, C peptide negative, *i.e.* they produced no significant amounts of endogenous mature insulin, our interpretation suggests that the newly regenerated pancreatic beta cells can secrete proinsulin but no insulin

Proinsulin in type 2 diabetes

Even if the increase of plasma proinsulin was reported in cross-sectional studies performed especially in long term T2DM patients[34–36, 45, 55, 79-81, 195, 201, 232], subsequently it was shown that this phenomenon is as frequent in newly diagnosed T2DM subjects[35–39, 71, 79–81]. Moreover, increased levels of proinsulin were reported also in patients with IFG or IGT[35, 36, 75, 202], and showed to have a predictive value for the evolution of these subjects towards clinically overt diabetes[54, 65, 67, 69, 76, 87, 202, 203]. The predictive value of the PI/I ratio has been well documented in the women's Health Study – a cohort of low moderate risk normoglycaemic

American women. In a survey of 4 years, Pradham *et al.*[76] found that PI/I ratio, but also PI only level are powerful predictors of progression to type 2 diabetes. After risk factor adjustment, women in the higher PI/I quartile at baseline were 10 times more likely to develop type 2 diabetes vs. age-matched women in the lower quartile group.

Studies carried out in Pima Indians and Japanese Americans with T2DM showed a direct correlation between the fasting PI/IRI ratio and the degree of fasting hyperglycaemia, suggesting that the PI/I ratio reflects the degree of β cell dysfunction[54], while Røder *et al.*[60] observed negative linear relationship between PI/I ratio and AIR-max in patients with T2DM. They considered that PI/I ratio might be a relatively easy parameter to obtain, indicating the degree of reduced β cell capacity in T2DM patients.

Proinsulin in offspring of diabetic subjects

One of the strongest arguments in favor of the proinsulin beta cell defect as a primary cause of diabetes is represented by the identification of increased proinsulin levels in offspring of both type 1[48, 58, 86, 88, 91] and type 2[70, 73, 92] diabetic subjects. The proinsulin secretory defect appears before or concomitantly with other beta cell defects recorded during the pre-hyperglycemic stage of diabetes and manifested by the loss of the physiological insulin secretion oscillations[95, 101, 191, 233–235] or the loss of the first phase insulin response[92, 96-98, 102, 236]. Indeed, in a Finnish prospective study, Røder *et al.* found elevated proinsulin levels in siblings with low first phase insulin response preceding the onset of type 1 diabetes[58]. In T1DM, the appearance of alterations of the insulin secretory pattern seem to be concomitant with the appearance of the anti beta cell antibodies[86] and possibly posterior to the increase in plasma proinsulin. Unfortunately we don't have yet precise data regarding the exact chronology of these alterations preceding the onset of T1DM and T2DM. Anyway, in a study that followed both the plasma proinsulin levels and plasma antibodies, Truyen *et al.*[86] reported that increased proinsulin and proinsulin/C peptide ratio can offer complementary information (to those provided by the antibody titer) for the prediction of type 1 diabetes.

To conclude, the precocity of increased plasma proinsulin in both two major diabetes phenotypes represents a strong argument in favor of the hypothesis that the beta cell proinsulin defect could be the first diabetogenic event in the beta cell. This offers also decisive argument in favor of the *unitary character* of the two diabetes phenotypes.

Proinsulin in other phenotypes of diabetes

Most studies that investigated plasma proinsulin levels in diabetic patients included only the two major diabetes phenotypes: T1DM and T2DM. Even if some older studies signaled the presence of increased proinsulin in gestational diabetes[111] or cystic fibrosis diabetes[112], it is surprising that this issue was not systematically analyzed in the different diabetes phenotypes included in the last classification of diabetes. Also lacking are the studies regarding the distribution of plasma proinsulin levels in the general population. However, even with all these minuses, taking into account the numerous studies published until now, we can state that increased plasma proinsulin levels could be seen as a *common denominator for all the diabetes phenotypes.*

