PROLEGOMENON TO THE EUROPEAN CONSTITUTION BOOK OF DIABETES MELLITUS

CONSTANTIN IONESCU-TÎRGOVIŞTE

National Institute of Diabetes, Nutrition and Metabolic Diseases “N. Paulescu”
Street Ion Movila, nr. 5-7, Bucharest, Romania, cit@paulescu.ro

Received December 16, 2008

The careful epidemiological analysis, the proper clinical and phenotypical characterization and rigorous follow-up for more than 3 decades of a couple of thousands of diabetic patients registered at “I Pavel” Diabetes Center in Bucharest allowed us to realize the extreme variability of diabetes phenotypes. It is far wider than enounced by the 4 “official” classifications of diabetes mellitus proposed between 1965 and 1998 by WHO, classifications that were subject rather to semantic than real conceptual changes. The progresses in the knowledge regarding the ensemble of tissues that regulate the energetic homeostasis of the human body, including the pancreatic beta cell from the Langerhans islets (a real center of “command and control”) and the 3 types of insulin dependent-cells from liver, muscles and adipose tissue (real “execution blocks”) allows for a new and better understanding of diabetes pathogenesis and for its close relatives, obesity and metabolic syndrome. The primary cause of diabetes and the secondary diabetogenic mechanisms can be found in these cell structures and action according to their position and role in the homeostatic system. By corroborating the epidemiological and clinical with the biochemical and hormonal data, we could find some solid proof for locating the primary cause of diabetes in the secretory function of the pancreatic beta cell. More precisely, it can be identified in the post-translational changes of the secretory proteins (proinsulin and proamylin) that take place mostly in the Endoplasmic Reticulum (ER), Golgi Apparatus (GA) and Secretory Vesicles (SV). The pathogenic effect of the pancreatic beta cell SV immaturity is expressed on one hand by the decrease of insulin secretion, the changes in the physiologic secretory pattern, the increase of proinsulin-to-insulin ratio and on the other hand by the amyloidogenic transformation of amylin. These four secretory alterations lead, on different pathways, to increased beta cell apoptosis and decreased beta cell regeneration, with the final result of progressive decrease of the beta cell mass. The onset of beta cell mass decrease is the best marker for the real start-point of the diabetogenic process and we propose that this event should be adopted as the main definition criterion for the diabetes syndrome.

Key words: Diabetes pathogenesis; Energy homeostasis; Endoplasmic reticulum function; Diabetes care.

PREAMBLE

Through the observations and proposals we are going to make in this prolegomenon, we shall try to correct a series of discordances evidenced during the last 4 decades between diabetological knowledge and the current definition of diabetes, as well as the classification of its various phenotypes and its pathogenesis. Before presenting these corrections, we should first mention some factors that were more harmful than beneficial to diabetologic thought: (1) maintaining of hyperglycemia as the main marker for diabetes and its sole diagnostic criterion; (2) introduction in the pathogenesis of diabetes of the concept of peripheral insulin-resistance that, despite the lack of an objective basis (or maybe just for this reason), was extended beyond the acceptable limits of a physiological judgment; (3) the rather overly facile interpretation of the pathogenesis of human diabetes based on the extrapolation of some data obtained in different animal diabetes
about the nature of diabetes as a dysfunction of control of energy metabolism

Art. 1 – Diabetologia, Diabetes & Metabolism, Diabetes Research and Clinical Practice, Diabetes & Vascular Disease Research, Diabetes Care or Diabetic Medicine are the titles of some specialized journals that try to sustain not only a medical specialty (diabetology) but also the science of metabolism and its branches. Delineating this specialty is however difficult since it should include all the disorders of the wide system that ensures the energetic homeostasis of the human body. If we leave out the large number of diseases/syndromes included in the category of “inborn errors of metabolism”, usually rare or very rare and found in the pediatric departments, the term “diabetology” defines, at least semantically, the “science that deals with diabetes and the care of diabetic patients”. By defining diabetes as a heterogeneous syndrome that expresses a complex disorder of the energy metabolism, overweight and obesity become automatically part of this syndrome and, by consequence, subject of study for “diabetology”.

Art. 2 – The previous statement is supported by the high number of components of the system that ensure the energy homeostasis of the body. They include apart the Bulls: the orexigenic and anorexigenic nervous centers (especially hypothalamic) that regulate alimentary behavior and the intake of various nutrients; the digestive tract that ensures the digestion and absorption of foods; the liver, that ensures the processing of the various absorbed nutrients and the interchanges between different metabolisms (carbohydrate, lipid and protein); the adipose tissue, that functions both as an energy deposit/reserve and as an “internal secretory gland” and the skeletal muscle that ensures a stable biochemical pressure inside the system by continuous energy consumption

Art. 3 – From this list we can derive the notion of “fundamental cells” involved in the diabetogenic process: hypothalamic neurons sensitive to glucose/lipids/insulin; gastro-intestinal epithelium with the specialized cells for digestion and absorption as well as for secretion of several hormonal messengers (ghrelin, GLP1, GIP, colecistokinin); hepatocyte (the primary site for various metabolic processes, including the production of apoproteins), myocyte (the main energy consumer) and adipocyte (that plays an
important role in the regulation of the energy metabolism both by its storing function and by secreting a large number of adipokines) (Fig. 1).

Art. 4 – The role of coordinating/integrating the function of the above mentioned cells/tissues belongs to the pancreatic beta cell. Its fundamental secretory product is insulin. Its function of metabolic regulation is performed by acting on the insulin dependent cells/tissues (skeletal muscles, liver, adipose tissue) but also on the nervous cells from the hypothalamic centers.

Art. 5 – Since the major chronic diabetic complications are mainly of vascular nature, a fundamental cell for the understanding of these complications is represented by the endothelial cell. This cell led the cardiologist to approach the diabetologist (or vice-versa) in the so called "cardio-diabetology". By this endothelial cell arise also the nephrological, ophthalmologic and neurological branches of diabetes. Thus, we could state that diabetology is one of the major turning points of modern medicine. It is the medical discipline that had the strongest effect in emulating the cellular and molecular biology, neurobiology, immunology and genetics.

Art. 6 – Diabetes mellitus is, from the clinic, pathogenic and genetic point of view, a heterogeneous syndrome, characterized by a complex alteration in the regulation of the energy metabolism of the human body, involving altogether the carbohydrate, lipid and protein metabolisms as well as other metabolisms. This syndrome is induced by a primary secretory defect of the pancreatic beta cells, accompanied in cascade by disorders in the function of the three insulin-dependent cells/tissues: liver, skeletal muscle and adipose tissue. The biochemical changes deriving from the alteration of the energy metabolism regulation lead to functional cell changes followed by irreversible anatomic lesions in numerous tissues and organs.

Art. 7 – The “brain” for the regulation of the energy metabolism is represented by the beta cell mass, i.e. the ~ 3 billion beta cells scattered inside the ~ 1 million Langerhans islets. The final product of each pancreatic beta cell is represented by the mature secretory vesicles (SV). Each of ~ 10,000 such SV from a beta cell contain ~200,000 insulin molecules (that we could call insulin „quanta”) and the same number (~ 2000) of each of the following molecules: C-peptide, amylin, un-split proinsulin and un-split proamylin. While non-islet cells can be genetically engineered to express insulin, the lack of secretory vesicles makes them nonfunctional.

Art. 8 – The major product of the pancreatic beta cell included in the SV is the insulin molecule. Each minute, a beta cell translates ~1 million of pre-proinsulin molecules which suffer a quick and precise posttranslational processing which starts in endoplasmic reticulum (ER), continued in Golgi apparatus (GA) and ends in secretory vesicles (SV). The primary cause of diabetes is located in this true command and control cell, i.e. in the inability of the β-cells to produce mature secretory vesicles.

Art. 9 – Insulin exerts its influences on the glucose, lipid and protein metabolisms by acting on the three insulin-dependent cells: hepatocyte, miocyte and adipocyte. They constitute three compact functional blocks of cells, each with its specific role: liver – the main organ for the processing of nutrients and for the regulation of the plasma concentration of circulating fuels; skeletal muscle – the most important energy consumer, in order to maintain the normal biochemical pressure in the system and finally the adipose tissue – main organ for the storage of the excess of circulating fuels resulted either from an increased food intake or from an under-utilization of fatty acids and glucose by muscles in conditions of decreased physical activity.

Art. 10 – Any alteration of insulin secretion will be promptly reflected in the function of the three blocks of cells as secondary disorders, with precise
diabetogenic significance. A defect in the function of these insulin-dependent cells can represent the basis of some additional diabetogenic mechanisms that involve either the hepatocyte (the increased output of glucose due to an increased gluconeogenesis, a decreased glycogenogenesis and/or a increase glycogenolysis), miocyte (decrease of overall mitochondrial oxidations or a preference towards oxidation of fatty acids in the detriment of glucose) and adipocyte, by its particular position in the energy system. We admit that each of these three insulin-dependent tissues could carry some distinct genetic defects, such as alteration of their proliferation potential (i.e. adipogenesis in the adipose tissue), inheritance of a particular cell phenotype, in skeletal muscle for instance, with “metabolic” inflexibility of miocyte to switch off substrate oxidation from lipid to carbohydrates and reduced mtDNA content of skeletal muscle or a propensity for fat accumulation in liver. These disorders could be related to the secretory dysfunction of beta cell, either directly by the under-activation of the metabolic pathways, or indirectly by the mithogenic function of insulin that can influence the gene expression of the functional molecules in these cells.

Art. 11 – The main two environmental factors responsible for the without precedent increase of diabetes mellitus incidence in the last half century are: (a) the increase in the global caloric intake, with the change of the ratio between the main nutrients (decrease of complex carbohydrates/increase of simple carbohydrates; increase of animal lipids and proteins/decrease of vegetal proteins and lipids) and (b) the decrease of physical activity. Their additive effect on the energy balance explains the increase in the prevalence of obesity and metabolic syndrome, disorders that precede the occurrence of diabetes mellitus. Their pathogenic nature is related to the constantly increased pressure of the various nutrients that enter the energy system.

Art. 12 – The diabetogenic effect of obesity can be mediated through three mechanisms: a) the constant increased solicitation of the pancreatic beta cell secretory function, proportional with the weight gain and secondary not only to the expansion of the adipose tissue but also of the other two insulin-dependent organs/tissues: liver and skeletal muscles; b) the second is related to the increased leptin secretion with a pro-apoptotic effect on the pancreatic beta cell and decreased adiponectin secretion, a complex hormone that is adequately secreted only by normal adipocytes, with the role to stimulate the oxidation of fatty acids in skeletal muscles, liver and pancreatic beta cells; c) the third is related to the inducement in the hypoxic adipose tissue of a pro-inflammatory reaction. The over-expanded adipose tissue becomes hypoxic and the hypertrophied adipocytes are subject to a constant biochemical stress at the ER level, leading to the generation of an inflammatory reaction, perpetuated not only by adipo-citokines but rather by cytokines produced by the monocytes/macrophages attracted in the profound regions of the adipose tissue that are more hypoxic, regions where adipocyte apoptosis is more intense. One of the factors that mediate the decreased expression of adiponectin mRNA is adipose tissue hypoxia, capable moreover to trigger a pro-apoptotic reaction mediated by CHOP.

Art. 13 – Ignoring the natural physiological thinking, many of the different metabolic derangements recorded in the function of these three insulin-dependent cellular blocks has been erroneously interpreted as signs of peripheral insulin resistance, an ambiguous but well-chosen term that made career due to some very good advocates. They built a virtual construction based on mathematical calculations and sophisticated formulas that provided not measurable parameters but rather more or less abstract indexes. The whole field of the insulin-resistance hypothesis as the primary cause of diabetes mellitus is too complicated to be discuss in detail here. The rhetoric question posed by the author of a recent review regarding the genetics of T2DM, “Where are the insulin resistance genes?” is relevant. It was not a surprise that T2DM has been associated with genes that encode beta cell structures but it would have been strange that geneticists would be able to identify genes for a “mathematical concept”. We observed that by omitting insulin resistance, the concept regarding the pathogenesis of diabetes became clearer. On contrary, if you try to avoid β-cell from the pathogenesis of diabetes remains nothing. This is the reason why in this discussion we shall omit the references for the hypothetical insulin-resistance, concentrating our attention on the pancreatic beta cells inside which, we have evidence, it is hidden the primary cause of diabetes mellitus.
**β-CELL – THE MAIN PLAYER IN THE REGULATION OF ENERGY METABOLISM**

Art. 14 – The genetic architecture of various phenotypes of diabetes as it is known at the end of 2008, showed that almost all genes involved in neonatal diabetes, in monogenic forms of diabetes, partially in type 1 diabetes (where some immune related genes are involved) and in type 2 diabetes, are related with beta cell structure and function. So, the primary cause of diabetes must be found somewhere inside this highly specialized β cell, with the reflection of its defect in the 3 main insulin-dependent cells.

