## Angiogenesis and lymphangiogenesis in experimental diabetes models

***Keywords***: angiogenesis, lymphangiogenesis, insulin, PROX-1, podoplanin

The ***main reasons*** for which we chose this research topic are: epidemiology of diabetes, according to the Atlas of the International Diabetes Federation (IDF) published at the end of 2019, there were 463 million adults living with diabetes, the prevalence of the disease has been increasing in recent years and by the end of 2030 it is expected that there will be 578 million adults living with diabetes. Morphological and immunohistochemical changes of the early stages of islet cell destruction have been very little studied, the combination of the inflammatory factor, angiogenesis and lymphangiogenesis in the preliminary stages of diabetes is ambiguous. The involvement of Podoplanin in the pathogenesis of diabetes is completely unknown, most experimental models of diabetes were obtained by chemical induction (method omitting the main type-2 diabetes inducing factor, improper diet). The characterization of angiogenic and lymphangiogenic markers from the preliminary stages of diabetes may be the approach of new treatment targets.

***Material and methods***

We used 13 Sprague Dowley rats, kept in the biobase of the University of Medicine and Pharmacy "Victor Babes" in Timișoara, under optimal conditions, according to the European norms in force. Their diet was hypercaloric (hyperglucidic - fast-absorbing, hyperlipidic carbohydrates, rich in saturated and trans fats, hyperproteic). About 2-3 times per week, they received fast food, a diet similar to that of human subjects who develop obesity with or without pre-diabetes/type 2 diabetes. Basal blood glucose was measured from the capillary blood by means of a tail puncture, using the Achtung glucometer initially (before exposure to the hypercaloric diet) and after 3 and 9 weeks post-exposure, respectively.

Pancreatic tissue was sampled before exposure and at 3, 6, 9, 12 and 16 weeks post-exposure. The sampled fragments were noted (N1, control 1), (OB1, obese1), (OB2, obese2), (OB3, obese3), (OB4, obese4) and OB5 (obese5). Then, the primary processing took place, hematoxylin-eosin staining that allowed the histopathological diagnosis and the selection of cases for immunohistochemical evaluation (anti-insulin antibody, anti-Factor VIII antibody, anti-AC-133 antibody, anti-OCT3/4 antibody, anti-Podoplanin antibody, anti-PROX1 antibody). After the immunohistochemical evaluation, we selected the cases for validation by RNAscope *in situ* hybridization assay.

***The first chapter of the thesis analysed the morphological changes of islets of Langerhans, in dynamics, before exposure, and in different stages of exposure to an obesogenic diet.***

In case (N1, control) the specific, normal distribution of the islets of Langerhans in the exocrine parenchyma was observed. Well-delimited round-oval islets, paler in colour, located

in the centre of the pancreatic lobes (compact islets), and at the periphery of the pancreatic lobes we observed paler structures, poorly delimited by the exocrine component, diffuse pancreatic islets. The smear test also contained spleen fragments, because the pancreas in rodents is difficult to harvest, as it is not organized into a well-defined organ, but consists of pancreatic tissue included in the fat from the abdominal cavity, posterior to the stomach.

In the case (OB1, 3 weeks post-exposure), the following differences were noted: hypertrophy of compact islets, increase of islet density per pancreatic section, heterogeneous distribution of pancreatic islets in the pancreatic parenchyma, more visible hyperemia of the peri-insular vessels.

In case (OB3, 9 weeks post-exposure), the pancreatic parenchyma was surrounded by an increased amount of white adipose tissue, and in (OB5, 16 weeks post-exposure), peri-insular fat degeneration was found.

Detailed changes in dynamics at the level of the islets of Langerhans consist of: in (N1) the intra-insular endocrine cells occupy almost the entire islet area, the vascularization is more pronounced in the centre of the islet, compared to the peripheral area; in case (OB1) hypertrophied compact islets were identified, with predominant optically clear endocrine cells (functionally active) arranged in cords, with the increase of peri-insular vascularization. In case (OB4) we noted a change in the intra-insular cell architecture (in the case of a hypertrophied islet), cell disorganization, decrease in cell density, accentuated vascularization, with obvious stasis.

In case (OB3) we observed an increase in the amount of white peripancreatic adipose tissue, with inflammatory infiltrate, diffuse location and follicular appearance. In addition, in the peripancreatic white adipose tissue, we noted isolated endothelial cells, endothelial cells delimiting non-perfused tubular-like structures and perfused mature vessels, which suggested a possible active angiogenic process (known stages of sprouting angiogenesis).