In this respect, it is interesting to note that increased plasma proinsulin levels in different phenotypes and different stages of diabetes were reported in various populations: European[35–39, 58–61, 67, 72, 79–81], Asian[65, 69, 70], Mexican-American[73, 202] or American[195, 201]. Thus, it could be stated that increased proinsulin was found practically in all categories of diabetic patients and for all populations and could be considered not only as a valuable marker for the beta cell dysfunction but also as an indicator of the site of the beta cell defect. Otherwise, the decrease of insulin secretion interpreted could be as a *consequence* of the decrease in the beta cell mass/function; however the increased beta cell proinsulin must be considered the *cause* for the decrease of the beta cell mass/function. From the pathogenic point of view, the increase in proinsulin in the beta cell could be as important as, or maybe even more important than the decrease of insulin secretion. This should not surprise us if we take into account that insulin results from the split of proinsulin and increased proinsulin expresses a defect of the main beta cell function, that to produce mature insulin.

Proinsulin, obesity, metabolic syndrome and cardiovascular disease

A recent paper of Kronborg *et al.* (Diabetologia 50:167-1614, 2007) offers the result of a prospective

study (mean follow up of 7 years) performed on a high number of subjects (3857 cases). This study showed that between the proinsulin-to-insulin ratio measured at baseline and the incidence of vascular disease (indicated by the carrotid plaque growth) there is a significant positive correlation. This important finding puts back into the spotlight the increased plasma proinsulin described in obesity[77, 237, 238], coronary heart disease[53, 92, 239–246], intima-media thickness of the carotid artery[203], stroke[241] or with various vascular risk factors[252]. All these have been associated to so called "insulin resistance syndrome"[8].

Although peripheral insulin resistance was evoked as the possible explanation for the beta cell "proinsulin" defect[8, 50, 64, 68, 79–81, 157, 247], the relationship between proinsulin and peripheral insulin sensitivity could not be proved[35–39, 248, 249]. In our view, the increased plasma proinsulin could be better explained by a supplementary beta cell load due to the supplementary body fat mass which will stimulate the production of a higher amount of insulin. This will increase the molecular flux through the ER and will potentially generate secretory vesicles with a higher content of proinsulin[157, 247, 250]. Because the early beta cell dysfunction is associated with a decreased insulin secretion (but not low enough to increase the BG values), the increased proinsulin will be associated rather with lipid alterations[251, 252], including weight excess[78, 237] and a proatherogenic lipid profile[92, 239, 242]. It is interesting to note that, in a clinical trial, the exogenous administration of proinsulin (as a potential source of slow release insulin) was early discontinued due to the excess of CV mortality[253]. This attempt confirmed the deleterious peripheral effect of high proinsulin levels. These might be mediated by the high-affinity proinsulin specific receptors identified on the endothelial cells[254].

However, the overload of the beta cell function in normal animals by continuous glucose infusion for three days did not change the plasma proinsulin levels, while the suppression of insulin secretion with somatostatin in hyperproinsulinemic patients reduced the plasma proinsulin level but did not normalize it[58]. In addition, the non-diabetic subjects, who are hyperinsulinemic, have a normal PI/I ratio[49, 255]. In fact, the whole concept of peripheral insulin resistance based on the evaluation of plasma insulin levels, during a period when the RIA method included in the provided results both insulin and proinsulin, should be carefully re-analyzed. Maybe we shall find out that, maybe not all, but many of the so called peripheral insulin resistant states defined on the basis of sophisticated but un-physiological investigations and complicated mathematical calculations[9, 256] could be in fact rather theoretical constructs than objective clinical realities. The recent re-activation by Wilkin[10] of the *accelerator hypothesis* operating during the diabetogenic process in different diabetes phenotypes[257] is convincing when it sustains the *unitary character* of diabetes, but absolutely non-convincing when it considers *insulin-resistance* as one of the most universal and diabetogenic accelerators.

Pancreatic amyloid and type 2 diabetes

At the beginning of the last century, Opie[138] described the presence inside the Langerhans islets of deceased diabetic patients of a hyaline substance, identified much later as amyloid[6, 131, 258]. The presence of amyloid in the pancreas of Type 2 diabetic patients, easy to evidence using the Congo red staining, was interpreted as an association or even a consequence of diabetes than a possible causal relationship[259]. Indeed, the presence of islet amyloid in non-diabetic patients suffering from various chronic diseases has been a strong counter-argument to its specific role in the pathogenesis of diabetes. Another counter-argument was the absence of detectable islet amyloid in 5–10% of patients with T2DM[133, 259–261].

