

THE ROLE OF THE PANCREATIC AMYLOID IN THE PATHOGENESIS OF TYPE 2 DIABETES

CONSTANTIN IONESCU-TIRGOVISTE^a, CRISTIAN GUJA^a, V. CRISTESCU^a and D. GUTU^a

^a National Institute of Diabetes, Nutrition and Metabolic Diseases, “N.C. Paulescu”

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Aretaeus of Cappadocia in the second century A.D. made a short and vivid characterization of diabetes as a “mysterious disease”. With its multiple phenotypes and the main “thin diabetes” and the “fat diabetes”, Lancereaux indicated to us where the secret of type 2 diabetes must be found, namely in the adipose tissue. The pathogenic discordance between beta-cell mass (which progressively decreases) and adipocyte mass (which increases and thereafter remains increased) will be the subject of another paper. Here we attempt to clarify the significance of pancreatic amyloid described by Lancereaux and Paulesco in 1912 by renaming the pancreatic hyalinosis mentioned in 1901 by the American Opie and Viennese Weichselbaum.

The interest in amyloid and diabetes was started by Westermark, who in 1986 discovered a second secretory line inside the beta-cells: pre-proamylin/proamylin/ amylin. However, despite the large amount of research on this topic, the amyloidogenic mechanism of amylin remains largely unknown. The importance of endoplasmic reticulum in post-translational molecular processing of proinsulin and proamylin could shed a light on the pathogenesis of type 2 diabetes. In our view, of the many other pathogenic factors (adipocytes, with their secretory function is one of them), an amyloidogenic mechanism could be related to the overload of pancreatic beta-cells, which, by trying to secrete more insulin, due to the processing defect, finally leads to the production of more proinsulin. This can have an adverse effect on beta-cell survival and by disequilibrium with amylin, can lead to the aggregation of monomers of amylin. Due to their toxic effects the insoluble amyloid aggregates could contribute to the progressive loss of the beta-cell mass, especially in the elderly.

Key words: Pancreatic amyloid; Proapoptotic mechanism; β -Cell apoptose

INTRODUCTION

The pancreatic amyloid deposits were mentioned as early as 1901 by the American Opie¹ and the Austrian Weichelspaun² and designated as hyaline or hyalinosis. In the paper of Opie is given the color drawing of a pancreatic islet made by the author from the optic microscope image. For its beauty and accuracy this drawing was reproduced several times in the last years in numerous papers. In a review paper from 2008³ is mentioned that Erlich *et al.* discovered in 1961⁴ that the material previously known as hyaline is in fact amyloid, a term used to describe the extracellular protein deposits that resemble amylopectin (amidona-mylopectin; “amyloid-like amylopectin”). However,

in volume III of the “*Traité de Médecine Lancereaux Paulesco*” published in 1912⁵, the authors included a whole chapter devoted to “*Pancreatic amyloidosis*” in which they describe the peri-vascular deposits of amyloid that do not alter the acinary tissue. Thus, the term of amyloidosis dates perhaps from the beginning of the last century but we don’t know if Lancereaux and Paulesco were the first to use it or if it had been used by other authors before them.

One of us⁶ presented in 1971 the kidney amyloid as one of the possible causes of renal cause hypertension. I mentioned that in these cases the kidneys are “heavy” – 200–400 g – because of the high density of the protein material.

The pancreatic islet amyloid is always abnormal. It is currently known that amyloid is an insoluble substance made of protein monomers assembled in a beta sheet, a structure evidenced by "spin labeling analysis"⁷ and atomic force microscopy⁸. Under electronic microscopy, amyloid aggregates appear as non-branched fibrils that can be evidenced in optical microscopy by staining with Congo red or Thioflavones³.

The presence of amyloid in the pancreas of type 2 diabetic patients was interpreted as an association or even a consequence of diabetes than a possible causal relationship⁹. 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^{10, 9, 11, 12}.

AMYLOID AS A CONFORMATIONAL PATHOLOGY

Conformational diseases occur when an endogenous protein undergoes a fibrillar change leading to a self-association (aggregation) and tissue deposition. Such fibrillar molecular aggregates appeared in several diseases such as Alzheimer, Parkinson, prion encephalopathy, amyotrophic lateral sclerosis and Huntington disease^{9,13}. These diseases has been included in the same category supposing that the amyloidic deposits has an toxic effect on the tissue where they have been found. If a such toxic effect exist this seams to be not induced by the extra cellular amyloid deposits, but by the small non fibrillar oligomers. In the beta cell they can be seen in the cytoplasmic compartment and even inside the secretory vesicles^{14,15}.