Art. 15 – Regulation of the energy metabolism by the beta cell depends on its excitable nature, evidenced in: the prompt reaction stimulus-response and on the distribution of its secretory products (insulin and amylin) in secretory vesicles (secretory „quanta”) which are an ingenious “anatomic” construct that allows the fine tuning in amount of insulin release according to the magnitude of the stimulus by variation in the number of exocytised SV. These characteristics depend on the presence inside the β-cells of some neuronal phenotypic characteristics.

Art. 16 – The main disorder that appears early in the natural history of metabolic disturbances (especially diabetes mellitus) is the incapacity of the beta cells to produce constantly mature secretory vesicles. An immature secretory vesicle contains higher quantities of un-split proinsulin and proamylin and, evidently, lower quantities of mature insulin and amylin. A disequilibrium between the two secretory lines (pre-proinsulin/proinsulin/insulin and pre-proamylin/proamylin/amylin) can represent the basis for the amyloidogenic transformation of amylin, a disorder that can contribute to the higher incidence of T2DM in older ages.

Art. 17 – The immaturity of the SV implies not only a defect in the processing of promolecules from the secretory vesicles, but also an incomplete development of its motor machinery, rendering more difficult their exocytose. This can explain the presence in the early stages of diabetogenesis of various changes in the physiological insulin-secretion pattern: attenuation or disappearance of insulin-secretion oscillations, disappearance of the first phase insulin response, prolongation of the interval between the glycemic stimulus and insulin response and decrease of the area under the curve for insulin-secretion in response to the higher glycemic increase. It is worthy to note that all these changes are associated with a decrease of the beta cell mass but without any effect on the fasting or postprandial level of blood glucose. We call this stage of diabetogenesis the pre-hyperglycemic stage of diabetes mellitus.

Art. 18 – The results of standardized beta cell morphometry studies on necroptic pancreases demonstrate that, when blood glucose alteration appears (IFG/IGT/clinical overt diabetes), the beta cell mass/function is already decreased to ~50% of its initial level. This finding forces us to reconsider the current definition of diabetes mellitus by replacing the epiphrenomenon hyperglycemia with the phenomenon itself which is represented by the decrease of the beta cell mass/function. The pathogenicity of the metabolic derangements from the pre-hyperglycemic stage of diabetes is sustained also by the presence of chronic complications of diabetes at time of its clinical diagnosis based on decompensation of blood glucose regulation.

Art. 19 – The decrease of the β-cell mass found by us occasionally in patients deceased with major cardio-vascular events (myocardial infarction or stroke) could result from the metabolic alterations (expressed in the various plasma lipid changes) acting before the onset of clinical diabetes, according to its current definition: fasting blood glucose ≥ 126 mg/dl. On arbitrary base it was decided that the obvious abnormal values for the blood glucose (defining IFG/IGT) to be considered as non-diabetic, despite their repeatedly confirmed association with the vascular pathology. In last years, diabetes is considered a true cardiovascular disease, but the reverse of this statement might be that atherosclerotic vascular disease could be looked as expressing a pre-hyperglycaemic stage of diabetes. The study of β-cell in this category of patients, could reveal the similar β-cell changes, differentiated from beta cell in diabetes only quantitatively, and not qualitatively.

**RELATIONSHIP BETWEEN OBESITY AND DIABETES**

Art. 20 – From the pathogenic factors associated with T2DM, obesity plays a major role, being present in ~85% of cases. Even if this association was well known as early as 1877, when Lancereaux described the two main forms of diabetes (“thin” diabetes and “fat” diabetes respectively), the molecular mechanisms that explain this association started to be elucidated only much later, after the discovery of the complex secretory function of the adipocyte.
Art. 21 – Epidemiological data regarding the evolution of food intake during the last 200 years evidenced numerous changes\textsuperscript{12}, four of which we consider can have a diabetogenic effect mediated by the increased load on the ER function, not only in β-cells, but also in all insulin dependent cells: (a) the first is represented by the progressive increase of the overall caloric intake, from ~2100 kcal/day at the end of the 18th century to ~2700 kcal/day in present; (b) the second change refers to the decrease of carbohydrate intake (from ~65% at the end of the 18th century to ~50% today), associated with an increase of the percentage of refined simple carbohydrates (sugar and derived, processed cereals, etc.) with the concomitant decrease of complex carbohydrates intake (from vegetables, fruits, etc.); (c) the third change was represented by a big increase in intake of protein and lipid from animal origin, and the decrease in those of vegetal origin; (d) finally, the fourth change is associated with the previous and is represented by the increase in fat intake (from 25% to 45%) and especially in saturated FFAs intake with the decrease of unsaturated or polyunsaturated FFA’s\textsuperscript{82}.

Art. 22 – Weight excess is encountered in ~85-90% of T2DM cases and precedes, usually, the glycemic decompensation by years or even decades\textsuperscript{78}. Despite the enormous research effort, the pathogenic mechanisms for this association still remains in the field of hypotheses. No one could answer yet the following two questions: Why do some subjects, obese from a young age, will never develop diabetes? Why is it that only 30% of obese subjects develop diabetes? Our interpretation is the following: weight excess indicates a supplementary tissue mass, consisting especially of insulin-dependent cells: adipocytes, miocytes and hepatocytes. This supplementary insulin requiring cell mass needs a proportionally higher insulin secretion. The subjects with a normally developed ER in the beta cells, will cope with the increased secretory demand. On the contrary, in those subjects with a constitutionally underdeveloped ER, the secretory overload will lead in time to a decompensation of the blood glucose regulation explained by the occurrence and then aggravation of the proinsulin β-cell defect. Since the insulin dependent cells belong to the same system, an excessive parallel overload for the ER from all these cells is predictable. Numerous metabolic disturbances (increased FFA and TAG, decreased HDL, alteration of lipoprotein particle dimensions) recorded in offspring of diabetic subjects that subsequently developed diabetes are valuable indicators of the parallel dysfunction in ER from the various dependent cells. The appearance and progression of the diabetogenic process, which operate decades before the blood glucose decompensation is in our view a true pre-hyperglycemic stage of diabetes.

Art. 23 – The incidence and prevalence of obesity has increased in the last 50 years and is associated with the increase in the GDP of different countries. Between the increase in the incidence of diabetes and that of obesity there is a striking parallel. The type of food intake and the traditional ratio between the various nutrients\textsuperscript{83} has changed, the global caloric intake increased markedly, while the energy expenditure diminished significantly. The most vulnerable populations were those with a rapid transition from deprivation to abundance\textsuperscript{15} while those more protected were those with a strong cultural basis, as are in Europe the Scandinavian countries. However, even for the countries in transition, a large part of the population remained normal-weight. We could accept the hypothesis that the subjects that became obese are those that inherited the „thrifty genotype” pattern\textsuperscript{84} or those with a more unstable neurologic-behavioral system\textsuperscript{15, 85}. The culprit for this true “metabolic disaster” is mainly the society based on “profit at any cost” principle. Only secondarily can the guilt be blamed on the uneducated individual who cannot protect himself from the media pressure of the strong industry of soft-drinks and high-calorie fast foods, that are cheap and attractive in aspect and taste. We totally agree with Niswender and Beech\textsuperscript{14} that state: „success in fight against obesity will arise from integration of finding from both homeostatic and non-homeostatic feeding models, from translational and educational work in communities and, perhaps, from partnerships with industry”.

Art. 24 – The long established\textsuperscript{78} relationship between T2DM and obesity should be analyzed in the broader context of the lipid disorders associated with diabetes mellitus\textsuperscript{86-89}. These disorders arise initially from the impact of the insulin-secretory defect on the liver\textsuperscript{20-28} followed by its influence on the utilization of fuels in the skeletal muscles\textsuperscript{1,9,25,90,91} and finally on the adipose tissue\textsuperscript{92-94}.

Art. 25 – The adipocyte seems to be the second important cell after the beta cell for diabetogenesis with numerous inputs but more numerous output messengers (Fig. 1). They group in the adipose tissue, which functions as an ingenious system for the controlled storage of fatty acids\textsuperscript{45}. Its storage capacity has an optimal, a maximal level\textsuperscript{95} and an
excessive (pathogenic) level. No one knows when optimal level evolves towards maximal and where the boundary between physiologic and pathologic is. It is obvious that this boundary is marked by the moment that triggers adipocyte dysfunction, expressed by excessive production of some adipokines with cytokine action, and by the decrease in the normal secretion of some adipokines with positive hormonal action. Of these, leptin and adiponectin seem to have a relation with diabetogenic mechanisms: the first, leptin, by its pro-apoptotic beta cell effect when overweight becomes chronic and its secretion remains constantly increased; the second, adiponectin, by the decrease of its production. These patients with severely decreased adiponectin production, could be included in the group of “adiponectin-dependent diabetes”. With similar significance we should note the decreased production of RBP4 (Retinol Binding Protein 4) and finally to skeletal muscles, by its pro-apoptotic beta cell effect when overweight becomes chronic and its secretion remains constantly increased; the second, adiponectin, by the decrease of its production. These patients with severely decreased adiponectin production, could be included in the group of “adiponectin-dependent diabetes”. With similar significance we should note the decreased production of RBP4 (Retinol Binding Protein 4) and finally to skeletal muscles, by its pro-apoptotic beta cell effect when overweight becomes chronic and its secretion remains constantly increased; the second, adiponectin, by the decrease of its production.

The role of adipocyte dysfunction in diabetogenesis is much more complex but it cannot be extracted (and separately analyzed) from the context of the metabolic ensemble it belongs to. The visible nature of overweight but invisible nature of the beta cell mass/function (and its decrease) leaves the impression that obesity precedes and induces beta cell dysfunction. In the absence of precise data regarding the early stages of diabetogenesis in respect to the beta cell mass/function, diabetes was interpreted to be secondary to a putative adipose insulin-resistance. This interpretation was logicless since weight gain and overweight persistence implied a good or even excessive adipocyte insulin sensitivity. In time, the site of the putative insulin-resistance was successively moved to liver and finally to skeletal muscles. In fact, adipose tissue is recognized as a source of proinflammatory cytokines, produced both by adipocytes (mainly) and by activated macrophages and other immune active cells invadeting the adipose tissues from obese subjects. Low grade inflammation is considered to be a link between obesity and T2DM. This type of inflammation could be related to hypoperfusion and hypoxia in adipose tissue, which has been related with high intra-abdominal pressure, due to a direct mass effect of the visceral obesity. It should not be forgotten that this pro-inflammatory reaction is late, thus it cannot be interpreted otherwise than just a supplementary mechanism, only secondary to the beta cell defect.

The uncertainties regarding the relationship diabetes-obesity are supported by two main findings: only ~25–30% of obese subjects develop diabetes; on the other hand, ~10–15% of T2DM cases appear in non-obese subjects. The association between these two metabolic disorders is frequent, but it is neither compulsory nor necessary. To this, we should add the attempt to generalize the diabetogenic mechanisms operating in extreme (morbid) obesities, with a BMI > 35, which represent only a small percentage from the total number of obesities. The strongest argument which supports the causal relationship between diabetes and obesity is represented by the spectacular effects of the bariatric surgery on the metabolic profile in a recently published randomized trial. Indeed, in the study of Dixon et al., the difference between the control group (with standard treatment of obesity) and the bariatric surgery group (including morbid obese people with a BMI >35), in a small interval of time (2–3 weeks) it was a striking expected – weight loss of 1 kg (in control treatment) vs. 21 kg (in the surgical group). It was no surprise that the evaluation after 2 years shows a significant decrease of weight, fasting glycemia, triglycerides and insulin sensitivity only in the bariatric surgery subjects. These results are impressive but they are not different from those recorded in obese diabetics that lose weight similarly by classic (non-surgical) methods. The diabetogenic mechanism in this morbid phenotype of obesity with its clinical and pathogenetic particularities, could be not extrapolated to diabetes associated with the more frequent, common, forms of obesity (BMI between 30–35 kg/m²) and less with those with a BMI between 25–30 kg/m².