The interest for the pancreatic amyloid was revived by the studies of Westermark[131] studies that peaked in 1986 with the identification of a new peptide from the family of calcitonin gene related peptides (CGRP), considered to be an amyloid fibrilary protein. Next year, Westermark *et al.*[6] and Cooper *et al.*[197] cloned this peptide that proved to be identical with that of the amyloid deposits. This peptide was named amylin, but the term Islet Amyloid Poly Peptide (IAPP) is also used. The amylin gene (chromosome 12) encodes a longer peptide known as pre-proamylin. From the 89 amino-acids of pre-proamylin, 22 amino-acids represent the signal sequence that is cleaved initially. The remaining peptide of 67 amino-acids known as proamylin is converted (as in the case of proinsulin) to mature amylin by cleavage at two basic amino-acid residues (Lys-Arg) flanking the amylin sequence, generating amylin and another two small peptides. One of these is a short polypeptide of 11–residues N terminal, while the second is a 16-residue C terminal. It is interesting

to know that proamylin is enzymatically processed by the same converting enzymes (CP3, CP2 and carboxypeptidase E) involved in proinsulin splitting[262]. Formation of a disulphide bridge between cysteine residues 2 and 7 of amylin and amidation of the terminal tyrosine are required for the full biological activity of mature amylin[133]. It is believed that in normal people, amylin play a paracrine role to attenuate insulin secretion[172, 263, 264] and to inhibit centrally the food intake.

The discovery of this second beta cell secretory line (pre-proamylin/proamylin/amylin) did not raised the deserved interest since the physiological effects of amylin were considered to be non-significant. Its only better studied physiological effect was that of appetite inhibition, which led to the therapeutically synthesis of the amylin analogue known as Pramlintide. Due to its nature (this product can be administered only parenterally but cannot be mixed in the same syringe with insulin), its utilization was quite limited and its therapeutical effects less known.

Two decades passed in order for the amylin significance to be seriously taken into account. It was suggested maybe by the parallel pathways of the amylin and insulin secretory lines. However, there are major differences between the 2 peptides: insulin is a bigger molecule (51 amino-acids) and has a complicated globular spatial structure; amylin is a smaller molecule (37 amino-acids) and has a linear spatial structure, being capable to polymerize in the form of fibrils, resembling those encountered in the amyloid deposits. Insulin has specific peripheral receptors that mediate its numerous functions while amylin has few and doubtful peripheral functions[6], supposedly to be mediated by 3 variants of receptors, considered to be part of the calcitonin receptors[264]. Finally, the concentration of amylin inside the secretory vesicles and its concentration in the peripheral circulation are in a ratio of 1:100 with that of insulin. This high difference in the concentration of insulin and amylin would suggest a role for amylin in the stabilization of insulin inside the secretory vesicles, a role similar to that of the C peptide, present also inside the secretory vesicles. The last is also a small peptide (31 amino-acids), has a linear structure, don't possess peripheral receptors and has doubtful peripheral physiological actions[85].

Despite these differences, insulin and amylin have also some resemblances and even common characteristics: both molecules are synthesized in the form of pre-pro-hormones (pre-proinsulin with 110 amino-acids and, respectively, pre-proamylin with 87 amino-acids). Both pro-hormones are transferred into the ER after removal of the signal peptide (23 amino-acids for pre-proinsulin and 22 amino-acids for pre-proamylin). Inside the ER and later in the secretory vesicles emerging from the GA, proinsulin and proamylin will be split by the same convertases: PC3, PC2 and carboxipeptidase E, which for both molecules split the chain between two basic amino-acid residues: Arg-Arg/Arg-Lys for proinsulin, Arg-Lys/Arg-Lys for proamylin. Normally only a small part (~1%) of proinsulin (and perhaps proamylin also) remain un-split and are exocytised as so into the peripheral circulation. Nothing is known regarding the intra-vesicular disequilibria between the pro-hormones (proinsulin and proamylin) competing for the same convertases as well as between the final secretory molecules (insulin and amylin). However, the fact that the both proinsulin and proamylin are produced inside β cell under a common regulatory promoter sequences[259, 265], the excess of proinsulin and proamylin inside the secretory vesicles may act as endogenous diabetogenic factors. Moreover, has been proposed[266] that β cell dysfunction may result for the alteration in ubiquitin-proteasome machinery which is a multimeric enzymatic complex involved in the disposal of defective proteins. A such alteration can explain the accumulation inside β cells of abnormal molecules such as proamylin or toxic oligomers that mediate the amyloid formation[266, 267]. Anyway, the amyloidogenic transformation of amylin must be related in a way with high proinsulin levels inside the β cells.