Alzheimer disease is characterized by the plaques of beta-amyloid (A β) formed by the amyloid precursor protein (APP) and neurofibrillar tangles made up by hyper-phosphoryllated tau protein¹⁶. Interestingly enough mRNA of APP and TAU is increased in the adipose tissue of obese patients, with 22% and 34% respectively, versus lean subjects. So, a relationships between obesity, type 2 diabetes and Alzheimer disease its made by the increase of several commune genes.

THE DISCOVERY OF THE AMYLIN AS A SECRETION OF β CELLS

The interest for the pancreatic amyloid was revived by the studies of Westermark¹⁷ that peaked in 1986 with the identification in the pancreatic amyloid deposits of a new peptide from the family of calcitonin gene related peptides (CGRP), considered to be an amyloid fibrillary protein. Next year¹⁸, Westermark *et al.* and shortly after him Cooper¹⁹ 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 (Fig. 1) 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 (Fig. 2).

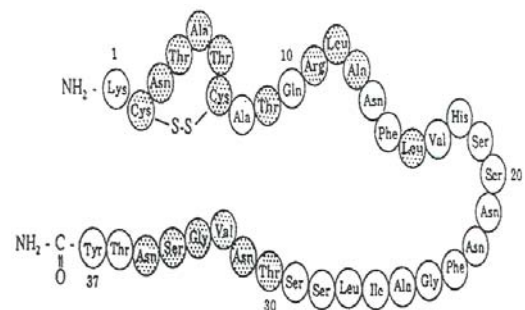


Fig. 1. The aminoacid structure of amylin (the 25-28 hydrophilic sequence: Ala-Ile-Leu-Ser is the amyloigenic sequence).

It is interesting to know that proamylin is enzymatically processed by the same converting enzymes (CP3, CP2 and carboxypeptidase E) involved in proinsulin splitting²⁰. Formation of a disulphide bridge between cysteine residues 2 and 7 of amylin is required for the full biological activity of mature amylin¹². It is believed that in normal people, amylin play a paracrine role to attenuate insulin secretion^{21,22,23} 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-

significant³. It's 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 these two peptides: insulin is a bigger molecule (51 amino-acids) and has a complex 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^{18,24} supposedly to be mediated by 3 variants of receptors, considered to be part of the calcitonin receptors²³. Finally, the concentration of amylin inside the secretory vesicle 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 vesicles²⁵. 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^{25,26}.

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 endoplasmic reticulum (ER) and later in the secretory vesicles (SV) emerging from the Golgi apparatus (GA), proinsulin and pro-amylin will be split by the same convertases: PC3, PC2 and carboxypeptidase 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 (Fig. 2).