The transport of lipids in the human body depends on its protein component (apoprotein) which characterized also the various lipoproteins. Also, the negative consequence of high cellular lipids may be related to the ability of various cells to regulate lipid storage and utilization, strongly linked to the PAT (perilipinic) proteins as surfactant of the lipid droplets surface. Packaging lipids in smaller units and restricting access to lipase, the role of protein components seems to be of great importance. Packaging lipids in smaller units and restricting access to lipase, the role of protein components seems to be of great importance. Packaging lipids in smaller units and restricting access to lipase, the role of protein components seems to be of great importance. Packaging lipids in smaller units and restricting access to lipase, the role of protein components seems to be of great importance. Packaging lipids in smaller units and restricting access to lipase, the role of protein components seems to be of great importance. Packaging lipids in smaller units and restricting access to lipase, the role of protein components seems to be of great importance.

European Constitution Book of diabetes mellitus
An increase of FFAs is a hallmark of diabetes, but also of obesity and metabolic syndrome and the high fat mass that is maintained for a long period of time is associated with high FFAs. The ‘‘omental’’ (central or abdominal) adipose tissue may be a supplementary risk factor for T2DM versus peripheral (subcutaneous) adipose tissue because the flux of FFAs seems to be higher from central fat deposits. The diabetogenic role of high FFAs has been attributed to a supposed increase in insulin resistance in adipose tissue preceding the development of T2DM. This mechanism is far to be substantiated and could be linked more with the increase of intramyocellular triglycerides, than to the associated level of FFAs. In this view, the higher level of plasma FFAs could result from a decrease in their utilization in peripheral tissues, especially in skeletal muscles.

Art. 29 – One of the main links between obesity and T2DM is made by the increase in FFAs. An increase of FFAs is a hallmark of diabetes, but also of obesity and metabolic syndrome and the high fat mass that is maintained for a long period of time is associated with high FFAs. The “omental” (central or abdominal) adipose tissue may be a supplementary risk factor for T2DM versus peripheral (subcutaneous) adipose tissue because the flux of FFAs seems to be higher from central fat deposits. The diabetogenic role of high FFAs has been attributed to a supposed increase in insulin resistance in adipose tissue preceding the development of T2DM. This mechanism is far to be substantiated and could be linked more with the increase of intramyocellular triglycerides, than to the associated level of FFAs. In this view, the higher level of plasma FFAs could result from a decrease in their utilization in peripheral tissues, especially in skeletal muscles.

Art. 30 – The increase in unsaturated FFAs seems to become pathogenic inducing a strong ER stress in β cells. Several studies demonstrated that palmitic acid (the most important plasma saturated FFA), when administered in high doses has an increased proapoptotic effect on the β-cells. In a series of studies performed by Laybutt et al., they demonstrated that the MIN6 cells, incubated in a palmitic acid reach medium present obvious signs of ER stress. Moreover, the activation of the genes involved in the ER stress was evidenced in the beta cells of the db/db mice as well as in the human islets from T2DM subjects. It was shown that the proapoptotic effect of palmitic acid is mediated by the increased expression of several molecules, the most important being CHOP. This is a transcription factor expressed normally at low levels in the ER, which markedly increases during ER stress, being considered an indicator of β-cell apoptosis. It has been demonstrated that palmitate selectively induces the pro-apoptotic protein CHOP in β-cells reducing β-cells viability in a time-dependent manner (6–12 h) and cause a dramatic alteration in the cells morphology, including marked distention of ER membranes. PERK signaling pathway of ER stress has been found activated by palmitate increasing the expression of the transcription factor ATF4 and CHOP. The treatment of MIN-6 cells with saturated FA palmitate, but not the unsaturated FA oleate, cause impairment of vesicular trafficking for ER to GA. This effect in trafficking is associated with ER stress and β-cells apoptosis and may contribute to the development of T2DM. Similarly, on INS-1E cell line, the palmitic acid activates the UPR, especially if accompanied by increased glucose level contributing to a precocious beta-cell apoptosis mediated by the caspases. Another pathogenic pathway induced by the circulating FFA is related to their inhibitory effect on the PC3, PC2 convertases and on carboxi-peptidase E partially explaining the increase of proinsulin percentage inside the secretory vesicles in T2DM. The same effect is recorded on the processing of proamylin: when β-cell vesicles from +hIAPP/–mIAPP MIN6 fed a high-fat diet were morphologically investigated, fibril structure resembling amyloid fibrils were identified in the halo region in a large proportion of islets. The toxic effect of these protofibrilles formed in β cells has been demonstrated.

Art. 31 – The supplementary diabetogenic loop induced by overweight is mainly indicated by the adipocyte secretory dysfunction which alters its adipokine profile, decreasing the production of adiponectin, thus depriving the beta cells of an important anti-diabetogenic factor and increasing the production of pro-inflammatory cytokines. In addition, the increased level of plasma leptin will have a pro-apoptotic effect on β cells. Since the post-translational processing of adipocyte secretory proteins takes place inside the endoplasmatic reticulum, adipocyte dysfunction was attributed to the adipocytary ER stress which alters adipocyte secretory dysfunction. A similar dysfunction can be evidenced also in other insulin-dependent cells (hepatocyte or miocyte) subject to increased work-load due to the biochemical pressure of the increased fuels in the energy system associated with the weight excess from obesity. The precise chronology of the pathogenic relationship between weight excess and T2DM remains one of the major draw-backs to diabetological research.

Art. 32 – The recent description of FTO gene which is associated with both BMI and T2DM, but also with high leptin and decrease adiponectin, might suggest one of the mechanisms linking this two metabolic syndromes. The high heritability of obesity suggests that the inter-individual differences in susceptibility to gain weight are related with a genetic defect, located not in adipocyte itself, but in the neuronal machinery operating in hypothalamus and regulating the energy balance affecting neurobehavioral pattern. It is worthy of note that the individuals who are monozygous for the high-risk allele (AA) of FTO weight on average 3 kg more than individuals with two low risk alleles, with heterozygote having a intermediate risk.
Art. 33 – It has recently been demonstrated that FTO encodes a nucleic acid demethylase, a member of a 2-Oxoglutatate-dependent dioxygenase family. Interesting enough is the fact that the highest level of expression FTO gene is in brain in the arcuate nucleus of the hypothalamus. Some data suggest that it seems most likely that intronic variation in the FTO gene influences obesity through its effects on some brain process relevant to energy balance. The influence of the cerebral cortex on food behavior is sustained by the influence of FTO genotype on the human cerebro-cortical response to insulin. This data switches the interest in understanding the genesis of obesity from the adipocyte to the central system control of food intake. Indeed, if we look at figure 1, the brain has a high number of input peptide messengers. However, these are not able to activate efficiently the anorexigenic mechanisms, those being in fact annulled by the magnitude of orexigenic sensory stimuli. In this manner we can explain the occurrence of obesity and its perpetuation thereafter. In fact, an increased adipocyte storage of triglycerides expresses its own function, that to store the various fuels which are increased in the circulation, when the ratio between their input and their utilization is increased. The adipocyte dysfunction appears only when the level of lipid stores in adipocytes crosses a pathogenic threshold.

Art. 34 – The heritability of BMI/waist circumference was found to be up to 77% in monozygotic twins up to the age of 11 years, suggesting that a genetic defect is important in this respect. The mechanisms involved in the concept of „thrifty genotype” or „thrifty phenotype”, are based on a proper judgment that could explain large part of the heritability of common obesity forms. The existence of a „thrifty metabolism” in some subjects and of a “wasteful metabolism” in others could explain the co-existence in the same population of obese and normal-weight subjects.

Art. 35 – T2DM genetic studies identified as associated with obesity not only FTO gene, but also PPARγ gene. The last encodes a transcription factor that influences adipocyte differentiation, while the second is associated with BMI through a nucleic acid demethylase, influencing by a not yet elucidated mechanism the central control of food intake. This finding should be associated with the large number of specialized hypothalamic receptors, including those for insulin, leptin, GLP-1 and many more. The presence of insulin receptors in different cerebral areas prove that, although the brain is considered to be an insulin-independent tissue, insulin (the messenger of the “metabolic brain”) plays an important role in the regulation of energy metabolism by acting not only on the hypothalamus but also on the cerebral cortex. It is possible that the common variants of the above mentioned two genes (for sure other will be discovered in the future) could play an important role in the obesity with onset in young ages or to explain the presence of some morbid obesities, both being only particular and rather extreme subtypes of the obesity syndrome.

β-CELL DEFECT IN THE PATHOGENESIS OF DIABETES

Art. 36 – The primary and fundamental disorder in diabetes mellitus is represented by the secretory beta cell defect. This defect is reflected both in proinsulin and proamylin processing. These two defects have different pathogenic significance. Since the whole beta cell machinery was constructed with the aim to exocytise the proper quantities of insulin required for the regulation of circulating fuels, the decrease of insulin secretion was more intensively and thoroughly studied. It can explain the various secondary disorders in the function of the three insulin dependent cellular blocks: in the liver – the fatty accumulation, in the skeletal muscle – increased triglycerides deposits, both intra and extra-miocytary, in the adipose tissue – des-inhibition of adipogenesis and excessive increase of lipid stores and adipocyte dysfunction. The real hyper-insulinism, i.e. increased insulin levels corrected for the level of blood glucose and for the percentage of proinsulin included in the standard non-specific radio-immunological assays, is hard to prove. Even if it will be proved to be real in some isolated cases, it is sure that it lacks from the natural history in the common forms of diabetes mellitus whose onset should be placed at the initiation of beta-cell decrease and not at the moment of blood glucose alteration. On the contrary, if we add proinsulin to insulin secretion, this implies a high secretory work load, but with a low peripheral insulin activity, since proinsulin has a minimal insulinic effect.

Art. 37 – A second major component of the beta cell secretory defect is the increase in the proinsulin-to-insulin PI/I ratio resulting from the both, the increase of proinsulin percentage (as a consequence of the defect in its processing) and from the decrease of insulin...
production (see 47,53). In these conditions, it is evident that the percentage of insulin in the immature SV decreases, with the metabolic consequences already described above. However, the pathogenic significance of un-split proinsulin remained in the beta cell secretory system (ER, GA, SV) as an independent diabetogenic mechanism that supplementary decreases insulin secretion was neglected until now.

Art. 38 – Our hypothesis is that the increase of intra β-cell proinsulin (reflected also in its increased plasma concentration) could represent the trigger for the anti β-cell autoimmunity in T1DM. Indeed, increased proinsulin will operate in subjects carrying the genes for the immune defect characteristic for T1DM, leading to an autoimmune process with increased beta cell apoptosis and inefficient beta cell regeneration (see 47,53). In the absence of immune defect, the increase in plasma proinsulin is supposed to be well tolerated when the increases in proinsulin are intermittent (during periods of increased secretory work-load) or when it is not surpassing a critical threshold. Without having definite proofs in this respect, we suggest that its negative effect will operate stronger on the most sensitive beta cell process: its regeneration and/or survival 69,138. Even if increased proinsulin will not influence beta cell apoptosis, decreased beta cell regeneration can be enough in order to trigger the progressive beta cell mass decrease 67. This decrease was neglected due to the lack of practical methods for its correct assessment and quantification 69. Evaluation of the beta cell function by laborious techniques 63 evidenced numerous defects present many years before the onset of alteration in blood glucose regulation. In this context, as we noted before, it is surprising that hyperglycemia remains the main (and maybe only) marker for diabetes mellitus, despite the fact that at its onset at least 50% of the beta cell mass/function is already irretrievably lost 66-69, 71.

Art. 39 – A supplementary argument for the pathologic/pathogenic significance of increased plasma proinsulin, this time reflected on the peripheral tissues, is represented by its correlation with the peripheral vascular disease 139. If this effect on blood vessels is real and proven, it can represent an argument for our hypothesis that increased intra β-cell proinsulin negatively influences the function and survival of the pancreatic beta cells themselves. In addition, the change of the proinsulin/proamylin or insulin/amylin ratio inside β-cells can represent the basis for the biophysical mechanisms that favor the oligomerisation of amylin and trigger the amyloidogenic process 57,58,60,140,141.