Among the similarities between these two sets of molecules (proinsulin/insulin, proamylin/amylin) we should mention also the particular characteristic of insulin from the Dego rat, a relative of Chilean hamster, to generate fibrils and produce a fibrilar hyaline substance around the beta cells, with the consequent appearance of a severe form of diabetes[268]. It would be interesting to analyze the structural particularities of the insulin molecule secreted by the pancreas of this animal model which could explain why it maintains a linear structure and later polymerizes. Contains the insulin molecule of this animal the sequence of 4 amino-acids characteristic for the amyloidogenic process in human, monkey and cat?

In the same year, 1986, when Westermark described amylin as identical to a linear peptide found also in amyloid fibrils, Howard[269] correlated islet morphology with the clinical and metabolic status of *Makka nigra* monkeys, along their

evolution from non-diabetes to diabetes. The authors observed that the appearance of islet amyloid coincided with (or immediately preceded) the onset of hyperglycemia and concluded that between islet β cell amyloid and metabolic progression to diabetes must be a causal relationship. The same relationship has been observed also in cats[270]. When the amino-acid structure of amylin was elucidated, it was observed that the[25–29] hydrophilic amino acids sequence (Ala-Ile-Leu-Ser-Ser) is common in humans, monkeys and cats, all of each develop both islet amyloid and diabetes. It has been considered that this amino-acid sequence is necessary for the formation of amyloid fibrils[6, 250, 270, 271]. The sequence of amylin in mice and rats is of a non-amyloidogenic sequence. Neither of these species develops type 2 diabetes without genetic manipulation[272]. On the contrary, in human-amylin transgenic mice, toxic amylin oligomers were detected intracellularly in 20–40% of human-amylin transgenic beta cells[172]. These data confirm Westermark's suggestions[131, 258] that the first amyloidic molecules leading to amyloid are intracellular and distinct from the extracellular amyloid deposits when present[133, 172]. It seems that the extracellular amyloid deposits are formed rather by the pathologically increased proamylin than by amylin itself.

Even if the pathogenetic relationship between amylin and type 2 diabetes was often suggested to be important[55, 156, 259–261], the difficulty in accepting this theory derived from the fact that the amyloid deposits were always evidenced extra-cellular and thus the mechanism for their generation was considered to be exterior to the beta-cell. On the other hand, the reported prevalence of islet amyloid in necroptic studies varied a lot, between 45–95%[149, 151, 258, 273]. Westermark[6, 133, 274] but also Borromers *et al*[149] pointed out some methodological factors that could lead to the underestimation of the amyloid deposits presence. Since the pancreatic amyloidosis is often not generalized, the analysis of the islets from a single pancreatic fragment does not reflect the anatomic status of the whole gland. A such subestimation is made more often in the early stage of diabetes when the presence of amyloid can be restricted to only one or two pancreatic lobules, so that the small islets deposits of amyloid can be easily passed over. Thus, an exclusion diagnosis for pancreatic amyloid cannot be made using sections from a single pancreatic lobe. Unfortunately, the histological studies performed on necroptic samples are quite few, most of them performed in the last century on limited series, usually including lower than 50 cases. Only a few studies included more than 100 cases and the data regarding the clinical characterization as type of diabetes or the disease duration were not available[149].

An important observation is that pancreatic amyloid is strictly localized to the endocrine pancreatic tissue and is not seen in the exocrine tissue. The deposits of amyloid situated either in the islet core or in the periphery can occupy up to 80% of the islet space. In the cases with slight decrease of islet volume, the deposits are found between islet cells and capillaries[6, 135, 149, 156, 261].