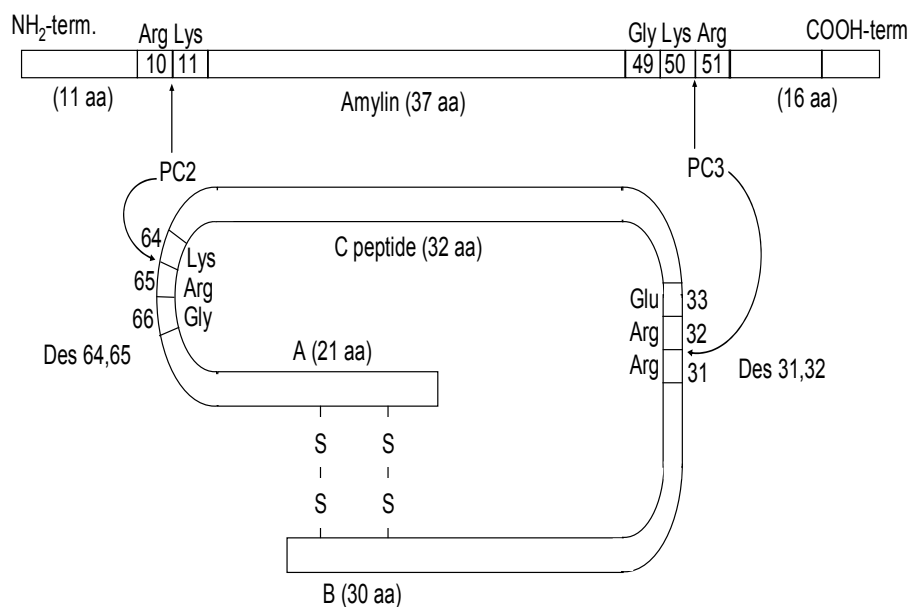


Fig. 2. The splitting of proinsulin and proamylin is mediated by the same proconvertases: PC2, PC3 and carboxypeptidase E. Initially, PC3 splits the link Glu33-Arg32 and then Arg 32-Arg 31. The amino acids Arg 32 and Arg 31 are splitted by carboxypeptidase's E (CPE). In a second step, PC2 split the link between Arg 65 and Gly 66, followed by the intervention of CPE, releasing Arg and Lys from the position 64 and 65. Finally, results the insulin molecule (51 aa), with the chain A (31 aa) and chain B (30 aa), and C-peptide (32 aa) and 4 basic amino acids (3 Arg and 3 Lys). In parallel, PC3 act on proamylin, splitting the link Lys50-Arg 51 from C terminal end, while in a second step, PC3 split the link Arg10-Lys11 from the N-terminal end. The remaining basic amino acids (Gly 49-Lys 50-Arg 51) are splitted by the intervention of CPE.

Normally only a small part (~1%) of proinsulin (and perhaps proamylin also) remain un-split and are exocytosed 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^{9,27}, the excess of proinsulin and proamylin inside the secretory vesicles may act as endogenous diabetogenic factors. Moreover, has been proposed²⁸ 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^{28,29,30}. Anyway, the amyloidogenic transformation of amylin must be related in a way with high proinsulin levels inside the β cells.

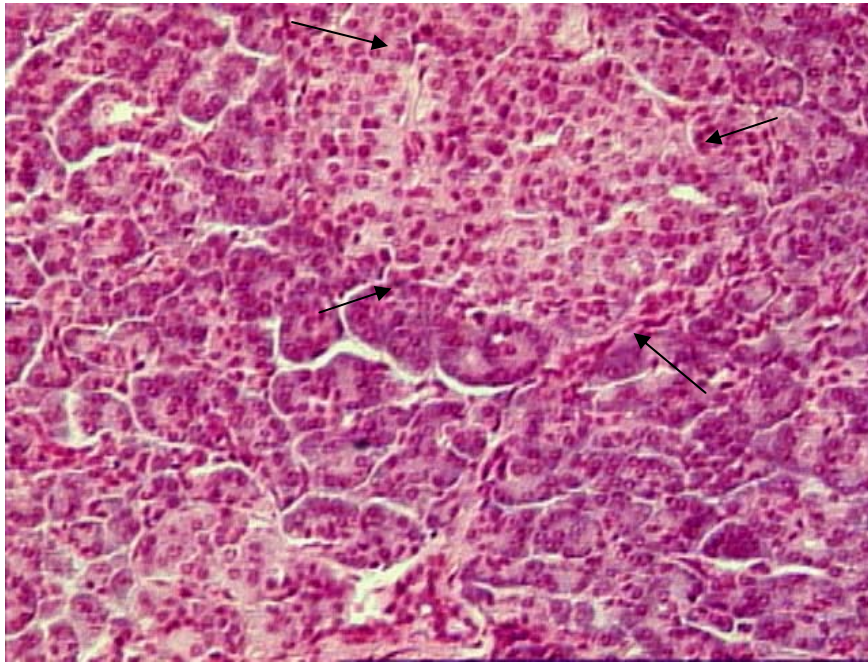
Among the similarities between these two sets of molecules (proinsulin/insulin, proamylin/amylin) we should mention also the particular characteristic of insulin from the DeGo mouse, a relative of Chilean hamster, to generate fibrils and produce a fibrillar hyaline substance around the beta cells, with the consequent appearance of a severe form of diabetes³¹. 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? Indeed, already in 1982 Palotay and Howard³² found typical amyloid deposits in non human primates.