The conclusions of the first chapter of the thesis are: the hypercaloric diet induces early major morphological changes in the pancreatic islets, the dynamics of morphological changes of the pancreatic islets is a complex process that not only affects the pancreatic β cells, but also the capillary network, as well as the inflammatory status dependent on the time elapsed since the feeding of the hyper-caloric diet. The vascular microdensity increases proportionally with the hypertrophy of the pancreatic islets, there are two types of angiogenesis in the pancreatic islets after the feeding of the hypercaloric diet: compensatory, physiological angiogenesis and inflammatory, pathological angiogenesis. The hypercaloric diet not only determines the accumulation of peripancreatic adipose tissue, but also its particular organization, by the existence of diffuse inflammatory infiltrate or organized in the form of pseudofolicules, as well as by the existence of an active capillary network subject to a permanent remodelling.

***In the second chapter of the thesis, we highlighted the variability of insulin expression and the correlation with blood sugar in capillary blood.***

In case (N1), insulin-positive cells were distributed across the pancreatic islets, with an intense, homogeneous cytoplasmic appearance. In (OB1), insulin immunoexpression was higher compared to (N1), insulin-positive cell density increased, occupying almost the entire islet area, in the case of hypertrophied compact islets. In the case of hypertrophied diffuse islets, the density of insulin-positive islet cells was significantly lower than the compact ones, and the intensity of the immunoexpression was moderate/low. In case (OB2), there was a significant increase in the intensity of insulin immunoexpression in the hypertrophied islets, compared to (N1), which coincides with the hypersecretory status and the decrease of blood sugar levels. In case (OB3) a drastic decrease of insulin immunoexpression was observed, which overlaps with the decrease of insulin secretion and the increase of blood sugar levels.

The hypercaloric diet determines early hypertrophy of the pancreatic islets by hyperplasia of insulin-secreting cells. There is an early decrease in blood sugar levels caused by the large amount of compensatorily secretion of insulin. Islet hypertrophy with increased density and insulin immunoexpression in pancreatic β-cells was correlated with the transient decrease in capillary blood sugar. Later on, pancreatic β-cell depletion causes hyperglycemia and diabetes. Islet degeneration, with the decrease in the number and intensity of insulin immunoexpression, resulted in the development of serum hyperglycemia at a late stage since the beginning of the hypercaloric diet.

***In the third chapter of the thesis we evaluated the microvascularization of pancreatic islets after feeding the hypercaloric diet.*** We used anti-FVIII antibodies. In case (OB2) there was an increase in intra-insular vascular density, dilation of peri-insular vessels, FVIII-positive vessels, the intensity of immunoexpression was higher in mature peri-insular vessels and discontinuous/inconsistent in intra-insular vessels. In case (OB2), the dilated vessels from the periphery of the islets invade the hypertrophied pancreatic islets, the vessels have a high degree of ramification, the cells at the tip of the sprout are FVIII-positive, the vessels coming from the vessel that invaded the islet have no patent lumen, appearance of cord at an early stage of islet angiogenesis.

In case (OB3) we noted microvascularization of white peripancreatic adipose tissue, increased density of the capillary vessels and their intense ramification, especially near the pancreatic tissue.

The hyper-caloric diet induces the increase of vascularization in pancreatic islets directly proportional to the time of feeding of this diet. Our data support the existence of two types of angiogenesis depending on the time of its evaluation: adaptive angiogenesis as an adjuvant factor of the transient increase in insulin secretion and its compensatory release, angiogenesis that can be considered physiological, given the interrelation between the

endothelial cell and pancreatic β cells in normal islets, an inflammatory angiogenesis, occurring in the late stages of hypercaloric diet feeding, angiogenesis that can be considered pathological, by maintaining macrophage accumulation and irreversible destruction of pancreatic islets.

***In the fourth chapter of the thesis we studied the vascular reaction of the pancreas implant on the chorioallantoic membrane (CAM), harvested at different stages of the feeding of the hypercaloric diet.***

During the grafting of pancreatic tissue from (OB3) on the CAM, we noted the area free of blood vessels around the implant.