Art. 40 – In 2006 when, based on the increased plasma proinsulin levels recorded in various diabetes phenotypes, I advanced the hypothesis that increased proinsulin could express the primary β-cell defect in processing the secretory molecules (proinsulin and proamylin) with its primary site in the ER 55, I didn’t think that very quickly several confirmatory arguments will come from various directions. The first was brought by Loos et al. 142 which established the correlation between TCF7L2, gene and plasma proinsulin levels. Also in 2007, Rulifson et al. 143 provided details regarding the role of the Wnt signaling system in the regulation of both beta cell proliferation and beta cell secretory function. These data has been completed and strongly amplified in the review of Jin 144,145 pertinently commented by Gustafson & Smith 146 in the same issue of Diabetologia. The relationship between TCF7L2 and proinsulin has been confirmed in other three papers published in 2007 and 2008 136,137,146. The group of Serrano-Rios 136 reminded that TCF7L2 could explain the alteration in processing of beta cell secretory proteins by decrease of the GLP1 production, noted in the carriers of TCF7L2 risk alleles 144,147,148. Since TCF7L2 is well expressed both in the pancreas and the adipose tissue, we should consider the existence of a parallel defect in the pancreatic beta cell and in adipocytes. This could be supported also by the fact that Wnt signaling system influences both the survival of beta cells and adipogenesis 144,145. Since this system operates also in the entero-insular axis, including liver, it could mediate the genetically determined functional relationship existent between the three insulin dependent cellular blocks belonging to the system that regulates the energy metabolism.

THE ARGUMENTS FOR ENDOPLASMIC RETICULUM AS THE MAIN LOCATION OF β-CELL DEFECT

Art. 41 – ER is a labyrinthic, canalicular and sacciform structure placed between the cell nucleus (with whom it communicates by numerous orifices) and the Golgi apparatus, which continues ER and opens in the cytoplasm. It is the most active cell compartment, the factory where the crude molecules translated in the ribosomes attached to its walls are subjected to numerous post-translational modifications. Following these
changes (folding, twisting, packaging), the initially translated linear peptide is shaped finally in the “mature” three-dimensional molecule, *i.e.* the molecule able to perform its specific physiologic function.149

Art. 42 – Even if we shall refer only to the post-translational changes of the two secretory molecules (pre-proinsulin and pre-proamylin), it is worthy of know that inside ER are processed all the molecules (~3000 different types) of the beta cell, including those that build the cell structures such as ER, GA, SV, mitochondria, lysosomes, peroxisomes, enzymes, cell cytoskeleton and cell membrane. All these different molecules suffer the same post-translational processing and are subjected to the same system of quality-control.

Art. 43 – The main functions of the ER are performed by various “functional” structures with particular physiologic properties: chaperones, enzymes, ion channels and transporters, as well as sensing and motor proteins. In order for the ER to function in optimal conditions, the internal milieu of this “living” cell structure is tightly controlled in regard to pH, temperature, ion concentration (especially Ca$^{2+}$ and Zn$^{2+}$), physical pressure and physical space required for the processing of different molecules. All these processes take place in conditions of high molecular transit.

Art. 44 – The maintenance of correct post-translational function in conditions of increased molecular flux, the ER possesses a homeostatic mechanism called *Unfolding Protein Response – UPR* (Fig. 2).119,120,150-153 When molecule processing becomes defect, the control system (BiP chaperone) de-blocks 3 molecules that will activate three different protective pathways: 1) they will induce an *attenuation of translation* in ribosomes in order to decrease the influx of new molecules inside the ER; 2) they will activate the *production of chaperones* that will be involved in the activation of proteins processing and finally; 3) they will activate *proteolytic machinery*, thus physically making more free space and proper physico-chemical conditions for a normal molecules processing. If the UPR reaction will not succeed to re-establish homeostasis inside ER, allowing for the correct post-translational processing of molecules, from within the ER itself will arise a *pro-apoptotic signal* represented by the expression of CHOP protein.154,155 This protein will enter the nucleus and will trigger (*via* the caspases pathway) the denaturation of nuclear DNA followed by the regulated cell destruction. Normally, the apoptotic cell will be replaced with a new cell by the parallel activation of the cell replication process.156

![Fig. 2. The UPR mechanism begin with activation of the three regulatory pathways starting by de-blocking the PERK, ATF6 and IRE1 molecules, previously kept inactive by their binding by the chaperone BiP. The results of these 3 pathways will be the attenuation of the new protein translation, the stimulation of chaperone production and the increase proteolysis of the unfolded or misfolded proteins. If these reactions couldn’t reestablish the homeostasis inside ER, the CHOP apoptotic pathway will be activated.](image-url)
Art. 45 – The ER dysfunction from the pancreatic beta cell has a particular place in the pathogenesis of diabetes since the beta cell is a highly specialized cell that functions continuously, with a rhythm imposed by the various changes in the circulating fuels concentration. For example, in conditions of important secretory demand (for instance in the postprandial state), ~50% of translated molecules that are processed in the ER are pre-proinsulin and pre-proamylin molecules. Each minute, the ER from the beta cell will be transited by ~1 million insulin molecules. This is a huge workload since the three-dimensional structure of the two mature molecules (insulin and amylin) is important for their physiological functions. It is hypothesized that the anatomic and functional capacity of the ER is limited despite the fact that their precise limits are hard to define and it is thought that there are substantial inter-individual differences. This view correspond in fact with the concepts of „thrifty genotype“ and „thrifty phenotype“. These theories suggest that the genetic program for the energy metabolism regulation system was adapted to the environmental conditions from the old ontogenetic period or closer phylogenetic period during fetal development. Both adaptations will influence the functioning of the metabolic assembly that includes mainly the pancreatic beta cell and the three insulin-dependent cellular blocks (liver, muscles, and adipose tissue).

Art. 46 – In normal environmental conditions, diabetes will appear either in the presence of a precise and well-defined monogenic defect of the beta cell or in the presence of multiple, polygenic defects that perform their diabetogenic effect only by cumulative addition. The genetic factors associate with the environmental factors promoted by the modern civilization, inducing a constant increase in diabetes incidence. This sort of civilization has in its basic socio-economic concept two strong pathogenic roots with additive effect: (a) increased caloric/lipid intake in comparison with the energy expenditure and (b) decreased physical activity. The excess of circulating fuels from the energetic system leads to obesity and many degenerative diseases and the senescence process. Their common pathogenic mechanism pointed out towards the post-translational changes of molecules that take place inside ER/GA, where we think it can be found also the end (or maybe the tip) of the diabetogenic thread.

Art. 48 – The direct study of the ER function in vivo and in situ is difficult since this cell structure is under continuous workload and molecular processing. However, some immuno-histochemical studies performed on human islets obtained from organ donors could evidence in diabetic patients an increased density volume of ER vs. non-diabetic subjects. Is considered that ER size is an early adaptive response to ER stress. Such increased ER density volume has been also noticed in the beta cells of New-Zeeland obese mice during the onset of T2DM. The same alterations are registered in the Akita mouse diabetes model. In the β cell of these mice, the volume density of ER was increased by 1.7 fold, which is similar with the data reported by Marchetti et al. in human diabetic beta cells obtained from multiorgan donors. Since among the markers of beta cell apoptosis ER stress proteins (BiP, CHOP) are well expressed, the alteration of ER could be seen as a pathogenic process preceding β-cell apoptosis.

Art. 49 – Amyloid was first described more than a century ago by Ehrlich & Ratner in 1961 due to its resemblance with amilopectin. It remained more a histologic curiosity frequently encountered in different heterogeneous clinical circumstances and was considered to be a non-specific change with a poorly defined systemic origin. After more than two decades of interest for the study of amyloid by the term of hyaline and named amyloid in 1961 by Ehrlich & Ratner due to its resemblance with amilopectin. It remained more a histologic curiosity frequently encountered in different heterogeneous clinical circumstances and was considered to be a non-specific change with a poorly defined systemic origin. After more than two decades of interest for the study of amyloid, Westermark et al. identified in 1986 in the islet fibril amyloid a new 37 amino acid peptide belonging to the family of peptides related to calcitonin gene. This molecule was cloned next year (1987) both by the team of Westermark, and that of Cooper et al. The first names this molecule Islet Amyloid Poly Peptide (IAPP), and the second names it amylin, term that we prefer due to semantic reasons.
Art. 50 – The two secretory lines: pre-proinsulin / proinsulin / insulin and the pre-proamylin / proamylin / amylin, have a similar intra-beta cell pathway. The two final mature molecules (insulin and amylin) are present in the SV and are co-exocytosed together. Moreover, the two secretory pro-molecules are split into the final products by the same enzymes (PC2, PC3 and CPH)

Art. 51 – Between the two secretory lines exist however some differences that render more difficult the precise understanding of the correlation between the two. The very good interspecies conservation of amylin molecule suggests an important physiologic role. However, its extra β-cell effects (inhibition of orexigenic hypothalamic centers, inhibition of gastric motility and secretion) are apparently minor, fact suggested also by the uncertainties regarding the specificity of its peripheral receptors. All these findings suggest that its major role should be sought moreover inside the β-cell. We consider that this role is to protect the main beta cell secretory product, insulin, and helping formation of zinc – insulin hexamers in the core of vesicles. This function is suggested also by the 100:1 ratio existent between the two secretory lines. The smaller number of amino-acids in amylin in comparison with insulin (37 vs. 51), its different tri-dimensional shape (somehow linear in comparison with the globular shape of insulin) suggest that amylin plays rather the role of chaperone (protector) for insulin than secretory product per se.

Art. 52 – If the physiologic function of amylin is still uncertain, its involvement in the activation of pro-apoptotic beta cell mechanisms revolutionized both the pathogenesis of T2DM, and the pathogenesis of some apparently unrelated diseases that were recently included in the group of "conformational diseases". These have the same molecular basis in the fibrillary amyloidic transformation of some structurally related proteins. In the pancreatic beta cell, the presence of the hydrophilic amino-acid sequence from position 25–29 (Ala-Ile-Leu-Ser-Ser) renders amylin molecules, in still unclear conditions, to bind one to another by a zipper type mechanism. This first association between two molecules triggers a process by which an important (but still not very high) number of molecules will attach one to another at the level of the 5 amyloidogenic amino-acids. This process leads to the generation of the toxic amylin oligomers or protofibrils that form inside cell a compact molecular block capable to induce a strong proapoptotic process. Irrespective of the place where they are generated (inside the SV or outside the SV, but inside the beta cells) the toxic oligomers release the apoptotic signal that is retrograde transmitted towards the cell nucleus via the CHOP molecule (originating from the ER) that activates the caspases 12 and 3. It was suggested that the initiation of the proapoptotic beta cell mechanism could be explained by a decreased action of matrix-proteinases 2 and 9 with the role to mediate proteolysis of the excess of normal or miss-folded fibrils.

Art. 53 – One of the intriguing feature of amilloydosis is the large variation in the number of islets affected in this process and the amount of amyloid which sometime is quite impressive. These might be related to the anatomic and functional heterogeneity of the pancreatic islets and beta cells, reflected at the anatomic-histological level by the scattering of amyloid deposits inside the islets and around the beta cells. Lessons learned from the various transgenic rodent models, using hIAPP (see) provided important information for the understanding of the pathogenic mechanism operating in human beta cells. Somehow it was established that the extra-beta cell amyloid has a different significance that could be non-pathologic, as suggested by the presence of islet amyloid deposits (it is true that with a small extent) in non-diabetic subjects. On the other hand, the theory of the intra beta cell toxic amylin oligomers pathogeny gained strength. Using transgenic mice for hIAPP, scientists could replicate the chain of pathogenic events that take place in the human pancreatic β-cell: the amyloidogenic process starts inside the pancreatic β-cell, initially inside the secretory pathway structures (ER/GA/SV), afterwards in the cytoplasm and only finally extracellularly.

Art. 54 – It was suggested that the mechanism for the toxic amyloid oligomers generation is related to ER stress. So, in conditions of exaggerated insulin demand, amylin can be disproportionately overproduced. This will...
generate a competition between the two secretory lines, as the same convertases mediate the final transformation of proinsulin into insulin and proamylin into amylin, respectively (Fig. 3). In obesity, the constant insulin secretory demand induced by the increased mass of insulin-dependent cells will expose the ER from the pancreatic beta cell to a continuous UPR but with an intensity below the threshold for triggering the beta cell apoptotic process. In these conditions, the UPR mechanism succeeds to attenuate the translation in ribosomes of new protein molecules, and to stimulate the production of chaperones required for the processing of secretory molecules and succeeds to degrade the miss-folded proamylin / proinsulin molecules thus limiting the oligomerization of amylin. In another set of obese subjects, with a limited functional capacity of ER, the pathogenic threshold can be reached in conditions of supplementary secretory demand, as encountered for example in conditions of increased intake of animal lipids. It is known that the saturated fatty acids have an inhibitory effect on the function of ER and diminish the action of protein convertases from the ER/GA/SV, both that will influence the processing of secretory molecules with the final effect of increased toxic amylin oligomers production. Their generation will activate the proapoptotic pathways due to the surpassing of the physiologic UPR reaction capacity for compensation. Scheuner și Kaufman underline the paradox of UPR that the response leads simultaneously alteration of both physiological (adaptive) and pathological (proapoptotic) pathways (Fig. 2). The proapoptotic mechanisms are tagged by ATP or calcium depletion, the increase in oxidative stress, altered glycosilation, saturation of folding capacity or increase in misfolding protein.