In the same year, 1986, when Westermark³³ described amylin as identical to a linear peptide found also in amyloid fibrils, Howard³⁴ 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^{14,34,35}. When the amino-acid structure of amylin was elucidated, it was observed that the 25-28 hydrophilic amino acids sequence (Ala-Ile-Leu-Ser) is common in humans, monkeys

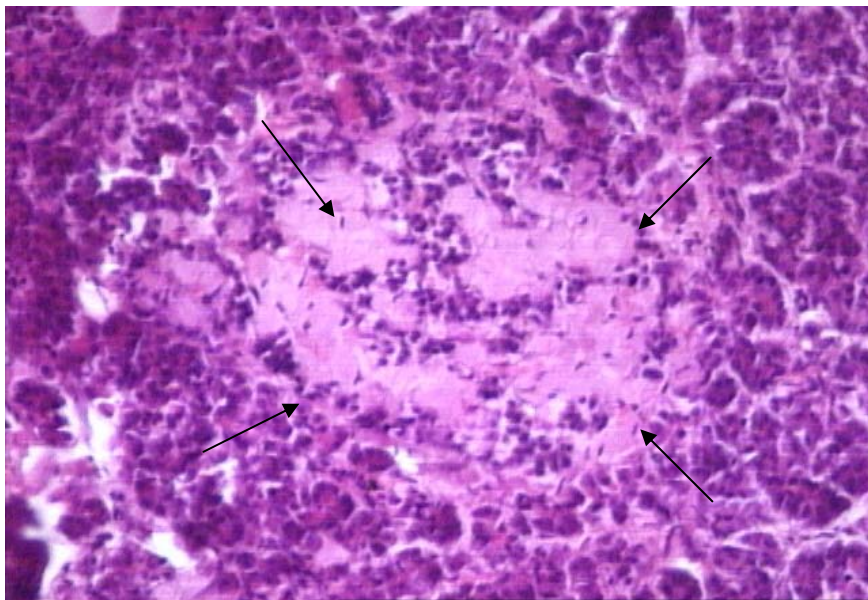
and cats, all of each develop both islet amyloid and diabetes. It has been considered that this amino-acid sequence is necessary for the formation of amyloid fibrils^{18,24,36,37}. The sequence of amylin in mice and rats is of a non-amyloidogenic sequence. Neither of these species develops type 2 diabetes without genetic manipulation³⁸. On the contrary, in human-amylin transgenic mice (h IAPP), toxic amylin oligomers were detected intracellularly in 20–40% of human-amylin transgenic beta cells²². These data confirm Westermark's suggestions^{17,39} that the first amyloidic molecules leading to amyloid are intracellular and distinct from the extracellular amyloid deposits when present^{12,22}. It seems that the extracellular amyloid deposits are formed rather by the pathologically increased pro-amylin than by amylin itself.

Even if the pathogenetic relationship between amylin and type 2 diabetes was often suggested to be important^{9,11,18,40}, the difficulty in accepting this hypothesis derived from the fact that the amyloid deposits were always evidenced extracellular 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%^{39,41,42,43}. Westermark^{12,18,43} 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, as a rule, not available⁴⁴.

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 (Fig. 3). In the cases with slight decrease of islet volume, the deposits are found between islet cells and capillaries^{11,12,18,45,44}.