The second day post-implant, we noted the angiogenic reaction induced by the pancreatic tissue implant from (OB3). The macroscopic exam revealed an increase of the peri-implant vascular density, the peri-implant vascular network consists of numerous small-sized blood vessels, vessels with a high degree of ramification, which create a dense network around the implant. In addition, the vascular density and the degree of ramification of the peri-implant vessels was higher on day 4 postimplant compared to day 2. These aspects were also validated by the images obtained with the stereomicroscope: on day 4 post-implant, at the time of the interruption of the experiment, the peri-implant vessels invaded the periphery of the implanted pancreatic tissue and were characterised by intense ramification and perfusion. In the peri-implant area, we noted the emergence of a large number of vessels from pre-existing vessels, with a sinuous appearance, with the free end dilated, which suggested a still active angiogenesis. The paler central area ofthe implant suggested lysis of the pancreatic tissue, but its periphery was viable, most likely due to the acquisition of blood vessels.

The microscopic examination revealed a central lysis of the implant (highly acidophilic mass) and the preservation of the tissue from the periphery of the implant. The implant adhered to the CAM and was vascularized at the periphery, while the peripancreatic adipose tissue was surrounded by inflammatory infiltrate. Massive inflammatory infiltrate was observed in the condensed chorion of CAM. At CAM level, we also noted besides the rich inflammatory infiltrate and the accentuation of the vascularization, vessels with high density, dilated blood vessels, with a rich content in inflammatory cells, aspects observed at a distance from the pancreatic tissue implant.

The pancreas implant on CAM has a low viability and requires particular attention to its harvesting and implantation on CAM. Pancreatic xenografts induce a massive inflammatory response in the CAM chorion, and inflammatory cells are present in large numbers also in CAM blood vessels. The peri-implant vascular reaction consisting of the acquisition of new blood vessels was due to the massive inflammatory infiltrate from CAM. The inflammatory

infiltrate was also found in the peripheral area of adipose tissue adjacent to the implant, similarly to microscopic preparations.

***In chapter 5 of the thesis we identified and characterized CD-133 positive and OCT ¾ positive cells (stem cells) in pancreatic changes after feeding the hypercaloric diet.***

In case (N1) rare CD-133 positive endothelial cells were detected on the pathway of the blood vessels in the exocrine parenchyma, star-shaped positive cells in the exocrine parenchyma and oval positive cells located in small or isolated groups, most often at the periphery of the exocrine pancreas. In case (OB3), the density of vessels and star-shaped CD-133 positive cells increased (compared to N1), with a tendency to get organised into a network in the exocrine component.

At islet level, in (N1), a single star-shaped CD-133 positive cell was observed, located at the periphery of the pancreatic islet. In case (OB1) there was an increase in the density of CD-133 positive intra-insular cells, insular hypertrophy correlated with the number of CD-133 positive cells inside the pancreatic islets. In (OB2) and (OB3), there was an increase in the number of intra-insular positive CD-133 cells, but also an increased number of CD-133 positive cells delimiting small intra-insular vessels, which suggests the immaturity of the vessels during this period of feeding a hypercaloric diet.

In case (OB2), OCT3/4 was expressed in rare cells from the periphery of the pancreatic islets, and in case (OB3) we observed the increase of the density of OCT3/4 positive cells, while the majority of the small vessels showed OCT3/4 nuclear expression cells inside the endothelium.

Pancreatic CD-133 positive cells undergo changes in distribution and density, depending on the time of feeding the hypercaloric diet. There are two subpopulations of CD-133 positive cells in the exocrine pancreas, but only star-shaped cells seemed to have increased in pancreatic islets at (OB3). CD-133 positive cells participate in the regeneration of pancreatic islets, as well as of intra-insular vessels. OCT ¾ is expressed in a small number of intra-insular cells at (N1), while the number of OCT3/4 expression cells was increased in the pancreatic islets from (OB3).

Our study demonstrates the involvement of the Podoplanin/PROX-1 axis in irreversible changes of endocrine pancreatic parenchyma.

***In Chapter 6 of this thesis we evaluated Podoplanin and PROX1 in pancreatic changes induced by the hypercaloric diet.***

In (N1) the expression of Podoplanin was homogeneous in the islets of Langerhans and in the cells of the central ducts, with increased immunoexpression in the peripheral area of the pancreatic lobes. Lymphatic endothelial cells from the peripancreatic adipose tissue were also positive. In case (OB2) the islet immunoexpression became heterogeneous, with

increased intensity at the periphery of the islets of Langerhans. In (OB2) an increase of Podoplanin expression was observed in the hypertrophied islets, with peri-insular inflammatory infiltrate. In case (OB3), an increase of Podoplanin immunoexpression was observed in the hypertrophied islets with peri-insular inflammatory infiltrate. Podoplanin expression was validated by the RNAscope method. In case (OB2), positive cells were detected at the periphery of the islet area. The cells on the periphery of the pancreatic islets were intensely positive for PROX1, in case (N1). In (OB1), positive PROX1 cells from the islet periphery decreased, rare positive cells were observed in the central area. In (OB2) the distribution of PROX1 positive cells became focal, in small groups at the periphery of the islet area. In the central area of the islet, the expression was isolated and with high density, both in the hypertrophic islets and in those with normal areas, with clustered expression of PROX1 mRNA in the hypertrophic islets. In case (OB2), in normal-sized islets, the distribution of positive cells was different from that of normal pancreas.