Although as early as 1973 Westermark[131] and subsequently Clark *et al.*[271] suggested a possible intracellular origin for the islet amyloid (hypothesis suggested by the presence of amylin inside the beta cells), the extensive presence of pancreatic amyloid in the extra-cellular space led to the idea that the formation of amyloid is typically extracellular. The amyloid deposits often created a gap between the beta cells, evidently affecting the communication between them. Even if the smaller amyloid deposits did not generate evident alterations of the islets, however a more careful analysis indicated the preferential decrease of the beta cell volume in comparison with the volume of the other islet cell types. The deposits of amyloid around the beta cells maintained constant the islet volume, generating the misleading idea that the islets are only a little affected[273]. This explain why, until recently, some authors[151, 273] continued to doubt the pathogenic role of amyloid in type 2 diabetes, arguing that important amyloid deposits are encountered quite often in older non-diabetic subjects. However, in most recent studies, intra-islet amyloid was evidenced in a high percentage of type 2 diabetic patients, reaching up to 95% of the cases analyzed post-mortem[133, 147, 149, 258, 259]. In addition, immuno-histo-chemical studies can evidence the presence of amylin inside the beta cells, amylin representing a normal component of the insulin-secretion vesicles. Also using immuno-histochemical methods, amylin can be evidenced inside the lysosomes, both in diabetic patients and normal subjects. It seems that lysosomes can take over some of the amylin translated in excess of proinsulin, maintaining a constant ratio of 1–2/100 between these two secretory molecules[133, 172, 271, 275, 276].

The uncertainty regarding the intracellular amyloid deposits derive from the impossibility to

evidence these deposits on necroptic material. However, as Westermark underlined[6, 131, 133], the development of the islet lesion in human diabetes is probably a very long lasting process and intracellular amylin may occur often at an early stage. In addition, the extracellular formation of an amylin fibrile is a nucleation-dependent process and preformed fibrils may catalyze the transformation of a soluble protein in a fibrillar form[133]. Because human amylin is a highly amyloidogenic peptide, the release of small insoluble intracellular aggregates into the extracellular space may induce the conversion of some small oligomers into amyloidic fibriles. These small oligomers are difficult to be detected ultrastructurally, needing an atomic force microscope approach[277]. However, these types of oligomers may have a cytotoxic effect inducing membrane instability and finally apoptosis[135, 172].

At present, it is obvious that islets with amyloid deposits have a small β-cell mass with evident cellular distortion and destruction[6, 131, 133, 147, 156, 259, 271]. The crucial question is which alteration comes first? Is it the generation of amyloid deposits an early event or does it occurs only after the hyperglycemic decompensation of diabetes? The answer seems to be unequivocal: amyloid deposits appear always before the hyperglycemic decompensation. Recently it was shown that the origin of extracellular amyloid can be found in the amyloid "protofibriles" represented by some very thin molecules that subsequently assemble (by a zipper type connections) to make up the mature and easy visible fibriles[275]. These protofibriles, which may be the most significant in causing beta cell injury, usually escape detection with regular electron microscopic studies and may well be present in islet apparently free of amyloid[133]. In a careful study made by Hé *et al.*[276] on 7 type 2 diabetic subjects and 8 non-diabetic controls, the authors showed that in normal pancreas no amylin oligomer deposition was found. On the contrary, in the islets with a reduced number of β cells, oligomer deposits were present. Oligomers were deposited in a scattered manner, and accompanied by a discrete fibrillar amyloid plaque. In islets with the total absence of β cells, oligomers were intermixed with amylin fibrillar amyloid. In islet cells, the oligomerization of amylin was associated with β cell apoptosis, induced by mitochondrial depletion and compromised oxidative phosphorilation. These data clearly showed that intracelullar oligomerization of amylin precede the development of diabetes and the formation of extracellular fibrillar amyloid[172, 276].

It is of interest that transgenic mice expressing human amylin do not always develop islet amyloid[172]. This suggests that the amyloidic transformation of amylin need the intervention of some additional factors. In our view, one of these factors (or maybe the main additional factor) might be the defect in the processing of proinsulin inside the secretory vesicles, which interfere with amylin properties. It is interesting to know that an increase in plasma free fatty acids could alter the processing, storage and release of both proinsulin[159] and amylin[278]. It has been assumed that a greater demand for insulin as a result of peripheral insulin resistance will force the β cells to produce proinsulin (and implicitly proamylin) at a faster rate then the one at which the converting enzymes can process these pro-hormones[180, 278]. However, this could be less probable then the possibility that some other conditions, such as the increased NEFAs, to alter the processing of the pro-hormones[159].