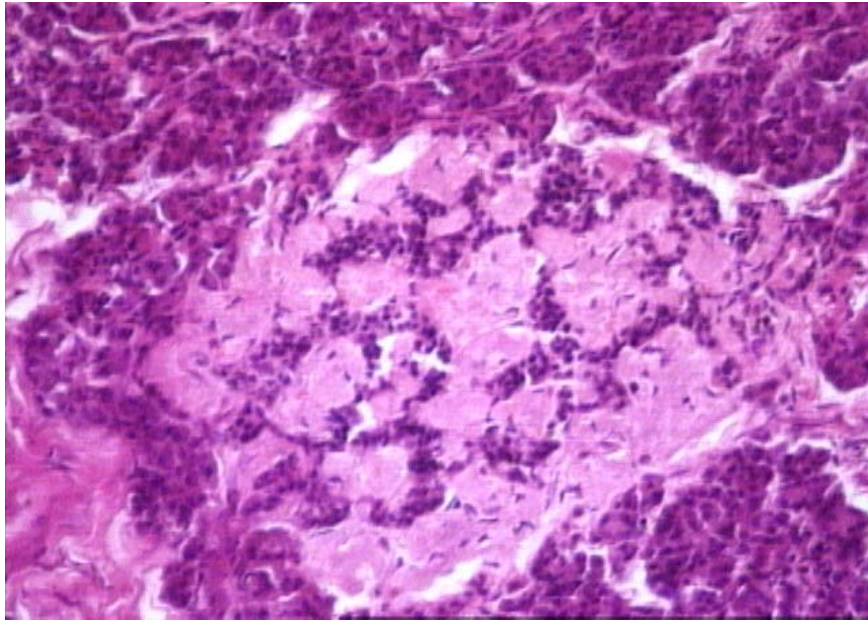


A. Normal pancreatic islets (*Hematoxylin Eosine* 20×).

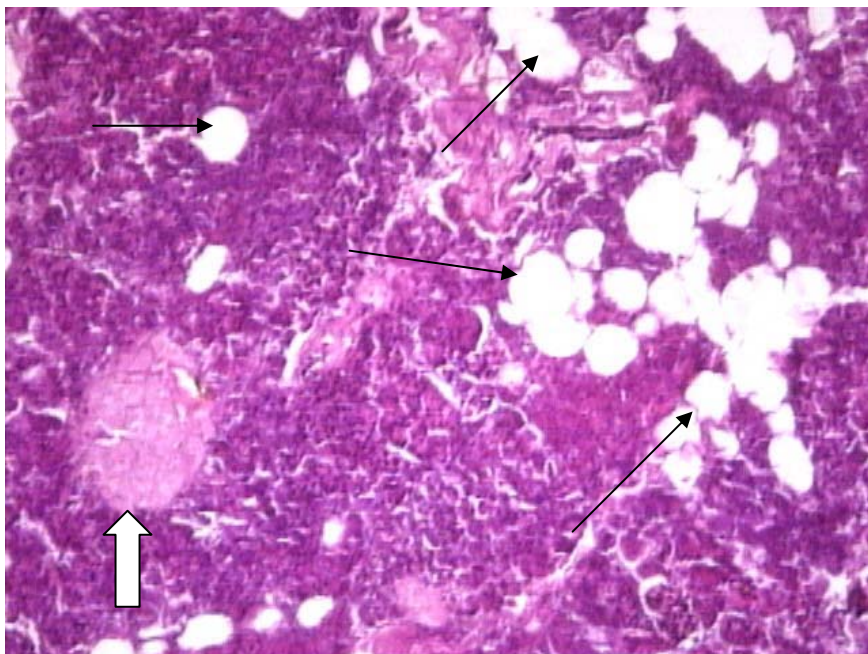


B. Type 2 diabetes with long-duration with important deposits of amyloid (arrows) (*Hematoxylin Eosine* 20×).

Fig. 3. A, B, C, D.



C. Type 2 diabetes with long-duration with important deposits of amyloid which enlarge the islets (*Hematoxylin Eosine 20x*).



D. Pancreas from a longstanding type 2 diabetes: Big arrow=an islet with amyloidosis and many lipidic depots (thin arrows) (*Hematoxylin Eosine 10x*).

Fig. 3. A, B, C, D.

Although as early as 1973 Westermark¹⁷, and subsequently Clark⁴⁶, suggested a possible intracellular origin for the islet amyloid (hypothesis suggested by the secretion 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 create 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 affected⁴². This explain why, until recently, some authors^{41, 42} 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^{9, 12, 39, 44, 47}. In addition, immuno-histochemical 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^{12, 22, 46, 48, 49}.

The uncertainty regarding the intracellular amyloid deposits derive from the impossibility to evidence these deposits on necroptic material. However, as Westermark underlined^{12, 18, 17}, 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 form¹². 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 approach⁵⁰. Protofilaments have about 4 nm in diameter (visible in electron microscopy), fibriles (2–4 protofilaments) about 10 nm in diameter and amyloid deposits, 10–100 μm or more. A small oligomer is < 10 nm in diameter. However, these types of oligomers may have a cytotoxic effect inducing membrane instability and finally apoptosis^{14, 22}.

It is of interest that transgenic mice expressing human amylin do not always develop islet amyloid²². 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 proinsulin⁵¹ and amylin⁵². 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 than the one at which the converting enzymes can process these pro-hormones^{52, 53}. However, this could be less probable than the possibility that some other conditions, such as the increased NEFAs, to alter the processing of the pro-hormones⁵¹.

THE PROAPOPTOTIC MECHANISM OF THE BETA CELL AMYLOID

At present, it is obvious that islets with amyloid deposits have a small β -cell mass with evident cellular distortion and destruction^{9, 12, 17, 18, 45, 46, 47, 54}. 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^{55, 56, 57}.