Art. 55 – There are some similarities in spatial configurations of various toxic amyloid oligomers in different cells. It is suggested by structural similarities between various toxic oligomers specific antibodies, despite their different cellular targets (i.e β-cells or neurons). In human-amylin transgenic mice, hIAPP toxic oligomers are detected intracellularly only. Lack of human pancreatic bioptic fragments makes these oligomers hard to be identified in humans. However they were found intracellularly in beta cells from human pancreases resected for insulinoma.

Fig. 3. 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.
THE GENETIC ARGUMENTS FAVORING THE ER ROLE IN THE PATHOGENESIS OF DIABETES

Art. 56 – Our hypothesis, that the primary beta cell defect in the processing of secretory pro-molecules has its origin inside the ER and progresses subsequently towards the GA and SV, received the first important confirmation from several genetic studies. The presence of non-classical genetic defect expressed in ER can explain both the increase of plasma proinsulin recorded by us in all diabetes phenotypes, and the amyloid transformation of amylin with peri-β cellular deposits in almost all T2DM patients. Both alterations can trigger pro-apoptotic beta cell mechanisms if the level of un-split molecules surpasses a critical threshold.

Art. 57 – At least 3 genes encoding structural or functional molecules from ER have been identified in association with some rare forms of diabetes: (a) Wolframin is a protein of 890 amino acids involved in maintaining calcium homeostasis inside ER. A monogenic defect in its gene (WFS1, on chromosome 4p) has been detected in Wolfram syndrome, known also as a DIDMOAD syndrome (diabetes insipidus, diabetes mellitus, optic atrophy and deafness). This complex syndrome is explained by the expression of wolfram in several cell types, especially in the pancreatic β-cells and neurons from the brain. In addition, the deletion of WFS1 in animal models is associated with decreased β-cell mass due to uncontrolled ER stress. Recently, a polymorphism has been detected in WFS1 gene in association with T2DM. This shed a new light in the role of ER from β cell in T2DM.

(b) PERK (Pancreatic ER Kinase) is a transmembrane protein that senses ER stress signals through its N-terminal/ luminal domain. This molecule is kept in a monomeric inactive form by its binding with the chaperone BiP inside the ER. PERK molecule has an important role in the UPR reaction. A genetic defect in PERK molecule has been associated with a reduced β-cell mass due to uncontrolled ER stress. The mutations reported (His to Asp or in position 10 of the B chain or a substitution of Arg65 with His, Pro or Leu) lead to a defect in proinsulin processing inside the ER. Other small defects in the processing proinsulin molecules inside the ER has been reported in the sand rat Psammomys obesus, or in the obese-fat mice and only occasionally in humans.

Art. 58 – Another genetic argument supporting the essential role of the anatomo-functional integrity of ER is provided by the Akita mouse diabetes model. In this model, the genetic defect was identified in a single mutation (Cys96Tyr) in the amino acid sequence of the proinsulin molecule. This mutation affects one of the disulphide bonds inside the proinsulin molecule, preventing its proper processing and packaging. The most impressive morphological changes were found in the ER which is blocked by the agglutinated unprocessed proinsulin molecules. The secretory vesicles, occasionally generated in the GA, contain high amounts of proinsulin. The level of plasma proinsulin is also very high. Diabetes in the Akita mouse model is a severe form of diabetes, induced by the progressive decrease in β cell mass. A genetic defect as that encountered in the Akita mouse was not reported in humans, but it offers an useful information on the great importance of the processes which take place in the ER. Other small defects in the processing proinsulin molecules inside the ER has been reported in the sand rat Psammomys obesus, or in the obese-fat mice and only occasionally in humans.

Art. 59 – In humans, the familial hyper-proinsulinemia is a rare form of “insulinopathy” which is associated with a minor diabetogenic potential. The mutations reported (His to Asp in position 10 of the B chain or a substitution of Arg65 with His, Pro or Leu) lead to a defect in proinsulin processing accompanied by high proinsulin plasma levels. In other various types of “insulinopathies” (described several decades ago), quite often diabetes is not part of the clinical presentation, since the involved amino acids don’t alter significantly the structure and function of insulin molecules.

Art. 60 – The monogenic defects in the ER functional molecules doesn’t represent an argument to sustain their involvement in the common diabetes phenotypes since they are very rare in humans and lead usually to severe forms of occurrence of a severe β-cell dysfunction; (c) A recent genetic study, analyzed the ATF6 (Activating Factor 6) gene on 1q, gene that encodes a molecule that functions as a proximal sensor in the ER (Fig. 3). This study showed that ATF6 is likely associated with T2DM. It is worthy to note that ATF6, as well as Wolframin and PERK molecules, has an important role in the UPR reaction.
disease, such as the Wolfram or Wolcott-Rallisson syndromes. Their rarity expresses the efficacy of natural selection processes. We could hypothesize that some of the aborted fetuses from diabetic mothers could carry some monogenic defects in some of the essential cell structures (such as the ER) that are not compatible with survival.

Art. 61 – We consider that in the common phenotypes of T2DM, another type of gene architecture should be actively sought, for example one which influence the maximal functional capacity of the ER. We think this could involve rather not the quality of the “components” of the ER from the beta cell (and also adipocyte or other insulin-dependent cells) but mostly the sheer volume of the whole ER assembled from these fragments. In this respect, our hypothesis meets the concept of „thrifty genotype” proposed by Neel \(^{84}\), applied to the capacity of the ER to process the specific secretory molecules of the beta cell (proinsulin and pro-amylin), of the adipocyte (leptin, adiponectin or other adipokines) essential for the function of these cells. Involvement of the ER stress in the apoptosis of the beta cells \(^{118,120}\), or adipocytes \(^{33}\), suggest that the chronic dysfunction of these cells could represent a diabetogenic mechanism operating at least in some insulin-dependent cells.

Art. 62 – Our hypothesis regarding the limited capacity for processing of functional molecules in the ER provides a logical explanation for the intervention of diabetogenic environmental factors (decrease of physical activity and increased intake of calories/lipids) on the incidence of diabetes mellitus, mediated mostly by weight gain. Weight gain precedes the onset of diabetes \(^{83}\), while weight loss is associated with regression or even complete stop of the diabetogenic process \(^{11,100}\). Very interesting are the data of Lindeberg et al. \(^{203}\) demonstrating that the use of a “Paleolithic diet” in our days has an evident effect on the decrease of the insulin-secretion demand, in a way similarly with the effects of bariatric surgery \(^{11,100}\). Both diabetogenic factors (obesity and the modern hypercaloric diets) can lead to an increased insulin-secretion demand with which, the beta cell cannot cope for a long time.

Art. 63 – Chronically elevated proinsulin inside the pancreatic beta cell will decrease the survival of these cells by increased apoptosis and delaying the physiological processes of beta cell regeneration. The decrease in beta cell mass can be explained not only by the presence of some major genetic defects, detectable by the current methods, but also by the discrepancy between the limited functional capacity of the ER and the increased workload it is subjected to. Although this capacity is genetically determined, the final elucidation would require the identification of the genetic architecture for the whole anatomo-functional system that controls the energy homeostasis: pancreatic beta cell and the three blocks of insulin-dependent cells.

ENDOPLASMIC RETICULUM STRESS IN NON-INSULIN DEPENDENT CELLS

Art. 64 – Similar amyloidogenic mechanisms operate from structurally related proteins from cortical neurons (Alzheimer disease), dopaminergic neurons (Parkinson disease), again cortical neurons (prionic fongiform encephalopathy), motor neurons (lateral amyothrophic sclerosis) or pyramidal neurons (Hungtinton disease) \(^{61}\). In all these conditions, the molecular aggregation is based on the pathogenic ability of some molecules from the above mentioned cells to form in aqueous medium amyloid fibrils. The interest for the study of “conformational diseases” increased exponentially since all these diseases are frequent and severe. Even if between the pancreatic beta cell and neurons there are some phenotypic resemblances that confer to the beta cells characteristics of excitable cell \(^{49}\), the fundamental functions of the two cellular types are essentially different. The nervous cell accomplishes its regulatory function by neuronal electrical impulses while the beta cells perform their regulatory function by insulin secretion. This makes that the progresses recorded in one field (in the study of Alzheimer or Parkinson diseases) cannot be extrapolated in the interpretation of T2DM pathogenesis. A common element is, for example, that both diseases appear more frequently in more advanced ages, a fact that connects their pathogenesis to the senescence process \(^{61,204}\).

Art. 65 – The common element for all these “conformational diseases” is the loss of functional cells (neurons, beta cells) due to an apoptotic process. The final effect will be more severe when this process takes place in the neurons, whose regeneration ability is practically null, while in T2DM, even in more advanced stages of its evolution, remains still a mass of more or less functional beta cells due to the intervention of the cell repair (regeneration) process.
ENDOPLASMIC RETICULUM DYSFUNCTION IN INSULIN DEPENDENT CELLS

Art. 66 – A strong proapoptotic ER stress in β-cell leading to a decrease in β-cell mass/function will be automatically reflected in the 3 main insulin-dependent cells, starting in adipocyte, and then in cascade in hepatocyte and miocyte. The chronic excess of fuels from the energetic system will lead to an increased secretory beta cell demand that will become permanent when the triglycerides deposits from the adipose tissue will surpass the physiologic threshold, variable from person to person. The first major regulatory solicitation will appear in the ER from the beta cell that has to secrete more insulin in order to operate a functional adjustment in all the three insulin dependent cells: hepatocyte, miocyte and adipocyte. This functional adjustment is made by myogenic function of insulin, i.e. activating the transcription of a set of genes from all the three blocks of cells; this means a supplementary functional effort in each of these cells. Reduced whole body lipid oxidation in offsprings of T2DM patients\(^7,10,24,25,81,82,101,102\). Reduced whole body lipid oxidation in offsprings of T2DM patients\(^9\) and reduced mitochondrial content of skeletal muscles in subjects with a family history of diabetes might support the existence of an early mitochondrial dysfunction. However, the GWA studies didn’t reveal any genetic defects related with skeletal muscles or mitochondria\(^{41-44}\) involved in T2DM susceptibility. In this case, an alternative explanation for the reduced skeletal muscle oxidations could be related to the early insulin secretory defects which might operate through the various defects in FFAs utilization\(^82,90,91\) or to the increase in the intramyocellular triglycerides content\(^7,90,91,114\). These alterations could be secondary either to the insulin secretory defect or to the low physical activity, a characteristic of modern society.

THE CHRONOLOGY OF THE DIABETOGENIC EVENTS

Art. 69 – One of the most important sources for the elucidation of the primary cause of diabetes and the chronology of the pathogenic events involving various tissues is offered by the study of descendents from diabetic parents (offspring) or of non-diabetic first degree relatives of diabetic subjects. The secretory, metabolic, or anthropometric alterations revealed the precocity and wide variability of alterations. It is interesting to note that in these offsprings many disorders (recorded in offspring progressors to diabetes vs. non-progressors) in respect of BMI, body fat, TG, HDL-cholesterol, insulin, proinsulin, were not statistically significant in the moment of analysis (many years before the clinical onset). However, the constant deviation of the analyzed parameters, all in the same direction, and the high number of disorders with diabetogenic significance stress their important predictive value for the future evolution towards diabetes. In addition, the wide variety of disorders suggests that not only beta cell dysfunction is precocious but also the defects induced in the three blocks of insulin-dependent cells are also precocious. This source of information was not yet fully exploited.