From all the data presented above, we can conclude that amyloidosis is a complex process that can have a different cause in diabetic patients in comparison with non diabetic subjects. In diabetic patients, the amyloid dysfunction (which we believe is closely linked with increased proinsulin) can have a pathogenic effect by acting on two pathways: a) the first is that of intracellular toxic amylin oligomer formation which induce beta cell apoptosis by an intrinsic mechanism; b) the second pathway could be represented by the extracellular transfer of proamylin/amylin after exocytosis of the secretory vesicles, followed by its transformation in amyloid deposits. These peri-β cell deposits will break off the physiological inter-beta cell connections, diminishing the secretory function of these cells. The direct effect of the amyloid fibrils on the integrity of the cell membrane can trigger beta cell apoptosis by an extrinsic (extra beta cell) mechanism[6, 110, 133, 259, 261].

In our view, both the proinsulin/insulin and proamylin/amylin dysfunction are *endogenous diabetogenic factors.* The origin of these dysfunctions can be located in the ER. Many secrets of the diabetogenic mechanism seem to be closely related to the conformational changes of proinsulin/proamylin and an initial defect in one of the molecules may induce an alteration in the function of the other one.

BETA CELL APOPTOSIS/REGENERATION

The main final diabetogenic mechanism operating in every diabetes phenotype is represented by the **decrease of the beta cell mass**[147, 149, 279–283]. Passing over the contradictory results regarding the beta cell mass published in the past, especially in type 2 diabetes, we shall stop only to the more recent studies[6, 133, 147, 149, 280], which, based on a more rigorous morphometric analysis, evaluated the relative beta cell volume reported to the acinnary cells/islets volume using modern immunohistochemical methods. Based on the results of these studies we can conclude that the decrease of the beta cell mass is a common denominator of all diabetes phenotypes[147, 279]. The onset of the beta cell mass decline takes place many years before the glycemic decompensation[6, 147, 259, 281, 284].

When we refer to beta cell mass, we have to take into account the balance between the two antagonist processes that determine the lifespan of a beta cell: beta cell *apoptosis* and beta cell *regeneration*. Then, when we take into account ~3 billion pancreatic beta cells distributed in ~1 million islets, we accept that the assessment of beta cell apoptosis/regeneration in a specific case is extremely relative due to the impossibility of a direct approach to the pancreatic islets. We should add that, although we have numerous data regarding the apoptotic mechanisms for the isolated beta cells, for the apoptotic mechanisms operating for the *in vivo/in situ/human beta cells* the data are more relative. Even more relative are the data regarding the mechanisms of beta cell regeneration. Finally, we don't know yet even the answer to an apparently simple question: for how long lives a beta cell in a normal subject and for how long in a diabetic patient? And in diabetic subjects, for how long lives a beta cell in the pre-hyperglycemic period and for how long in the hyperglycemic period?

The apoptotic process is supposed to be more active in the dysfunctional cells, especially if some environmental factors (increased fatty acids intake for instance) create the "pro-apoptotic" conditions well documented in some studies performed on isolated islets or isolated beta cells[109, 159, 281, 285-287]. Since the beta cell mass disposes of a complex system of regulation[146, 288–290], an increased apoptosis can be compensated by an increased process of beta cell regeneration. In young people (before the age of 30), beta cell regeneration seems to be active and efficient in conditions of normoglycemia. In a recent study performed on isolated human islets, Maedler *et al.*[281] reported that the capacity of beta cell regeneration can be altered very early, especially in conditions of hyperglycemia, and with a magnitude higher than the increase of beta cell apoptosis. This means that in young ages what counts more in the end in determining the fate of the beta cell mass is the incapacity of initiating the mechanism for beta cell regeneration, *i.e.* replacing an apoptosed beta cell with a new, eventually normal, beta cell.