However, studies performed on isolated human or rat pancreatic islets exposed for long periods of time to high levels of glucose showed that amylin synthesis increases in hyperglycemic conditions more than insulin synthesis⁵⁸. It was suggested that this disequilibrium can be also present in human diabetes. The ratio of 1:100 between amylin and insulin molecules can substantially increase when the production of insulin molecules

decreases while that of amylin remains constant. The dissociation between the production of the two secretory molecules is possible since their transcriptional control is different^{58, 59}.

Moreover, if (as suggested by us) the defect of insulin processing inside the beta cell ER is the primary defect in T2DM^{60, 61, 62}, it can be hypothesized that the laborious post-translational processing of insulin would require an increased biological work-load, leading also to an increased transit time through ER/GA. The simpler (and with minor post-translational processing) pro-amylin molecules will reach the GA faster and in higher number. As a consequence, at the time of SV generation inside the GA, a higher number of proamylin/amylin molecules will be included in the SV in comparison with proinsulin/insulin molecules. The increased ratio between the two secretory lines from the normal of 1:100 to 2 or even 3:100 could represent the seeds of the oligomerization process of amyloid fibrils that seems to originate from the secretory vesicles^{56, 63}.

Westermarck *et al.*^{39, 64} described an important deposition of amyloid in transplanted islets. This can explain the marked decrease of the number of functional islets longer time after transplantation. The decrease recorded in the first days after transplantation could be related to the inability of the islet vessel bunch to connect itself to the intrahepatic circulation. Since smaller islets proved to be more resilient than larger islets, it was suggested that the death of islets could be due to increased hypoxia of larger islets (higher distance for oxygen diffusion to the center of the islet). A possible relation between hypoxia and amyloid formation was not described but it could be taken into account.

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 to make up the mature and easy visible fibriles^{14, 48, 65}. 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 amyloid¹². An analysis of amyloid using the atomic force microscope⁸ do not added to much informations. In a careful study made by Hé *et al.*⁴⁹ 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 phosphorylation. These data clearly showed that the intracellular oligomerization of amylin precede the development of diabetes and the formation of extracellular fibrillar amyloid^{22, 49}. However, the link between amyloid formation and β -cell destruction is not very clear. The propose mechanism includes: pseudo channel structure in plasma membrane^{14, 65}; interaction of hIAPP fibrils with components of β -cell membranes such as heparin sulphate proteoglycan⁶⁶; increase in intracellular calcium do to interference with ion channels⁶⁷ or activation of proapoptotic caspase⁶⁸ and Fas-ligand⁶⁹ pathways.

This last mechanism have been indirectly proved using two *ex vivo* models of islets amyloid formation (cultured human islets and hIAPP expressing mouse islets lacking caspase-3). In these models prevention of caspase-3 activation protects islet β -cells from apoptosis induced by fibrillogenesis, irrespective of their endogenous or exogenous origin. It has been suggested that amyloid formation may contribute to β -cell death through a similar mechanism both, in type 2 diabetes and in cultured or transplanted islets^{55, 70}. The involved in this processes of endoplasmic reticulum stress^{3, 57} is an attractive hypothesis^{61, 62}, because a such pathophysiological mechanism might explain the close interrelation between the two β -cell secretory lines: proinsulin/insulin and proamylin /amilin^{61, 62}.

Despite of large number of researches devoted to the amyloidogenesis, the mechanism operating in vivo and in situ inside the human β -cells, remain still unclear^{56, 57}. Several unanswered question remains why the amyloidogenesis affect only the β -cells and not also the α -cells^{55, 70}? Why apparently the same amyloid occurred sometimes in humans not known to have diabetes^{17, 57}? Are these subjects with hyaline islets undiagnosed or potential diabetes as suggest Bell⁷¹ half a century ago? We must admite that the mechanism responsible for β -cell cytotoxicity during the process of islet amyloid formation is still largely unknown.