Both Podoplanin and PROX-1 are expressed differently, depending on the feeding of the hypercaloric diet. Podoplanin mediates the destruction of pancreatic islets most likely by means of an immune and/or autoimmune mechanism.

PROX-1 has a double role in the pathogenesis of early changes in endocrine parenchyma in diabetes, and our results support the following pathways: (a) PROX-1 reactivation induced by hyperglycemia may initially result in an increased number of immature pancreatic β cells, which is also supported by the increase in the number of CD-133 positive cells described in our study, in the previous chapter; (b) its repression by hyperglycemia supported by the feeding of the hypercaloric diet determines the inhibition of the GSIS gene that supports insulin secretion. This explains the disappearance of PROX-1 expression and the decrease in insulin immunoexpression in the pancreas harvested at 9 weeks.

This study is the first description of the variability of expression of D2-40 in the pancreatic islets during the feeding of the hypercaloric diet, known as responsible for the induction and maintenance of diabetes. The correlation between the low PROX-1 and a low insulin immunoexpression and transient increase in CD-133 supports an inefficient compensatory mechanism, also aggravated by the immune response initiated and supported by D2-40.

***Final conclusions:***

The pancreatic parenchyma undergoes early changes after the start of the hypercaloric diet.

Pancreatic changes affect the cell population in the endocrine pancreas, but also that of the exocrine pancreas.

The factors involved in degenerative changes of the endocrine pancreas during the hypercaloric diet are multiple: angiogenic, inflammatory, activation of transcription factors, suppression of the expression of possible regenerative factors.

Insulin hypersecretion is transient and transiently suppresses the hyperglycaemia induced by the hypercaloric diet.

The angiogenesis observed in the pancreatic changes induced by the hypercaloric diet plays a dual role in pancreatic degeneration.

Early onset angiogenesis, in the stages of hypertrophy of pancreatic islets has a compensatory role of participating in increased insulin release and is directly related to the number and secretion of pancreatic β-cells. Angiogenesis associated with inflammation in late stages of pancreatic degeneration supports the inflow of inflammatory cells that support pancreatic destruction.

Angiogenesis is not restricted only to pancreatic islets, it is present and exacerbated in dynamics and peripancreatic adipose tissue, as well as in the peri-insular exocrine parenchyma.

The origin of newly formed vessels due to the pancreatic changes induced by the hypercaloric diet has not been elucidated and requires further studies.

The angiogenic effect was certified on the experimental model of implant on the chorioallantoic membrane (survival until day 4 is exceptional), certifying the pathological, inflammatory angiogenesis which contributed to the destruction of the implanted parenchyma.

Early changes induced by the hypercaloric diet determine the activation of CD-133 positive stem cells, most likely compensating for pancreatic β cell regeneration, but this activation is transient and inefficient.

Activation of the PROX-1 transcription factor occurs in the early stages of pancreatic changes following a hypercaloric diet and controls D2-40 expression in the affected pancreatic islets.

Inactivation of PROX-1 results in failure to obtain mature insulin-secreting pancreatic β-cells, most likely by suppressing the GSIS gene responsible for stimulating insular secretion.

The originality elements of this thesis are: (a) induction of pancreatic tissue changes by feeding food similar to that consumed by human subjects and not drug-induced; (b) correlation of the insulin immunoexpression with the blood sugar level; (c) dynamic evaluation of vascular density variability during pancreatic changes; (d) study of positive CD-133 cells in pancreatic parenchyma and related vessels after feeding of the hypercaloric diet and not after drug induction.

Reporting for the first time the expression and variability of expression of D2-40, but also of PROX-1 in pancreatic islets affected by the hypercaloric diet, as well as their certification by applying the *RNA-scope* method for identifying PROX-1 mRNA, since the literature states that suppression of insulin secretion is caused by a decrease in mRNA, but not in protein expression.