The evolution of the beta cell mass changes dramatically in the presence of some pro-apoptotic beta cell defects. In our view, these defects involve either the quantity or the quality of the two beta cell secretory proteins: proinsulin/insulin and pro-amylin/amylin. Supporting this point of view, we have multiple arguments provided by recent researches. The pro-apoptotic beta cell mechanism can be initiated from two beta cell structures: the ER and the secretory vesicles. The pro-apoptotic signal released from the ER appears when the misfolded proinsulin molecules block the secretory traffic towards the Golgi apparatus[114]. In this situation, the total alteration of the beta cell function will trigger the apoptotic process by enhancing the transcription of the DNA-damage Inducible Transcript 3 (known as CHOP) and activation of junk1 (c-jun NH_2-terminal kinase) and caspase12[104, 106–108, 162]. The other signal, released from the secretory vesicles, is related to the cytotoxic effect of small amylin oligomers formed inside the secretory vesicles[135, 291] that have the ability to induce membrane instability, calcium accumulation inside the beta cell and apoptosis[110, 133, 136, 137, 172]. Evidently, when apoptosis is markedly increased by the two mentioned mechanisms and beta cell regeneration decreased, a diabetogenic mechanism with slow evolution, silent for years or decades, can become suddenly so strong that, even in older ages (after 60 years for instance), the clinical onset of the disease resembles closely that recorded classically in type 1 diabetes: polyuria, polydipsia, weight loss, marked metabolic decompensation. This clinical form represents however an exception for the classical phenotype of type 2 diabetes. Most cases (including those with an apparently explosive onset) follow a long diabetogenic process that can start at the age of 20 and become clinically overt in the form of glycemic decompensation only at the age of 60 years.

A supplementary diabetogenic mechanism operating sometimes in type 2 diabetes can be of inflammatory nature, but not mediated by the T cells as in T1DM. It can be represented by the cytokine proinflammatory reaction induced by some previous diabetogenic factors, especially obesity or fatty liver disease associated with weight excess[128, 228, 292–295]. This reaction will affect altogether beta cell apoptosis (stimulation) and beta cell regeneration (inhibition). Since this forth diabetogenic mechanism is chronologically posterior to the other three, the decrease of the beta cell mass in this case will be more rapid and diabetes will take sometimes a severe and progressive curse, resembling a form of type 1 diabetes in older ages[293, 297], but in the absence of classical plasma anti beta cell antibodies[298].

The slow evolution of T2DM can have a logic explanation: the initial proinsulin defect represents in fact the expansion or amplification of a "physiological process" as can be considered the incomplete conversion of proinsulin into insulin and C peptide or of proamylin into amylin. It is known that even in the normal, mature, secretory vesicles, ~1% of proinsulin remains un-split, a fact reflected by the plasma proinsulin levels of 5–10 pmol/l recorded in non-diabetic subjects[35–39]. Exceeding this threshold or even a doubling of plasma proinsulin can be well tolerated since this protein is a normal beta cell molecule. The dysfunctional character of the beta cell becomes evident only when the proinsulin-to-insulin ratio increases very much.

The moment for the evolution of beta cell proinsulin dysfunction from a state of relative equilibrium in a state of disequilibrium is reflected by the progressive decrease of the beta cell mass and of the beta cell secretory capacity[34, 147, 284], which, in order to reach the threshold of glycemic decompensation, has to involve more than 50% of the beta cell mass/function[147]. In our view, the slow (and clinically silent) beta cell loss during this *pre-hyperglicaemic diabetes stage* might be due to the decrease of the regenerative processes induced by the high proinsulin levels inside the beta cells. If the rate of apoptosis increases, following the intervention of some exogenous mechanism (overweight, excess in animal fat intake, etc.) or by activation of a supplementary endogenous mechanism (proamylin dysfunction), the loss of beta cells can increase dramatically. The stepwise increase in the incidence of T2DM[17, 32] after the age of 30 years reflects the intervention of an additional pathogenic factor on top of the proinsulin defect. This supplementary mechanism may be between the age of 20–30 years the decrease of the beta cell regenerative capacity, between 30–40 years the acceleration of apoptosis (in the presence of reduced regenerative processes) and between 50–60 years the intervention of the amyloidogenic mechanism.

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