THE ENDOPLASMIC RETICULUM STRESS

The evolution of the beta cell mass changes dramatically in the presence of some genetic determined pro-apoptotic beta cell defects^{61, 62}. In our view, these defects involve either the quantity or the quality of the two beta cell secretory proteins: proinsulin/insulin and proamylin/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 miss-folded proinsulin molecules block the secretory traffic towards the Golgi apparatus⁷². 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 NH₂-terminal kinase) and caspase12^{73, 74, 75, 76, 77, 78, 79}. The other signal, released from the secretory vesicles, is related to the cytotoxic effect of small amylin oligomers formed inside the secretory vesicles^{14, 50} that have the ability to induce membrane instability, calcium accumulation inside the beta cell and apoptosis^{12, 22, 63, 80, 81}. 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 sometimes 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^{61, 62}.

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^{9, 12, 18, 36, 80}.

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 molecular network forming 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^{44, 47, 82, 83, 84, 85, 86}. 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^{18, 84, 47, 44, 12}, which, based on a more rigorous morphometric analysis, evaluated the relative beta cell volume reported to the acinary 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^{82, 47}. The onset of the beta cell mass decline takes place many years before the glycemic decompensation^{18, 9, 47, 87, 86}.

Beta cell apoptosis induced by amyloid deposits likely play an important role in the progressive loss of beta cell which can explain the high prevalence of T2DM in older age⁶¹. Recently Marzban *et al.*⁸⁸ demonstrate that inhibition of amylin synthesis prevents amyloid formation and beta cell death in cultured human islets from cadaveric organ donors transfected with small interfering RNA designated to suppress human proamylin. Thus, the islet amyloid area decreased by 65% compared with that of non transfected cultured islets. The rate of beta cell apoptosis decreased also⁸⁸.

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 and, finally, the absolute number of beta cells that remain active at a precise moment in the pancreas: 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 and in situ* 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^{89, 51, 90, 91, 92, 86, 93}. Since the beta cell mass disposes of a complex system of regulation^{94, 95, 96, 97}, 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.*⁸⁶ 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 and normal, beta cell.

LOW GRADE INFLAMMATION AND BETA CELL APOPTOSIS

A supplementary diabetogenic mechanism operating sometimes in type 2 diabetes, potentially influencing the amyloid formation can be the

proinflammatory reaction associated with obesity^{98, 99, 100, 101, 102, 103}. This reaction is mediated by macrophages attracted inside the adipose tissue by various adipokines. Some of these adipokines have a powerful pro-apoptotic and ant regenerative effects on the beta cell.

The pathogenic role of the adipose tissue is related to the *adipocytary dysfunction* expressed by the decrease of the adipokines with positive metabolic effects (mainly adiponectin) or by the increase of the adipokines with negative effects (leptin, resistin, RBP4-Retinol Binding Protein, TNF α , etc.). Many of these adipokines have cytokine and pro-inflammatory roles^{104, 105}. The mediators of this "low grade inflammation" process have a double negative effect on the regulation of the energy metabolism: (a) on one hand, they interfere with the already dysfunctional intracellular biochemical processes altered by the excess of fuels from the system. These alterations were erroneously interpreted as reflecting the peripheral insulin-resistance^{106, 107}. The multiple metabolic alterations (of carbohydrates, lipids and proteins) are not due to defects of the insulin receptor. In fact they are secondary to some distal metabolic defects, possibly located even at the level of mitochondrial oxidative processes¹⁰⁸. The fact that these disorders are secondary and do not represent a primary diabetogenic mechanism (as claimed by the peripheral insulin resistance supporters) is proved by their rapid remission after weight loss induced by bariatric surgery procedures^{109, 110, 111}. Such a remission could not take place in the presence of genetically encoded peripheral insulin resistance, independent from the presence of obesity; (b) on the other hand, the pro-inflammatory cytokines (both systemic and locally produced) proved to have negative effects on the insulin secretion by their beta cell pro-apoptotic actions¹¹². In other words, the weight excess expose the beta cell to an increased insulin secretion demand and, in the same time, the dysfunctional adipocytes deprive the pancreatic beta cell from the positive effect of adiponectin and expose it to the negative effects of pro-inflammatory adipokines, often in the presence of fat tissue accumulation inside the pancreatic islets¹¹³.

SLOW OR VERY SLOW DECREASE IN BETA CELL MASS

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 higher than 5–10 pmol/l recorded in non-diabetic subjects^{60,61}. 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^{47, 114, 61}, which, in order to reach the threshold of glycemic decompensation, has to involve more than 50% of the beta cell mass/function⁴⁷. In our view, the slow (and clinically silent) beta cell loss during this pre-hyperglycaemic 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 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^{115, 116} 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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