Art. 70 – Similar biochemical/hormonal changes can be found precociously in the obese adolescents\(^{205}\). When analyzing adolescents with increased BMI...
according to the tertiles of the visceral adipose mass, those from the third tertile (in comparison with those from tertile 1) had significantly increased levels of plasma insulin (41.3 vs. 29.4 µU/ml), plasma proinsulin (27.5 vs. 19.1 pmol/l), plasma C peptide (1.4 vs. 1.0 pmol/l), triglycerides (103.9 vs. 71.6 mg/dl) and decreased HDL-cholesterol (41.8 vs. 45.4 mg/dl). Although not significant, increases of FFAs (545.7 vs. 463.8 µmol/l) and decreases of adiponectine (6.2 vs. 8.1 µg/l) were also recorded. We should mention that these data include results from both the obese adolescents that will evolve to diabetes and those that will not evolve towards diabetes. As in the cases of diabetic parent’s offspring, this group included adolescents with normal glycemia, considered according to the current diagnosis criteria as being non-diabetic. It is interesting to note that differently from the obese adults, for whom the markers of inflammation are strongly expressed, in this group of obese adolescents they were absent.

Art. 71 – A part of metabolic defects recorded in first degree relatives of T2DM, is related to so called „metabolic inflexibility” of skeletal muscle, which reflect impaired switching of substrate oxidation from lipid to carbohydrates and reduced mtDNA content in skeletal muscles. These metabolic defects might be related to the high fat intake and low physical activity in the presence of an intrinsic genetic defect which has not been detected during WGA studies.

GENETIC VERSUS ENVIRONMENTAL FACTORS

Art. 72 – The genetics of diabetes has been one of the most debated topics because attempts to explain its heritability, known from a long time, couldn’t be included in the frame of Mendelian model of transmission. The progresses made in molecular genetics have lead to the identification in 1973–1974 of the beta cell autoimmunity genes by which was the main and strong argument of the immunogenetic theory of this phenotype. Along the time, the number of genes associated with this phenotype increased, but the genetics of T1DM remains an open topic. At least for this diabetes phenotype, the past genetic “nightmare” evolved into an only annoying dream. In contrast, the genetic “nightmare” was transferred in the field of type 2 diabetes. The genetic data in this last phenotype could be considered provisional having in view the high movement in the field, each month appearing new and sometimes very useful information.

Art. 73 – Some success in deciphering the genetics of T2DM has been preceded by the discovery of 6 monogenic forms of diabetes known today as MODY types. Completion of the human genome project in 2003 and the Haplotype Mapping (HapMap) Project in 2005 opened the way for the GWA studies, not only for diabetes, but also for many common diseases. The explosion of studies on genetic of diabetes counted by Khoury to over 170 in 2007–2008, was accompanied by many review articles. After the inventory mentioned by Khoury at 1st August 2008, 431 genes have been studied in relation with T2DM with more than 1340 publications and 50 meta-analysis, as well as 15 GWAS.

Art. 74 – Maturity Onset Diabetes of the Young (MODY) is a heterogeneous group of diabetic disorders. Clinically, MODY is characterized by nonketotic diabetes resulting from a primary defect of beta cell function, onset usually before the age of 25, frequently in childhood or adolescence, and presentation usually as mild asymptomatic hyperglycemia in non-obese subjects. The 6 classical MODY types are monogenic forms for which the gene defects affect beta cell proteins, given in Table 1. Genetically, MODY is characterized by a monogenic autosomal dominant pattern of transmission. Multigenerational involvement is common. In the recent years, MODY has been an invaluable model for genetic and physiologic studies of diabetes. Genetic studies of MODY have led to the identification of genes that play a key role in the development and function of beta cells. The lessons learned from the study of MODY also improved the understanding of the insulin secretory defect in type 2 diabetes. MODY phenotypes can explain ~2–5% of the total number of diabetes cases.
Table 1
Genetic defects associated to the various MODY phenotypes

<table>
<thead>
<tr>
<th>MODY type</th>
<th>Gene</th>
<th>Chromosome</th>
<th>Gene function</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>MODY 1</td>
<td>HNF-4α</td>
<td>20q12-13.1</td>
<td>Transcription Factor/Nuclear Receptor</td>
<td>Neonatal hyperinsulinism Diabetes (early adulthood)</td>
</tr>
<tr>
<td>MODY 2</td>
<td>Glucokinase</td>
<td>7p13-15</td>
<td>Hexokinase IV</td>
<td>Mild hyperglycaemia (in early childhood)</td>
</tr>
<tr>
<td>MODY 3</td>
<td>HNF-1α (TCF1)</td>
<td>12q24.2</td>
<td>Transcription Factor / Homeodomain</td>
<td>Diabetes (early adulthood)</td>
</tr>
<tr>
<td>MODY 4</td>
<td>IPF-1</td>
<td>13q12-1</td>
<td>Transcription Factor / Homeodomain</td>
<td>Diabetes (pancreas agenesis in homozygote)</td>
</tr>
<tr>
<td>MODY 5</td>
<td>HNF-1β (TCF2)</td>
<td>17q21.3</td>
<td>Transcription Factor / Homeodomain</td>
<td>Diabetes, RCAD Pancreas hypoplasia</td>
</tr>
<tr>
<td>MODY 6</td>
<td>Neuro D1</td>
<td>13q</td>
<td>Transcription Factor</td>
<td>Diabetes (infancy and early adulthood)</td>
</tr>
</tbody>
</table>

Recently, were described MODY forms induced by mutations of INS (insulin gene) and CEL (encoding the lipolytic enzyme carboxyl-ester lipase). Additional unknown MODY loci (MODY-XX) may represent, depending on the population studied between 11%43 and 50% of the cases, being more prevalent in German and Spanish families44.

Art. 75 – Diabetes diagnosed in the first few months (usually before 6 moths) of life is defined clinically as neonatal diabetes. Neonatal diabetes mellitus is very rare, with incidence of ~1 case per 300,000-to-500,000 live births218. Neonatal diabetes can be stratified in permanent neonatal diabetes mellitus (PNDM) or transient neonatal diabetes mellitus (TNDM), depending on whether the diabetes resolves in time. The available, combined data indicate that somewhat over half (~57%) of neonatal diabetes cases are transient, require insulin treatment initially and spontaneously resolve in less than 18 months, only to relapse in later years. Neonatal diabetes is part of the group of monogenic diabetes. While TNDM is usually associated with mutations at 6q ZAC locus43, PNDM is usually associated with monogenic defects of K<sub>ATP</sub> channels, both Kir6.2 subunit encoded by KCNJ11210 and SUR2 subunit encoded by ABC28220, or proinsulin gene43. Rarely, PND is associated with homozygote mutations in GCK (glucokinase gene), HNF-1β, IPF-1, PTF-1A (Pancreas Transcription Factor 1α) or other genes43,44. All these forms have a variable clinical severity and interestingly, some of them (those induced by K<sub>ATP</sub> channel defects, encoded both by KCNJ11 or ABCC8) can be treated with sulphonylureas221.

Art. 76 – Other monogenic forms of diabetes include rare genetic syndromes whose phenotype include in familial forms of diabetes associated with extra-pancreatic anomalies. In this group we could include: 1) Diabetes and Pancreatic Exocrine Dysfunction (DPED) associated with single-base deletions in the carboxyl ester lipase (CEL) gene44; 2) Diabetes associated with mitochondrial defects – Maternally Inherited Diabetes and Deafness (MIDD). This form was associated with a 10.4 kb deletion in the mitochondrial genome44 or with an A to G transition at base-pair 3243 affecting tRNA (Leu) 43,44. Prevalence studies have suggested that 3243 A/G mutation accounts for ~1–2% of diabetes in Japanese and 0.2–0.5% in European series221; 3) Finally, diabetes associated with the Wolfram Syndrome, also known as DIDMOAD, which is a rare progressive neurodegenerative disorder, inherited in an autosomal recessive manner, associated with mutations of WFS1 gene on chromosome 4p16.1 that encodes (as we discussed above) an 890-amino-acid polypeptide named wolframin.

Art. 77 – The lifelong T1DM risk in the general population is 0.4% but increases to 6% in the first degree relatives of T1DM subjects222. The 15 times higher risk in first degree relatives demonstrate the strong family aggregation of T1DM cases and sustains the importance of genetic/hereditary factors for this diabetes phenotype. Genetically speaking, T1DM is a complex, polygenic disease, with many predisposing/ protective gene variants, interacting with each other in generating the global genetic disease risk 223. Historically, the study of candidate genes in T1DM identified the major two susceptibility genes for T1DM: IDDM1 encoded in the HLA region of the Major Histocompatibility Complex (MHC) on chromosome 6p21214,224,225.
and mapped to the DRB1, DQB1 and DQA1 loci and IDDM2 encoded by the insulin gene region mapped to the VNTR region 5’ of the insulin gene on chromosome 11p15. The same candidate gene approach also revealed the association of other three loci, all with smaller contributions to T1DM susceptibility: IDDM12 – the CTLA4 (Cytotoxic T Lymphocite Associated Antigen 4) gene on chromosome 2q33, the PTPN22 (Lymphoid Tyrosine Phosphatase 22) gene on chromosome 1p13 and the IL2RA/CD25 gene region on chromosome 10p15. Using linkage analysis strategies by whole genome scanning (GW Linkage Studies), some other regions of the human genome were linked with T1DM but none of the putative diabetogenic genes from these regions has been identified yet. Finally, in the last two years, the Genome Wide Association (GWA) studies led to the identification of a sixth T1DM gene, IFIH1 (Interferon Induced Helicase 1) on chromosome 2q24, as well as other four T1DM associated chromosome regions – 12q13, 12q24, 16p13 and 18p11 – for which the identification of the causal genes is still not elucidated.

Art. 78 – The classical form of T2DM, with its various subtypes (normal weight vs. overweight for example), was associated with multiple gene defects. As for the common T1DM phenotype, in T2DM also susceptibility loci were identified by studying candidate genes or by scanning the whole genome using linkage (GW Linkage) or association (GWA) approaches. The candidate gene approach included traditional candidates, such as the K<sub>ATP</sub> – KCNJ11, AdipoQ (adiponectin) or PPARγ loci, as well as candidates revealed by the study of the monogenic forms of diabetes (KCNJ11, HNF1β, HNF4α, WFS1). Currently ~17 loci (Table 2) were confirmed in various studies, including Candidate Gene Studies, Large Scale Association Studies or GWA’s (Genome Wide Scan Association). This is only a provisory figure that explains <5% of the overall risk of T2DM from the genetic basis of this polygenic disease. Currently, common risk variable for T2DM do not provide strong predictive values at population level. Tens or maybe hundred novel susceptibility genes can be identified in future in association with T2DM, taking carefully into account the ethnic origin of this population.

Table 2
The first 17 loci with the proven role in T2DM genetic susceptibility (Adapted after43)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chr</th>
<th>SNP</th>
<th>Risk allele freq</th>
<th>Effect size</th>
<th>Method</th>
<th>Hypothesized Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARG</td>
<td>3</td>
<td>Rs1801282</td>
<td>0.85</td>
<td>1.23</td>
<td>Candidate gene</td>
<td>Adipocyte differentiation and function</td>
</tr>
<tr>
<td>KCNJ11</td>
<td>11</td>
<td>rs5219</td>
<td>0.40</td>
<td>1.15</td>
<td>Candidate gene</td>
<td>β-Cell K&lt;sub&gt;ATP&lt;/sub&gt; channel</td>
</tr>
<tr>
<td>TCF7L2</td>
<td>10</td>
<td>Rs7901695</td>
<td>0.40</td>
<td>1.37</td>
<td>Large-scale association</td>
<td>Incretin signaling in the islet</td>
</tr>
<tr>
<td>WFS1</td>
<td>4</td>
<td>rs10010131</td>
<td>0.60</td>
<td>1.11</td>
<td>Large-scale association</td>
<td>Endoplasmic reticulum stress</td>
</tr>
<tr>
<td>TCF2/HNF1B</td>
<td>17</td>
<td>Rs757210</td>
<td>0.43</td>
<td>1.08</td>
<td>Large-scale association</td>
<td>β-Cell development and function</td>
</tr>
<tr>
<td>HHEX</td>
<td>10</td>
<td>Rs5015480</td>
<td>0.63</td>
<td>1.13</td>
<td>GWA</td>
<td>Pancreatic development</td>
</tr>
<tr>
<td>SLC30A8</td>
<td>8</td>
<td>rs13266634</td>
<td>0.72</td>
<td>1.12</td>
<td>GWA</td>
<td>Zn transport in β-cell insulin granules</td>
</tr>
<tr>
<td>FTO</td>
<td>16</td>
<td>Rs8050136</td>
<td>0.45</td>
<td>1.23</td>
<td>GWA</td>
<td>Hypothalamic effect on weight regulation</td>
</tr>
<tr>
<td>CDKAL1</td>
<td>6</td>
<td>rs10946398</td>
<td>0.36</td>
<td>1.16</td>
<td>GWA</td>
<td>β-Cell function and mass</td>
</tr>
<tr>
<td>CDKN2A/B</td>
<td>9</td>
<td>rs10811661</td>
<td>0.86</td>
<td>1.19</td>
<td>GWA</td>
<td>Cell cycle regulation in the β-cell</td>
</tr>
<tr>
<td>IGF2BP2</td>
<td>3</td>
<td>Rs4402960</td>
<td>0.35</td>
<td>1.11</td>
<td>GWA</td>
<td>mRNA processing in the β-cell</td>
</tr>
<tr>
<td>JAZF1</td>
<td>7</td>
<td>Rs864745</td>
<td>0.50</td>
<td>1.10</td>
<td>GWA</td>
<td>Transcriptional repression in the islet</td>
</tr>
<tr>
<td>CDC123/</td>
<td>10</td>
<td>rs12779790</td>
<td>0.18</td>
<td>1.09</td>
<td>GWA</td>
<td>Cell cycle regulation (CDC123)</td>
</tr>
<tr>
<td>CAMKID</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSPAN8</td>
<td>12</td>
<td>Rs7961581</td>
<td>0.27</td>
<td>1.09</td>
<td>GWA</td>
<td>Cell surface glycoprotein</td>
</tr>
<tr>
<td>THADA</td>
<td>2</td>
<td>Rs7578597</td>
<td>0.90</td>
<td>1.12</td>
<td>GWA</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>ADAMTS9</td>
<td>3</td>
<td>Rs4607103</td>
<td>0.76</td>
<td>1.06</td>
<td>GWA</td>
<td>Metalloprotease</td>
</tr>
<tr>
<td>NOTCH2</td>
<td>1</td>
<td>rs10923931</td>
<td>0.11</td>
<td>1.11</td>
<td>GWA</td>
<td>Pancreatic development</td>
</tr>
</tbody>
</table>

Constantin Ionescu-Tirgoviste
ARGUMENTS FOR THE UNITARY CHARACTER OF DIABETES

Art. 79 – The genetics of diabetes is important not only for understanding the pathogenesis of the various diabetes phenotypes (neonatal diabetes, MODY, T1DM, T2DM and gestational diabetes) but also in influencing the age for the disease onset, that can become apparent from birth until the older ages. Neonatal diabetes (with onset in the first 6 months of life) is induced by some monogenic defects of glucokinase, proinsulin or K\textsubscript{ATP} channels (KCNJ11 and ABCC8), rarely in other genes also \textsuperscript{42,44}. All these forms have a variable clinical severity and interestingly, some of them (those induced by K\textsubscript{ATP} channel defects) can be treated with sulphonylureas \textsuperscript{42}.

Art. 80 – By advocating the unitary nature of diabetes mellitus, we should explain the pathogenetic particularity of its autoimmune phenotype (T1DM), for which the gene defect is localized in several genes related to the immune system, rather than in beta cell genes. In fact, T1DM is a complex and heterogeneous disease resulting from the interaction between a small number of genes with large effects (for example HLA and insulin) and a large number of genes with small effects. This conclusion has been clear after the publication of the results of the first type 1 diabetes GWA scans \textsuperscript{238}. The most important genes for all subtypes of the autoimmune phenotype (mainly related to the age at onset) remain those located in the HLA region on chromosome 6p21 (IDDM1). For instance, it was found that the risk for islet autoimmunity drastically increased in DR3/DR4-DQ2/Q8 siblings who share both HLA haplotypes, identical by descent with their diabetic proband siblings (63% by age 7 years and 85% by age 15 years) compared with siblings who didn’t share both HLA haplotypes with their diabetic proband siblings. This is important since HLA genotyping at birth may identify individuals at very high risk for T1DM before the occurrence of clear signs of islet autoimmunity or overt diabetes \textsuperscript{241}.

Art. 81 – By analyzing the pathogenesis of T1DM, we can realize that, if autoimmunity is the predicate, the subject is in fact the pancreatic beta cell. Three arguments can be brought to support this statement: The first is related to the genetic basis that could not be completely identified. However, one fact remains: beside the autoimmunity genes, at least one gene (IDDM2 – pre-proinsulin gene on chromosome 11p15) indicates the beta cell as a major player in the autoimmune process. The involvement of this gene points out that, despite the fact that the major defect in T1DM is of the immune system, the target of autoimmunity is very precise – the pancreatic beta cell. It is the only one to be destroyed from the Langerhans islets. Although in cadaveric pancreases from subjects with long standing disease the islet architecture is so badly damaged that hardly someone can identify the various cell types, in the early stages of the disease the alpha, delta and PP cells are present and apparently they are not modified \textsuperscript{242}. The second is related to beta cell antigens. It is known that insulin has antigenic epitopes both on the A chain (in DR4 subjects, the susceptibility allele for T1DM is related with insulin epitope 1–15) and the B chain (amino-acid residues 9–23 and/or 15–23). All these epitopes are present also in the proinsulin molecule. In addition, proinsulin itself has some specific antigenic epitopes, located also on the B chain, amino-acids 22–23 or 24–36 \textsuperscript{112}. Moreover, Wagner et al \textsuperscript{243} proved that post-translational protein changes can potentially create new antigenic epitopes, which may trigger the autoimmune reaction induced by T lymphocytes hyper-reactivity. In our view, the incomplete processing and packaging of proinsulin inside the ER can be one of the explanations for the antigenic protein changes that can explain the initiation of the anti beta-cell autoimmune response. In this respect, it was shown that a post-translational change in the conformation of the A chain is sufficient to expose a new epitope that could be recognized by the T cells \textsuperscript{244}. Recently, autoimmunity against proinsulin has been studied in relation with its decreased expression in the thymus, a finding that could explain its increased antigenicity \textsuperscript{245,246,247}. Since in the chronology of the autoimmune anti beta-cell process the anti-insulin antibodies are the first to be identified \textsuperscript{248}, the secret of the anti beta-cell autoimmunity could be found in the interaction between the immune system and the main function of the beta cell, that to produce insulin from proinsulin. Finally, the third argument is related to the trigger for the autoimmune process. After 4 decades of intense research regarding the intervention of some various environmental factors (viral, chemical, nutritional, etc.), despite the sophisticated studies, no conclusive remarks could be drawn in this respect \textsuperscript{249,249,250}. In our previous papers \textsuperscript{47,53} we emitted the hypothesis regarding the potential role of increased beta cell
proinsulin, identified even in descendants of T1DM patients. If proinsulin and insulin are natural antigens, the increase of one or another inside the beta cell could represent the sought but not yet identified trigger for the anti-beta cell autoimmunity. Such a possibility is supported by the fact that the defect in proinsulin processing into mature insulin and C peptide is, perhaps, the most important event on the insulin-secretion pathway that takes place inside the ER. Moreover, one of the mechanisms for the beta cell apoptosis in T1DM could be triggered by the excessive and uncontrolled ER stress via the CHOP-caspase pathway leading to DNA disorganization and cell death. It is known that IL1-β and IFNγ induce ER stress through a chain of reactions such as NO-mediated depletion of ER calcium and inhibition of ER chaperones, inhibiting beta-cell defense and augmenting the apoptotic pathway. From this perspective, the pathogenesis of the two main diabetes phenotypes (T1DM and T2DM) is unitary not only by involving the beta cell, but also its most important secretory process, splitting of proinsulin into mature insulin and C peptide into the endoplasmic reticulum.

Art. 82 – The developments in the field of genetics are rapid, but useful information for clinical practical purpose is however still very low. The combined analysis of various known genes associated with diabetes in general population showed that the information offered by the presence of the genes polymorphisms related with T2DM, have a weak predictable ability. Of course, the subjects carrying more risk alleles had a higher risk of T2DM (with the figure for ROC of 0.60). This indicator is even lower than the risk indicated by age, sex and BMI (ROC of 0.78). This increases only to 0.81 by addition of genetic risk. From all these, it can be concluded that, for the moment, the genetic polymorphisms, only marginally improved the prediction of T2DM beyond clinical characteristics such as age, BMI and family history of diabetes. If more variants will be identified, tests with better credibility performance should become available for clinical practice. Currently, common risk variants for T2DM do not pose strong predictive value at a population level. The recent discovery of 17 genes involved in T2DM (Table 2) can explain a fraction lower than 5% of the overall risk of T2DM. Tens or maybe hundreds of susceptibility genes can be identified in future in association with T2DM in various populations. This, because there are some genes present only in some ethnic groups and not in others.

THE β-CELL MASS/FUNCTION

Art. 83 – The major issue in diabetology, irrespective if studying the autoimmune or non-autoimmune phenotypes of the disease is represented by the difficult access to human pancreatic tissue. Currently, this is available only occasionally following some surgical procedures (for acute or chronic pancreatitis, pancreatic cysts, malign or benign pancreatic tumors, etc.), from organ donors but also as necroptic pancreatic tissue. This should be obtained as soon as possible after death that currently can be well and definitively documented in less than 8 hours after the sorrowful event. Unfortunately, “laws” protects the body of the deceased, even if all that it’s left after death is his intellectual and spiritual legacy, in fact the only thing that would require legal protection. By excessively and uselessly protecting the bodies of the deceased, we deprive in fact the whole humanity of the information that could be retrieved from the analysis of the pancreases or other organs, information that in time could save many human lives. In a recent review article referring to the pathogenesis of type 1 diabetes, the authors (Pietropaolo et al.) state: “There is a need to have access to pancreatic and lymphoid tissue from cadaveric donors with signs of autoimmunity before disease onset to uncover the role of T-cell responses against islets auto-antigens in disease pathogenesis.” The same need is obvious for the early pre-clinical stage of the other diabetes phenotypes, mainly T2DM. In this respect, an adequate and wise legislative initiative from the European Parliament is not only appropriate but also necessary. This could include the possibility to obtain cadaveric tissues, at least in larger university centers and under a clear regulatory conditions.

Art. 84 – The beta cell mass is rather a theoretic notion because of our current impossibility to directly evaluate how many beta cells there are in a pancreatic islet and how many islets a particular subject carries. The high heterogeneity of the pancreatic islets, and inside them of the pancreatic beta cells renders impossible the prediction of the beta cell mass based on the information provide by an eventual in vivo pancreatic biopsy puncture. Diabetologists wait
with great interest for the development of some practical methods for the evaluation, even indirect, of the beta cell mass 69.

Art. 85 – When we try to evaluate the beta cell mass, we should make a distinction from the beta cell function, evaluated indirectly by studying both quantitatively and qualitatively the secretory beta cell function63,259. A secretory beta cell defect identified by studying the insulin-secretion response to diverse stimuli is usually already associated with a decreased beta cell mass. However, will be difficult to assess the beta cell loss only by the study of insulin secretion. For instance, at a decrease of β-cell mass with 25%, the remaining 75% cells may or may not be able to maintain the control of blood glucose regulation. If the remaining cells have only half of their normal functional potential, the blood glucose regulation will be evidently affected.

Art. 86 – Irrespective of the diabetes phenotype, the direct cause for the alteration of the energy metabolism regulation is represented by the decrease in beta cell mass/function67,69,138,188. In T1DM phenotype, due to the particularities of the autoimmune process, which once initiated will be consequently amplified by successive “waves” of autoimmune attacks241,250, the decrease of the beta cell mass occurs rapidly. On the contrary, in T2DM phenotype the decrease of the beta cell mass occurs slowly or even very slowly260. The results of standardized morphometry studies (the quantified pancreatic fractional β cell area and immuno-histochemical identification of β cell replication and apoptosis using the markers (Ki67 and TUNEL respectively) performed on necroptic pancreases showed important changes in the architecture of pancreatic islets, with a decreased number of pancreatic beta cells while the space between them are occupied by amyloid or lipid deposits or even fibrous tissue. In the presence of this complex and advanced pathological picture, scientists cannot decipher anymore neither the primary start point for the pathogenic process, nor its nature or its time evolution.

Art. 87 – The fundamental mechanisms for the decrease of the beta cell mass are apoptosis and only rarely beta cell necrosis, recorded especially (but not only) in the autoimmune T1DM phenotype. In the T1DM phenotype, the massive aggression induced by different cytokines produced by the T cells leads rapidly to beta cell death263. This is an extrinsic β cell aggression, acting through the cell surface death receptors and leading to a cytokine-induced cell death563. IL1β, TNFα and IFNy released by the lymphocytes and macrophages invading the islets have powerful apoptotic effects260. Even if the beta cell regeneration processes are still active69,201 the newly generated cells will be born in a “cytokinic” milieu and will die before reaching complete maturity and expressing their function. All these explain the rapid decrease of the beta cell mass and the explosive nature of this diabetes phenotype. In T2DM phenotype, the pro-apoptotic beta cell mechanism is of intrinsic nature. Several mechanisms have been proposed to explain the increased β cell apoptosis in T2DM, including oxygen free radicals264,266, free fatty acids toxicity266,267, gluco-lipo-toxicity89,268 and formation of toxic amyloidic oligomers60,269,270. The last mechanism is related to ER stress, occurring by the increased production of miss-folded secretory proteins inside the ER54,269.

Art. 88 – The more precise data regarding the beta cell apoptotic process, much more easily to study on beta cell lines in culture, contrast strongly with those more elusive regarding the beta cell regeneration. It is currently estimated that a beta cell can live for a few months or a few years. In this view, maintaining the homeostasis of the beta cell mass implies the presence of an efficient process of beta cell regeneration. This can be done by the replication of existing adult beta cells, by neogenesis (from the islet mesenchymal stem cells or ductal stem cells) or by trans-differentiation from acinar cells71. The contribution of these mechanisms in maintaining the beta cell mass is still unclear71,272, as are in fact the data regarding the life-span of a human beta cell (estimated between months or years!).

Art. 89 – The regenerative capacity of the pancreatic islets/beta cells from rodent diabetic animal models led to a premature enthusiasm regarding the possibility to stimulate this process in humans182,273. However, after reaching adult life (20–25 years), the potential for regeneration of the human pancreatic beta cells decreases dramatically138, explaining why diabetes defined
as the presence of overt hyperglycemia is still considered to be an un-curable disease, since at this time already half of the beta cell mass is irremediably lost. We wait with interest that one or other from the new classes of anti-diabetic drugs will bring some solid-proof regarding a potential effect on the regeneration of the beta cell mass, or at least on stopping its decline.

Art. 90 – The hope regarding a potential increase of the human beta cell mass induced by pharmacological means started from the observation that in some particular physiologic conditions, such as puberty, pregnancy or weight gain, the beta cell mass can increase significantly. It should be mentioned that such an adaptative response is encoded in the genetic code of the beta cell specifically for these periods. During puberty, beta cell mass increase is done by beta cell replication while in pregnancy (a transient state) and in weight gain (genetically encoded as a still transient state) it is done mainly by increase of the beta cells volume. Unfortunately, only normal pancreatic beta cells can react in such a manner. In diabetic subjects, the regenerative capacity is decreased or even abolished due to the defects already present in the fundamental function of these cells. If we refer to obesity, we could anticipate that obese subjects that don’t inherit the beta cell defect will adapt to the increased secretory demand, preventing the occurrence of blood glucose increases. This is the category of obese diabetic subjects the beta cell mass is invariably decreased.

THE GENETICS OF β-CELL REGENERATION

Art. 91 – A recent study showed that the normal embryogenesis and development of the Langerhans islets involves not less than 1029 genes, of which 237 encode regulating transcription factors. This process is very complex and can become inadequate in the presence of specific gene defects. Such a defect can be represented by that identified in TCF7L2 gene (chromosome 10q, SNPs rs1255372 and rs7903146) by Grant et al. The importance of this gene, identified using the large-scale association method, has a double significance: the first, it is the strongest associated T2DM gene; the second it is involved in the Wnt signaling pathway, with a specific role in the regulation of cell growth mechanisms. The protein encoded by this gene is an ubiquitary transcription factor involved not only in the growth and development of the pancreas but also of the entero-insular axis and of some insulin-dependent cell types. The diabetogenic polymorphisms of this gene are located in the non-coding (intronic) region. Their presences predict T2DM and contribute to the diabetogenic risk by 10–25%.

DIABETES MELLITUS AND SENESCENCE

Art. 92 – More than 50% of diabetes mellitus cases are diagnosed after the age of 50 years, while the maximum increase in the slope of diabetes incidence is recorded between the ages 40–55 years (Fig. 4).
Art. 93 – Even if the T1DM autoimmune phenotype is predominant in younger ages while the T2DM non-autoimmune phenotypes in older ages, frequent exceptions from this rule are recorded. Neonatal diabetes includes most often non-autoimmune phenotypes, while the so called form of “maturity diabetes” (currently described as T2DM) is encountered more and more frequently in adolescents or even children1,14.

Art. 94 – The age at diabetes onset is related both with the genetic component and the intervention of various environmental factors. Epigenetics still has to explain how the different environmental factors, acting through transcription factors, can activate the diabetogenic mechanisms that otherwise could have remained silent even until the 9th decade of life184. The most important diabetogenic mechanism operating after the age of 50 years is perhaps the amyloidogenic transformation of amylin. It can explain the peak in T2DM incidence (diagnosed clinically through overt hyperglycemia) recorded around the age of 60 years. Since the amyloidogenic process starts inside the secretory pathway of the beta cell and only afterwards progresses in the cytosol and eventually outside the beta cell (without the possibility to be evidenced until the extra-cellular stage), its real onset can take place years before the hyperglycemic stage of diabetes. We consider that the extra-cellular amyloid deposits could represent the late consequence of some pro-apoptotic mechanisms operating inside the beta cells185. The lack of a parallelism between the magnitude of the amyloid deposits and the beta cell function could be due to the rather inoffensive nature of the extra-cellular beta cell amyloid deposits184, but the definite pathogenic nature of the toxic amylin oligomers127 present inside the beta cell but not identifiable in the necrotic pancreatic tissue since they cannot be evidenced by classic staining techniques with Congo red.

Art. 95 – The association of pancreatic amyloidosis with similar alterations in other tissues and their increased incidence in older ages place type 2 diabetes mellitus among the diseases related to the senescence process. This process is based on the decreased capacity of the ER from the β cells to promptly and efficiently perform their own specific posttranslational functions. This process can be influenced by the inhibition of gene expression due to the DNA methylation at PPARG gene level184,267.

Art. 96 – Related to the aging process, we mention the recent identification of the LMNA gene which encodes laminin A, a component of the network of proteins composing the nuclear lamina inside the nuclear membrane281. The majority of Hutchinson-Gilford progeria syndrome subjects had an identical mutation in one allele of this gene, C-1824-T substitution204. Other LMNA mutations are associated with other sever monogenic diseases, including forms of generalized lipodistrophy. In the Hutchinson-Gilford syndrome, LMNA mutations result in the production of a protein designated „progerin” with
an internal deletion of 50 amino acids as compared with normal laminin A. After its transcription, laminin molecules are post-translationally processed in the ER and released in the cytosol in order to be finally imported into the nucleus. The study of progeria mechanism could lead to a better understanding of the senescence process and possibly the way by which the age influences the incidence of diabetes.

CONCLUDING REMARKS

Art. 97 – In the broader domain of metabolic disorders, diabetes mellitus remains a peculiar disease due to its high complexity, the presence of its multiple phenotypes and its complex pathogenesis, that it is well understood in some aspects, less understood in others and sometimes, unfortunately, mistakenly understood. That is why, many years from now its content will need periodic revisions for adjustments and additions.

Art. 98 – In fact the medical speciality Diabetes, Nutrition and Metabolic Diseases is one of medical specialties only recently defined. In 1934, the famous Romanian diabetologist Prof. Ion Pavel (1897–1992), grouped his diabetic patients from a Clinic of Internal Medicine in two halls from the same ward. Eight years later, in 1942, during hard war conditions, he had the genial inspiration to make an announcement in the press that diabetic patients could receive meat tickets, a product hard to find during those times. This is how he managed to start a diabetes registry including in one month the first 800 patients from Bucharest, Romania. With good clinical intuition, he noticed the socio-medical peculiarities of diabetic patients needing good and specific education, social help and long term surveillance. These peculiarities sustained the need for a distinct medical specialty in order to assemble a complex medical team with responsibility both for the prevention of various chronic complications but also for their treatment if they couldn’t be avoided. Registration continued permanently thereafter. Currently the number of diabetic patients included in this registry has reached the figure of 155,000.

Art. 99 – The medical speciality of diabetes, nutrition and metabolic diseases found its way into the official list of medical specialties and in university teaching with difficulty. It emerged from the specialty of internal medicine since in Romania it was from the onset never associated with endocrinology. It is significant that in one Internal Medicine Textbook from the 1950’s diabetes mellitus represented a chapter 10 times smaller than the chapter dealing with rheumatic fever. Now the main classical textbooks of diabetes have more than 1000 pages and new editions are published approximately every 5 years. We don’t know any other medical specialty that had such a rapid evolution as diabetology, which probably in the future will be even faster. Its destiny was strongly mixed with that of molecular biology or genetics as one of the most active fields in medicine.

Art. 100 – One of our generation’s responsibilities is to make a correct analysis of the different systems for diabetes care currently operating in Europe, and, based on this, to find an appropriate adjustment for the list of medical specialties. This should be done according to the current predictions regarding the increasing trend in prevalence of metabolic disorders. Finally, this should provide a better solution than that existing now, in which, in an artificial manner, under the cover of endocrinology, two different groups of medical specialists are at work: one treats only diabetic patients while other deals only with the various endocrine diseases. The first is evidently the more numerous and should receive a title in accordance with the disease they deal with, that of diabetologist.

Art. 101 – As direct participants to all the successes and failures of diabetology, we felt the need to include in a succinct manner those scientific data we considered to have remained valid. From this code the younger generations of diabetologists can begin deciphering the issues that still remain unclear, and especially to illuminate those areas still shrouded in mystery.

ACKNOWLEDGMENTS

Thanks to Solomon Marcus, Cristian Guja, Sorin Ioacara and Lawrence Chukwudi Nwabudike for their useful suggestions.

REFERENCES


European Constitution Book of diabetes mellitus


Florez J.C., Newly identified loci highlight beta cell dysfunction as a key cause of type 2 diabetes: where are the insulin resistance genes? Diabetologia 2008 51:1100–1110.


Giordano I., Brigatti C., Podini P., Bonifacio E., Meldolesi J., Malosio M. L., Beta cell chromogranin B is partially segregated in distinct granules and can be released separately from insulin in response to stimulation Diabetologia 2008, 51:997–1007


Ionescu-Tírgoviște C., Guja C., Vladica M., Ioacara S., Bojin A., Filip I., Proinsulin levels are significantly increased both in type 1 and type 2 diabetes compared with normal subjects; Diabetes 55, 2006, Suppl. 1, A573.


European Constitution Book of diabetes mellitus


75. Hanefeld M., Koehler C., Fuecker K. et al., Insulin secretion and insulin sensitivity pattern is different in isolated impaired glucose tolerance and impaired fasting glucose: the risk factor in Impaired Glucose Tolerance for Atherosclerosis and Diabetes Study. Diabetes Care 2003, 26:868–874.


97. Saltevo J., Vanhala M., Kautiainen H., Laasko M., Levels of adiponectin, C-reactive protein and interleukin-1 receptor antagonist are associated with the relative change in body mass index between childhood and adulthood. Diabetes & Vascular Disease Research 2007, 4:328–331.


127. Thörn K., Bergsten P., Fatty acid-induced lipid handling and apoptosis in MIN6 cells is determined by fatty acid saturation and chain length. Diabetologia 2007, 50 [Suppl.1] S174.


European Constitution Book of diabetes mellitus


Wang J., Xu J., Finnerty J. et al., The prohormone convertase enzyme 2 (PC2) is essential for processing pro-islet amyloid polypeptide at the NH2-terminal cleavage site. Diabetes 2001, 50:534–539.


213. European Constitution Book of diabetes mellitus


193. diabetes mellitus: a disease linked to multiple mechanisms. Orphanet J Rare Dis 2007, 2:12.


262. Marchetti P., Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that β-cell death is caspase mediated, partially dependent on ceramide pathway and Bcl-2 regulated. Diabetes 2002; 51:1437–1442.


278. Lapi R., Dotta F., Marselli L., Del Guerra S., Masini M., Santangelo C., Patane G., Boggi U., Piro S., Anello M., Bergamini E., Mosca F., Di Mario U., Del Prato S.,